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

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

Inaugural dissertation

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I. Introduction

During the last decades, the emergence of antibiotics, the generalisation of hygiene and food reliability has tackled much of pathogen-related diseases. However, some illnesses remain difficult to treat, including fungal infections. Because fungi are eukaryotic organisms, their physiology is close to humans. Furthermore, fungi are versatile and able to resist treatment. In addition, because of AIDS, cancers and progress in modern medicine (organ transplantation), the number of immuno-compromised patients has risen. For these patients, opportunistic pathogens that would present no threat for healthy individuals can be a deadly hazard. The ascomycete fungus *Candida albicans* is one of such pathogens. *C. albicans* is present in the normal gastro-intestinal flora of a large portion of the population (≈50%; Eggiman *et al.,* 2003). In healthy individuals, *C. albicans* usually behaves as a commensal; it can nevertheless cause benign skin or vulvo-vaginal infections (Kim *et al.,* 2011). However, if the immune defences are low or after heavy surgery, *C. albicans* can lead to disseminated candidiasis, which often is a fatal disease. Because hospitals cumulate risk factors for candidiasis, *C. albicans* is responsible for a large portion of nosocomial (hospital-acquired) infections. C*. albicans* has an arsenal of virulence factors that make it a very dangerous and hard to treat pathogen. The survival rate for disseminated candidiasis is low (40-60 %; Eggiman *et al.,* 2003). One of *C. albicans'* key virulence factor is its ability to grow as yeast (unicellular form similar to baker's yeast) and as a hypha (multicellular filamentous form; Lo *et al.,* 1997). The yeast-to-hypha transition is linked with the capacity of *C. albicans* to disseminate, to adhere to host cells and to form resistant biofilms (Mayer *et al.,* 2013). Filamentation is regulated by complex signalling pathways that will be described in detail below. Because these regulatory pathways are very complex, advances in biomolecular tools are essential; until today, progress in the understanding of human pathogens has been associated with progress in molecular biology techniques. In particular, efficient gene reporters that make the expression of a gene easily measurable and allow identification of *cis*- and *trans*-acting elements are dearly needed. In the case of fungal filamentation, because changes in gene expression are abrupt, a gene reporter that would allow the monitoring of multiple genes in real time would be an important asset.

This work describes new luminescent gene reporters for *C. albicans*, the click beetle luciferases. After a short description of *C. albicans*, its filamentation regulation pathways will be described with particular attention given to the crucial cAMP/PKA pathway. The existing gene reporters available for *C. albicans* will then be presented followed by a description of luminescence in nature and the underlying chemical mechanisms.

1. *C. albicans*, a pathogen

C. albicans is a very common microorganism in humans; studies have estimated that approximately 50 % of the population is colonised (Eggimann *et al.,* 2003). In healthy individuals, *C. albicans* exists as a commensal and is part of a non-problematic gastro-intestinal flora. In some cases, *C. albicans* can cause localised surface infections; classically in the mouth, vulvo-vaginal tract or on the nails. These types of non-life threatening infections are commonly found in new-borns, elderly people or immunocompromised patients. However, *C. albicans* can also cause candidemia (presence of *Candida* cells in the blood) or disseminated candidiasis (deep-seated infection in

multiple organs), which are life threatening diseases. In US hospitals, between 1995 and 2002, *C. albicans* was isolated in the blood of 9 % of the patients; *Candida spp.* was the 3rd cause of nosocomial blood infection, after *Staphylococcus spp*. and *Enterococcus spp*. (Wisplinghoff *et al.,* 2004).

A number of risk factors increase the probability of developing disseminated candidiasis. The risk factors can roughly be divided in two groups: immune system deficiencies and hospital-related risks. An efficient immune system is essential for protection against *C. albicans*. Patients with HIV are at risk (reduction in LT CD4+, macrophages and dendritic cells), as are transplanted patients. Heavy surgery is also a risk factor, especially in the case of abdominal surgery. Generally, a rupture of the gut barrier presents a risk, because of the presence of *C. albicans* in the gastro-intestinal tract. The use of intravenous catheters, or other invasive man-made devices, drastically increases the probability of systemic candidiasis (Kojic *et al.,* 2004). This is related to the ability of *C. albicans* to adhere to artificial surfaces and to form resistant biofilms. Studies have even demonstrated the positive impact of catheters exchange on remission after *Candida spp*. infection (Rex *et al.,* 1995). Antibiotic therapy, especially a prolonged treatment with broad spectrum antibiotics, is also a risk factor. This is explained by the disturbance to the normal mucosal flora (Colombo *et al.,* 2000). Altogether, a long stay in intensive care unit increases significantly the risk of developing a disseminated candidiasis. It is noteworthy that colonisation of the intestinal tract by *C. albicans* often precedes the infection. Indeed, the *Candida* strain causing the blood infection is, in a high proportion of cases, the same as the one previously found in the gastro-intestinal tract (Saiman *et al.,* 2000). Moreover, the colonisation rate by *C. albicans* increases with the length of stay in intensive care unit, as demonstrated by the presence of *Candida* in the stools (Petri *et al.,* 1997). Disseminated candidiasis is a critical illness, with an overall mortality rate as high as 50 % (Eggimann et al., 2003). Mortality rate of critically ill patients with a *Candida spp.* blood infection is of 59 % compared to 19 % for a similar non-infected group of patients (Wey *et al.,* 1989). After contamination of the blood, whether by a sullied catheter or a rupture of the gut barrier, *C. albicans* will spread through the blood to different organs and colonise them, ultimately causing multiple organ failure (Eggimann *et al.,* 2003).

The importance of *C. albicans* as a pathogen and its virulence can be explained by a set of virulence factors. First, *C. albicans* is able to adhere to artificial surfaces and human cells. The Als family (agglutinin-like sequence) are cell wall proteins that contain domains close structurally to the immunoglobulins. Als proteins have a preponderant role in adhesion; they are responsible for adhesion to collagen, fibronectin, laminin and to endothelial cells, as well as to epithelial cells (Karkowska-Kuleta *et al.,* 2009, Filler *et al.,* 2006). Another cell wall abundant protein, Hwp1, is a substrate for the transglutaminase of epithelial cells and is therefore implicated in covalent attachment to host cells. Virulence of *C. albicans* is linked with the expression of lipases, especially phospholipases (phospholipase B), and secreted aspartic proteases (SAPs; Karkowska-Kuleta *et al.,* 2009). In particular, SAPs represent a family of at least 10 proteins in *C. albicans* and is described as having a key role in *C. albicans* virulence (Schaller *et al.,* 2005). *C. albicans* are able to pass through epithelial and endothelial cell layers to invade deeper tissues. This characteristic is crucial for its dissemination and pathogenicity: from the blood, it can disseminate and invade multiple organs. The invasion mechanism can be linked with the production of lytic enzymes (Filler *et al.,* 2006). *C. albicans* can also induce endocytosis in normally non-phagocytic cells, epithelial and endothelial cells. In particular, the interaction of Als3 protein with the cadherin of the host epithelia induces endocytosis and therefore permits invasion (Phan *et al.,* 2007). Iron limitation is a passive defence mechanism of mammals against infection. *C. albicans* has adapted and has the ability to acquire iron

from host proteins like haemoglobin (Almeida *et al.,* 2009). *C. albicans* has also adapted to resist killing by the host immune system through mechanisms including detoxification of reactive oxygen species, shielding of pathogen associated molecular patterns (PAMPs), regulation of the complement and resistance to killing by macrophages (Miramón *et al.,* 2012; Luo *et al.,* 2013; Mayer *et al.,* 2013). It has also been suggested that *C. albicans* is able to modulate the host immune response by preventing inflammation to avoid being detected as a pathogen (Cheng *et al.,* 2012). A key virulence factor is the ability of *C. albicans* to adapt its morphology to different conditions. The phenotypic switching from normal yeast cells to rode-like opaque cells allows *C. albicans* to adopt different niches of the host ecosystem (Soll, 2014). Importantly, *C. albicans* is able to switch between two growth forms: yeast and hypha. This yeast-to-hypha transition is crucial for pathogenicity and virulence; non-filamentous *C. albicans* have a drastically reduced virulence (Lo *et al.,* 1997).

2. Filamentation in *C. albicans*

The yeast-to-hypha transition has a crucial role in the virulence of *C. albicans* (Jacobsen *et al.,* 2012). Hyphae allow *C. albicans* to penetrate epithelial cell layers permitting invasion of the organs (Weide *et al.,* 1999; Dalle *et al.,* 2010). Filamentation is linked with adherence, hyphal cells express adhesins (Heilman *et al.,* 2011). Hyphae also have a key role in allowing *C. albicans* to escape the immune system. When a *C. albicans* yeast cells is phagocytised by a macrophage, the specific environmental condition induces hypha formation. The hypha can pierce the phagocyte's cellular membrane, killing the macrophage and letting *C. albicans* escape (Lorenz *et al.,* 2004; Jacobsen *et al.,* 2012). The yeast and hypha form have also been described as having different immunological profiles. Lowman *et al.* showed that the immune system reacts more actively to the hyphal form, presumably because of specific glucans (Lowman *et al.,* 2013). The authors hypothesised that this differential reaction is due to the distinction between colonisation and invasion (hypha being associated with pathogenicity). Filamentous forms of *C. albicans* are also important for biofilm formation; biofilms consist typically of three types of cells: yeast, pseudohypha and hypha (Douglas, 2003). The yeast form is similar in shape to the baker's yeast *S. cerevisiae* which buds and separate after mitosis. The two filamentous forms, pseudo-hypha and hypha, are close in aspect but different at the molecular level. Pseudohyphae are chains of elongated cells remaining attached after mitosis. In contrast, hyphae are complex multicellular structures. Starting with an initial yeast cell, provided that the conditions are hypha-inducing, a bud will develop. The bud will then grow apically in elongated form, and when a definite segment size is reached, a septum will form backwards of the apex. Septae separate segments of the hypha but are porous and allow transfer between the compartments. Apical growth is supported by the Spitzenkörper, an accumulation of vesicles coming and going to the apex (Berman, 2006). In hypha, vesicles coming from the Golgi apparatus contain the necessary components for apical extension and are transported on actin cables. True hypha do not present constriction near the septum and have parallel sides. In contrast, pseudohypha present constriction at the cell separation and have curved sides (Stoldt *et al.,* 1997; Crampin *et al.,* 2006).

A variety of conditions can induce filamentation in *C. albicans*; this diversity of induction factors explains the versatility of *C. albicans* hypha formation in the host. Hyphae are induced by the presence of serum, high temperature (37°C), pH>6.5, nutrient limitation (absence of glucose or nitrogen; Ernst, 2000). Filamentation also occurs when *C. albicans* is embedded in agar or at high CO_2 concentration (5 %, concentration found in the host; Shapiro *et al.,* 2011). The cAMP/PKA signal

transduction pathway is needed for filamentation in most conditions; it is therefore considered as the main regulation pathway for yeast-to-hypha transition in *C. albicans*.

The presence of serum at physiological temperature, 37°C, is a strong and robust signal for hyphal induction and has been commonly used to study yeast-to-hypha transition (Ernst, 2000). Through the GTPase Ras1, the presence of serum activates the two main signal transduction, pathways for yeast-to-hypha transition, namely the cAMP/PKA (cyclic AMP/protein kinase cAMP dependent) pathway and the MAPK pathway (mitogen-activated protein kinase)(Fang *et al*., 2006). Moreover, the cAMP/PKA pathway is activated by direct interaction of the bacterial peptidoglycan (present in the serum) with Cyr1 (Xu *et al*., 2008). Cyr1 is an adenylate-cyclase and is a key component of the cAMP/PKA pathway. It integrates the information coming from a variety of environmental cues (Rocha *et al*., 2001). Cyr1 forms a complex with G-actin and Srv2 protein (Zou *et al*., 2010). The activation of Cyr1 leads to an increase of the intracellular cAMP. The cAMP-dependent protein kinase (PKA) isoform Tpk1 and Tpk2 are activated by the rise in cAMP concentration (Bockmühl *et al*., 2001; Sonneborn *et al*., 2000). The PKA complex is constituted of these two protein kinases and of a regulatory subunit, Bcy1. Bcy1 is a repressor of Tpk1 and Tpk2 and therefore of hyphal growth (Cassola *et al*., 2004). The activated Tpk1 and Tpk2 phosphorylate Efg1, the major transcription factor of the cAMP/PKA pathway (Enhanced Filamentous Growth), presumably at threonine 206 (Bockmühl *et al*., 2001). Besides, Tpk1, Tpk2 and Bcy1 all have specific and only partially overlapping genomic binding sites (Schaekel *et al.,* 2013). Activated Efg1 will in turn activate the Flo8 transcription factor and the Cdc28-Hgc1 complex (Cao *et al.,* 2006; Zheng *et al.,* 2004). The activation of Flo8 leads to the expression of Ume6, among other genes (Zeidler *et al.,* 2009). The transcription factor Ume6 is responsible for the activation of numerous hypha-specific genes and the repression of yeast-specific genes. In particular, Ume6 inhibits Tup1-based repression of hypha-specific genes (Banerjee *et al.,* 2008). Tup1 is a global repressor of gene expression and is associated with proteins like Nrg1, Ssn6 or Tcc1. *C. albicans* mutants without Tup1 or Tcc1 are thus hyper-filamentous. Hgc1 is a hypha-specific G1 cyclin; it is associated with the cyclin dependent kinase Cdc28 (Zheng *et al.,* 2004). The complex Hgc1-Cdc28 also integrates informations from the cell cycle (Wang *et al.,* 2009). The activated Cdc28-Hgc1 complex will in turn phosphorylate Efg1 at threonine 179. T179-phosphorylated Efg1 is responsible for the repression of Ace2 specific genes, a set of genes implicated in growth of *C. albicans* as a yeast and in cell separation (Wang *et al.,* 2009). Cdc28-Hgc1 complex is also responsible for the inhibition of Rga2, a GTPase activating protein for the GTPase Cdc42. Active Cdc42 is crucial for apical extension and polarised growth (Court *et al.,* 2007). Activated Cdc28 in conjunction with Ccn1 or Hgc1 cyclins will lead to the phosphorylation of Cdc11, a septin involved in cytoskeleton reorganisation.

Altogether, the activation of the cAMP/PKA pathway influences the expression of genes implicated in morphology and hyphal growth (polarised growth, cell separation, apical extension) but the activation of the pathway also influences the expression of virulence factors like secreted aspartyl proteases and proteins implicated in the adhesion to host cells (Als family, Hwp1). Of all the proteins, of which expression is activated during the hyphal induction, Hwp1 (Hyphae Wall Protein) has the highest expression level (Nantel *et al.,* 2002; Kadosh *et al.,* 2005; Sudbery, 2011). Hwp1 is present at the surface of *C. albicans* cell wall and mimics mammalian transglutaminase substrates; it is therefore implicated in adhesion, especially to epithelial cells (Staab *et al.,* 1999). Because Hwp1 is hypha-specific, is strongly expressed and is a virulence factor, it has been used in high throughput studies as a reporter of hyphal growth (Heintz-Buschart *et al.,* 2012).

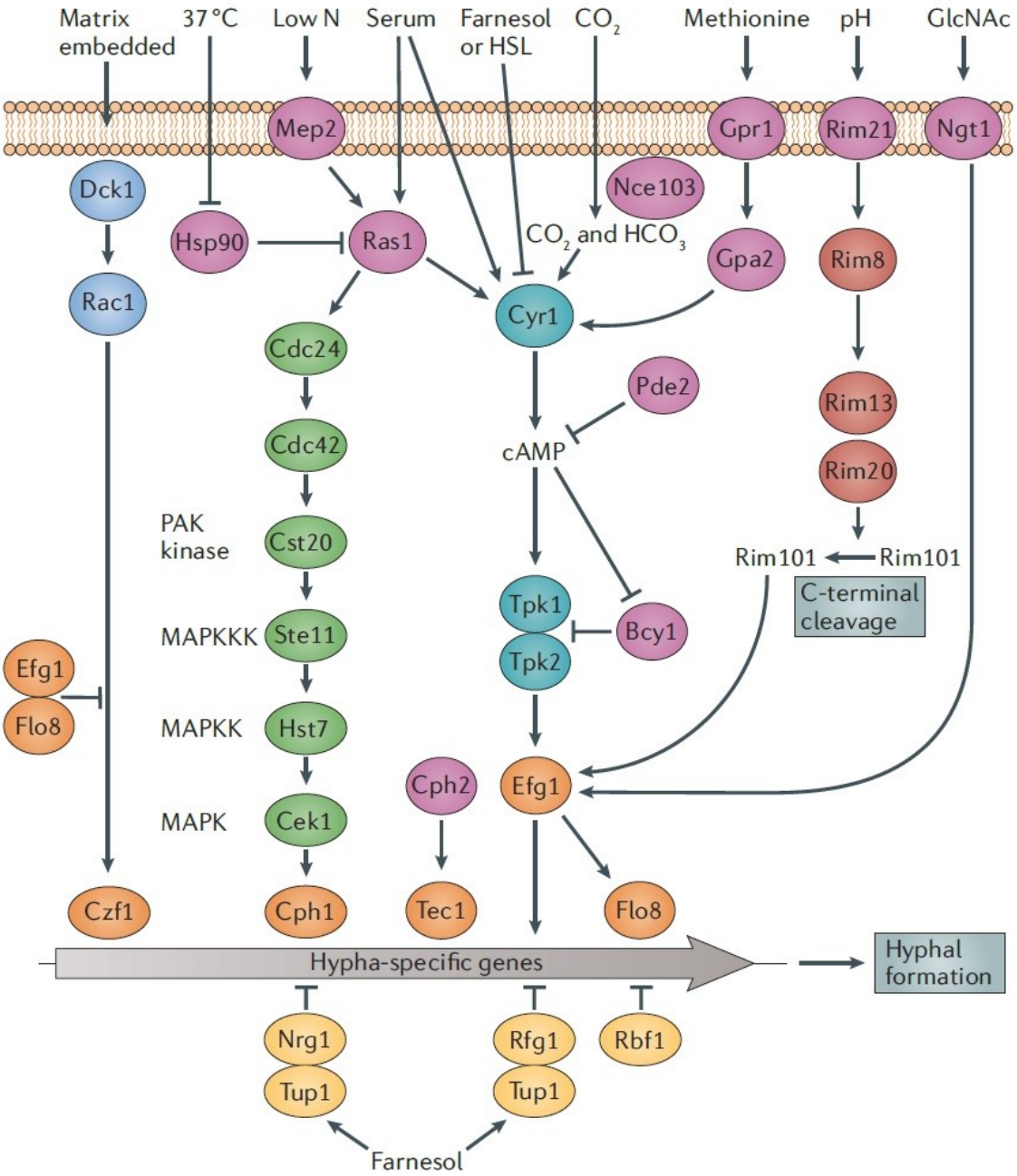


Figure I.1. Signal transduction pathways leading to expression of hypha-specific genes (Sudbery, 2011)
Illustration of filamentation two main signal transduction pathways (MAPK pathway and cAMP/PKA pathway). Some other regulation pathways or regulators are also shown.

The MAPK pathway is responsible for yeast-to-hypha transition in starvation condition, on "Spider" medium for example (Shapiro *et al.,* 2011). The MAPK pathway shares with the cAMP/PKA pathway the activation through Mep2 ammonium permease and Ras1 GTPase. Activation of the MAPK cascade downstream of Ras1 leads to the activation of the central transcription factor in this pathway, Cph1 (Brown *et al.,* 1999). Cph1 will in turn activate a set of hypha-specific genes. Other minor pathways also have a role in filamentation in *C. albicans*. The pH perception via the Rim21 sensor and the Rim101 transcription factor influences the yeast-to-hypha transition (Davis, 2003). Like Rim101, which activates Efg1 in the cAMP/PKA pathway, other sensors or regulation pathways are "branched" on the cAMP/PKA pathway or the MAPK pathway (Figure I.1; Sudbery, 2011). For example, temperature is a key induction factor for hyphal induction. It is regulated through the

chaperone protein Hsp90 (heat shock protein). At a temperature of 37°C, Hsp90 repression on Ras1 is relieved, thus leading to filamentation (Shapiro *et al.,* 2009).

The formation of hyphae in *C. albicans* is also linked with the cell density and quorum sensing: filamentation is inhibited at high cell concentration. A small organic compound, farnesol, has a major role for quorum sensing in *C. albicans*. Farnesol is produced by *C. albicans* at a concentration proportional with cell density. At high concentration farnesol inhibits hyphal formation, presumably through inhibition of the adenylate cyclase Cyr1 (Lindsay *et al.,* 2012, Shareck *et al.,* 2011). It is also hypothesised that the impact of farnesol on filamentation is mediated by the global repressor Tup1 (Kebaara *et al.,* 2008). Another important molecule for quorum sensing and filamentation in *C. albicans* is tyrosol. Unlike farnesol, tyrosol promotes hyphal formation (Alem *et al.,* 2006).

The regulation of yeast-to-hypha transition in *C. albicans* involves intricate signal transduction pathways where the information coming from the environment are integrated at multiple crossroads, like Cyr1 or Efg1 for the cAMP/PKA pathway (Hogan *et al.,* 2011). This reflects the capacity of *C. albicans* to form hyphae in a range of conditions present in the host. The study of yeast-to-hypha transition is relevant medically because of the impact of filamentation and associated virulence factors on virulence. Given the complexity of these pathways, it is crucial for their study to have efficient reporter systems.

3. Luminescence and luciferases

Bio-luminescence can easily be observed in the wilderness, for example in sea water or with fireflies during summer nights. The first mention of bioluminescence dates back to ancient China (Roda, 2011). Until recently, however, these cold light sources were poorly understood. Some of these bio-luminescence phenomena gave birth to legends, like the milky sea phenomenon described by Jules Vernes or the saprophyte fungus luminescence (foxfire). During the XIXth century, the chemical process behind luminescence was partially solved. The Italian scientist Spallanzani observed that the luminescence declined in the absence of air and could be restored by the introduction of oxygen, but not hydrogen or nitrogen, thus demonstrating the need for oxygen. A French physiologist, Raphaël Dubois, used extract from the clam *Pholas dactylis* to experiment on luminescence (Harvey, 1957). He observed that heat extinguished luminescence from a clam extract. However, heated extract was able to restore the luminescence of an untreated clam extract which luminescence had worn out. He thereby demonstrated that two components were needed, one thermo-labile and the other thermo-stable. As this coincided with the first discoveries of enzymes, the thermo-labile part was called luciferase, literally meaning light enzyme, and the stable compound luciferine (the –e being dropped afterwards). Darwin mentioned several luminescent organisms but was unable to explain the diversity of luminescent organisms and of light-emitting organs. The last big step in the understanding of the basic mechanism of luminescence was made by William McErloy who demonstrated the role of ATP in firefly luminescence (Roda, 2011). In the middle of the XXth century, the luminescent organisms had been accurately described and the fundamental principles of the luminescent reactions understood.

In the various bioluminescent organisms, luciferases and luciferin differ in structures. The evolution process has selected several enzymatic mechanisms that can sometimes share the same luciferin, acquired through food (coelenterazine). However, the fundamental chemical principle of the formation and destruction of a peroxide bond (providing the energy for photon emission) is broadly shared among bioluminescent organisms (Wilson *et al.,* 1998). A selection of bioluminescent

organisms will be described below with particular attention given to beetle luminescence mechanism.

3.1. Bacterial luciferase

A number of bacteria from the plankton are able to produce light. Bacteria producing light are often found in symbiosis with fish allowing them to hide or hunt. Astonishingly, some fish use luminescent bacteria to hide from their predators, although it seems counter-intuitive that producing light could help hiding. When looked at from underneath fishes are visible as a dark patch over the light of the surface; by producing light they blend with the light coming from the surface becoming invisible. The "milky sea" phenomenon mentioned by Jules Verne is also due to planktonic bacteria.

In the case of bacterial luciferase, the energy for luminescence comes from the reduced flavin mononucleotide. The first step of bacterial luminescence is the catalysis by the luciferase of the oxidation of $FMNH_2$ (flavin mononucleotide) and of a long chain fatty aldehyde (Wilson *et al.,* 1998).

$FMNH_2+O_2 \rightarrow E\bullet FOOA + R–CH=O \rightarrow E\bullet F^* + R–COH=O \rightarrow FMN + H_2O + h.v$

The luminescence can subsist for some time without oxygen, as the intermediate of the reaction (FOOA) is relatively stable. The enzyme is constituted of two subunits coded on two genes, *luxA* and *luxB*. The *luxC,D,E* genes are responsible for the synthesis of the aldehyde. Together with *luxA* and l*uxB* these genes are part of the *lux* operon. The luminescent bacterium *Vibrio fischeri* had a key role in the discovery of quorum sensing. In this environment, as a planktonic bacterium (free in the sea), *V. fischeri* does not produce light. It is however luminescent in the photophore of a squid. Scientists studying this phenomenon discovered that *V. fischeri* will only produce luminescence at high cell concentrations. The authors discovered that above a threshold level, the auto-inducer homoserine lactone induces luminescence, thereby showing quorum sensing for the first time (Meighen *et al.,* 1991). The lux system has been expressed In *S. cerevisiae*, the genes *luxA*, *-B*, *-C*, *-D*, *-E* and *frp* have been transformed in the yeast using plasmids (Sanseverino *et al.,* 2005). The resulting auto-luminescent yeast system was used for detection of oestrogen in drinking water (Bergamasco *et al.,* 2011).

3.2. Dinoflagellates

Some species of Dinoflagellates (planktonic protists) are bioluminescent. The dinoflagellates are responsible for most of the luminescence observed in the oceans. The luciferase of dinoflagellates catalyses the oxidation of a tetrapyrrole derived from chlorophyll (Dunlap *et al.,* 1980). The production of luminescent proteins follows a circadian rhythm and has therefore been the object of thorough studies by chrono-biologists (Wong *et al.,* 2005).

3.3. Fungi

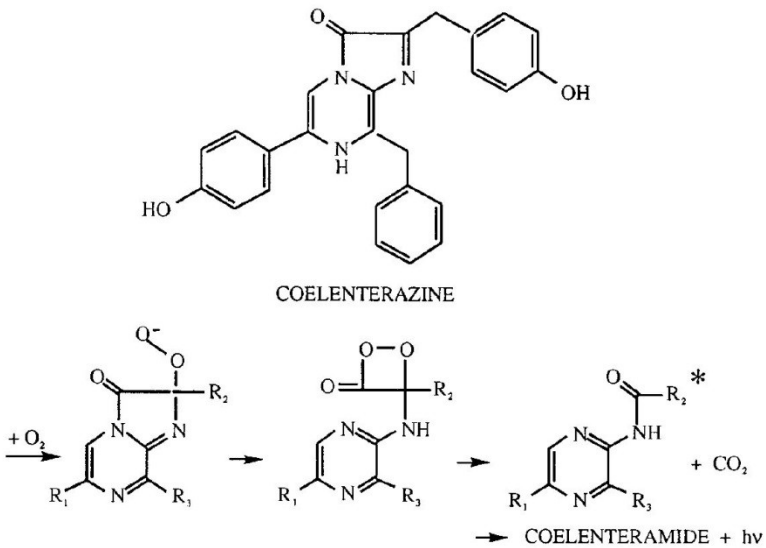
Bioluminescent fungi are very common among the saprophyte basidiomycetes. They produce a dim luminescence. The true function of this luminescence remains unclear; it has however been hypothesised that luminescence might ward off predators, in a similar fashion as the bright colours of some toxic fungi. Another hypothesis is that the light may attract insects that would spread the spores of the fungus. The chemical mechanism is thought to be close to bacterial luminescence. The reaction is, however, not activated by the addition of $FMNH_2$ so the reactions are not strictly identical

(Desjardin *et al.,* 2008). Because their luciferases and luciferins are cross-reacting, it is likely that the different species of luminescent fungi share the same enzymatic mechanism (Oliveira *et al.,* 2012).

3.4. Coelenterazine-based luminescence

The luminescence of many marine organisms is based on coelenterazine. Several sea-organisms have adapted to use the coelenterazine present in their diet (Haddock *et al.,* 2010). This adaptation explains why several sea-organisms have structurally unrelated luciferases using the same substrate. The case of cnidaria and crustaceans illustrates this phenomenon. Some of the coelenterazine-based luciferases, such as the luciferase of the sea pansy *Renilla reniformis*, are well known because of their use in molecular biology. In nature, the soft coral *R. reniformis* produces flashes of luminescence to ward off predators. The *Gaussia princeps* luciferase (Gluc) is also commonly used in molecular biology. This copepod uses luminescent pellets as a decoy while swimming away from his predators; interestingly, the *Gaussia* luciferase is secreted into the environment. The most prominent luciferase using coelenterazine as a substrate certainly originates from the jellyfish *Aequorea victoria*. The luminescence of *A. Victoria* is due to aequorin, a complex of luciferase enzyme and oxidised coelenterazine (Shimomura, 1978). In nature, the jellyfish stocks granules of aequorin; the luminescence is then triggered by the binding of calcium, producing a flash of light. In the jellyfish, the aequorin is associated with the green fluorescent protein (GFP). In the presence of GFP, the light emitted by the luciferase of *A. victoria* shifts from a peak at 470 to 508 nm (Tsien, 1998). The energy emitted by the aequorin is transmitted to the fluorescent protein by Förster resonance non-radiative energy transfer (FRET), which requires the two proteins to be in close vicinity (Morise *et al.,* 1974). The GFP was discovered by Shimomura *et al.* (1962) and proved very useful in many fields of biology, as a gene reporter and a protein tag. Shimomura, Chalfie and Tsien were awarded the Nobel Prize in 2008 for their discovery.

For all coelenterazine-based luciferases, the first step is the oxidation of coelenterazine by the luciferase. This oxidation leads to the formation of a peroxide bond. The rupture of the peroxide bond creates an excited state of coelenteramide, which energy is then released by liberation of a photon (Figure I.2).



<u>Figure I.2.</u> Production of light by coelenterazine-based luciferases
Coelenterazine is oxidised, leading to the formation of a dioxide bond. A photon is emitted after the rupture of this energy rich bond.
(Wilson *et al.,* 1998)

3.5. Beetle luciferases

Luminescent insects, mainly fireflies and click beetles, are the most common form of luminescence in nature outside the sea. Most of the luminescent insects are part of the coleoptera order (commonly called beetles). Insects can use their luminescent organs to attract sexual partners, like fireflies, for communication or even to lure preys. The best example of luminescence used for hunting is *Arachnocampa luminosa* (Diptera order). The larvae of this gnat uses the light to lure preys into its sticky filaments (Meyer-Rochow *et al.,* 1990). The luminescence mechanism, like for coelenterazine-using luciferases, is based on the formation of a peroxide bond. The rupture of the energy-rich peroxide bond creates an excited state and thus light. Insects have specialised structures for luminescence like the luminescent organs of fireflies or the lanterns of click beetles. The oxidised state of beetle luciferin is extremely unstable and cannot be stored; the luminescence reaction therefore needs a permanent supply of oxygen. Some insects regulate their luminescence by limiting oxygen supply via contraction of the tracheae providing oxygen to the luminescent organs. The luminescence emitted by insect luciferases can vary from green to red, depending of the species and sometimes the individuals. The cDNA for firefly luciferase (Fluc) and click beetle luciferase (CBluc) were cloned, thereby opening a new era of gene reporters (wood *et al.,* 1989). The firefly luciferase is the most commonly used and the mechanism of the reaction and light emission has been extensively studied. The firefly and click beetle luciferases evolved from a common ancestor, an acyl-coA ligase (Viviani, 2002). The firefly and click beetle luciferase genes have a 45% amino acid-sequence homology and a very high domain homology (Viviani, 2002). Both enzymes catalyse the adenylation and oxidation of beetle luciferin (Figure I.3). The resolution of the crystallised firefly luciferase led to a better understanding of its activity (Conti *et al.,* 1996).

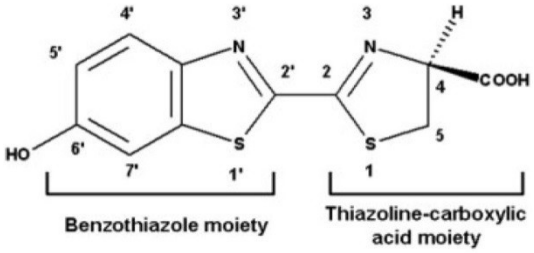


Figure I.3.: Chemical structure of firefly luciferin, D-LH2.
The two principal moieties of the molecule are shown, with the atoms numbering.
(Marques *et al.,* 2009)

The first reaction step catalysed by the beetle luciferase is the adenylation of the luciferin. The carboxyl group undergoes a nucleophilic attack on ATP. The reaction is made possible by a Mg^{2+} ion, which masks the two charges from the β and γ-pyrophosphate groups of the ATP (Marques *et al.,* 2009). The carbon C_4 of the luciferin is made acidic by the presence of the AMP group (Figure I.3). After loss of the proton, the C_4 reacts with molecular oxygen *via* nucleophilic attack. From the resulting intermediate, the peroxide bond is formed (internal nucleophilic attack on the carboxyl group) resulting in the loss of the AMP group. The subsequent rupture of the peroxide bond leads to the liberation of CO_2 and oxyluciferin in an excited state. In the proper environment, excited oxyluciferin returns to ground state energy by emitting a photon (Figure I.4; Navizet *et al.,* 2011). The wavelength of the photon depends on the energy difference between the excited state and the ground state (the larger the energy difference, the shorter the wavelength).

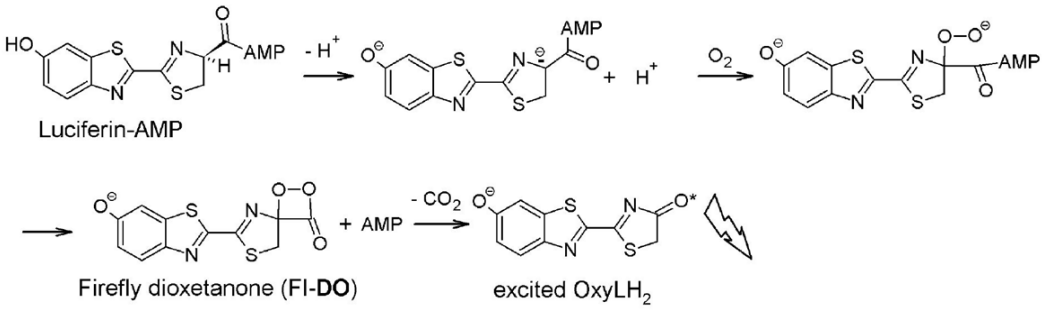


Figure I.4. Mechanism of light emission by oxidation of beetle luciferin
In the presence of ATP and Mg^{2+}, luciferin is oxidised by beetle luciferase leading to the formation of a peroxide bond. A photon is emitted after rupture of this bond. (Navizet *et al.,* 2011)

Beetle luciferase catalyses the two steps of the reaction (adenylation and oxygenation) and provides the suited environment for the relaxation of the excited state by charge transfer and thus the emission of light. The analysis of the crystal structure of the firefly luciferase revealed that the luciferase is composed of two domains. The bigger one (N-terminal domain) is linked to the C-terminal domain by a loop (Conti *et al.,* 1996). The reaction site, where luciferin binds, is thought to be located between the two domains. The pocket has two compartments, one for the benzothiazole moiety and the other for the carboxylic moiety (Figure I.3; Navizet *et al.,* 2011). Beetle luciferases have a binding site for ATP, thereby facilitating the adenylation step. Other key features of beetle luciferases are the presence of a basic residue in front of the luciferin C_4, helping deprotonation before the oxidation step. The active site provides the hydrophobic environment necessary for a high yield light emission. The luciferase enzyme also facilitates the last reaction step (rupture of the peroxide bond and formation of excited oxyluciferin) by stacking with the aromatic π-conjugated system of the luciferin (Viviani *et al.,* 2002; Navizet *et al.,* 2011).

The major difference between firefly and click beetle luciferases is that click beetle luciferases are not pH-sensitive. The pH-sensitivity is due to the stabilisation of the active site by basic residues; while the pH-insensitive luciferases have an active site stabilised by non-pH-sensitive hydrophobic interactions (Ohmiya *et al.,* 1995; Viviani *et al.,* 2001; Viviani *et al.,* 2002). Another particularity of click beetle luciferases is the colour spectrum of their light emission. In nature, the click beetle *Pyrophorus plagiophthalamus* emits green light from its dorsal organ (wavelength from 548 to 565 nm). The colour of the light emitted by the ventral organ ranges from green to orange (547 to 594 nm). There is currently no consensus concerning the cause of the different emission wavelenths. The three most common hypotheses are: (i) differences in the active site polarity; (ii) tautomerisation of luciferin; (iii) rotation of the C2-C2' bond (Viviani *et al.,* 2002). However, according to Navizet *et al.* (2011), none of these hypotheses are able to fully explain the differences in colour emissions. Navizet *et al.* (2011), hypothesised that luciferin is in keto form ($C_{6'}$–O^-) and that the colour depends on the polarity of the micro-environment near the benzothiazole moiety. The assumption is that the changes in polarity impact the π-conjugated electron field, thus changing the resonance energy levels. The green and orange alleles of the click beetle luciferases have been further engineered to widen the gap between their emission peaks (Almond *et al.,* 2003). The resulting luciferases have an emission peak at 537 nm for the green luciferase and 613 nm for the red luciferase.

Luciferase activity is strongly inhibited by a product of a side reaction: dehydroluciferyl-adenylate (L-AMP). However, in a similar manner to the ancestral acetyl-coA synthetase, beetle luciferase can catalyse the reaction of L-AMP with acetyl-coA, thereby producing the non-inhibitory dehydroluciferyl-CoA. Consequently, the adverse effect of the L-AMP side-reaction can be avoided by adding acetyl-coA to the reaction.

4. Reporter proteins in *C. albicans*

Historically, the adaptation of reporter proteins to *C. albicans* has been difficult; *C. albicans* like a number of other *Candida* species is part of the CUG clade. The fungi of this clade translate the CUG codon to serine instead of the classical leucine (Santos *et al.,* 2011). Therefore, gene reporters containing CUG codons are not correctly expressed. This was demonstrated by the attempt to introduce the *Aequorea victoria* green fluorescent protein (GFP) in *C. albicans* (Morschhäuser *et al.,* 1998). Only GFP with adapted CUG codons showed significant fluorescence in *C. albicans*. Some alternatives were found, for example using *Kluyveromyces lactis* or *Streptococcus thermophilus* β-galactosidase genes instead of the CUG-rich *Escherichia coli LacZ* gene (Leuker *et al.,* 1992; Uhl *et al.,* 2001). After the identification of the CUG limitation, a number of reporters were introduced for *C. albicans*. The first class consists of open reading frames (ORF) whose expression products can be quantified easily, because of their enzymatic activity, for example β-galactosidases or OMPs (orotidine 5'-phosphate decarboxylase). The second class of reporters comprises the fluorescence proteins like the green fluorescent protein (GFP). Although these proteins cannot be as easily quantified as β-galactosidases, the high intensity of the fluorescence signal makes it possible to visualise the protein localisation. The third class of reporters in *C. albicans* consists in bioluminescent proteins. Bioluminescent proteins catalyse the oxidation of a substrate to produce light. In the following, the three classes of reporters available in *C. albicans* will be summarised.

4.1. Colorimetric reporters

β-galactosidases are very common gene reporters, which can conveniently be used in *C. albicans,* as it does not naturally express them. β-galactosidases catalyse the hydrolysis of β-galactosides. The hydrolysis by these enzymes of alternative substrates like X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or ONPG (ortho-nitrophenyl-β-galactoside) generates a coloured product. The colour change can be quantified by measuring optical density modifications (colorimetry). β-galactosidases were the first reporters introduced in *C. albicans*. The first attempts using *E. coli lacZ* gene were unsuccessful; indeed, the *E. coli lacZ* gene includes 51 CUG codons, making its expression problematic in a member the CUG clade. In 1992, Leuker *et al.* described the use of *K. lactis LAC4* in *C. albicans* (Leuker *et al.,* 1992). The *LAC4* gene has only two CUG codons, but these two CUG codons might still prevent optimal expression. Leuker *et al.* noticed that β-galactosidase activity was observed only when the *LAC4* gene is highly expressed. The later introduction of *Streptococcus thermophilus lacZ* ORF in *C. albicans* solved this potential problem (Uhl *et al.*, 2001). *S. thermophilus lacZ* gene only contains one CUG codon at the very end of the ORF; moreover, the β−galactosidase activity was unaffected by the modification of the CUG codon to UUG (Leu; Uhl *et al.,* 2001). β-galactosidase is an effective tool to evaluate a promoter activity in *C. albicans*; however, lysis of the cells is required to quantify enzymatic activity.

4.2. Fluorescent reporters

In nature, bioluminescence enzymes that produce light from a chemical reaction are often associated with fluorescent proteins that enable a shift in the emission wavelengths (Introduction 3.4). Fluorescent proteins can absorb light at a specific wavelength and re-emit at a longer wavelength. Using optical filters, fluorescence is easily visible in the cells, making it a valuable tool for molecular biology. For example, the GFP ORF can be fused with a gene of interest. Because of the strength of the fluorescence signal, the localisation of the tagged protein can then be observed at the single cell level. The promoter activity of a gene promoter can also be monitored; however, because of the long half-life of GFPs in the host cells, fluorescent proteins are not ideal for that purpose (Corish *et al.,* 1999). The fluorescent proteins must fold correctly and the chromophore be created by internal reaction. This time lag between gene expression and the fluorescence peak is a disadvantage for the study of quick events like the yeast-to-hypha transition in *C. albicans* (Gordon *et al.,* 2007). Moreover, because the fluorescent proteins must be excited by a photon beam, the background signal is high ($1/4^{th}$ of the fluorescence signal for FbFb in *C. albicans*; I. Eichhof, personal communication).

GFP consists of 11 β-strands forming a hydrophobic barrel, in which the chromophore is secluded. Like for all luminescent proteins, the active site or chromophore must be protected from water to avoid quenching (Tsien *et al.,* 1998). The synthesis of the chromophore occurs internally after proper folding of the protein. Molecular oxygen is needed for chromophore formation, which can be a limitation in some environments. In the case of disseminated candidiasis, for example, deep-seated infection sites or the inside of biofilms are anoxic. The chromophore absorbs light from an excitation source (e.g. a bioluminescent enzyme or a laser) and re-emits a photon at a longer wavelength. The emission wavelength of the fluorescent protein is separated from other wavelengths using optical filters with a narrow transmittance window (e.g. between 500 and 550 nm in the case of CaFbFP). A codon-optimised version of the *A. victoria* GFP was first used in *C. albicans* in 1997; the single CUG codon contained in GFP ORF was modified to insure correct expression (Cormack *et al.,* 1997). Later on, *C. albicans* adapted GFP was employed by Morschhäuser *et al.* (1998) as a gene reporter used to measure the activity of *SAP2*, a gene regulable in known conditions. After chromosomal integration, the fluorescent activity was induced in *SAP2*-inducing medium. The authors also demonstrated that non CUG-optimised GFP was not able to fluoresce in *C. albicans*, thereby demonstrating that most gene reporters must be adapted for the *C. albicans*-specific genetic code. Other fluorescent proteins were adapted for use in *C. albicans*, for example the yellow and cyan fluorescent proteins (Gerami-Nejad *et al.,* 2001). Gerami-Nejad *et al.* also described a PCR-based approach to integrate GFP reporters in *C. albicans* genome. Recently, Tielker *et al.* (2009) described a fluorescence-based system using the protein FbFP that does not need oxygen to be able to produce luminescence, in contrast with other GFPs and bioluminescence enzymes.

4.3. Luminescent reporters

As described above, luminescent reporters catalyse the oxidation of a luciferase to produce light (Introduction 3.). The *Renilla* luciferase (coelenterazine-based; Introduction 3.) gene does not contain any CUG codons and was therefore used by Srikantha *et al.* (1996) as a gene reporter. The authors demonstrated the effectiveness of the reporter by fusing it to a galactose-inducible promoter (*GAL1*); luminescence was only observed in inducing conditions. Unlike fluorescent proteins, luminescent

enzymes are excellent gene reporters. Because there is no background signal, luminescent reporters are extremely sensitive and the detection limit is set by the detection system (Contag *et al.,* 2006). The dynamic range of luminescent proteins is extremely wide, offering detection of both very low luminescence and high activity levels (Fan *et al.,* 2007). In practice, luminescence can be accurately and easily quantified using a luminometer. The major drawback is that the absolute light signal (photon flux) is weak compared with fluorescence. While this is not a problem when using cultures, the signal cannot be seen under the microscope at the single cell level. Fluorescence is therefore a better tool for protein localisation studies. Luminescent proteins have also been used for *in vivo* assays in animals to monitor the progression of a disease. However, this kind of experiments has been unsuccessful in *C. albicans* until recently, allegedly because of the low permeability of *C. albicans* cells to luciferin. However, Enjalbert and colleagues developed an innovative system where the *Gaussia princeps* luciferase is fused to a GPI-anchored protein (Enjalbert *et al.,* 2007). Because of the fusion, the enzyme is displayed at the surface of *C. albicans,* where it is in contact with the reagent; this is important because *C. albicans* cells are not permeable to coelenterazine. This elegant system has been used to monitor the progression of vaginal candidiasis, which would have been impossible with β-galactosidases or GFPs, since tissue sections would have been needed (Enjalbert *et al.,* 2007; Pietrella *et al.,* 2012). However, only surface activity may be measured, because coelenterazine does not enter all mice body tissues.

Another luciferase was used in *C. albicans*, the firefly luciferase; it catalyses the oxidation of beetle luciferin in the presence of ATP and $MgCl_2$ (Introduction 3.5). A CUG codon-corrected luciferase was used in *C. albicans* (Doyle *et al.,* 2006). The firefly luciferase ORF (*Fluc*) was cloned downstream of the *ACT1* promoter and activity from a cell culture in the presence of beetle luciferin was measured. The luminescence of *C. albicans* transformants strains showed that the firefly luciferase is an effective gene reporter and that *C. albicans* cells are permeable to beetle luciferin. Because *C. albicans* cells are permeable to beetle luciferin, assays using live cells are possible. Live cell assays open perspectives for *in vivo* studies, high-throughput screening and the monitoring of gene activation during rapid morphological changes like the yeast-to-hypha transition in *C. albicans*.

5. Goals

Because of the challenges offered by the study of complex regulation pathways, new reporters of gene activity in *C. albicans* would be a precious asset. Luciferases have been used in mammals, plants and yeasts as sensitive reporters of gene activity. In *C. albicans*, the *Renilla* luciferase has been used routinely but presents issues in terms of practicality and results scattering (Srikantha *et al.*, 1996). The click beetle luciferases have the specificity of being available in two colours, red and green (Almond *et al.*, 2003). The emission peaks of these click beetle luciferases are separated by 75 nm and can be isolated by optical filters. Moreover, given the permeability of *C. albicans* cells to beetle luciferin, click beetle luciferases could potentially be used in live cells assays (Doyle *et al.,* 2006).

The click beetle red and green luciferases were adapted for *C. albicans* specific genetic code and codon usage. The luminescence of the resulting luciferases was tested using multi-copy plasmids in *S. cerevisiae* and *C. albicans*. The emission spectra of red and green click beetle luciferases expressed in *C. albicans* were verified. To evaluate the potential of click beetle luciferases as gene reporters in *C. albicans*, the red and green luciferase ORFs were integrated genomically downstream of constitutive or inducible promoters and the luciferase activity was measured. The instruments for the measurement of click beetle luciferases activity in *C. albicans* were evaluated and the he system for

red and green signal separation was established. The regulation of genes involved in the cAMP/PKA signal transduction pathway was then analysed using the click beetle luciferases as reporters in several hypha-inducing conditions and in the presence of quorum sensing molecules. Furthermore, *C. albicans* strains were constructed in which the activities of the two luciferases were measured simultaneously.

II. Material and methods

1. Chemical products and enzymes

Chemicals products or enzymes where obtain from the following companies:
Difco (Detroit, USA); Merck AG (Darmstadt, Germany); Thermo Fisher Scientific (Whaltham, USA); New England Biolabs (Ipswich, USA); Promega (Madison, USA); Qiagen (Hilden, Germany); Roche Applied Science(Penzeberg, Germany); Sigma-Aldrich (St Louis, USA); Promega (Madison, USA); Carl Roth (Karlsruhe, Germany); VWR (Radnor, USA); Serva (Heidelberg, Germany); AppliChem (Darmstadt, Germany); Biomol (Hamburg, Germany); Grüssing (Filsum, Germany); GE Healthcare (Little Chalfont, UK); Becton Dickinson (Franklin Lakes, USA); Caesar and Loretz (Hilden, Germany); Macherey-Nagel (Düren, Germany).

2. Instruments

Fluoroskan ascent FL and NanoDrop 2000c, Thermo Fisher Scientific (Whaltham, USA); TriStar LB 941, Berthold Technologies (Bad Wildbad, Germany); 510/60 and 610LP filters, Chroma Technology (Bellows Falls, USA); Mastercycler pro, Eppendorf (Hamburg, Germany); FastPrep-24, MP Biomedicals (Santa Ana, USA); ELx800, BioTek (Winooski, USA); Axioskop 40 and axiocam, Zeiss (Jena, Germany).

3. Strains and media

3.1. Strains

Strain	Reporter	Genotype	Reference/source
C. albicans			
CAI4	none	*ura3*Δ::*imm*434/ *ura3*Δ::*imm*434	Fonzi *et al.*, 1993
BWP17	none	*ura3::imm434/ura3::imm434 iro1/iro1::imm434 his1::hisG/his1::hisG arg4/arg4*	Wilson *et al.,* 1999
CR1	*ACT1p-CBRluc*	As CAI4 but transformed with pD-CBR	This work
CG1	*ACT1p-CBGluc*	As CAI4 but transformed with pD-CBG	This work
ipTGL	*TCCp-CBGluc*	As CAI4 but *TCC1/TCC1::pTGL*	This work
ipDG	*ACT1p-CBGluc*	As CAI4 but *LEU2/LEU2::pD-CBG*	This work
ipDR	*ACT1p-CBRluc*	As CAI4 but *LEU2/LEU2::pD-CBR*	This work
HwK7GU	*HWP1p-CBGluc*	As CAI4 but *HWP1/hwp1::CBGluc*	This work
UmK7GU	*UME6p-CBGluc*	As CAI4 but *UME6/ume6::CBGluc*	This work
ipTGL-B	*TCC1p-CBGluc*	As BWP17 but *TCC1/TCC1::pTGL*	This work
ipDR-B	*ACT1p-CBRluc*	As BWP17 but *LEU2/LEU2::pD-CBR*	This work
ipDG-B	*ACT1p-CBRluc*	As BWP17 but *LEU2/LEU2::pD-CBG*	This work
AcK7RH	*ACT1p-CBRluc*	As BWP17 but *ACT1/act1::CBRluc*	This work
HwK7RH	*HWP1p-CBRluc*	As BWP17 but *HWP1/hwp1::CBRluc*	This work
HwAc	*ACT1p-CBGluc* *HWP1p-CBRluc*	As ipDG-B but *HWP1/hwp1::CBRluc*	This work
HwTc	*TCC1p-CBGluc* *HWP1p-CBRluc*	As ipTGL-B but *HWP1/hwp1::CBRluc*	This work

S. cerevisiae			
MC45-5A	none	*MATα*, *leu2-3,112, trp1-289, ura3-52, GAL*	Kötter *et al.,* 1990
SG1	*ACT1p-CBGluc*	As MC45-5A but transformed with p4-CBG	This work
SR1	*ACT1p-CBRluc*	As MC45-5A but transformed with p4-CBR	This work
E. coli			
DH5αF‘	none	F^- [ϕ80Δ (*lacZ*)M15] Δ (*lacZYA-argF*) *U169 recA1 endA1 hsdR17* $r_k^- m_k^+$ *supE44 thi-1 gyrA1 relA*	Hanahan *et al.,* 1983

3.2. Growth conditions

For yeast growth of *C. albicans* and *S. cerevisiae*, strains were grown at 30°C with shaking in YPD medium (1 % yeast extract, 2 % pepton, 2 % glucose) or in SD medium (6.7 g/l Yeast Nitrogen Base without amino acids, 2 % glucose). For solid medium, 2 % agar was added. For solid SD medium, pH was adjusted to 6.9 with NaOH.

S-Galactose medium (6.7 g/l Yeast Nitrogen Base without amino acids, 2 % galactose) was used as an inducing medium for galactose/glucose regulated promoters (*C. albicans PCK1* promoter and *S. cerevisiae GAL1* promoter).

For hyphal induction in *C. albicans*, cells were grown at 37°C in YP medium (10 g/l yeast extract, 20 g/l pepton) supplemented with 10 % horse serum. Alternatively, cells were grown in SLAD medium (1.7 g/l Yeast Nitrogen Base without ammonium sulfate or amino acids, 6 mg/l amonium sulphate, 0.165 mol/l MOPS buffer, 0.2 % Glucose, 0.1 % maltose, pH adjusted to 7.25 with NaOH) or in RPMI 1640 (Sigma-Aldrich).

E. coli strains were grown at 37°C with shaking in LB medium (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl). For solid medium, 2 % agar was added to the LB medium. When necessary, strains were selected by adding 50 µg/ml ampicillin or 50 µg/ml kanamycin

For conservation, 700 µl of cells from an overnight culture (when necessary in selective medium) were mixed with 700 µl of 50 % glycerol and frozen at -80 °C

3.3. Estimation of cell concentration

To estimate the concentration of *C. albicans* cultures, the OD_{600} of a dilution of the cells was measured.

To accurately evaluate the concentration of a *C. albicans* cultures, 100 µl of a dilution of the cultures are plated on YPD plates (10^{-4}>OD_{600}>10^{-7}). After 2-3 days growth, the Colony Forming Units (CFU) were counted and averaged.

4. PCR and primers

4.1. PCR reactions

4.1.1. Expand HiFi PCR system

The expand HiFi Polymerase Chain Reaction (PCR) system (Roche) was used according to the recommendations of the user manual. The mixes were made as indicated in the following table.

	stock solution	volume for 50 µl mix (µl)	end concentration
Buffer(3) with $MgCl_2$	5X	10	1X
$MgCl_2$	25 mM	3-4-6-8	1.5 -2.5-3.5-4.5 mM
dNTP	2mM each	5	0.2 mM each
Forward primer (Fw)	10 µM	1.5	0.3 µM
Backward primer (Bw)	10 µM	1.5	0.3 µM
Polymerase	3,5 U/µl	0,75	2.6 U/50µl
H_2O		27.25-26.25-24.25-22.25	V_f=50 µl
DNA		Plasmid: 20 ng; genomic: 150 ng	

The amplification was then done using the Mastercycler pro thermocycler (Eppendorf) with the following cycle:

94 °C 2 min
94 °C 30 sec
Tm 30 sec } *30
72 °C 1 sec per 1.5Kb
72 °C 10 min
4 °C ∞

With Tm the annealing temperature.

4.1.2. Colony PCR

The one Taq hot start DNA polymerase kit (NEB) was used as recommended in the user manual. Cells were grown for 2-3 days on YPD agar and a number of cells were picked using the point of a 10 µl tip. Cells were then re-suspended in the following PCR mix.

	stock solution	volume for 50 µl mix (µl)	end concentration
Buffer	10X	5	1X
dNTP	2 mM each	5	0.2 mM each
Forward primer (Fw)	10 µM	1.5	0.3 µM
Backward primer (Bw)	10 µM	1.5	0.3 µM
Polymerase		**NONE**	
H_2O		36.75-34.55-32.35	
$MgCl_2$	25 mM	0-2.2-4.4	2-3-4 mM

The mix was then put in the Mastercycler pro thermocycler (Eppendorf) for the following cycle:

5 min 24°C
5 min 94°C
≥5 min 4°C

The mix was then kept on ice and the polymerase added: 0.25 µl polymerase per sample if the amplification product size was < 3 Kb. If the amplification product size was between 3-6 Kb, 0.5-1 µl enzyme per probe was added. The amplification was then realised with the following cycle:

94 °C 30 sec
94 °C 30 sec
Tm 30 sec
68 °C 1 min/Kb
68 °C 5 min
*30
4 °C ∞

4.2. Primers

The primers detailed in the following table were used in this work. All primers were obtained from Sigma-Aldrich; primers >50 bp of length were HPLC purified.

for	primer name		sequence, 5'-3'
ACT1	Check ipD-R	Fw	AGCGAGCATGAGGTTGTG
		Bw	CCGCTGTTGAGATCCAGTTC
	AcK7RH	Fw	GCTGCTTTAGTTATCGATAACGGTTCTGGTATGTGTAAAGCCGGTTTTGCCGGTGACGACG CTCCAAGAGCTGTTTTCCCATCTCTTGTTATGGTTAAAAGAGAAAAAAACG
		Bw	CAACAAAACTGGATGTTCTTCTGGAGCAACTCTCAATTCATTGTAAAAAGTGTGATGCCAG ATTTTTTCCATATCGTCCCAGTTGGAAACTTAAACTCTACAATTTGATATCTCG
	AcK7RH Dia	Fw	CAATATCCTATGGCCAAGGG
TCC1	TCC1 2k	Fw	TCCCCCCGGGATTGTTAATGGTTCGGATTC
		Bw	CTAGCTAGCTTTAGTATTGAAAATATTGTTCAG
	C1 ipTGL	Fw	TTGGTTGGTTGCGGTTTC
	C2 ipTGL	Bw	CACCAGCAGTCAAATCTTCC
CBGluc	CBGlucNheI	Fw	CTAGCTAGCATGGTTAAGAGAGAAAAAAACGTTATC
	CBGlucXbaI	Bw	CCGTCTAGATTAACCACCAGCTTTTTCC
HWP1	HwK7GU	Fw	ATGAGATTATCAACTGCTCAACTTATTGCTATCGCTTATTACATGTTATCAATTGGGGCCAC TGTCCCACAGGTAGACGGTCAAGGTGAACATTCAGATCTATGGTTAAGAG
		Bw	GGATTGTCACAAGGAACATCAGGTTGAGGAGGATTGTCACAAGGAACATCAGGTTGAGG TGGATTGTCGCAAGGTTCTTGTGGTTGTTGTGGGTAGTCACACATTTATAATTGGCCAGTC
	Check iHwK7Gu	Fw	CCGGGATAAGTTAGTTAGCC
		Bw	TGGAGCAACGATCATACC
	HwK7RH	Fw	ATGAGATTATCAACTGCTCAACTTATTGCTATCGCTTATTACATGTTATCAATTGGGGCCAC TGTCCCACAGGTAGACGGTCAAGGTGAAATGGTTAAAAGAGAAAAAAACG
		Bw	TGGTTGTTGTGGGTAGTCACAAGGTTCTTGTGGCTGTTGTGGATAGTCACATGGCTCTTGT GGTTGTTGTTGTGGGTAATCACAAGGTTCTTAAACTCTACAATTTGATATCTCG
	HwK7RH Dia	Fw	TGGCTCACAACCGGGATAAG
Ume6	UmK7GU	Fw	ATGATTACCCATATGGTTACACCCGATTCAACTTCTTCAGCACCAAATTCGCCTTATGGAGA AGATACAATAAAGTTGAACTCGTCAGTGCATTCAGATCTATGGTTAAGAG
		Bw	CCTAGTCCCAACTCCAGATCCAGTAGCAGTGCTGGGGGTGGCTGTGGTTGCAAATCACTA TGATTTGTAAGTGGAGCCAGATGTAAATATACATTTATAATTGGCCAGTC
	Check iUmK7GU	Fw	CCCGGGAGTTGCTTATTA
		Bw	GGAGCAACGATCATACCA
	Check K7G	Bw	TGGAGCAACAATCATACC

5. Plasmid construction

5.1. Plasmid list

Plasmid	Reporter	markers	others	Reference/source
pES2	Rluc	*CaURA3, ampR*	-	Eva Szafranski-Schneider
pDS-1044	-	*CaLEU2, CaURA3, ampR*	*CaACT1p*	Dominique Sanglard
p426-GAL1	-	*ScURA3, ampR*	*ScGAL1p*	Mumberg *et al.,* 1994
pGEM	-	*ampR*	-	pGEM-T system (Promega)
pBI 1	-	*CaLEU2, CaURA3, ampR*	*CaPCK1p*	
pGEM-HIS1	-	*CaHIS1, ampR*	-	
pTCL	Rluc	*CaURA3, ampR*	*CaTCC1p*	This work
CBRluc-pMK-RQ	CBRluc	*kanR*	-	This work, GeneArt
CBGluc-pMK-RQ	CBGluc	*kanR*	-	This work, GeneArt
p4-CBR	CBRluc	*ScURA3, ampR*	*ScGAL1p*	This work
p4-CBG	CBGluc	*ScURA3, ampR*	*ScGAL1p*	This work
pD-CBR	CBRluc	*CaLEU2, CaURA3, ampR*	*CaACT1p*	This work
pD-CBG	CBGluc	*CaLEU2, CaURA3, ampR*	*CaACT1p*	This work
pB-CBG	CBGluc	*CaLEU2, CaURA3, ampR*	*CaPCK1p*	This work
pGEM-HIS-CBR	CBRluc	*CaHIS1, ampR*	-	This work
pGEM-HIS-CBG	CBGluc	*CaHIS1, ampR*	-	This work
pTGL	CBGluc	*CaURA3, ampR*	*CaTCC1p*	This work

5.2. Restriction

All restriction digestions were done with NEB enzymes according to the user manual recommendations. The digestion reactions were incubated for 2 h at the appropriate temperature. Whenever available, the High Fidelity version of the enzymes was used.

5.3. Dephosphorylation

Dephosphorylation was done with the Antarctic Phosphatase kit (NEB). DNA was mixed with 2 µl Antarctic phosphatase buffer, 0.5 µl of Antarctic phosphatase enzyme filled up to 20 µl with water. After 30 min digestion at 37 °C, the phosphatase was inactivated for 5 min at 65 °C. The digested DNA was then purified on columns (Material and methods 6.2)

5.4. Ligation

5.4.1. Quick ligation kit

For ligation of DNA fragments, the Quick ligation kit (NEB) was used. 50 ng of vector DNA were mixed with 3 times more insert DNA (molar ratio), 10 µl of 2X Quick Ligation buffer and water *quantum satis* (qs) 20 µl. The reaction was incubated for 5 min at room temperature.

5.4.2. T4 DNA ligase

In cases where quick ligation did not give satisfactory results, the T4 DNA Ligase was used (Roche). Up to 1 µg DNA was mixed with 3 µl 10X ligation buffer and 2 µl T4 DNA Ligase (H_2O qs 30 µl). Incubation was 15 °C overnight.

5.5. pGEM-T Vector Systems

When needed, the PCR amplifications were ligated with the pGEM-T Vector (Promega) as described above and according to the user manual.

6. DNA purification and isolation

6.1. Plasmid purification from *E. coli*

6.1.1. Mini-preparation

2 ml of an overnight culture in LB medium were centrifuged and resuspended in 300 µl P1 buffer (50 mM Tris-HCl pH=8, 10 mM EDTA, 100 µg/ml RNase A). After addition of 300 µl of P2 buffer (200 mM NaOH, 1 % SDS), tubes were mixed by inversion and incubated for 5 min maximum at room temperature (RT). P3 buffer was then added (3.0 M KAc, pH adjusted to 5.5 with acetic acid) and tubes were mixed by inversion. After 15 min centrifugation at 13 000 rpm, the supernatant was transferred to a new tube. Precipitation of the DNA was achieved by mixing the supernatant with 450 µl isopropanol and by centrifugation at 13 000 rpm for 30 min. The pellet was then cleaned with 70 % ethanol and after evaporation of the ethanol resuspended in 100-200 µl water.

Alternatively, when an optimal purity was required, the mini preparation kits Plasmid Mini Kit (Qiagen) or NucleoSpin Plasmid (Macherey-Nagel) were used according to the user manuals.

6.1.2. Midi-preparation

Midi-preparations were made according to the user manual with the Plasmid Midi Kit (Qiagen).

6.2. Genomic DNA extraction from *C. albicans*

Cells from an overnight culture in YPD were centrifuged and the pellet was washed with water and resuspended in 400 µl SCE/DTT/zymolyase solution (5 mM DTT; 350 µl zymolyase; 9.6 ml SCE [1 M sorbitol; 0.1 M Na-citrate; 10 mM EDTA; pH adjusted to 5.8 with HCl]). After an incubation of at least 1 hour at 37 °C, cells were centrifuged and the pellet resuspended gently with 500 µl EDTA (50 mM) + 50 µl SDS 10 %. After an incubation at 65 °C for 30 min, cells were cooled at room temperature for 10 min and 100 µl KAC 5 M (pH=6) was added. Following an incubation step of 30-90 min the suspension was centrifuged and the supernatant was kept. Genomic DNA was then precipitated by adding 900 µl of absolute ethanol (at -20°C) and by centrifugation for 15 min at 13 000 rpm. RNA was digested by a 30 min incubation at 37 °C with RNase A (200µl RNase A at 10 mg/ml; 500 µl NaAc at 3 M, pH=5.9; TE qs 10 ml). DNA was then purified by phenol/chloroform extraction, washed with chloroform and precipitated over night at -20 °C by adding 800 µl absolute ethanol. After drying of the ethanol, genomic DNA was resuspended in 100 µl water.

6.3. DNA purification

For DNA purification, the QIAquick PCR Purification Kit (Qiagen) was used according to the user manual.

6.4. Agarose gel purification

For separation of DNA fragments of different sizes, gel purification was performed. DNA was loaded in an agarose gel at a concentration dependant on the size of the target fragment:

0.5 %	1 000–30 000 bp
0.7 %	800–12 000 bp
1.0 %	500–10 000 bp
1.2 %	400–7 000 bp
1.5 %	200–3 000 bp
2.0 %	50–2 000 bp

After size separation by electrophoresis, the target fragment was cut out and purified using QIAquick Gel Extraction Kit (Qiagen) according to the user manual.

6.5. DNA concentration and purity

DNA concentration and purity was assessed using a NanoDrop 2000c (Thermo Fisher)

7. Transformation

7.1. *E. coli*

Competent *E. coli* cells were prepared using the rubidium chloride method. A fresh 50 ml *E. coli* culture in LB medium with OD_{600}= 0.1 was grown for 1.5 h at 37 °C ($0.4<OD_{600}<0.6$). Cells were then cooled 15 min to 2 h on ice. After centrifugation at 7 500 rpm for 10 min at 4 °C, the pellet was resuspended in RF1 solution (100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc,10 mM $CaCl_2$, 15 % glycerin, pH adjusted to 5.8 with CH_3COOH, sterile filtration) and incubated for 2 h on ice. Cells were then centrifuged and the pellet re-suspended in 4 ml RF2 (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15 % glycerin, pH adjusted to 6.8 with NaOH, sterile filtrated). After an incubation of 15 min on ice, competent cells were aliquoted and frozen at -80 °C for conservation.

For transformation, 150-200 ng DNA of the ligation products were added to 100 µl competent cells. The competent cells and the DNA were incubated for 20 min on ice, preceding a 90 sec heat shock at 42 °C. After the heat shock, 700 µl LB medium was added and the cells were left for at least 30 min at 37 °C to recover. Cells were then plated on selective media.

7.2. *S. cerevisiae*

S. cerevisiae cells were transformed using the lithium acetate method. A fresh culture of *S. cerevisiae* cells was incubated at 30 °C in YPD medium (OD_{600}=0.2) up to OD_{600}=0.6-0.8. Pelleted cells were then washed with 10 ml water and resuspended in LiAc 0.1 M. Aliquots of 50 µl were then centrifuged (5 min at 3 500 rpm). The following ingredients were added to the pellet (in the following order): 240 µl 50 % PEG 3 350 (or 4 000); 36 µl LiAc 1M; 25 µl carrier DNA (herring sperm) 2 mg/ml

(cooked for 10 min at 95°C, then shortly cooled on ice); 70 µl H_2O + plasmid DNA (0,1-10 µg). After gentle mixing, cells were incubated for 30-60 min at 30 °C. After a heat shock for 15 min at 42 °C, cells are cooled on ice, pelleted, resuspended in SD medium and plated on selective medium.

7.3. *C. albicans*

C. albicans cells were transformed using a modified version of the *S. cerevisiae* lithium acetate method (Walther *et al.,* 2003). Cells from an overnight culture in YPD were diluted 1/100 in YPD and grown for 4 h at 30 °C (up to OD_{600}= 0.5-0.8). Pelleted cells were then washed with 5 ml LATE buffer (0,1 M LiAc; 10mM TrisHCl at pH=7.5; 1 mM EDTA at pH=8; pH adjusted to 5.0 with HCl) and resuspended in 500 µl LATE buffer. Aliquots of 100 µl of cell suspension were mixed with herring sperm DNA (final concentration 2mg/ml; cooked 10 min at 98°C, then cooled on ice) and the transforming DNA (80 µl of PCR reaction or 2-10 µg plasmid DNA). Cells were then incubated for 30 min at 30 °C. After incubation, 700 µl of PLATE solution (40 g PEG 3350; LATE buffer qs 100 ml) was added and the cells were re-incubated for 12-16 h at 30 °C. After a heat shock at 44 °C for 15 min, cells were cooled on ice and washed with SD medium before being plated on SD medium (when needed, cells were incubated for 3-4 h in YPD at 30 °C before plating).

8. Luciferase assays

8.1. Sample preparation methods

8.1.1. Crude extract

From an overnight culture in YPD (or S-Galactose) of *C. albicans* or *S. cerevisiae,* 2.5 ml cells were centrifuged. The pellet was then resuspended in lysis buffer (20 mM HEPES, 250 mM NaCl, 10 % glycerol, 0.1 % Tween 20) and half the volume of glass pearls was added. Cells were then broken at 4 °C using FastPrep-24 (MP Biomedicals) 6 times 40 seconds at 6.5 m/s. After centrifugation for 15 min at 13 000 rpm, the supernatant was kept.

8.1.2. Flash lysis

A sample of 100 µl of cells from a *C. albicans* culture was centrifuged and the pellet resuspended in Glo-Lysis Buffer (Promega). Probes were then briefly frozen in liquid nitrogen and thawed at room temperature before assessing the luciferase activity.

8.1.3. Live cells

For live cell luciferase assays, a sample of 100 µl was taken from the cell cultures and directly subjected to luciferase assay

8.2. Luciferase reaction

8.2.1. Luciferin

Probes were mixed with Luciferin-EF (Endotoxin-Free; Promega) at an end concentration of 160 µg/ml. For use in petri dishes, the luciferin was added (160 µg/ml) after autoclaving, when the medium had cooled.

8.2.2. Chroma-Glo reagent

Probes were mixed with an identical volume of the Chroma-Glo reagent before measuring luminescence activity.

8.3. Luminescence measurements

After starting the probes and reagent, luminescence was read using either the Fluoroskan Ascent FL (Thermo-Scientific) or Tristar (Berthold) luminometers. Probes were dispatched in microtiter plates of either 96 or 384 wells. The probe volume was 200 µl for 96 well plates and 65 µl for 384 well plates. Plates were shaken for 10-15 sec at 1 m/s before the first luciferase activity measurement and for 10 sec before each measurement. The reaction temperature was 30 °C. For each time point, three measurements were made: NoFilter, where no optical filters were used; 510/60, the green optical filter 510/60 (Chroma Technology Corporation) was used; 610LP, the red optical filter 610LP (Chroma Technology Corporation) was used. For all measurements, unless otherwise specified, the exposition time was 1 sec. When the luminescence signal was strong counting time could be reduced to 100 msec. For measurements with the red and green filters, the luminescence values were corrected as detailed in Results 2.1.3. Unless otherwise specified, luminescence was measured over time (> 250 min) and the maximal luminescence value (L_{max}) was reported.

9. Statistics

The error bars displayed on the graphics show the standard deviation, calculated with Microsoft Excel (Albuquerque, USA). The stars displayed on graphics illustrate the results of an unpaired t-test (with Microsoft Excel): *, p-values ≤0.05, ** p-values ≤0.01, *** for p-values ≤0.001.

III. Results

1. Activity of click beetle luciferases in yeast

In 1964, Seliger *et al.* described the ability of a Jamaican click beetle to produce light by bioluminescence at different wavelengths (Seliger *et al.,* 1964). The cDNA coding for green (546 nm) and orange (593 nm) click beetle luciferases was cloned in 1989 (Wood *et al.,* 1989). Unlike other luciferases commonly used as gene reporters, the wide gap between the emission wavelengths of the click beetle luciferases and their use of the same substrate allows simultaneous measurement of multiple colours. Mutagenesis of the luciferases widened the emission wavelengths difference between the red and green click beetle luciferases. The original green click beetle luciferase (CBGluc) emits light at 510 nm. The original red click beetle luciferase (CBRluc) emission wavelength is 610 nm (Almond *et al.,* 2003). The sequences of both luciferases open reading frames (ORF) were adapted for strong expression in mammalian cells. Furthermore, to improve the expression levels of the luciferases and the growth rate of the cells, the peroxisome targeting sequence present at the end of insect luciferase sequences was removed (Leskinen *et al.,* 2003). In order to avoid interaction of transcription factors with the luciferase ORF, the potential consensus transcription factor binding sites were mostly removed. A Kozak consensus sequence was introduced at the ATG initiation codon sequence and the codon usage was adapted for mammalian cells to increase the expression of the luciferases in mammals. The amino acid sequence of both click beetle luciferases shows that CBGluc and CBRluc differ in 8 amino acids (Figure III.1-1). Here, in order to use these reporters in the ascomycete fungus *C. albicans*, the sequences encoding the luciferases were adapted and, after preliminary tests using plasmids, the click beetle luciferase genes were integrated into the genome of *C. albicans*. The click beetle luciferases adapted for use in mammalian cells are referred to as original CBRluc and CBGluc; the *C. albicans*-adapted luciferases are named CBRluc and CBGluc respectively.

```
1   mvkreknviygpeplhpledltagemlfralrkhshlpqalvdvvgdeslsykeffeatv
1   ............................................................
61  llaqslhncgykmndvvsicaenntrffipviaawyigmivapvnesyipdelckvmgis
61  ............................................................
121 kpqivfttknilnkvlevqsrtnfikriiildtvenihgceslpnfisrysdgnianfkp
121 ............................................................
181 lhfdpveqvaailcssgttglpkgvmqthqnicvrlihaldprygtqlipgvtvlvylpf
181 ...........................................v................
241 fhafgfhitlgyfmvglrvimfrrfdqeaflkaiqdyevrsvinvpsvilflsksplvdk
241 ......s.....................................................
301 ydlsslrelccgaaplakevaevaakrlnlpgircgfgltestsaiiqtlgdefksgslg
301 ..............................................n.hs.r.........
361 rvtplmaakiadretgkalgpnqvgelcikgpmvskgyvnnveatkeaidddgwlhsgdf
361 ............................................................
421 gyydedehfyvvdrykelikykgsqvapaeleeillknpcirdvavvgipdleagelpsa
421 ............................................................
481 fvvkqpgteitakevydylaervshtkylrggvrfvdsiprnvtgkitrkellkqllvka
481 .......k....................................................e..
541 gg
541 ..
```

Figure III.1-1. Click beetle red and green luciferase protein sequence
Comparison between the amino acid sequences of the mammalian cell-adapted CBRluc (upper strand) and CBGluc (lower strand) amino acid sequence; the amino acids differentiating the two click beetle luciferases are highlighted.

1.1. Adaptation of the click beetle luciferases for *C. albicans*

Every organism has a specific codon usage bias, which favours certain codons for a specific amino acid. The attempt to express a gene that does not match this codon usage will likely yield poor protein production levels (Kurland, 1991). A gene reporter must be produced at maximal levels to measure the activity of weakly expressed genes. Hence the original luciferase genes were modified for *C. albicans* codon usage using GeneOptimizer (GeneArt). The Codon Adaptation Index (CAI) measures how accurately a gene matches the codon usage of an organism; a CAI of 1 indicates a perfect match. The CAI of the original luciferases for *C. albicans* was 0.54. After adjustment it was 0.9, higher than the strongly expressed *C. albicans ACT1* gene (Figure III.1-2). Moreover, it had to be considered that *C. albicans* is part of the CUG clade; members of this clade translate the CUG codon to a serine and not a leucine residue like most other fungi (Santos *et al.,* 2011). Therefore all CUG codons in the luciferases ORF were replaced by an equivalent leucine codon (18 CUG codons for the original *CBRluc*, 19 for the original *CBG68luc*). The hereby modified genes were named *CBRluc* and *CBGluc*, encoding respectively the red and green click beetle luciferase. A sequence alignment comparing the original *CBGluc* and *CBRluc* genes with *CBRluc* and *CBGluc* genes adapted for expression in *C. albicans* is available in the annex (Figure S1). Both genes were synthetised *in vitro* (GeneArt) and inserted into the pMK-RQ plasmid (Figure III.1-3)

	CAI-1	CAI-2	% G+C
CBRluc original	0.543	0.536	49.6
CBGluc original	0.543	0.537	50.0
CBRluc	0.918	0.933	33.9
CBGluc	0.899	0.915	34.3
CaACT1	0.796	0.817	39.1

Figure III.1-2. Codon adaptation index values (CAI)
The codon adaptation index values were calculated using the CAIcal program (genomes.urv.es/CAIcal/). The values for the adapted *CaCBluc* are compared with the original sequences. As reference, the CAI values of the strongly expressed *C. albicans ACT1* gene are also provided (exons only). The percentage of G+C of the sequences is shown (average in *C. albicans* is 36,1 %). CAI-1: codon usage reference table from *Candida* Genome Database (Figure S2). CAI-2: codon usage reference table from Kazusa DNA research Institute Codon Usage Database (www.kazusa.or.jp/codon/; Figure S2).

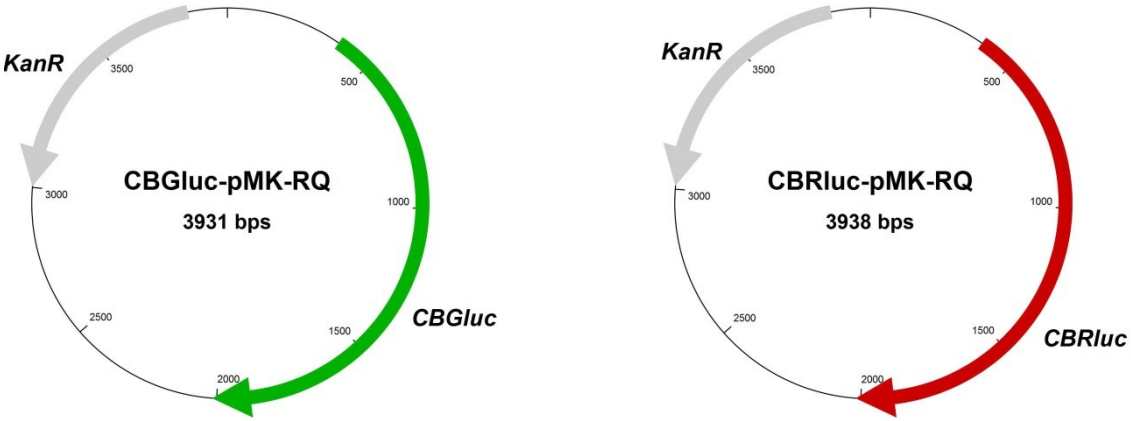


Figure III.1-3. Plasmids carrying the *C. albicans*-adapted genes encoding the click beetle luciferases
The *in vitro* synthetised *CBRluc* and *CBGluc* genes in the pMK-RQ plasmid are shown. *CBGluc*, *C. albicans*-adapted Click beetle Green Luciferase; *CBRluc*, *C. albicans*-adapted Click beetle Red Luciferase; KanR, kanamycin resistance marker.

1.2. Activity of click beetle luciferases in yeast

The first step for evaluating the new click beetle luciferase reporter genes was to verify their expression and ability to produce bioluminescence in *S. cerevisiae* and *C. albicans*. A set of plasmids was constructed with the luciferase ORFs under the control of strong promoters. Given the adaptations made to the gene sequence and the expression in a new organism, it had to be confirmed that the emission spectra of click beetle luciferases produced in *C. albicans* matched the original luciferases.

1.2.1. Luminescence in *S. cerevisiae*

The ascomycetous yeast *S. cerevisiae,* genetically close *to C. albicans,* allowed a rapid testing of the function of the luciferases. Multi-copy plasmids were constructed containing *CBRluc* or *CBGluc* under the control of a galactose-regulated promoter (*GAL1p*; Johnston *et al.,* 1984). The *GAL1* promoter is activated in presence of galactose and repressed by glucose (Kötter *et al.,* 1990). The plasmids, named p4-CBR and p4-CBG (Figure III.1-4), were transformed in the *S. cerevisiae* MC45-5A strain. The resulting transformants were named SR1 and SG1. To determine luminescence, cells from *S. cerevisiae* SR1 and SG1 strains were grown in the inducing S-Galactose medium for 48 h. To start the luminescence reaction, 100 µl of cells were mixed with the commercially available Chroma-Glo reagent. Chroma-Glo reagent contains luciferin and the appropriate buffer for optimal luminescence (Chroma-Glo™ Luciferase Assay System, Promega). Cells grown in glucose medium showed no luminescence; likewise, the parental strain did not show any luminescence (Figure III.1-5). The expression level of the luciferases in *S. cerevisiae* in these conditions was very high and the luminescence could be seen with bare eyes.

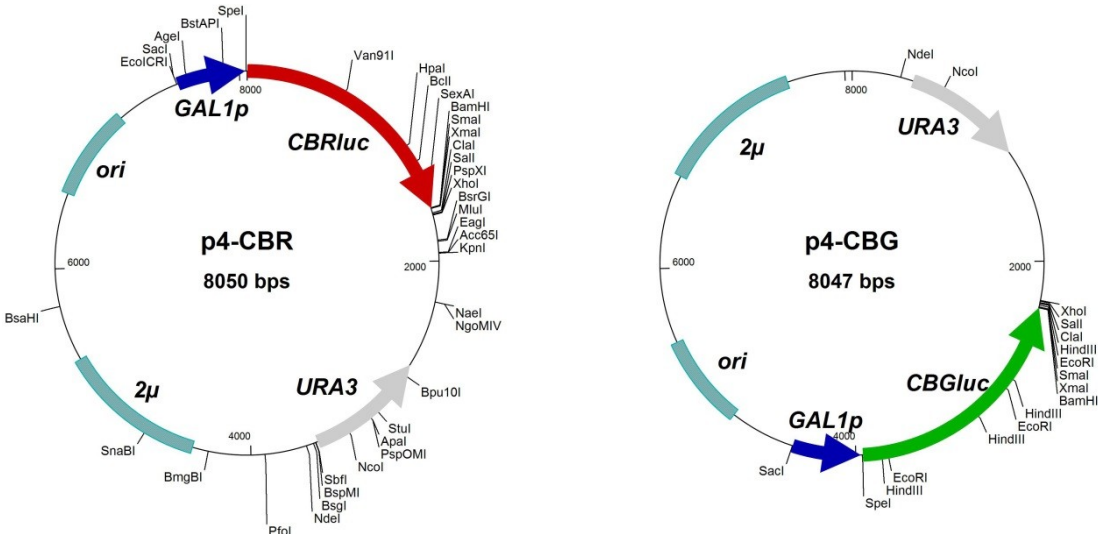


Figure III.1-4. Plasmids for click beetle luciferase expression in *S. cerevisiae*
CBRluc or *CBGluc* are under the control of the galactose-inducible *GAL1* promoter. Relevant restriction sites are shown. *CBRluc*: click beetle red luciferase; *CBGluc*: click beetle Green luciferase; *GAL1p*: *S. cerevisiae* galactose−inducible promoter; *URA3*: gene coding for *S. cerevisiae* orotidine-5'-phosphate decarboxylase; *2 µ*: yeast multicopy plasmid replication origin; *ori*: *E. coli* replication origin.

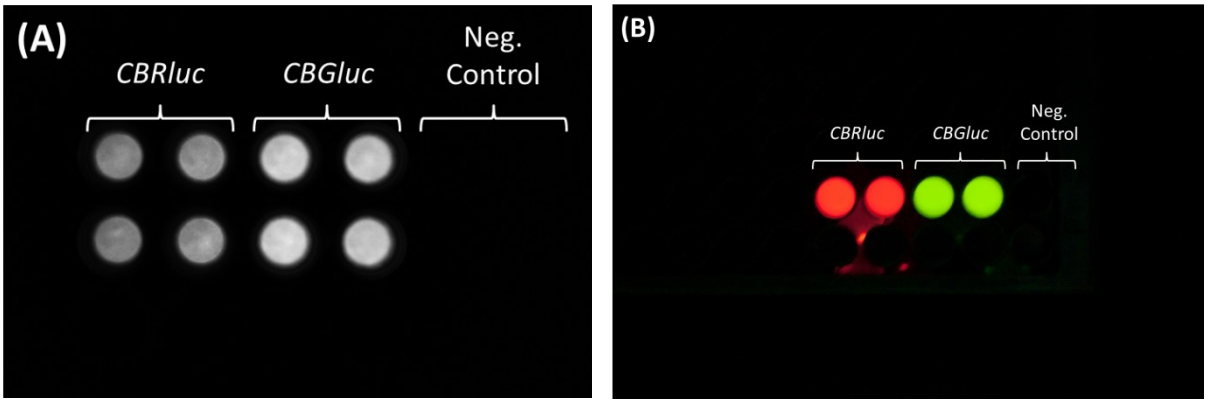


Figure III.1-5. Luminescence of click beetle luciferases in *S. cerevisiae*
S. cerevisiae (MC45-5A) was transformed with either p4-CBR (*CBRluc,* strain SR1), p4-CBG (*CBGluc*, strain SG1) or an empty plasmid (p426, negative control). In both cases the luciferase-encoding genes are under the control of the *GAL1* promoter. The photograph was taken after mixing 100 µl of a 48h culture (OD_{600}≈2) grown in inducing medium (S-Galactose medium) with 100 µl Chroma-Glo reagent. (A) Photographs were taken using Fujifilm luminescent image analyser LAS1000. (B) Photograph taken using a Canon EOS 450D digital camera.

1.2.2. Luminescence in *C. albicans*

Initially, the click beetle luciferases were tested in *C. albicans* using multi-copy expression plasmids containing the luciferase genes under the control of the strong *ACT1* promoter. The constructed plasmids named pD-CBR (*CBRluc*) and pD-CBG (*CBGluc*) are shown in Figure III.1-6. The *ACT1* housekeeping gene is constitutively expressed when cultures are growing (Swoboda *et al.,* 1994). Transformants of *C. albicans* CAI4 strain were selected on SD minimal medium lacking uridine. The strains were kept in this medium to maintain selection pressure and avoid plasmid loss. Bioluminescence was measured in crude extracts of transformants carrying either pD-CBR (strain CR1, red luciferase) or pD-CBG (Strain CG1, green luciferase). The luminescence reaction was started by mixing 50 µl of crude extract with 50 µl Chroma-Glo reagent. In both strains, a strong luminescence was observed (Figure III.1-7A).

The adapted click beetle luciferases encoded by plasmids were able to produce light in *C. albicans*; yet, a gene reporter must be regulable. Therefore, another plasmid was constructed with the *CBGluc* gene downstream of the *C. albicans PCK1* promoter (pB-CBG; Figure III.1-6). *PCK1* codes for PEP carboxykinase, which is part of gluconeogenesis; expression of *PCK1* is repressed by glucose and de-repressed on other carbon sources e.g. galactose (Leuker *et al.,* 1992). The *C. albicans* CAI4 strain was transformed with pB-CBG using selection on SD minimal medium lacking uridine; the resulting transformants were named CG−PCK1. Crude extracts were prepared from CG-PCK1 cells grown either in glucose or in galactose containing medium and luminescence was assessed. No luminescence was measurable when cells were grown in glucose; in contrast, when grown in an inducing medium (S-Galactose) a clear luminescence signal was observed (Figure III.1-7B).

These assays prove that the click beetle luciferases are effectively expressed in *C. albicans* and that the light signal they produce is easily measured. Moreover, the signal is only observed when the promoter upstream of the luciferase gene is activated.

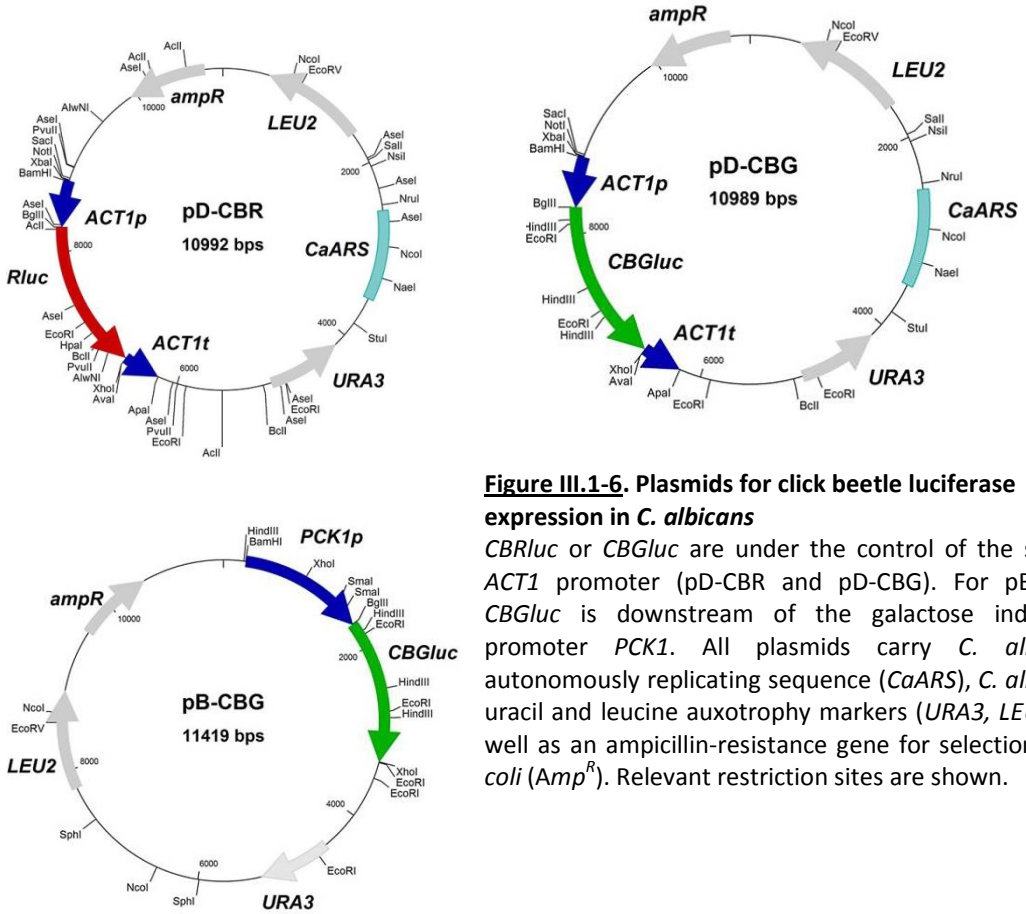


Figure III.1-6. Plasmids for click beetle luciferase expression in *C. albicans*
CBRluc or *CBGluc* are under the control of the strong *ACT1* promoter (pD-CBR and pD-CBG). For pB-CBG, *CBGluc* is downstream of the galactose inducible promoter *PCK1*. All plasmids carry *C. albicans* autonomously replicating sequence (*CaARS*), *C. albicans* uracil and leucine auxotrophy markers (*URA3, LEU2*) as well as an ampicillin-resistance gene for selection in *E. coli* (A*mp*^{R}). Relevant restriction sites are shown.

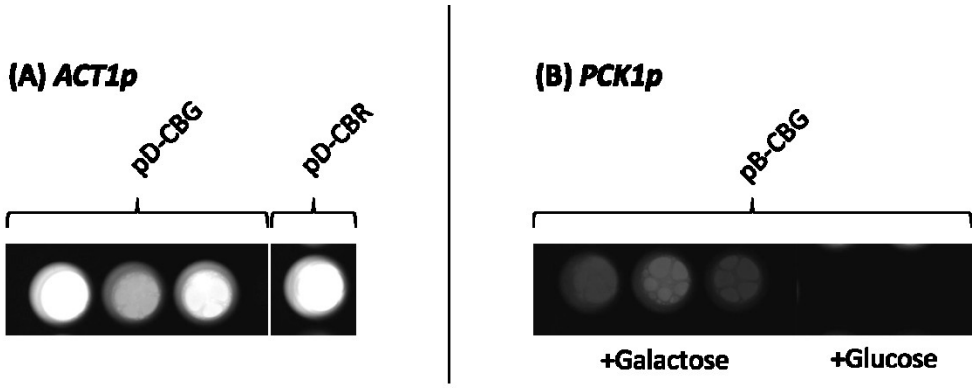


Figure III.1-7. Luminescence of click beetle luciferases in *C. albicans*
C. albicans CAI4 strain was transformed with either pD-CBR (*ACT1p-CBRluc*) , pD-CBG (*ACT1p-CBGluc*) or pB−CBG (*PCK1p-CBGluc*) plasmids. The transformants were grown overnight (up to OD_{600}≈2) in S- minimal medium containing either glucose or galactose. Crude extracts were then prepared (Tebarth *et al.,* 2003) and the luminescence reaction was started by mixing 50 µl crude extract (5 ml culture yielded 500 µl crude extract) with 50 µl Chroma-Glo reagent. Photographs were obtained using the Fujifilm luminescent image analyser LAS1000.

1.2.3. Light emission spectrum of click beetle luciferases produced in *C. albicans*

The CBRluc and CBGluc luciferases have known specific luminescence spectra; the difference in their emission wavelengths makes separation of the two signals possible (Almond *et al.,* 2003; Davis *et al.,* 2007). The red luciferase has an emission peak at 613 nm and the green luciferase at 537 nm. Because gene sequences were adapted for expression in *C. albicans*, it was necessary to verify the wavelengths emitted by CaCBRluc and CaCBGluc. The previously described *C. albicans* CR1 and CG1 strains (carrying plasmids with *ACT1p-CBRluc* and *ACT1p-CBGluc* expression cassettes) were used to prepare crude extracts from an overnight culture in SD medium. After mixing 100 µl of crude extract with 100 µl of Chroma-Glo reagent, a PerkinElmer LS 55 spectrophotometer was used to measure the light emission spectrum between 400 and 750 nm. Figure III.1-8 shows the measured values expressed in percent of the maximal luminescence for each luciferase. The luminescence emission spectra are characterised by: (i) the wavelength of the emission peak; (ii) the half-bandwidth (HbW), the wavelengths window were the emission is ≥50 % of the peak. CaCBRluc has an emission peak at 606 nm with a half-bandwidth of 56 nm. The green luciferase peak is at 530 nm with a HbW of 61 nm. The measurements match the spectra of unmodified click beetle luciferases indicating that the modification of gene sequence and luciferase production in *C. albicans* has no impact on the emitted wavelengths (Almond *et al.,* 2003). The luciferases have distinct peaks that should allow separation of the two luminescence signals.

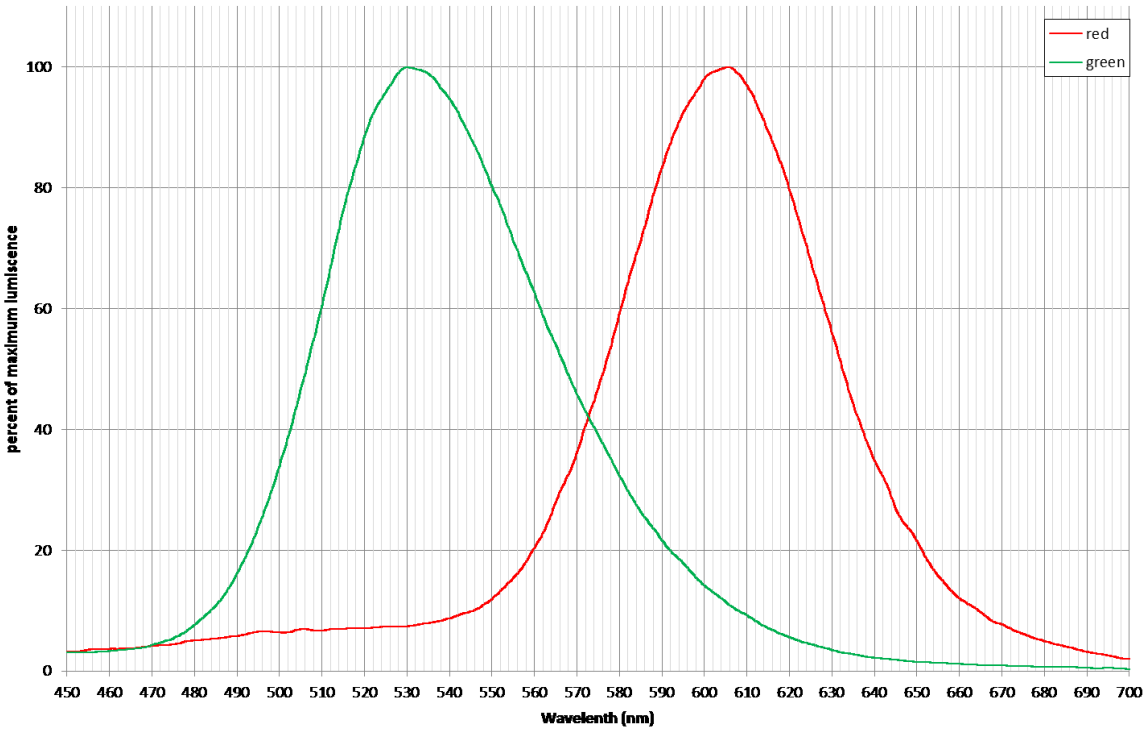


Figure III.1-8. Emission spectrum of CaCBRluc and CaCBGluc expressed in *C. albicans*
C. albicans cells from the CR1 or CG1 strains (red and green click beetle luciferases) were grown in SD medium overnight at 30 °C. Crude extracts were prepared (500 µl of crude extract were obtained from 5 ml culture at OD_{600}≈2) and the luminescence reaction was started by mixing 100 µl of crude extract with 100 µl Chroma-Glo reagent. Light emission spectra were read using the PerkinElmer LS 55 spectrophotometer.

1.3. Genomic integration of click beetle luciferase genes in *C. albicans*

The aim of this work was to demonstrate the potential of click beetle luciferases as reporters in *C. albicans*. To evaluate their sustainability, strains were constructed containing luciferase ORFs genomically integrated downstream of relevant genes encoding components of the PKA pathway. This pathway is of special interest because it regulates metabolism and morphogenesis in *C. albicans* (Ernst, 2000). In addition, the luciferase genes were inserted into the genome downstream of the *ACT1* promoter, which is constitutively active during growth (Swoboda *et al.,* 1994). Furthermore, to obtain a reporter of the yeast-to-hypha transition, the click beetle luciferase gene was integrated downstream of the *HWP1* promoter, which is strongly activated during hyphal formation (Loeb *et al.,* 1999).

The first method used to integrate luciferase gene into the genome was plasmid based; integration occurred by homologous recombination at a genomic sequence present on the plasmid. In addition, a PCR based approach was used. A cassette containing the luciferase gene and a selection marker gene was amplified by PCR using primers with homologous ends for integration at specific genomic sites. This latter method is advantageous because the homology sequence on the primers can easily be changed and the cassette amplified for integration at a different target sites.

1.3.1. *ACT1* promoter

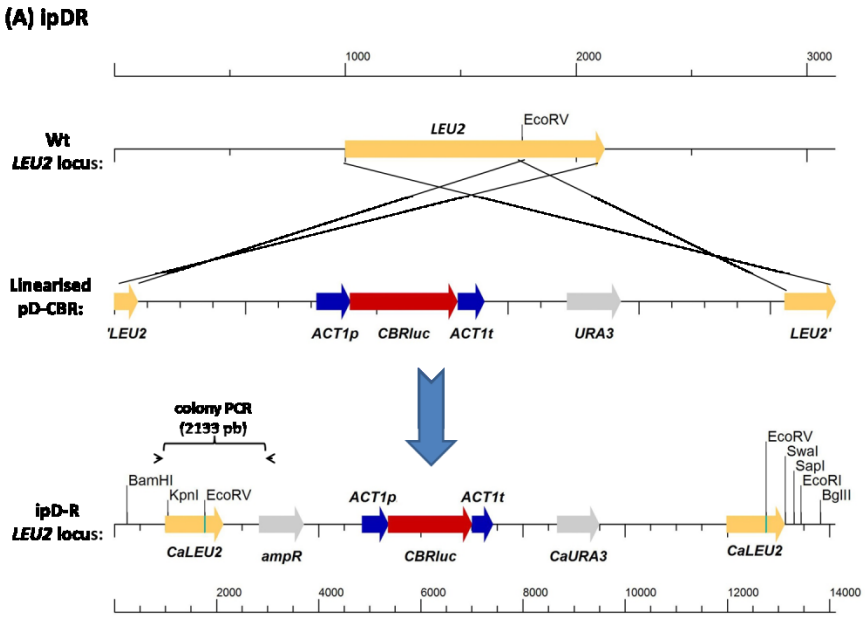
Because the *ACT1* promoter is often used as a reference, the click beetle luciferase ORF were chromosomally integrated after *ACT1p* in the genome of *C. albicans*. The click beetle luciferase ORFs were first cloned downstream of the *ACT1* promoter on plasmids and the plasmids were subsequently integrated genomically into the *LEU2* locus (*URA3* selection marker). In addition, the *CBRluc* gene was integrated into the genome at the *ACT1* locus using a PCR-generated fragment carrying the *HIS1* selection marker.

1.3.1.1. Integration of click beetle luciferase genes into the *LEU2* locus

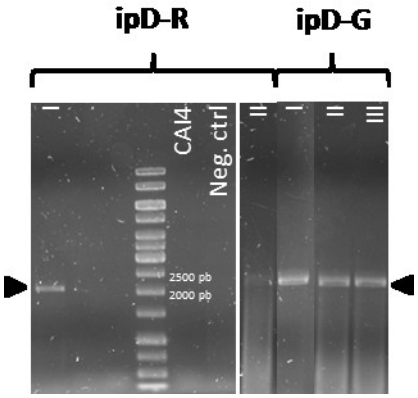
Plasmids pD-CBG and pD-CBR were used to chromosomally integrate the gene encoding the red and green click beetle luciferases into the *LEU2* locus of the *C. albicans* genome (Figure III.1-9). The plasmid was linearised using *Eco*RV and the linearised plasmid was transformed into the *C. albicans* CAI4 strain (selection for uridine prototrophy). The homology of *LEU2* on the plasmid and chromosomal *LEU2* sequences allowed homologous recombination. Transformants were selected on SD-medium. The integration was verified using colony PCR with primers check ipDR Fw/Bw (transformants I and II for ipD-R and I-III for ipD-G). The forward primer binds in the *ACT1* promoter upstream of the homology region used for integration, the backward primer binds in the click beetle gene (Figure III.1-9A). The PCR products matched the expected size (Figure III.1-9B).

The luminescence of ipD-R and ipD-G strains was measured as described below. All luminescence experiments described in this work were performed in accordance with the following protocol unless otherwise specified: 100 µl of cell culture (whole cells) was mixed with 100 µl of Chroma-Glo reagent (Promega). Luminescence was then read using the Berthold Tristar luminometer. The Tristar luminometer measures relative quantities of light (instrument specific) and the results are therefore expressed in Relative Light Units (RLU). The luminescence is read over time (200-250 min) and the maximal luminescence value, referred to as L_{max}, was recorded. The details of this protocol are described in Results 2.1 and 2.2.

In the case of ipD-R and ipD-G strains, luminescence of an overnight culture was measured. All clones exhibited a strong luminescence with an average of 1.85×10^5 RLU for ipD-R and 5.99×10^5 RLU for ipD-G (Figure III.1-9C).



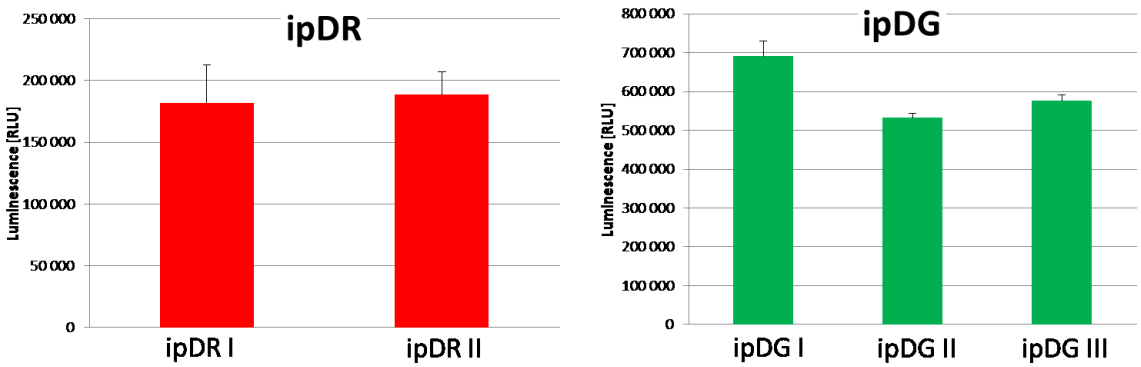
(B)



<u>Figure III.1-9</u>. Integration of click beetle luciferase genes into the *LEU2* locus

(A) The plasmids pD-CBR and pD-CBG were used for integration of *ACT1p-CBRluc* or *ACT1p-CBGluc* into the *C. albicans LEU2* locus. The integration process by homologous recombination is shown for pD-CBR; the *Eco*RV insertion site and colony PCR amplification primers are shown. (B) Candidate transformants were checked for correct integration by colony PCR using primers check ipDR Fw/Bw (independent transformants I and II for ipD-R and I-III ipD-G); the expected product size is 2133 bp (black arrow). (C) Luminescence of the transformants was also measured after mixing 100 µl of overnight cultures (SD medium at 30°C; OD_{600}≈2) with 100 µl of Chroma-Glo reagent. Luminescence was measured using the Berthold Tristar luminometer. The luminometer measures relative light signal (instrument specific), expressed in Relative Luminescence Units (RLU), not an absolute photon count. Error bars show the standard deviation for 3 technical replicates. *LEU2*: *C. albicans LEU2* gene; *ACT1p*: *C. albicans ACT1* promoter; *ACT1t*: *C. albicans ACT1* terminator; other genes are as indicated previously.

(C)



1.3.1.2. Integration of click beetle luciferase genes into the *ACT1* locus

The use of the red and green click beetle luciferases allows the monitoring of two genes simultaneously. Therefore, another set of plasmids carrying a click beetle luciferase gene and the *HIS1* auxotrophy marker was constructed in addition to the *URA3* plasmids. The cassette containing a click beetle luciferase gene and the *HIS1* selection marker can then be amplified by PCR and integrated chromosomally in genome of *C. albicans*. The two click beetle luciferase ORFs, were excised from CBRluc-pMK-RQ or CBGluc-pMK-RQ (Results 1.1) using *Bam*HI and *Bgl*II restriction enzymes. The fragments were then inserted in the linearised pGEM-HIS1 plasmid which contains the *HIS1* selection marker (*Bam*HI restriction). The resulting plasmids, pHIS-CBR and pHIS-CBG, were checked by restriction and sequencing (Figure III.1-10). These plasmids carry a cassette suited for integration of the click beetle luciferase into the genome of *C. albicans* with *HIS1* auxotrophy marker, for selection of transformants.

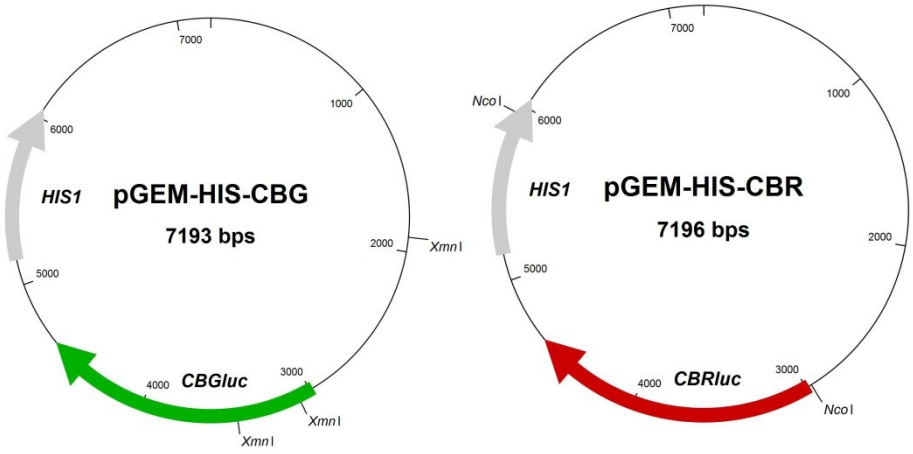
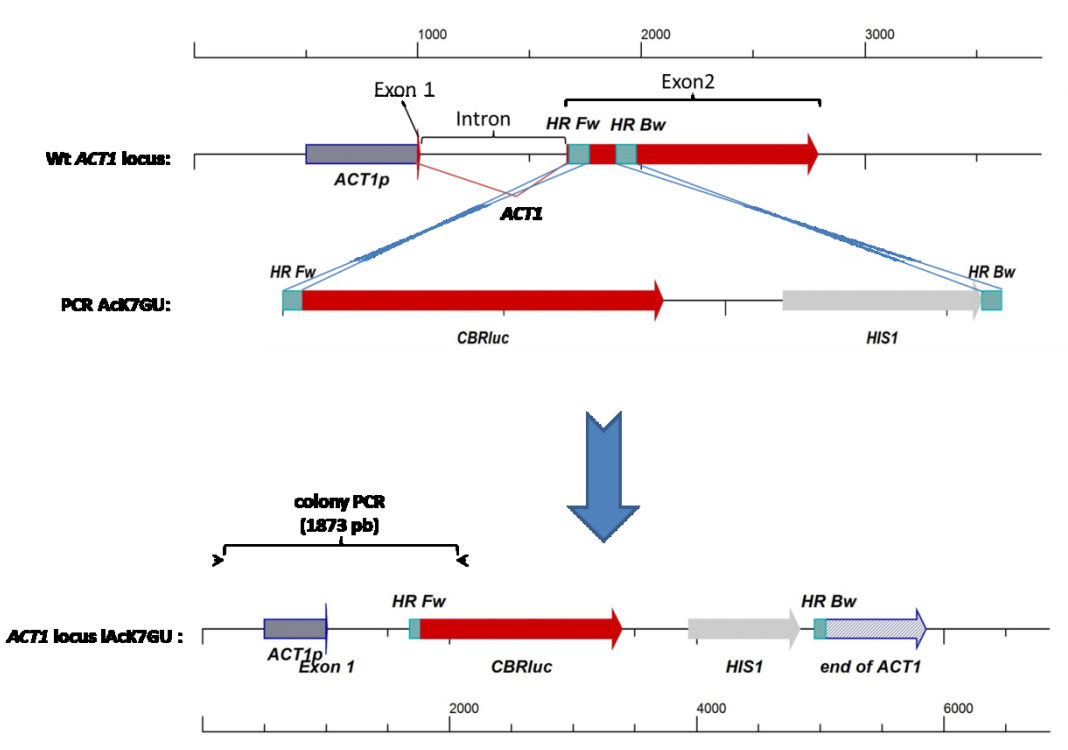


Figure III.1-10. pGEM-HIS-CBG and pGEM-HIS-CBR plasmids
Plasmids were constructed by inserting the ORFs of both click beetle luciferases (*CBGluc* and *CBRluc*) in the single *Bam*HI restriction site of the pGEM-HIS plasmid.

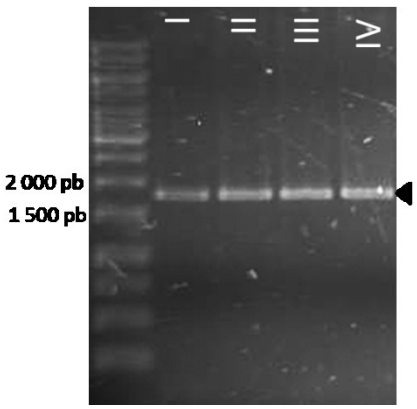
The *CBRluc-HIS1* cassette was amplified by PCR with AcK7RH Fw/Bw primers (plasmid pHIS-CBR). The *ACT1* ORF consists of a short exon, an intron and a large exon. The sequence homology between the primer and the 5'-end of *ACT1* gene, in the second exon, allows for genomic integration by homologous recombination. A scheme of the integration is shown in Figure III.1-11A. The N−terminal end of the resulting protein is shown in Figure III.1-11B. *C. albicans* BWP17 was transformed using selection for histidine prototrophy; the resulting transformant strain was named AcK7RH-B. Integration was verified using colony PCR. The colony PCR primers (AcK7RH Dia Fw, check K7G Bw) bind in the *ACT1* promoter and in the *CBRluc* gene (Figure III.1-11A). The colony PCR products matched the expected product size (Figure III.1-11C). The luminescence of the resulting strains was measured as described previously. Three independent transformants displayed strong luminescence, $113x10^3$ RLU in average (I, II and IV; Figure III.1-11D). The transformant II did not show luminescence and has therefore not been used for further experiments.

(A)

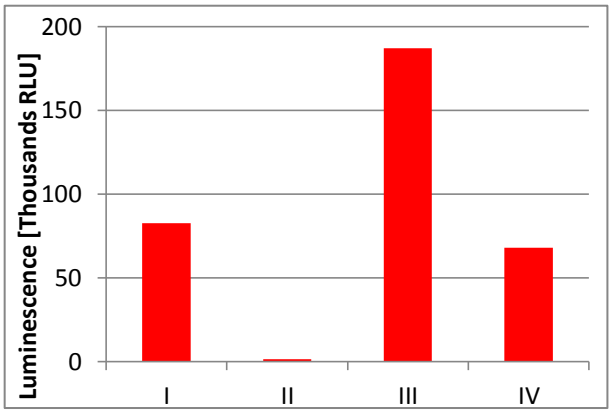


(B)

(C)



(D)



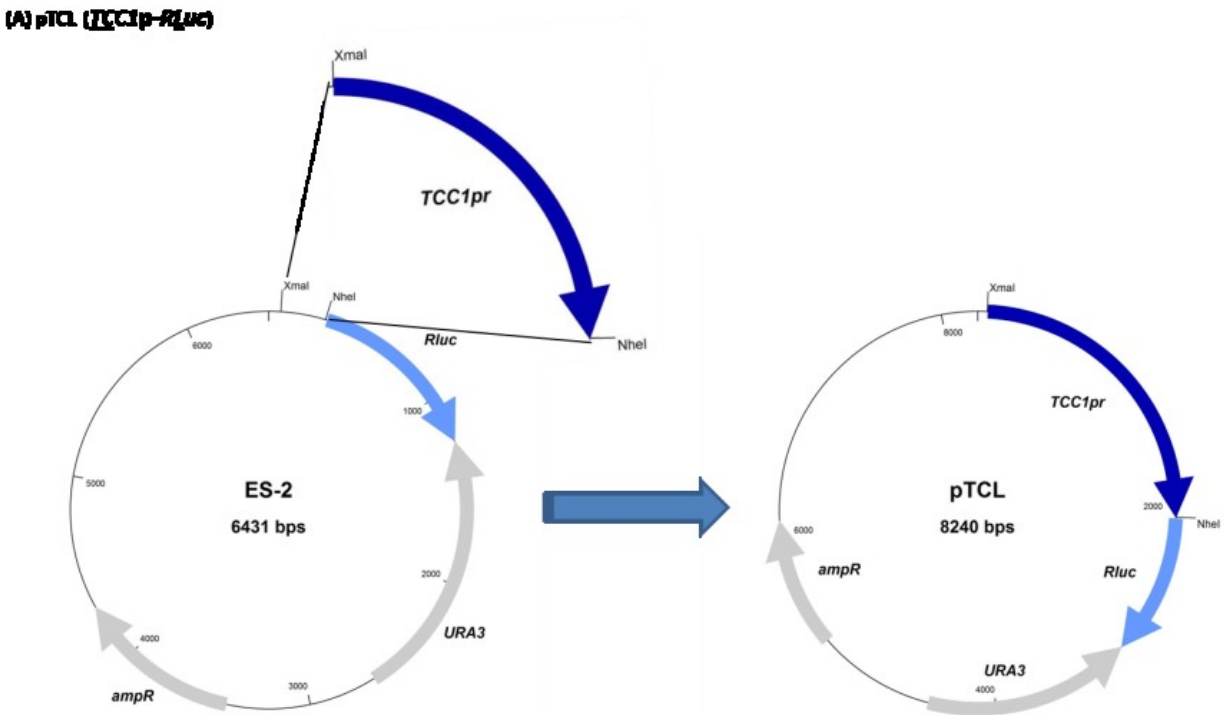
<u>Figure III.1-11</u>. Construction and activity of the strain AcK7RH-B

(A) After amplification of *CBRluc* ORF and *HIS1* selection marker by PCR using the AcK7RH Fw and Bw primers, the sequence was integrated in *C. albicans* BWP17 genome by homologous recombination (HR Fw and Bw sequences). After integration, *CBRluc* ORF is downstream of the *ACT1* promoter. (B) Translation of the N-terminal end of Act1 and CBRluc fusion after integration, exon-derived sequences are indicated. (C) Chromosomal integration was verified in transformants I-IV using colony PCR with primers AcK7RH Dia Fw and Check K7G Bw; expected product size of 1843 bp was obtained (black arrow). (D) Luminescence was measured after mixing 100 µl of cells (overnight culture in YPD at 30°C; OD_{600}≈6) with 100 µl of Chroma-Glo reagent.

1.3.2. Integration of the green click beetle luciferase gene into the *TCC1* locus

Previously, the Efg1 transcription factor had been shown to bind to the *TCC1* promoter during yeast growth (Lassak *et al.,* 2011). Therefore, *TCC1* constitutes an interesting regulatory target for use of click beetle luciferases. The *TCC1* promoter sequence (2 kb upstream of the start-codon) was amplified by PCR on genomic DNA using primers *TCC1* 2k Fw/Bw that introduce *Xma*I and *Nhe*I restriction sites, respectively. The amplified fragment was then digested with *Xma*I and *Nhe*I and inserted in the digested pES2 vector to generate plasmid pTCL (*TCC1-RLUC*; Figure III.1-12A). The correct structure of pTCL was verified by restriction analysis and sequencing. Subsequently, the green click beetle luciferase gene was amplified by PCR (template: pMK-RQ-CBGluc plasmid; primers CBGlucNheI Fw/Bw introducing *Nhe*I and *Xba*I restriction sites). The amplification product was first sub-cloned in pGEM (resulting plasmid pGEM-*CBGluc*). After excision of the *CBGluc* insert with *Nhe*I and *Xba*I, it was ligated in the digested pTCL resulting in plasmid pTGL (*TCC1* Green Luciferase; Figure III.1-12B). The pTGL plasmid sequence was verified by sequencing.

For integration in *C. albicans*, plasmid pTGL was linearised with *Swa*I (single restriction site in *TCC1p* region) and transformed into strain CAI4 and BWP17 using selection for uridine prototrophy. The resulting transformant strains are named ipTGL for CAI4 and ipTGL-B for BWP17 parental strain. A map of the *TCC1* locus after plasmid integration is shown in Figure III.1-13A. To verify the integration, colony PCR was performed with primers C1 and C2 ipTGL binding respectively in *TCC1* promoter upstream of the homologous sequence and in *CBGluc* gene. The resulting PCR products matched the expected product sizes (Figure III.1-13B). Independent transformants I to IV were tested for luminescence as previously described. The average luminescence for the 4 transformants was $7x10^3$ RLU (Figure III.1-13C).



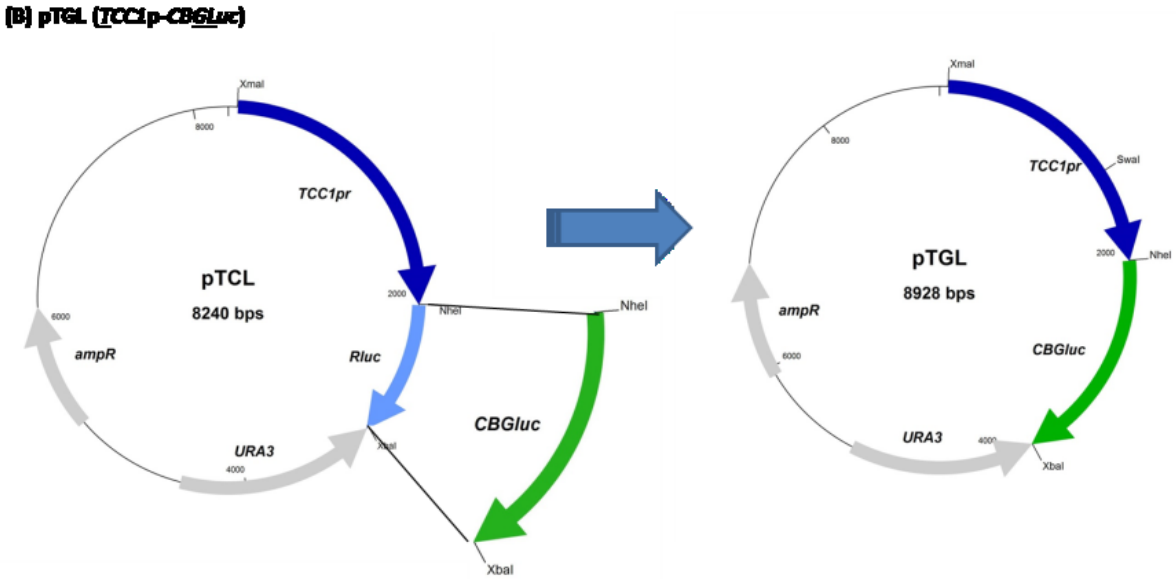


Figure III.1-12. Construction of pTCL and pTGL plasmids
(A) The pTCL plasmid was constructed by insertion of a 2 kb fragment of the *TCC1* promoter between *Xma*I and *Nhe*I restriction site. (B) To generate the pTGL plasmid, the *Renilla* luciferase reporter gene (*Rluc*) was replaced by *CBGluc*. Relevant restriction sites are shown. *TCC1p*: a 2 kb fragment upstream of *TCC1* start codon sequence; *Rluc*: Renilla luciferase reporter gene; other genes are as previously described.

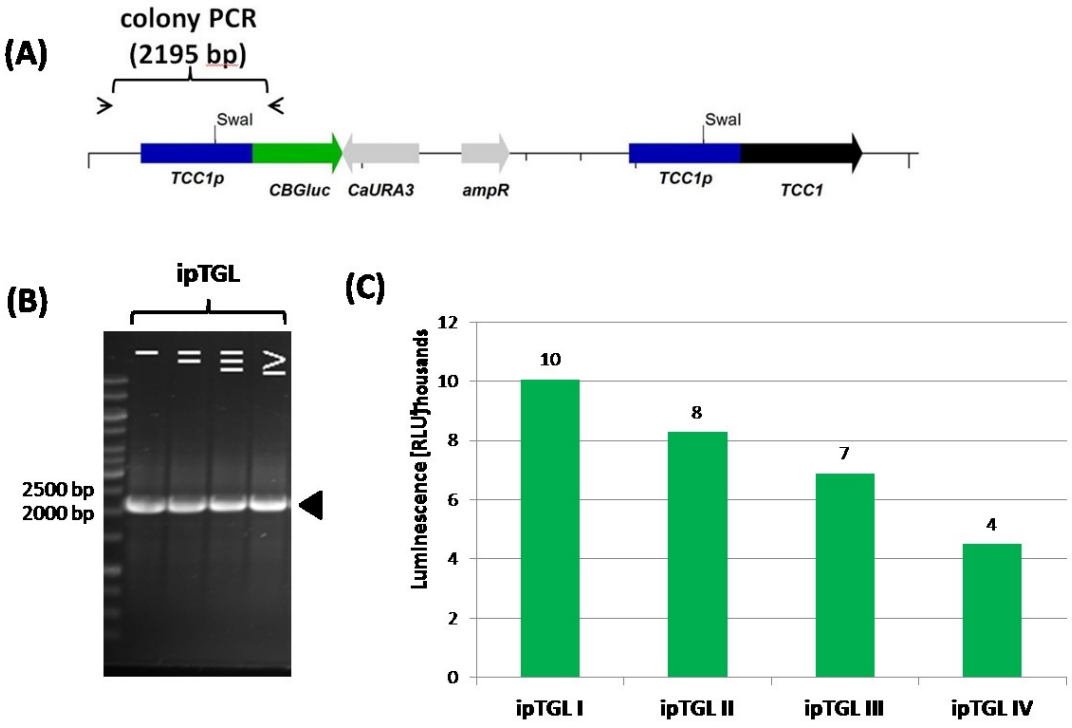


Figure III.1-13. Genomic integration of pTGL
(A) After digestion of pTGL with *Swa*I, the plasmid was integrated in the *C. albicans* CAI4 genome by homologous recombination (homology with *TCC1p*). After integration, the *CBGluc* ORF is situated downstream of *TCC1* promoter. (B) Integration was verified for transformants I-IV using colony PCR with primers C1 and C2 ipTGL; the expected band size is 2 195 bp (black arrow). (C) Luminescence was measured after mixing 100 µl of cells (overnight culture in YPD at 30°C) with 100 µl of Chroma-Glo reagent.

1.3.3. Integration of click beetle luciferase genes into the *HWP1* locus

Some proteins are strongly synthesised during hyphal induction; this is the case for Hwp1, a cell wall protein abundant in hypha but absent in yeast form. This increase is due to a strong induction of the activity of the *HWP1* promoter upon hyphal growth (Staab *et al.,* 1998). Since the yeast-to-hypha transition is a key virulence factor in *C. albicans*, it is particularly important to have a reporter of hyphal growth. Because the activation of the *HWP1* promoter is characteristic of the hyphal growth, a *HWP1* and β-galactosidase fusion has been used as a marker for hyphal growth. *HWP1* is also regulated by the cAMP/PKA signal transduction pathway. Therefore, the click beetle luciferase genes were integrated downstream of *HWP1* promoter, either using a *URA3* or *HIS1* selection marker.

1.3.3.1. *URA3* selection marker

In order to insert the *CBRluc* reporter gene downstream of *HWP1* promoter, the *CBRluc-URA3* cassette was amplified using primers HwK7GU Fw/Bw with pD-CBG as template DNA. The forward primer has a 90 bp-homology to the 5'-end of the *HWP1* ORF (HR Fw) and the reverse primer a 100 bp homology with the *HWP1* ORF between bp 311 and bp 411 (HR Bw). After homologous recombination, the *HWP1* allele is inactivated. The *C. albicans* strain CAI4 was transformed with the amplification product (Figure III.1-14A). The constructed strains are named HwK7GU (*HWP1-CBGluc URA3*). The integration was verified using colony PCR. The primers Check iHwK7Gu Fw/Bw used for colony PCR bind, respectively, in the *HWP1* promoter upstream of the homologous sequence used for integration and in the *CBGluc* ORF. For the 4 independent transformants tested (I-IV), the PCR product size matched the expected length (Figure III.1-14B). As *HWP1p* is only induced during hyphal growth, luminescence was assessed after 1 h incubation in hypha inducing conditions (YP+10 % serum; 37°C; initial OD_{600}=0.2). For transformants I and II, luminescence was $78x10^3$ RLU in average. For transformants III and IV, the average was $17x10^3$ RLU; these two transformants were not used for further experiments (Figure III.1-14C). The activity in yeast form is negligible (Figure III.3-2).

The *HWP1-CBGluc* junction in transformants was amplified by genomic PCR to verify its sequence. The sequencing of the resulting fragment revealed that the ATG sequence of *HWP1* and *CBGluc* ORF were not in frame, which was caused by the faulty sequence of primer HwK7GU Fw. The HwK7RH strain, constructed subsequently, corrected this and the *HWP1* ATG and *CBRluc* ATG are in frame. The regulation of the *HWP1* promoter by hyphal induction in both strains is identical; however the luminescence is in average 95 fold lower in the HwK7GU strain compared to the HwK7RH strain (Figure III.1-15).

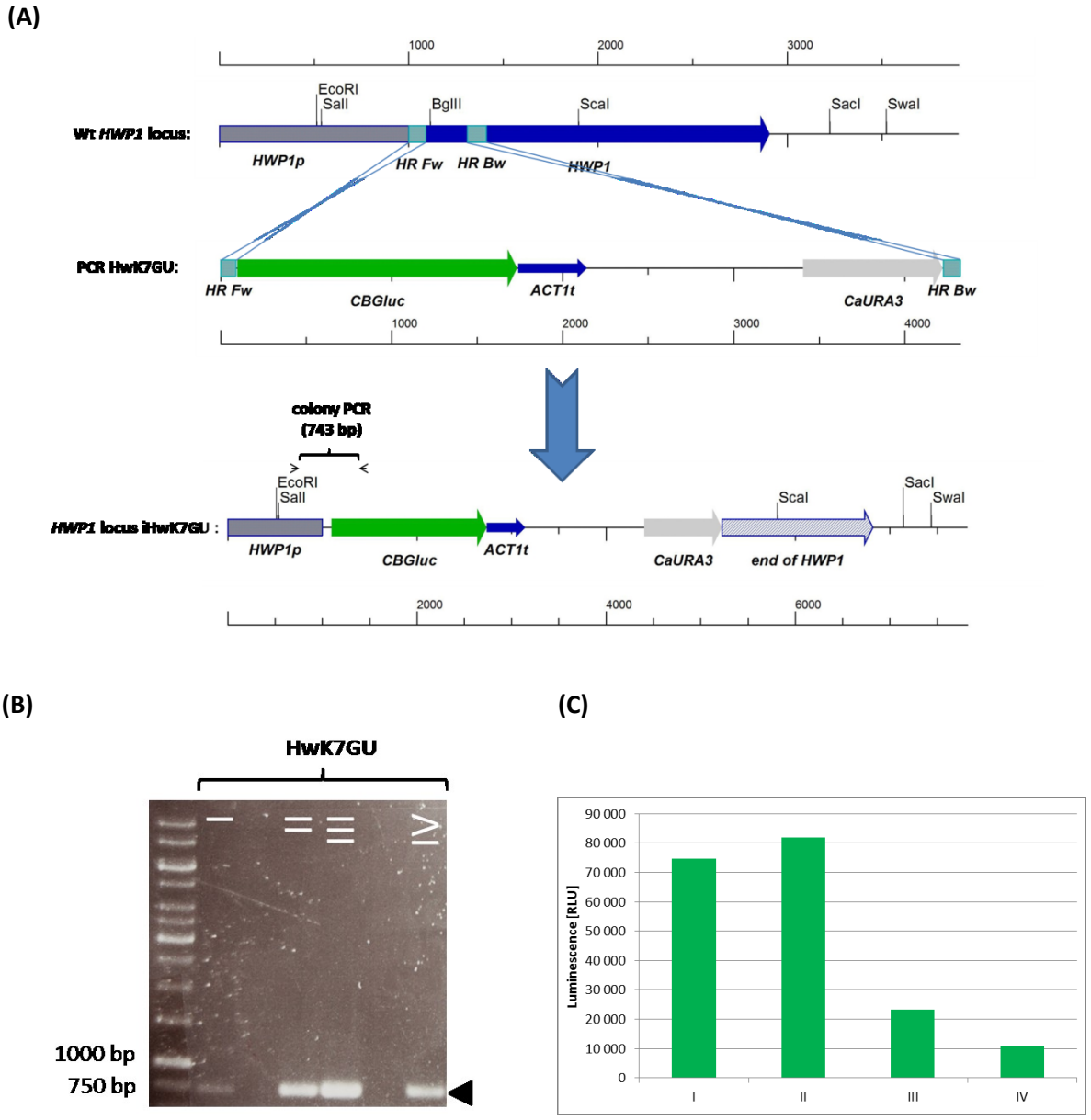


Figure III.1-14. Insertion of *CBGluc* downstream of *HWP1* promoter

(A) After amplification of the *CBGluc* ORF and the *URA3* selection marker by PCR using the HwK7GU Fw/Bw primers, the sequence was integrated in *C. albicans* CAI4 genome by homologous recombination (HR Fw and Bw sequences) using selection for uridine prototrophy. After integration, *CBGluc* ORF is situated downstream of the *HWP1* promoter. (B) Integration was verified (transformants I-IV) using colony PCR with primers Check iHwK7Gu Fw/Bw; the expected band size of 743 bp was obtained (black arrow). (C) Luminescence measured by mixing 100 µl of cells induced to form hyphae (1 h in YP+ 10 % serum at 37°C; initial OD_{600}=0.2) with 100 µl of Chroma-Glo reagent.

1.3.3.2. *HIS1* selection marker

As described for AcK7RH strain (Results 1.3.1.2), the pGEM-HIS-CBR plasmid was amplified by PCR with HwK7RH Fw/Bw primers and transformed into *C. albicans* BWP17 using selection for histidine prototrophy. The resulting *HWP1-CBRluc* strain was named iHwK7RH-B (Figure III.1-15A). Transformants were checked using colony PCR using primers HwK7RH Dia and Check K7G Bw. The forward primer binds in *HWP1* promoter upstream of the homologous region and the reverse primers in *CBRluc* ORF. For all transformants (I-IV), the obtained PCR products match the expected length (Figure III.1-15B). Luminescence during hyphal growth was measured as previously described (Results 1.3.3.1). In contrast with the previous integration method, the CBRluc ORF is in frame with *HWP1* ATG (Results 1.3.3.1). All transformants therefore displayed very strong luminescence of 4.5 million RLU in average (Figure III.1-15C). The luminescence during yeast growth was 1 300 fold weaker than for hyphal growth (data not shown).

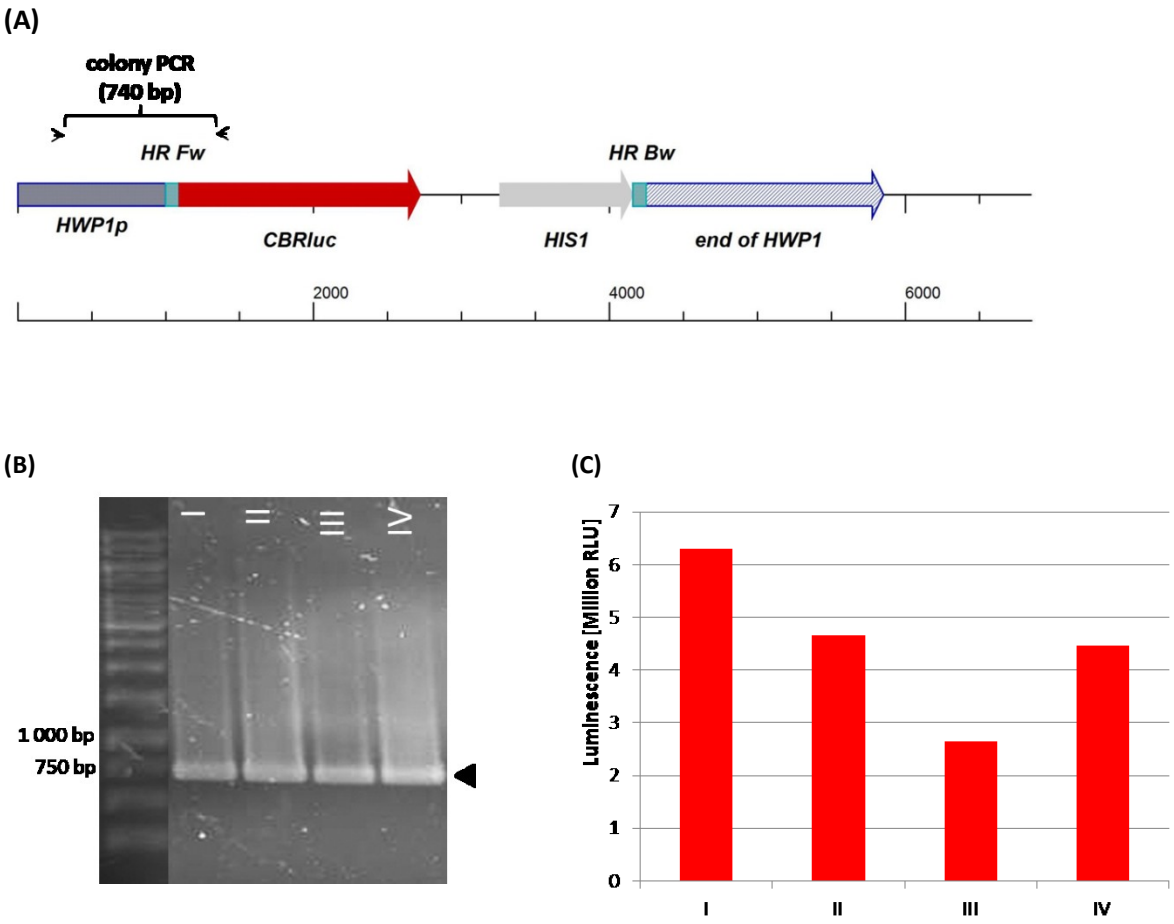


Figure III.1-15. Construction of HwK7RH strain
(A) After amplification of *CBRluc* ORF and *HIS1* selection marker by PCR using the HwK7RH Fw and Bw primers, the sequence was integrated in *C. albicans* BWP17 genome by homologous recombination (HR Fw and Bw sequences). After integration, the *CBRluc* ORF is situated downstream of *HWP1* promoter. (B) Chromosomal integration was verified for transformants I-IV using colony PCR with primers HwK7RH Dia and Check K7G Bw (expected product size: 740 bp; black arrow). (C) Luminescence was measured after mixing 100 µl of cells induced to form hyphae (YP+10 % serum at 37°C for 1 h; initial OD_{600}=0.2) with 100 µl of Chroma-Glo reagent.

1.3.4. Integration of the green click beetle luciferase gene into the *UME6* locus

Ume6 is a transcription factor necessary for filamentation and biofilm formation; it is a downstream element of the cAMP/PKA pathway (Banerjee *et al.,* 2008). As the *UME6* promoter is one of the Efg1 targets during hyphal induction (Lassak *et al.,* 2011), it was used for testing the click beetle luciferases and study yeast-to-hypha regulation. The insertion of the *CBGluc* ORF downstream of the *UME6* promoter follows the same method as for *HWP1p* described above (results 1.3.3.1.). For this purpose, the *CBGluc* ORF was amplified from pD-CBG with primers UmK7GU Fw/Bw. A map of the *UME6* locus after insertion is shown in Figure III.1-16. The constructed strain is named UmK7GU. As it is the case for HwK7GU, sequencing revealed that the *CBGluc* ORF is not in frame with the beginning of *UME6* ORF due to a faulty forward primer. Integration was verified by colony PCR (primers Check iUmK7Gu Fw/Bw). The forward primer binds in *UME6* promoter upstream of the homology sequence, the reverse primer binds in *CBGluc* ORF. The 3 transformants tested showed the expected 1 034 bp fragment (Figure III.1-16B). As Hwp1, Ume6 is only expressed during hyphal induction and the luciferase activity was therefore measured after 1 h of hyphal induction in YP+10 % serum (Figure III.1-16C). The luminescence measured was $4x10^3$ RLU for transformant II and $2.2x10^3$ RLU in average for transformants III and IV. The luminescence in yeast form was <200 RLU (Figure III.3-3)

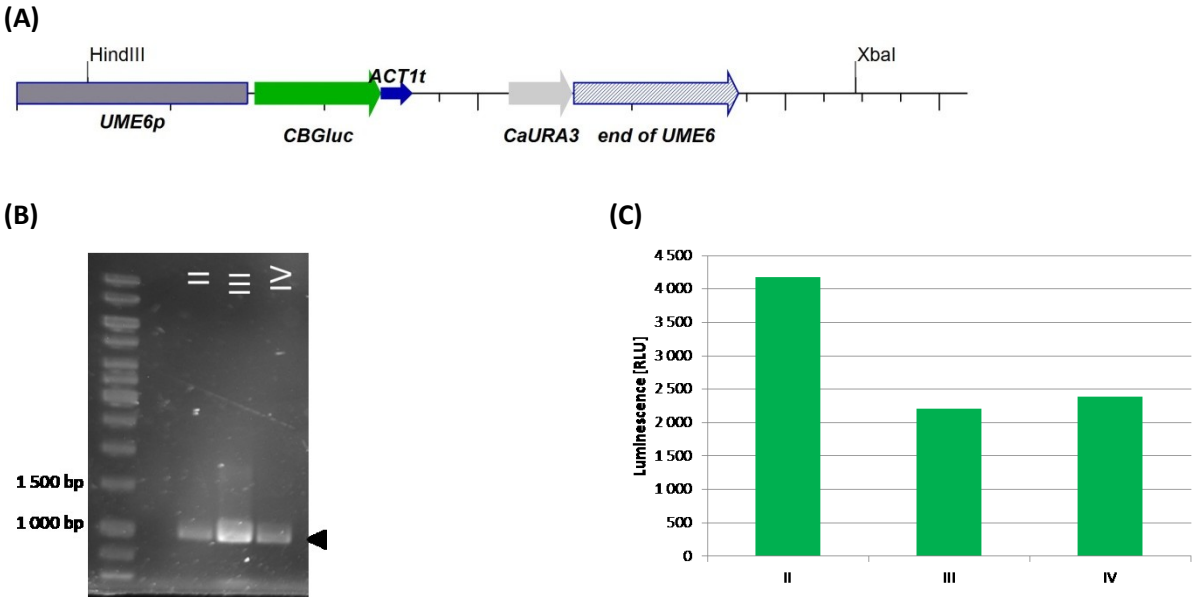


Figure III.1-16. Integration of *CBGluc* downstream of *UME6* promoter
(A) After amplification of *CBGluc* ORF and *URA3* auxotrophy marker by PCR using the UmK7GU Fw/Bw primers, the sequence was integrated in *C. albicans* CAI4 genome by homologous recombination. After integration, *CBGluc* ORF is downstream of *UME6* promoter. (B) Integration was verified (transformants II-IV) using colony PCR with primers Check iUmK7Gu Fw/Bw; the expected band size is 1034 bp (black arrow) and luminescence measured by mixing 100 µl of cells (1h in YP+ 10 % serum at 37°C; initial OD_{600}=0.2) with 100 µl of Chroma-Glo reagent (C).

1.3.5. Double chromosomal integration of red and green click beetle luciferase genes

Because the luminescence emitted by red and green click beetle luciferase can be read simultaneously in a cell, it is potentially possible to monitor the activity of two promoters at the same time. To test this possibility, the two click beetle luciferases ORFs were integrated into *C. albicans*' genome using the *URA3* and *HIS1* selection markers. Two dual-luciferase strains were constructed. In the first strain the *HWP1* and *ACT1* promoter were tagged simultaneously; in the second strain, both the *HWP1* and *TCC1* promoters were tagged.

1.3.5.1. Double chromosomal integration of click beetle luciferase genes downstream of *HWP1* and *ACT1* promoters

Using the activity of the *ACT1* promoter as a reference is particularly useful, especially if a regulated gene is tested simultaneously. To integrate the first luciferase gene, the pD-CBG plasmid was integrated into the *C. albicans* BWP17 strain as described in Results 1.3.1.1 (integration of *ACT1p-CBGluc* sequence into *LEU2* locus, using the *URA3* selection marker). Transformants were selected on SD plates supplemented with histidine and arginine. The resulting strain, named ipDG-B, contains the *ACT1p-CBGluc* sequence. The integration was verified by colony PCR using the primers check ipDR Fw/Bw (results 1.3.1.1., Figure III.1-9). In the three transformants (I-III) the amplified product size matched the expected PCR product length of 2 133 bp (Figure III.1-17A). The first transformant (I) was taken for the second transformation step. A scheme of the integration is shown in Figure III.1-15A. The second transformation step was performed as described in Results 1.3.3.2. The *CBRluc-HIS1* sequence was amplified by PCR and integrated by homologous recombination into the *HWP1* locus downstream of *HWP1* gene ATG (primers HwK7RH Fw/Bw; corrected sequence). The transformants were selected on SD plates supplemented with arginine. The strain resulting from this second transformation was named HwAc (*HWP1-CBRluc, ACT1-CBGluc*). The integration of *CBRluc-HIS1* was verified by colony PCR using the HwK7RH Dia and Check K7G Bw primers (results 1.3.3.2., Figure1-15). The PCR products size matched the expected length for the 4 transformants tested (743 bp for transformants I-IV, Figure III.1-17B). The scheme for this second integration step is shown in Figure III.1-11A. The luminescence of the HwAc strain is shown in Figure III.3-11.

(A) **(B)**

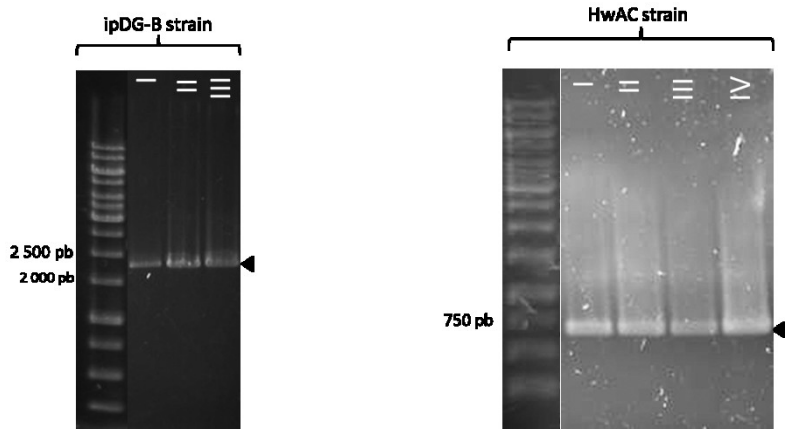


Figure III.1-17. Verification of HwAc strain
(A) Integration of *ACT1p-CBGluc* sequence was checked in strain ipDG-B using colony PCR with the primers check ipDR Fw and Bw (expected product size 2133 bp, black arrow). (B) the HwAc strain (*HWP1p-CBRluc*, *ACT1p-CBGluc*) was verified by colony PCR with the HwK7RH Dia and Check K7G primers (product size 743 bp, black arrow).

1.3.5.2. Double chromosomal integration of click beetle luciferase genes downstream of *HWP1* and *TCC1* promoters

The plasmid carrying the *TCC1-CBGluc* sequence, pTGL, was used for the first transformation step of the HwTc strain. The integration was carried out as described in Results 1.3.2. Transformants were selected for uridine prototrophy on SD plates supplemented with arginine and histidine. The resulting strain was named ipTGL-B. The integration was verified by colony PCR using the C1 and C2 ipTGL primers (Figure III.1-13). A scheme of the integration in the *TCC1* locus is shown in Figure III.1-13A. The colony PCR amplification product had the correct 2175 bp-size for the 4 transformants tested (I-IV; Figure III.1-18A). Transformant I was taken for the second transformation step. The second transformation step, using a PCR generated *CBRluc-HIS* sequence, was performed as described above for the HwAc strain (corrected sequence). The transformants were selected for histidine prototrophy on SD plates supplemented with arginine. The resulting strain was named HwTc. The size of the colony PCR product was correct for 3 of the transformants tested (743 bp for clones I-IV, Figure III.1-18B). The luminescence of the HwTc strain is shown in Figure III.3-12.

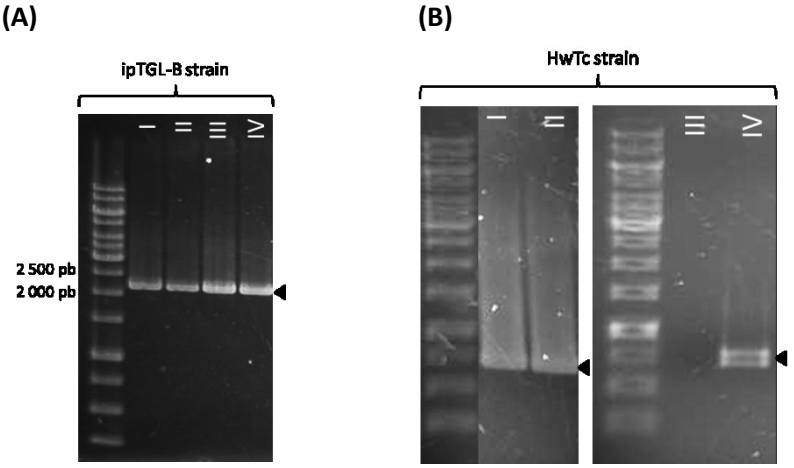


Figure III.1-18. Verification of HwTc strain
(A) Integration of *TCC1p-CBGluc* sequence was checked in strain ipTGL-B using colony PCR with the primers C1 and C2 ipTGL (expected product size: 2175 bp, black arrow). (B) The HwTc strain (*HWP1p-CBRluc*, *TCC1p-CBGluc*) was verified by colony with the HwK7RH Dia and Check K7G primers (product size 743 bp, black arrow).

2. Optimisation of click beetle luciferase activity measurements in *C. albicans*

The use of click beetle luciferases has been extensively described in mammalian cells (Almond *et al.,* 2002) but the methods for testing in *C. albicans* must still be established. The light signal emitted by the luciferases is detected using a luminometer. The choice of the luminometer and related parameters, including optical filters and calibration constants, are described in the following. The assays with luciferases in *C. albicans* (*Renilla* luciferase) relied on the use of crude extract, where cells had to be broken (Srikantha *et al.,* 1996). However, in contrast with coelenterazine, the substrate of the *Renilla* luciferase, beetle luciferin is able to enter *C. albicans* cells (Doyle *et al.,* 2006). The different methods for the testing of click beetle luciferase in *C. albicans* were investigated, including the use of crude extracts and of live cells. Once the detection method for click beetle luciferase assays in *C. albicans* had been established, its sensitivity was evaluated. Because the variation of the luminescence over time can have a big impact on the comparability of the results, the kinetics of the luciferase reactions were assessed.

2.1. Instrument parameters

The accurate and quantitative measurement of luciferase activity is possible with a luminometer. A luminometer quantitatively measures the light emitted by a sample. The chosen luminometer must have an optimal sensitivity for both luciferases. The CBRluc and CBGluc have different emission spectra (Figure III.1-8); therefore, the signal of a specific luciferase can be followed using a set of specific filters. Optic filters have a window of transmittance, allowing only photons with a specific wavelength to go through the filter. The transmittance of a filter is the percentage of the original light signal of the correct colour going through this filter (CBRluc for red filter; CBGluc for green filter). The crosstalk is the amount of undesired signal going through a specific filter (CBRluc with green filter; CBGluc with red filter). Figure III.2-1 shows an illustration of crosstalk and transmittance. The choice of filters for the simultaneous measurement of both the red and green click beetle luciferases is described in the following. Despite the quality of the filters, the signal obtained after filtration is not perfectly selective and must therefore be corrected.

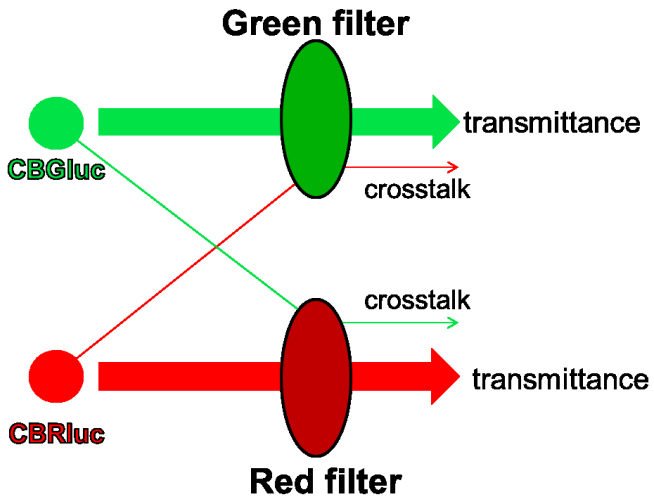


Figure III.2-1. Illustration of light signal separation using optical filters

2.1.1. Luminometer

The click beetle luciferases were first tested with the Fluoroskan Ascent FL luminometer (Thermoscientific). Using this luminometer, both luminescence signal and background noise observed were low. The signal-to-noise ratio (L_{max}/L_{noise}) of the Fluoroskan luminometer was measured using *C. albicans* CR1 and CG1 strains (*ACT1p-CBluc*). The filter used for this experiment was the red filter 610LP; filters are described in detail in Results 2.1.2. For the click beetle red luciferase the signal-to-noise ratio without filter was 1 175.0 but it decreased drastically to 1.2 when using the red filter (Figure III.2-2A and B, Fluoroskan). As shown in Figure III.2-2C, the Fluoroskan luminometer coupled with red filter yielded a very poor transmittance of only 0.25 %, which only allows measurement of very strong red luminescence signals. Therefore, another luminometer, the Berthold Tristar, was used. In Figure III.2-2A the raw data show the difference for red luminescence values between Fluoroskan and Tristar. The signal/noise ratio without filter was higher with the Tristar luminometer with $23.7x10^3$ (Figure III.2-2B). Importantly, the signal-to-noise ratio when using the red filter for Fluoroskan measurement was poor (1.19) and barely above the noise. In the same conditions with the Tristar luminometer, however, the measured signal/noise ratio was excellent with $14x10^3$ (measurements were made using the same samples). Using the Tristar luminometer the transmittance is around 60 % of the unfiltered signal. The level of transmittance with the Tristar luminometer corresponds to the expected yield, when comparing with the overlay of the filter window and CBRluc emission spectrum (Figure III.2-3).

		Fluoroskan	Tristar
A/ Luminescence ± SD (RLU)	**Red filter**	$0.015 \pm 5.25x10^{-5}$	671 145 ± 3 590
	NoFilter	6.232 ± 0.298	1 180 659 ± 477
B/ Signal/noise ratio	**Red filter**	1.19	13 821
	NoFilter	1 175.10	23 737
C/ 610LP transmittance		0.25 %	58.23 %

Figure III.2-2. Comparison of the Fluoroskan Ascent and Tristar luminometers
The luminescence of *C. albicans* CR1 strain (*ACT1p-CBRluc*, multi-copy plasmid) grown in SD was measured after mixing 100 µl of cells (OD_{600}= 0.6) with 100 µl of Chroma-Glo reagent. (A) Raw level of luminescence measured with the red filter (610LP) or without filter for both luminometers in RLU. (B) Ratio of luminescence to background noise of an empty well. (C) Measurement of the luminometer of the amount of unfiltered light passing through the red filter (ratio red filter/no filter). The standard deviation (SD) is calculated from at least 3 technical replicates.

Using the Tristar luminometer, the luminescence of an empty well of a microtiter plate generated a small signal, the background noise (Figure III.2-3). This very low noise signal is dependent on the luminometer and the temperature. An empty well had the same level of luminescence signal as wild type *C. albicans* CAI4 (Figure III.2-3).

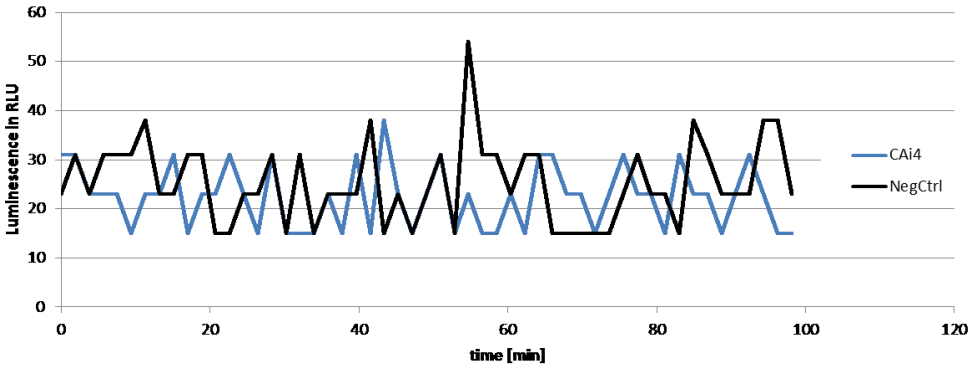


Figure III.2-3. Illustration of the background noise of the Tristar luminometer (Promega)
Comparison of the luminescence of an empty well (NegCtrl) or 100 µl of *C. albicans* CAI4 strain mixed with 100 µl Chroma-Glo reagent (room temperature; 1 second counting time; OD_{600}≈1).

2.1.2. Optical filters

Optical filters are needed to separate the emission signal emitted by the red (CBRluc) and the green (CBGluc) luciferases. The choice of filters is critical to the sensitivity and specificity of the experiment; using broad filters the "yield" would be increased but the signal coming from the other luciferase would also increase (crosstalk). The Figure III.2-4 illustrates the window of transmittance of the filters described below overlaid with the light spectrum emitted by the luciferases.

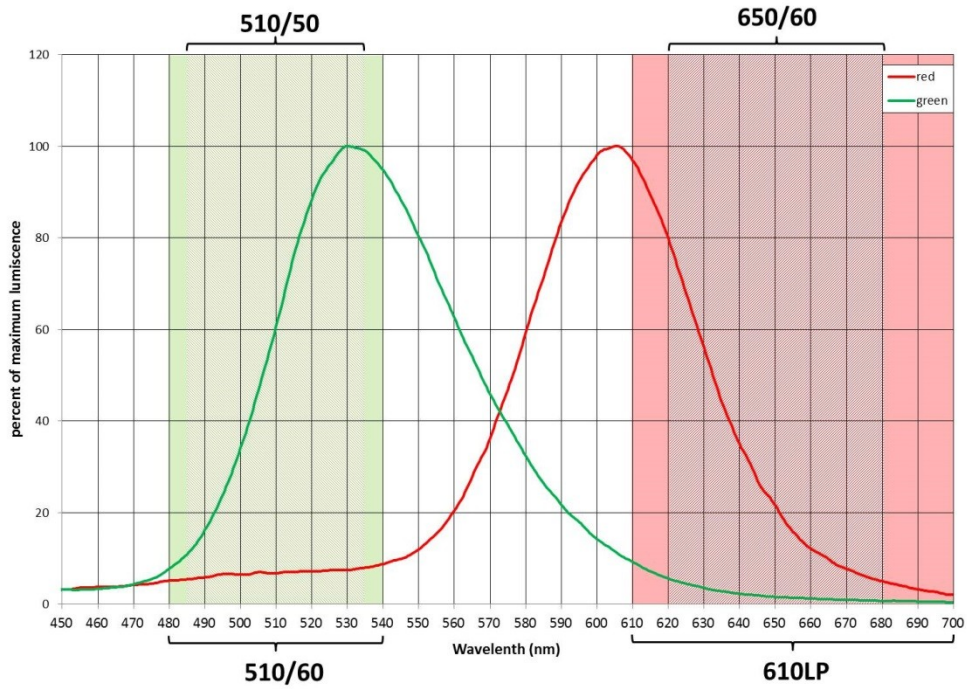


Figure III.2-4. Click beetle luciferases emission spectra and optical filters transmittance windows
The emission spectra of both CBRluc and CBGluc luciferase are displayed. The transmission windows of the filers are shown in overlay. Green filter: 510/50 and 510/60. Red filter: 650/60 and 610LP.

Given the emission wavelength of the click beetle luciferase, the filter choice was narrowed to a set of two filters for both the red and the green luciferase. Figure III.2-5 shows the transmittance and crosstalk characteristics for these filters. The optical filters are described by their peak of transmittance, 510 nm for the green filter for example, followed by a slash and the half-bandwidth. The half-bandwidth is the wavelength where transmittance is at 50 % of its peak value. Because the

considered filters are of high quality, the slope of the transmittance curve at the beginning and the end of the transmittance window is almost 100 %. The half-bandwidth value is therefore equivalent to the transmittance window. A red filter with an emission peak of 650 nm and half-bandwidth of 60 nm will be named 650/60 and have a transmittance window ranging from 590 nm to 710 nm. Another type of filters, the long pass (LP), let through all photons with a wavelength higher than their indicated threshold value.

All filters were obtained from the Chroma Technology Corporation. For the red filters two filters were envisaged, the 650/60 and 610LP. Both 650/60 and 610LP had a good transmittance, around 50 % of the unfiltered signal (Figure II.2-5). Both red filters displayed a similar crosstalk with green luciferase at 2.25 % and 2.13 % for 650/60 and 610LP filters respectively. Although the difference seems marginal the filter 610LP with the lowest crosstalk was chosen. The two green filters tested were the 510/50 and the 510/60. For the green filters, the 510/50 filter performed better than the 510/60 filter by 3 %, despite a smaller transmittance window (Figure II.2-5). The higher quality of the 510/50 filter coating lets a bigger proportion of the light through in its wavelength window. Given the similar crosstalk the 510/50 filter was chosen.

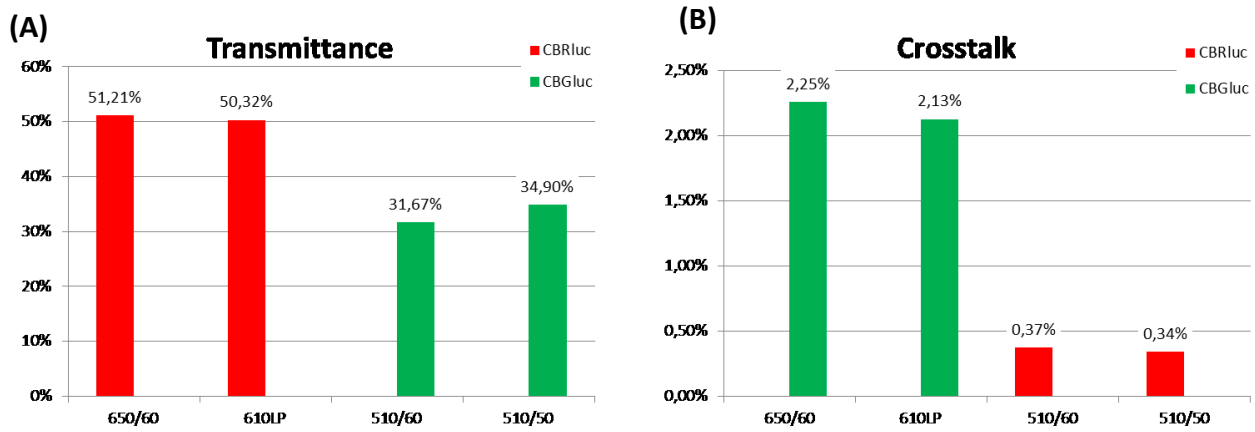


Figure III.2-5. Filter comparison for use with the red and green click beetle luciferases
(A) The graph shows the ratio between the signal measured with the specified filter and the unfiltered signal. Transmittance (CBRluc with red filter, CBGluc with green filter). (B) Crosstalk (CBRluc with green filter, CBGluc with red filter). *C. albicans* CR1 or CG1 (*ACT1p-CBluc*, multicopy plasmid) were grown in SD medium. Luminescence was assessed by mixing 100 µl of cells with 100 µl Chroma-Glo reagent. Measurements were made using the Berthold Tristar luminometer

2.1.3. Calibration constants

When measuring the red and green luciferase activity simultaneously, the filtered signal must be corrected for transmittance and cross-talk (Figure III.2-1 and III.2-4). The correction factor depends on the luminometer/filter couple and the luciferase used. To calculate the correction constants, 3 parameters for each luciferase were measured using pure culture of either the red or the green click beetle luciferase. After these values have been established once on pure cultures, they remain constant as long as the trio luciferase, luminometer and filters are unchanged.

R: unfiltered luminescence for red click beetle luciferase; Rrf: luminescence for red luciferase, red filter (610LP); Rgf: red luciferase, green filter

G: unfiltered luminescence for the green luciferase; Ggf: green luciferase, green filter (510/60); Grf: green luciferase, red filter.

Using these fixed parameters and the measured experimental values Lrf (luminescence red filter) and Lgf (luminescence green filter), it is possible to calculate the corrected red signal (R') from the signal measured through the 610LP filter (Davis *et al.*, 2007):

$$R' = \frac{Lrf - Lgf \times \frac{Grf}{Ggf}}{\frac{Rrf}{R} - \frac{Rgf}{R} \times \frac{Grf}{Ggf}}$$

Similarly the corrected green signal (G') can be calculated with the signal passing through the 510/60 filter (G) and the previously calculated R' signal:

$$G' = \frac{Lgf - R' \times \frac{Rgf}{R}}{Ggf/G}$$

As previously mentioned, in order to measure the correction constants, the luminescence of pure cultures of strains expressing either the red or the green click beetle luciferase must be measured without filters and with both red and green filters. Thus, pure cultures from the HwK7GU (*HWP1p-CBGluc*) and ipDR (*ACT1p-CBRluc*) strains were made. Their luminescence was measured by mixing cells with the Chroma-Glo reagent. Luminescence was read with the Berthold Tristar luminometer using either no Filters (R and G), the 610LP filter (Rrf and Ggf), or the 510/60 filter (Ggf and Grf). The constants (R, Rrf, Rgf, G, Ggf and Grf) shown in Figure III.2-6 were measured using these pure cultures.

R	134 391	G	60 123
Rrf	81 237	Grf	2 788
Rgf	41	Ggf	14 319

Figure III.2-6. Calibration constants
Cells from the *C. albicans* strains HwK7GU (*HWP1p-CBGluc*) and ipDR (*ACT1p-CBRluc*) were incubated for 3 h at 37 °C in YP+10 % serum at an initial OD_{600}=0.2. Luminescence was measured after mixing 100 µl of cells with 100 µl Chroma-Glo reagent using the Tristar luminometer, either with no filter, the 510/60 or the 610LP filter. The correction constants shown are values at time-point t=31 min where values were closest to the average. The constants are measured in RLU.

2.2. Methods for testing click beetle luciferases in *C. albicans*

Previously, the most common luminescence reporter in *C. albicans* was the sea pansy *Renilla* luciferase. Coelenterazine, the reaction substrate for *Renilla* luciferase, is not able to enter *C. albicans* cells. Luminescence was therefore measured in crude extract after breaking *C. albicans* cells. On the contrary, the substrate of click beetle luciferase (beetle luciferin) had been described by Doyle *et al.* (2006) as being able to enter *C. albicans* cells, making a quick and easy assay of luciferase activity in live cells possible. The click beetle luciferase produces light by oxidation of beetle luciferin in the presence of ATP (Viviani *et al.,* 2002). Other co-factors like $MgCl_2$ are also required. Different reagents were assessed, the intensity of their light signals was compared and their impact on *C. albicans* growth assessed. Different sample preparation methods were tested and the luminescence of the resulting probes was compared with crude extracts.

2.2.1. Luciferase substrates

The first reagent tested is the luciferin-EF (Endotoxin Free) from the company Promega. This reagent contains only beetle luciferin without added substances. To measure the luminescence, *C. albicans* cells transformed with the pD-CBG plasmid (*ACT1p-CBGluc*) and expressing the green click beetle luciferase were mixed with various concentrations of luciferin-EF. As both luciferases use the same substrate, the luciferin concentration (160 µg/ml) used for assays with the firefly luciferase by Doyle *et al.* (2006), was included in this experiment. As shown in Figure III.2-7, the level of luminescence did not increase significantly with the luciferin concentration, suggesting that in presence of approximately $4x10^7$ cells, the luciferin is in excess in the reaction. A concentration of 160 µg/ml was found to be optimal.

The commercial Chroma-Chroma-Glo reagent (Promega) had been previously developed for use of beetle luciferases with mammalian cells (Almond *et al.,* 2003; Davis *et al.,* 2007). This reagent was designed to lyse the mammalian cells and provide the optimal buffer environment for luciferase activity; the exact composition of this reagent has however not been disclosed by the company. The Chroma-Glo reagent efficiency was compared with pure luciferin-EF (Figure III.2-8). In *C. albicans* cells expressing the click beetle red luciferase under the control of the *ACT1* promoter (ipD-R strain), the luciferase activity was 26 fold higher when using Chroma-Glo reagent compared to luciferin-EF. The Chroma-Glo reagent was therefore used as the luciferase substrate for further experiments.

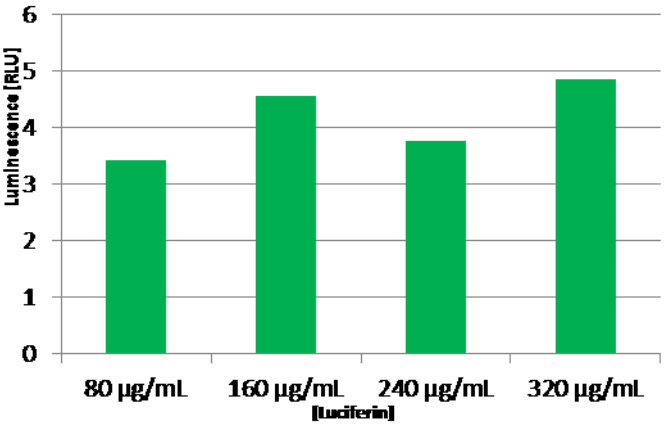


Figure III.2-7. Luminescence of *C. albicans* live cells with different luciferin concentration
C. albicans cells were transformed with the pD-CBG plasmid and grown in SD medium. 200 µl of live cells (OD_{600}=6.5) were mixed with 5 to 20 µl of luciferin-EF (Promega) at 3,6 mg/ml. Luminescence was measured with the Fluoroskan Ascent FL.

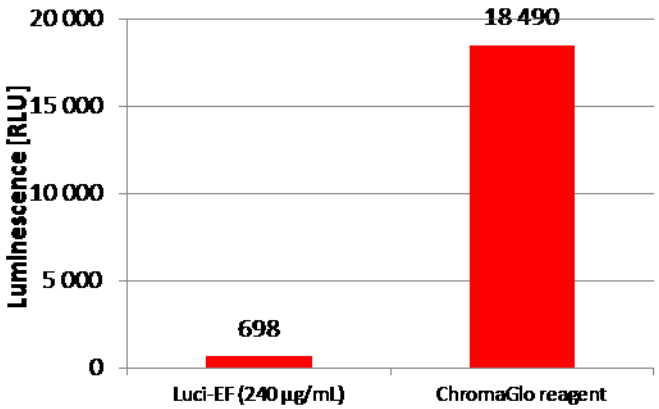


Figure III.2-8. Comparison of luminescence using luciferin-EF and Chroma-Glo reagents.
Live cells of *C. albicans* ipDR strain (*CBRluc-ACT1*) were grown in YPD. The luminescence was measured with Berthold Tristar after mixing 100 µl of an overnight culture (OD_{600}=0,2) with either 15 µl of luciferin-EF (3,2 mg/ml) or 100 µl of Chroma-Glo reagent.

2.2.2. Effects of the Chroma-Glo reagent on *C. albicans* cells

The ability of *C. albicans* cells to survive treatment with Chroma-Glo reagent was tested. For this purpose, *C. albicans* CAI4 cells were incubated for 1 h with Chroma-Glo reagent and were then plated on YPD medium to check for growth. No differences in survival were observed when compared with water, indicating that *C. albicans* is able to survive treatment with the Chroma-Glo reagent (Figure III.2-9).

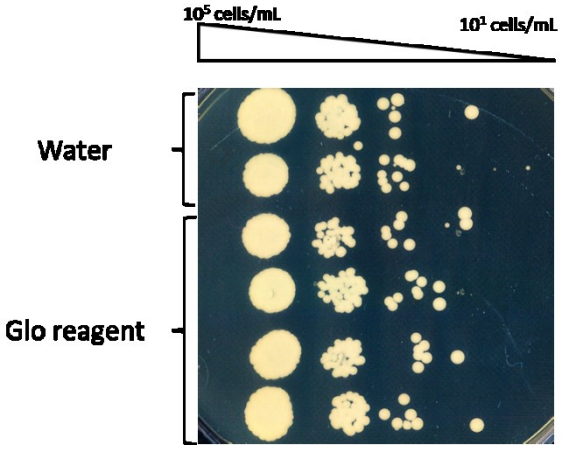


Figure III.2-9. Drop test assessing Chroma-Glo reagent toxicity for *C. albicans* cells
100 µl of cells from an overnight culture of *C. albicans* CAI4 were incubated with 100 µl of Chroma-Glo reagent or 100 µl of water for 1h at 30°C. Following this treatment, cells were diluted in water to 10^5 cell/ml and plated in series of tenfold dilution on YPD (10 µl drops).

To verify growth of in the presence of Chroma-Glo reagent, cultures of *C. albicans* were inoculated in YPD medium with either Chroma-Glo reagent or water. Probes were taken repeatedly and plated on YPD to measure the Colony Forming Units (CFU). Cells treated with Chroma-Glo reagent were unable to multiply, whereas cells treated with water grew normally (Figure III.2-10). Chroma-Glo reagent stopped the growth of *C. albicans.* These results show that Chroma-Glo reagent is unable to lyse *C. albicans* cells but can prevents growth.

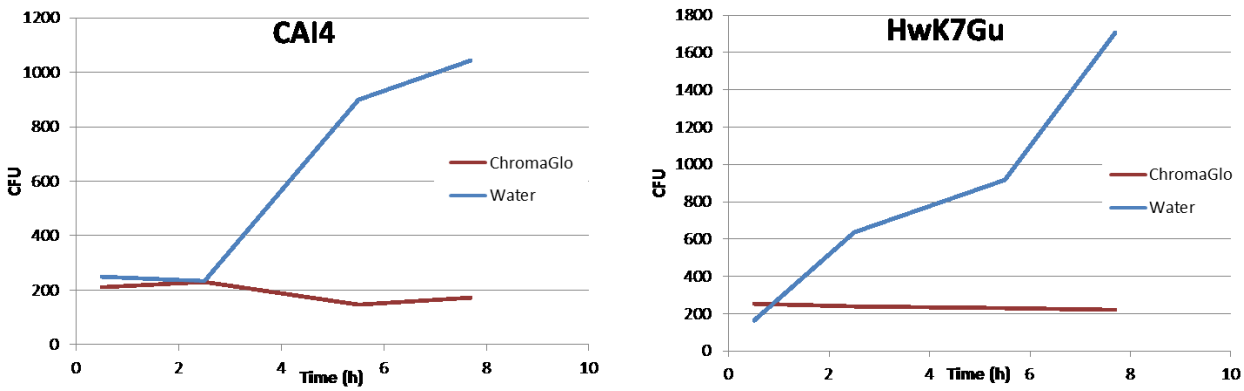


Figure III.2-10. Influence of Chroma-Glo reagent on the growth of *C. albicans*
A pre-culture of *C. albicans* cells CAI4 or iHwK7GU (*HWP1p-CBGluc*) was grown in YPD overnight. The main culture was inoculated at OD_{600}=0,2 in YPD. 500 µl of either Chroma-Glo reagent or water was added to 500 µl cells. Cultures where then incubated at 30°C for 8 h. Probes were repeatedly taken and plated on YPD. Colony Forming Units (CFU) were counted after 3 day of incubation at 30°C.

The capacity of *C. albicans* cells to form hyphae in presence of Chroma-Glo reagent was also tested. Cells were incubated at 37 °C in hyphal inducing medium, (YP+10 % serum, RMPI or 0.5 % N−acetylglucosamine) and examined under the microscope. After 1-3 h of growth in hypha-inducing conditions, cells handled with water showed normal hyphal development in all three media, whereas cells treated with the Chroma-Glo reagent did not form hyphae (Figure III.2-11).

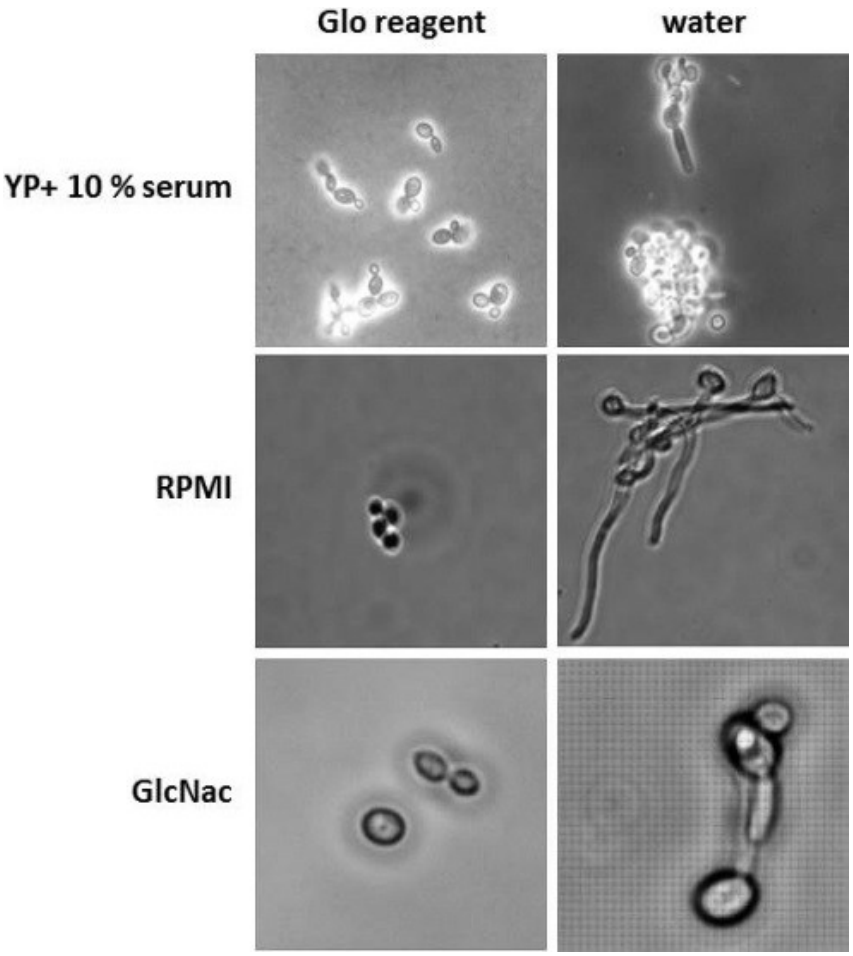
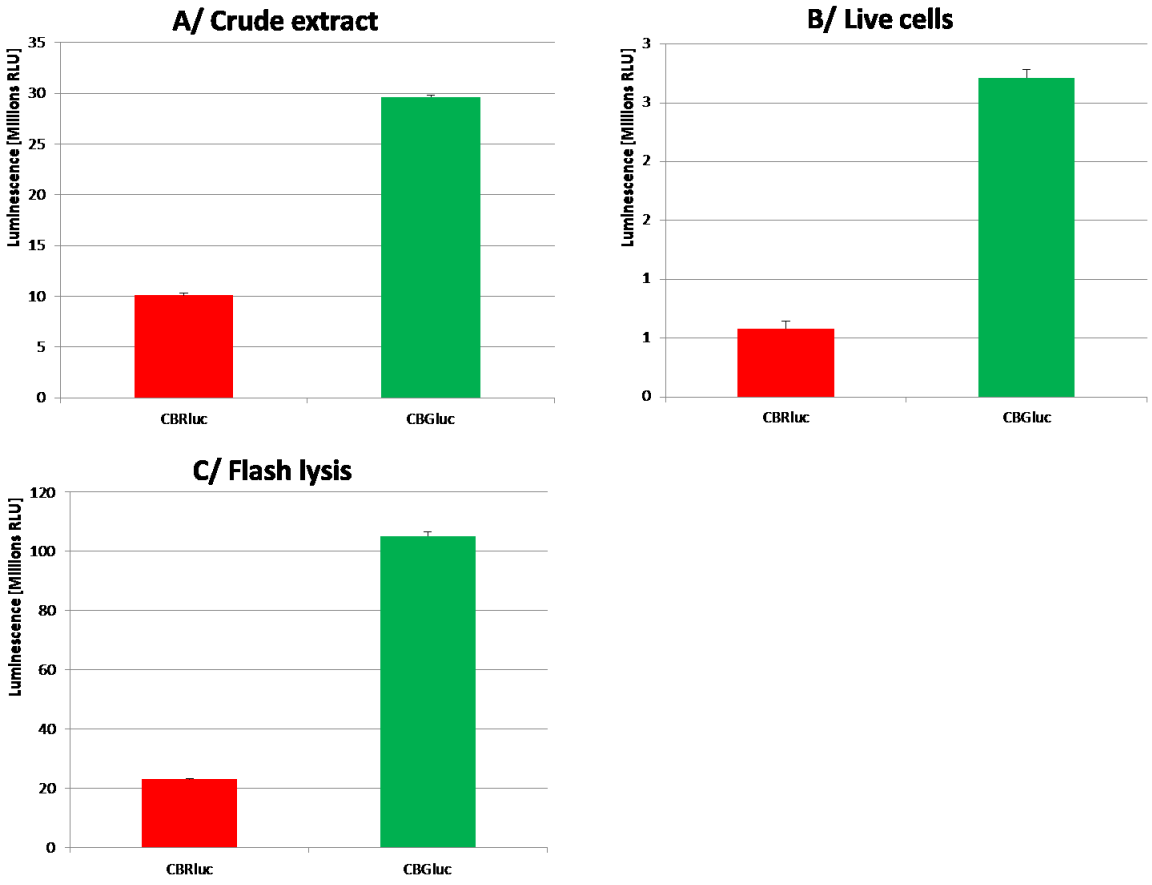


Figure III.2-11. Impact of Chroma-Glo reagent on hyphal induction
C. albicans cells were inoculated in the specified medium and mixed with Chroma-Glo reagent or water (500 µl cells with 500 µl Chroma-Glo reagent). Cells were incubated during 1.5 h for YP+ 10 % serum; 3 h for RMPI and 1h for 0.5 % N−acetylglucosamine (GlcNAc; 37°C with shaking). Photography were taken using Axioskop 40 microscope (Zeiss) combined with Axiocam camera (Zeiss).

2.2.3. Sample preparation methods

Because coelenterazine, the reagent for Renilla luciferase (Rluc), is unable to enter the cells, the method for assessing *Renilla* luciferase luminescence in *C. albicans* was based on the use of crude extracts (Tebarth *et al.,* 2003). The click beetle luciferase can be assayed in live cells because *C. albicans* cells are permeable to beetle luciferin (Doyle *et al.*, 2006). The ipDR and ipDG strains (*ACT1p-CBluc*), in which the click beetle luciferase gene is inserted downstream of the *ACT1* promoter in the *LEU2* locus, have been used to compare the different sample preparation methods. For crude extracts, cells were broken using mechanical disruption with glass beads and re-suspended in lysis buffer supplemented with protease inhibitor (Material and methods 8.1.1). The concentration factor inherent to crude extract preparation was taken into account and the luminescence values reported are for the equivalent of 100 µl of cell culture. Using crude extracts, the luminescence was $1.1x10^7$ RLU for ipDR strain and $3x10^7$ RLU for ipDG (OD_{600}≈7; Figure III.2-12A)

The preparation of crude extract is time extensive and live cells culture assays could be a valuable alternative. Cell samples were taken from the same ipDR and ipDG cultures used for the afore-mentioned crude extract evaluation. Their luminescence was assessed after mixing 100 µl of cells with 100 µl of Chroma-Glo reagent. The ipDR strain displayed a luminescence of $5.8x10^5$ RLU and ipDG $2.7x10^6$ RLU (Figure III.2-12B). Because the crude extract luminescence values were calculated for an equivalent of 100 µl cell culture and because the samples came from the same cultures, the luminescence values listed above for crude extract and live cell assays are directly comparable. These results demonstrate that live cell luciferase assays are a very effective way to measure click beetle luciferase activity in *C. albicans*. The permeability of *C. albicans* cells to beetle luciferin is also confirmed. The luminescence reaction can be performed in smaller volumes, with a ratio culture/Chroma-Glo reagent of 1/1. For smaller volumes, smaller wells were needed to insure that the reagent and sample can mix properly (386 well plates for V_f =50 µl). 100 µl final volume was the limit for optimal use of 96 well-plates (data not shown). Use of smaller volumes is particularly useful when screening large numbers of transformants, but the scattering of the measurements using smaller samples was higher (data not shown).



<u>Figure III.2-12</u>. Luminescence using 3 different methods of sample preparation

C. albicans cells from ipDR and ipDG strains (*ACT1*p-*CBRluc* and *ACT1*p-*CBGluc*) were grown overnight (YPD; 30°C; OD_{600}≈7). After sample preparation as listed below, luminescence was assessed after adding 100 µl of Chroma-Glo reagent to the probes. Error bars show the standard deviation for 3 technical replicates. (A) Crude extract: cells were re-suspended in *C. albicans* lysis buffer and broken using glass beads. 1 ml of crude extract were yielded from 2 ml of cell culture. Crude extracts luminescence values were then divided by 2 to take into account the concentration factor. (B) Live cells: 100 µl of untreated cells were mixed with 100 µl Chroma-Glo reagent (C) Flash lysis: 200 µl of cells were re-suspended in the same volume of Glo-Lysis buffer and (after 5 min incubation) frozen in liquid nitrogen. After thawing at room temperature, 100 µl of the resulting suspension were mixed with 100 µl of Chroma-Glo reagent.

As the luminescence of live cells was lower than crude extracts, a method more sensitive than live cell assay without the inconveniences of crude extracts preparation would be advantageous when maximal sensitivity is critical.

Three approaches were tested: (i) treating cells with Chroma-Glo lysis buffer (Promega); (ii) freezing cells in liquid nitrogen; (iii) freezing cells previously treated with the Chroma-Glo lysis buffer. A comparison of these three methods is shown in Figure III.2-13. The addition of Chroma-Glo lysis buffer did not change the light output compared to water. The congelation of the cells in liquid nitrogen followed by thawing at room temperature improved the luminescence considerably (4.4 fold compared to water). For cells treated with Chroma-Glo lysis buffer (5 min) and frozen in liquid nitrogen, the luminescence was the strongest with a 33 fold increase compared to untreated cells. This "flash lysis" method was tested with the same ipDR and ipDG cultures used for Figure III.2-12 to assess luminescence of crude extract and live cells. With flash lysis, the luminescence was $2.3x10^7$ for ipDR culture and $1.5x10^8$ for ipDG. Moreover, the scattering of the measurements between replicates was low (Figure III.2-12).

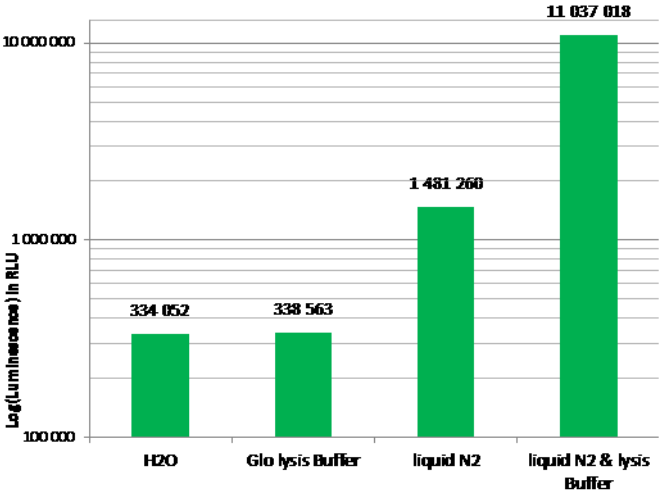


Figure III.2-13. Comparison of sample preparation methods in *C. albicans*
Cells from *C. albicans* ipDG (*ACT1p-CBGluc*) were grown overnight to OD_{600}=5.5 (YPD; 30°C). 100 µl of cells were centrifugated cells were either (i) resuspended in water (ii) treated with Chroma-Glo lysis buffer (Promega); (iii) frozen in liquid nitrogen; (iiii) re-suspended in Chroma-Glo lysis buffer and frozen in liquid nitrogen. Cells treated with liquid nitrogen where left to thaw at room temperature before luminescence measurement. Luminescence was measured after adding 100 µl of Chroma-Glo reagent to the samples.

A direct comparison of the three sample preparation methods is shown in Figure III.2-14 (ipDR strain, *ACT1p-CBRluc*). Because the luminescence values are expressed for 100 µl of the original cell culture, the three measurements are directly comparable. Overall, concentration factors taken into account, crude extracts yielded 7±0.7 fold higher luminescence than live cells assay. Flash lysis was 37±3.3 fold more efficient than live cell assay. However, it should be considered that the crude extract can be concentrated to increase the output (e.g. using 50 ml culture for 1 ml final volume). In conclusion, the live cell assays are a valid approach to asses click beetle luciferase signal in *C. albicans*. When maximum sensitivity is needed, the flash lysis method is a good alternative to live cell experiments, while being easier and quicker than crude extracts preparation.

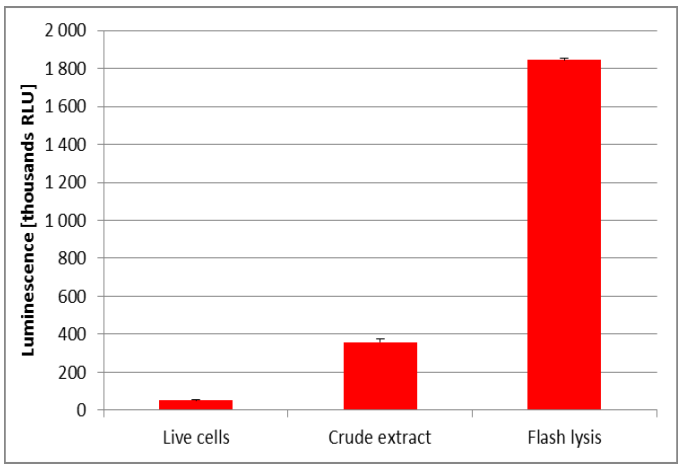


Figure III.2-14. Luminescence comparison of 3 sample preparation methods
C. albicans ipDR strain (*ACT1*p-*CBRluc*) cells (grown in YPD at 30°C; OD_{600}≈7) were either: (i) left untreated (live cells); (ii) broken in crude extract (II.8.1.1; concentration factor taken into account); flash-lysed (II.8.1.2). Luminescence was then assessed by adding 100 µl Chroma-Glo reagent to the samples. Errors bars show standard for 3 technical replicates. Exposition time: 100 ms instead of the usual 1 sec to avoid sensor saturation. Luminescence for 100 µl original culture is shown.

2.3. Sensitivity

The detection of weakly active promoters can be challenging for a gene reporter system. One of the advantages of luminescent reporters is their wide range of sensibility. Because there is no background light signal, the sensitivity threshold is low. The optimal counting time (or exposition time) was assessed and the sensitivity threshold of the click beetle luciferase gene reporter in *C. albicans* was evaluated.

2.3.1. Counting time, noise and signal strength

Using the Mithras luminometer (Berthold), the exposition time (counting time) is typically 1 sec but can be set from 50 msec to 10 sec. A longer counting time will increase also the signal strength; however, the noise level rises as well. To evaluate the optimal counting time and to measure where the noise/signal ratio is the highest, ipDR and ipDG strains were used (*ACT1p-CBluc*). The luciferase activity of diluted cultures were measured with counting times from 50 msec to 4 sec and related to the corresponding noise signal. As previously mentioned, luminescence was measured over 200 min of luciferase reaction and the maximal luminescence value (L_{max}) was recorded. Measurement results are shown in Figure III.2-15A. The ratio signal/noise increased until 0.5 sec and remained stable thereafter (Figure III.2-15B). According to these results, 0.5 sec is the optimal counting time and there is little difference in signal strength compared to the standard 1 sec exposition time.

(A)

Exp. Time (sec)	Noise average (RLU)	CBGluc L_{max} (RLU)	CBRluc L_{max} (RLU)
0,05	1,6	276	77
0,1	2	583	169
0,5	8,8	6 897	1 412
1	23,9	14 017	2 808
2	46,9	27 858	5 708
3	68,0	41 676	8 278
4	84,4	55 478	11 186

(B)

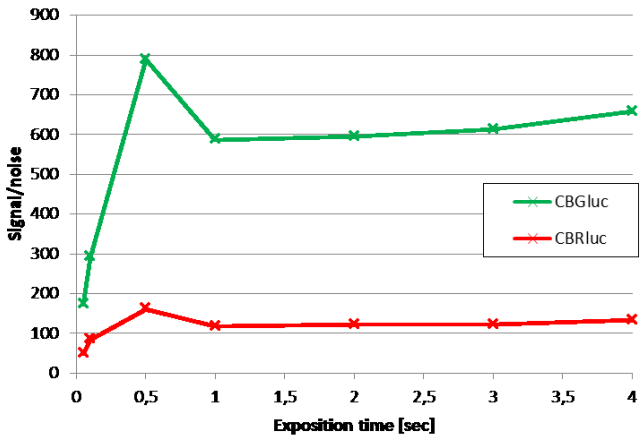


Figure III.2-15. Variation of the exposition time and its impact on noise and signal strength
C. albicans cells of the strains ipD-R and ipD-G (*ACT1p-CBRluc* and *ACT1p-CBGluc*) were grown in YPD medium and diluted to OD_{600}=0.1. Luminescence was then measured by mixing 100 µl cells with 100 µl Chroma-Glo reagent. The exposition time was set from 50 msec to 4 sec. (A) background noise average and L_{max} values for CBRluc and CBGluc. (B) Ratio signal/noise as a function of the counting time (exposition time).

2.3.2. Sensitivity threshold with a strong promoter

The sensitivity threshold was evaluated using serial dilutions of exponentially growing *C. albicans* cells of ipDR and ipDG strains (*ACT1p-CBRluc* or *ACT1p-CBGluc*). After mixing the diluted cells with Chroma-Glo reagent, luciferase activity was measured. To verify cell concentration, dilutions of the cell cultures were plated on YPD medium and the Colony Forming Units (CFU) were counted. Figure III.2-16 shows the luminescence signal in function of the number of CFU per well. No luminescence distinct from the background could be seen at 6 CFU/well. Remarkably, at a concentration of only 60 CFU/well (OD_{600}=2x10^{-5}), a luminescence signal distinct from the background could be measured: 184 RLU for the red luciferase and 222 RLU for the green click beetle luciferase (background noise=54 RLU). At concentration >600 CFU/well, the luminescence signal increased steadily with a maximum luminescence of 10^5 RLU and 3.5x10^5 RLU for the red and green click beetle luciferases at 6x10^5 CFU/well.

A concentration above 100 CFU/well (1 000 cells/ml) insured accurate measurements, but in optimal conditions, the detection threshold for click beetle luciferases was only of a few dozen cells.

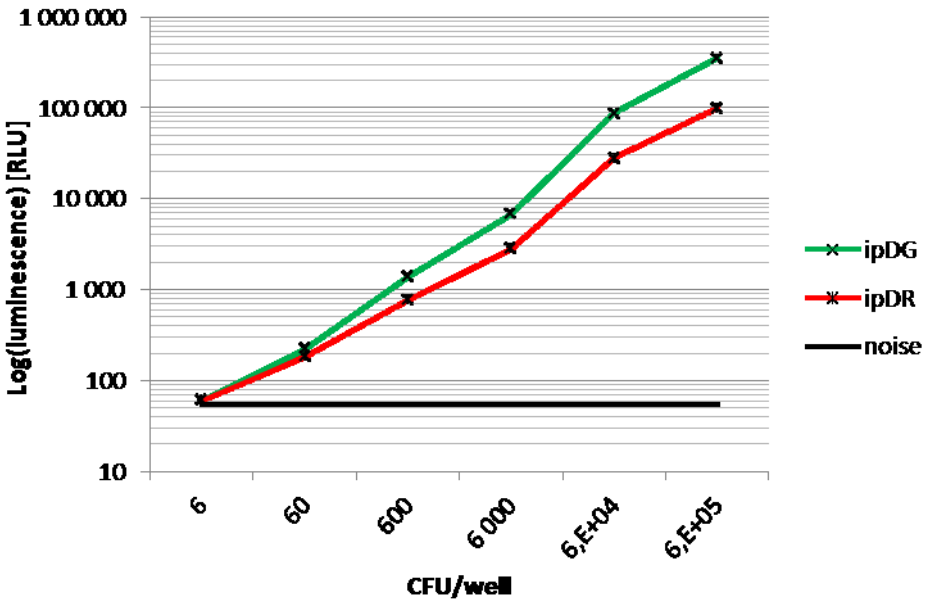


Figure III.2-16. Detection threshold of click beetle luciferases in *C. albicans*

C. albicans strains ipDR (*ACT1p-CBRluc*) and ipDG (*ACT1p-CBGluc*) were grown to OD_{600}=0,6-0,8 in YPD (exponential growth). The cultures were then diluted to the indicated concentrations and luminescence measured after mixing 100 µl of cells with 100 µl Chroma-Glo reagent. The background noise was measured on an empty well.

2.4. Kinetics of the luciferase reactions

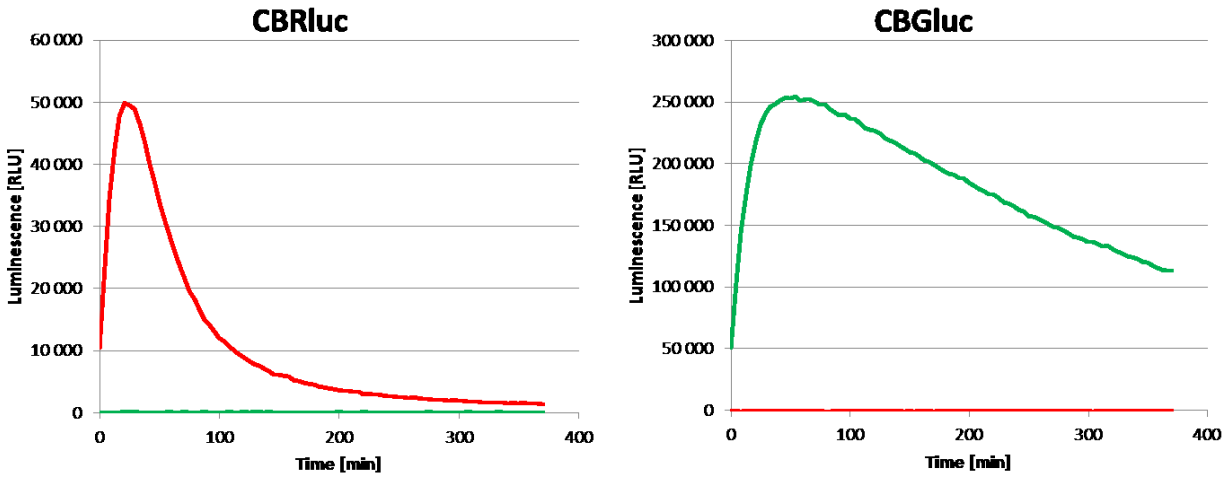
The primary objective of this work was the use of click beetle luciferases in live cell assays and the monitoring of the activity of a target promoter over time *via* luminescence measurements. In the following, the kinetics of the luminescence reaction and its repercussions on the experimental protocol are described. In order to verify if the components of the reagent are limiting factors in the luciferase reaction, the impact of the addition of Chroma-Glo reagent during the luminescent reaction was also evaluated. The impact of cycloheximide was investigated in order to determine the role of newly synthetised proteins on the kinetics of luminescence.

2.4.1. Kinetics of luciferase reaction in live cells

In order to understand the dynamics of the luciferase reaction, the luminescence of *C. albicans* cells expressing the click beetle luciferases was measured over time.

The time-dependent luminescence activity of *C. albicans* live cells transformants producing either the red or green click beetle luciferase is shown in Figure III.2-17A (CR1 and CG1 strains: *ACT1p-CBRluc* or *ACT1p-CBGluc*, multicopy plasmid). The peak luminescence (L_{max}) was reached after 40 min reaction time. A variation in L_{max} time-point of ±10 min was observed from one experiment to the other (data not shown). After the peak, the light signal decreased slowly in an asymptotical manner with a half-life of approximately 50 min for the red click beetle luciferase (CBRluc). The half-life of the light signal was significantly higher, 250 min, for the green click beetle luciferase (CBGluc). The light signal of the green click beetle luciferase is more stable than CBRluc. Since the exact timing of the peak varied from one experiment to the other, measuring the light signal at a definite time point after mixing the reagents did not give the most accurate results, while the maximal luminescence peak value (L_{max}) was reproducible. In *S. cerevisiae*, the maximal luminescence was higher than in *C. albicans* and occurred after a longer reaction time (50 min and 130 min for the red and green click beetle luciferases; Figure III.2-17B). The signal half-life was also higher in *S. cerevisiae.*

(A) *C. albicans*



(B) *S. cerevisiae*

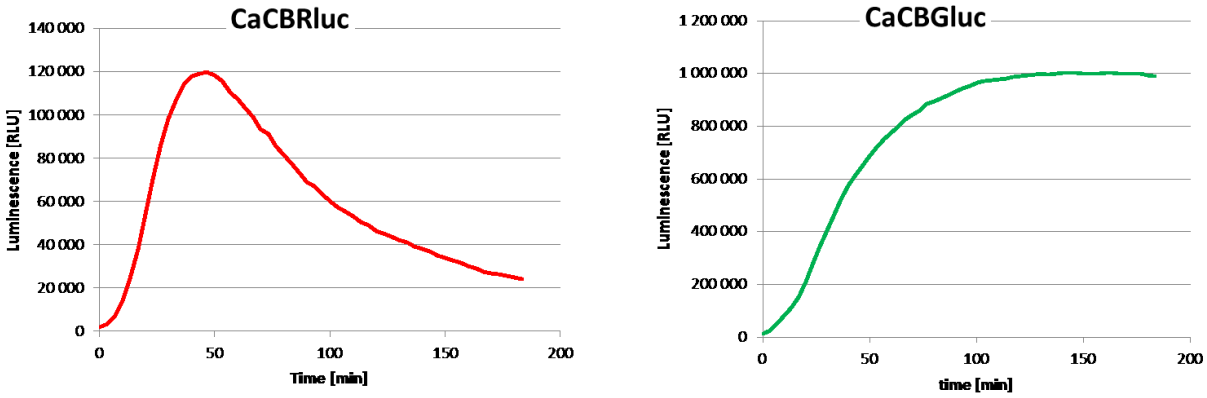


Figure III.2-17. Kinetics of click beetle luciferase activity in *C. albicans* and *S. cerevisiae*

(A) *C. albicans* cells from CR1 and CG1 strains (*ACT1p-CBRluc* or *ACT1p-CBGluc*, multicopy plasmid) were grown in SD medium (30 °C) and luminescence assessed by mixing 100 µl of diluted cell cultures (whole cells; OD_{600}=0,02) with 100 µl of Chroma-Glo reagent. (B) *S. cerevisiae* cells from the strains SCR1 and ScG1 (*GAL1p-CaCBluc*) were grown in S-Galactose (inducing medium). Luminescence was measured after mixing 100 µl of diluted cell cultures ($OD_{600}=10^{-3}$) with 100 µl Chroma-Glo reagent.

To assess the effect of cell concentration on the kinetics of the luciferase reaction, luminescence of cells from the CR1 and CG1 strains (*ACT1p-CBRluc* or *ACT1p-CBGluc*, multicopy plasmid) was measured. Cells were first grown in SD medium and diluted in water at different concentrations (10^4 to 10^6 cell/ml). The luminescence was measured after mixing 100 µl of diluted cells with 100 µl Chroma-Glo reagent. In the concentration range tested here, the dynamics of the reaction is unrelated to cell concentration, only the amount of light emitted changed (Figure III.2-19). The luminescence kinetics were very similar when *CaCBRluc* or *CaCBGluc* were expressed in *S. cerevisiae* (Figure III.2-17B). In comparison, the peak occurred very early with the *Renilla* luciferase, just a few seconds after mixing the reagents and the signal decreased rapidly (half-life ≈ 60 sec; Figure III.2-18). These results show that the signal of the *Renilla* luciferase was much less stable than the click beetle luciferases.

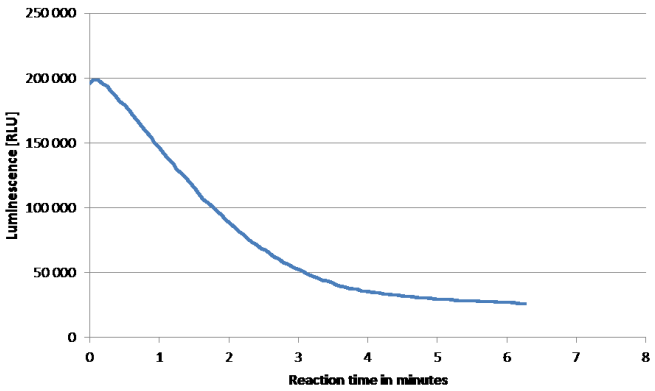


Figure III.2-18: Kinetics of *Renilla* luciferase activity in *C. albicans*

Crude extracts of *C. albicans* cells expressing the *Renilla* luciferase (*PMT1*p-*RLUC*) were prepared. Luminescence reaction was then started by mixing 20 µl of crude extract with 100 µl of *Renilla* Luciferase Assay Reagent (Promega). *Strain courtesy of Julia Koopmeiners*

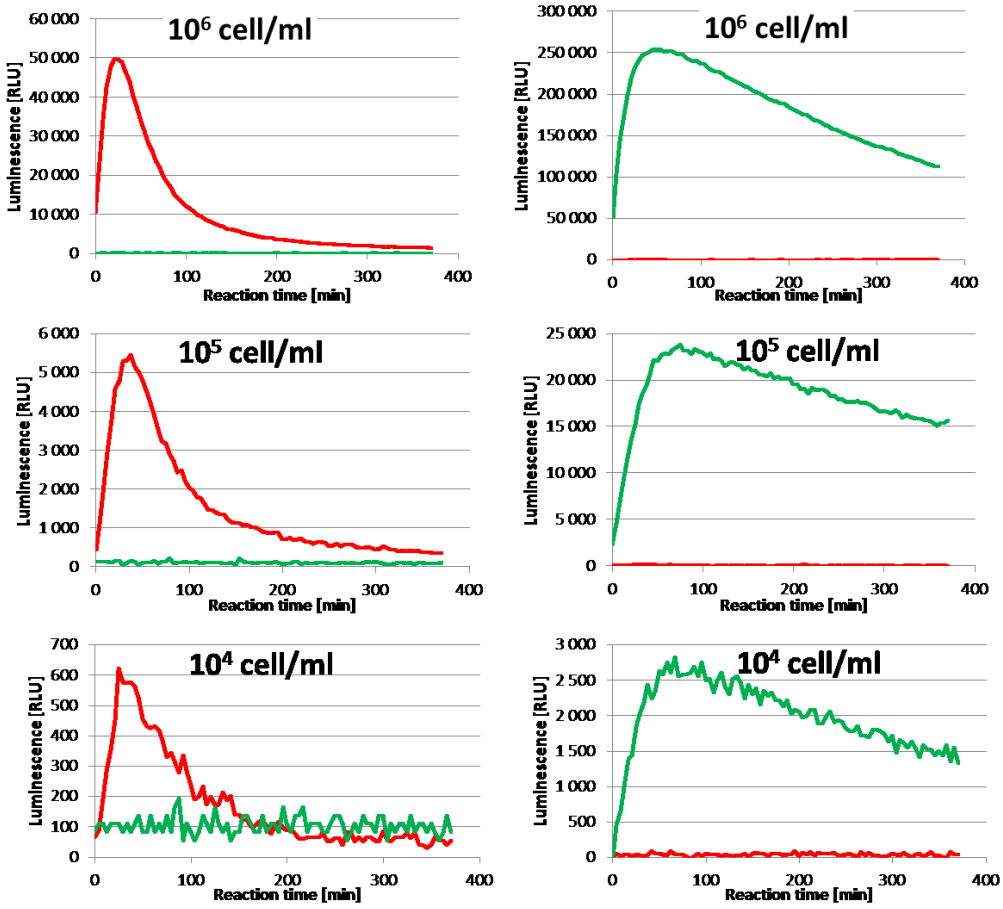


Figure III.2-19. Comparison of luminescence over time at different cell concentrations
C. albicans cells from CR1 (Left column; *ACT1p-CBRluc*) and CG1 strains (Right column; *ACT1p-CBGluc*) were grown overnight in SD medium (30 °C) and diluted to the indicated concentration in water (10^4-10^6 cell/ml). Luminescence was then measured after mixing 100 µl of diluted cell (whole cells) with 100 µl of Chroma-Glo reagent. The cell concentration was verified by plating dilution of the cells on YPD medium and counting the CFU.

2.4.2. Impact of the addition of Chroma-Glo reagent on luciferase reaction kinetics

The addition of fresh reagent could have an impact on the kinetics of the click beetle luciferases' signal, especially if one of the components of the reagent is a limiting factor. Therefore, to assess the influence of the addition of reagent during the reaction, Chroma-Glo reagent was repeatedly added (at 10 min intervals). Injection of fresh Chroma-Glo reagent had no impact on the reaction kinetics (Figure III.2-20). The luminescence with regular addition of Chroma-Glo reagent was similar to the control samples (without injection or with water). These results show that the reagent was in large excess compared to the luciferase. The decrease in the light output was thus not due to the lack of substrate. The *ACT1* promoter used in this experiment is a strong promoter and the reagent most likely will not be a limiting factor for weaker promoters.

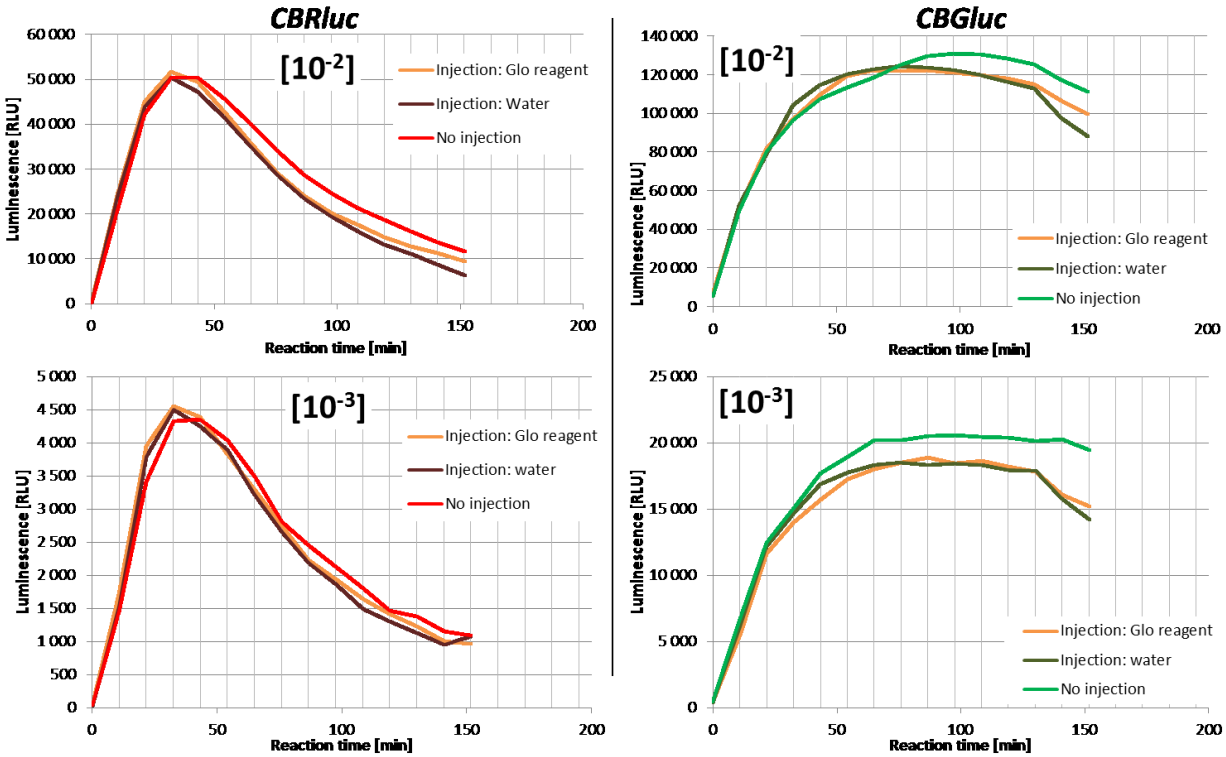


Figure III.2-20. Repeated addition of fresh Chroma-Glo reagent

Cells from the *C. albicans* CR1 and CG1 strains (*ACT1p-CBRluc* or *ACT1p-CBGluc*, multicopy plasmid) were grown in SD medium to OD_{600}=0.6. After a dilution in water (OD_{600}=10^{-3} or 10^{-2}), luminescence was measured after mixing 100 µl of cells with 100 µl of Chroma-Glo reagent. Every 11 min, 10 µl of Chroma-Glo reagent or water was injected in the reaction well. As a control, the luminescence was measured without injection.
(Experiment done in collaboration with S. Von Alen).

2.4.3. Impact of cycloheximide

To test if *de novo* protein synthesis would have an impact on the light output, especially on signal decay, cycloheximide was used (CHX). CHX binds to ribosomes and prevents translation; it is thus an inhibitor of protein synthesis in eukaryotes (Schneider-Poetsch *et al.,* 2010). *C. albicans* CR1 and CG1 strains (*ACT1p-CBRluc* or *ACT1p-CBGluc*, multicopy plasmid) were grown in SD medium. These cells were mixed with Chroma-Glo reagent and luminescence over time was measured. Light emission was monitored for two biological replicates for both luciferases at two different dilutions and compared the level of luminescence in the presence or absence of CHX (10 mg/ml final concentration; Imanishi *et al.*, 2004). The inhibition of protein synthesis by CHX at the concentration used was verified by observing the consequences of a treatment on hyphal induction. The cells treated with CHX (10 mg/ml) were unable to form hyphae, in contrast to cells handled with water (YP+ 10 % serum; 37°C; initial OD_{600}=0.2 - data not shown). Figure III.2-21 shows that treating cells with CHX had no impact on luminescence dynamics. The impact of the treatment on maximal luminescence was also analysed. The ratio $Lmax_{CHX}$/ $Lmax_{untreated}$ was 0.99 (±0.11) indicating that CHX had no impact on maximal luminescence or dynamic. *De novo* protein synthesis did not seem to occur during the luciferase measurements; this is probably due to the impact of Chroma-Glo reagent

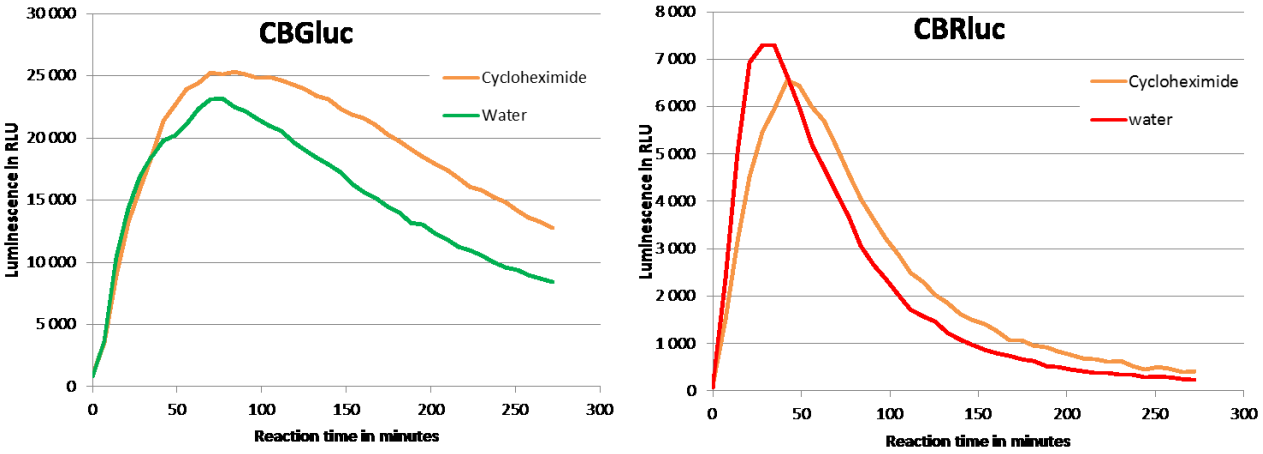


Figure III.2-21. Effect of cycloheximide on luminescence kinetics

Cells from the *C. albicans* CR1 and CG1 strains (*ACT1p-CBRluc* or *ACT1p-CBGluc*, multicopy plasmid) were grown in SD medium to OD_{600}=0.6. Cultures where then diluted to OD=0.2 in SD medium. Cells were treated with either cycloheximide (10 mg/ml) or water. 100 µl of treated cells were mixed with 100 µl Chroma-Glo reagent and luminescence measured over 5 h (at 30°C, with shaking).

3. Application of click beetle luciferase to monitor gene expression in *C. albicans*

After having set up the click beetle luciferase reporter in *C. albicans*, the potential of the system to bring new insights in *C. albicans* biology and specifically in the cAMP/PKA pathway was examined. The luciferase activity of strains with the luciferases genes downstream of different promoters relevant for this pathway was examined. The *HWP1* gene is expressed exclusively during hyphal growth; therefore its expression can be used as a marker of hyphal growth (Heintz-Buschart *et al.,* 2012). Using a fusion of the *HWP1* promoter and the click beetle luciferase gene, the effectiveness of hyphal induction in different media was assessed. Moreover, the impact of small quorum sensing molecules on filamentation was measured. The two colours of the click beetle luciferases theoretically allow measurement of two promoter activities simultaneously. To verify that two genes can indeed be monitored at the same time, the light signal of two cultures expressing each one of the luciferases was compared with the signal of a strain expressing both luciferases simultaneously.

3.1. Promoter activity of relevant PKA pathway genes during hyphal induction

In order to validate click beetle luciferases as new reporter in *C. albicans,* the behaviour of a set of selected genes was analysed in both yeast and hyphal forms. The cAMP/PKA pathway was chosen to test the system because of the potential application of click beetle luciferases for the study of these complex pathways, especially the measurement of two promoter activity simultaneously. Genes implicated in the yeast-to-hypha transition were chosen, including *HWP1*, *TCC1* and *UME6*. The constitutively expressed *ACT1* promoter was also used, as a control (Swoboda *et al.,* 1994). For each promoter, the expression was measured during yeast and hyphal growth. The relative expressions of the promoters were also compared using *ACT1* as a reference. Because changes in gene expression during yeast-to-hypha transition can be abrupt, the capacity of click beetle luciferase to monitor quick changes in gene expression was examined. For this, the expression of *HWP1* and *UME6* promoters was monitored during the first minutes of the hyphal induction to verify the reactivity of click beetle luciferase reporters. In addition, the influence of the promoter upstream of the click beetle luciferase on the reaction kinetics was inspected. The kinetics of the click beetle luciferase reaction was examined for *HWP1*, *UME6* and *TCC1* promoters.

3.1.1. *ACT1*

The strain ipD-R (*CBRluc-ACT1p*) was used to measure *ACT1* expression during hyphal growth. Cells from an overnight culture in YPD medium (30°C; final OD_{600}=6.6-8.5) were re-suspended at OD_{600}=0.2 and grown either in YPD medium at 30°C (yeast) or in YP+10 % serum at 37°C (hyphae). In the yeast form, the luminescence level was strong: 5.5±1.3x10^5 RLU (Figure III.3-1). In hyphal growth conditions, the luminescence activity was significantly higher at 1.3±0.16x10^6 RLU. A 2.4x increase was observed, when inducing hyphae in comparison with yeast growth conditions.

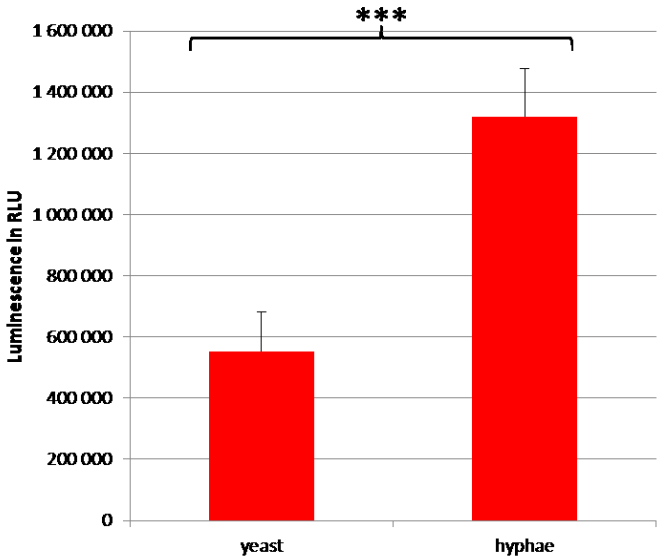


Figure III.3-1. *ACT1* promoter activity during yeast or hyphal growth
C. albicans cells from the ipDR strain (*ACT1p-CBRluc*) were grown for 2 hours at an initial OD_{600}=0,2 either in yeast or hyphae growth conditions (YPD at 30°C or YP+10 % serum at 37°C). Two different transformants were analysed and for each 3 technical replicates were assayed. Luminescence was started by mixing 100 µl of cells with 100 µl of Chroma-Glo reagent; the displayed results show the unfiltered measurements. The error bars show the standard deviation. The luminescence activity in yeast and hyphae were significantly different (t-test p-values <10^{-5}).

3.1.2. *HWP1*

To measure the impact of hyphal growth on *HWP1* promoter expression, luminescence of the strain HwK7GU (*HWP1-CBGluc*) was measured in yeast and hypha growth conditions (YPD at 30°C or YP+10 % serum at 37°C). A weak luminescence signal was observed in the yeast form (234±58 RLU), while activity is hyphae form was high (43±1.2x10^3 RLU). The 184 fold increase in luminescence observed between yeast and hyphae form shows the strong activation of *HWP1p* in the HwK7GU strain (Figure III.3-2). As a control, a similar experiment was performed on the HwK7RH strain (*HWP1p-CBRluc*) in which the click beetle luciferase gene is in frame with the *HWP1* ATG (Results 1.3.3). Using these strains, the luminescence was 6.7±4x10^3 RLU in yeast form and 5.5±1.3x10^6 RLU in hyphal form, corresponding to an 816 fold increase. The luminescence measured is higher in the HwK7RH strain, but a similar increase in luminescence was observed during hyphal growth for both strains. The HwK7GU strain is therefore valid to study *HWP1* promoter activity despite the click beetle luciferase gene being out of frame with the *HWP1* ATG.

In order to compare *HWP1* promoter activity with other genes, the *ACT1* promoter was used as a reference. The strains HwK7GU (*HWP1p-CBGluc*) and ipDR (*ACT1p-CBRluc*) were grown in strictly identical culture conditions; either in YPD at 30°C (yeast form) or in YP+10 % serum at 37°C (hyphae). The luminescence of both strains was then measured and the relative expression of *HWP1* (L_{HWP1}/L_{ACT1}) was calculated. The *HWP1* relative expression was $3.6x10^{-4}$ in yeast and $3x10^{-2}$ in hyphal growth conditions (Figure III.3-2B). The expression of *HWP1p* when considering relative expression levels (L_{HWP1}/L_{ACT1}) rather than the absolute value was 118 fold higher in hypha than in yeast.

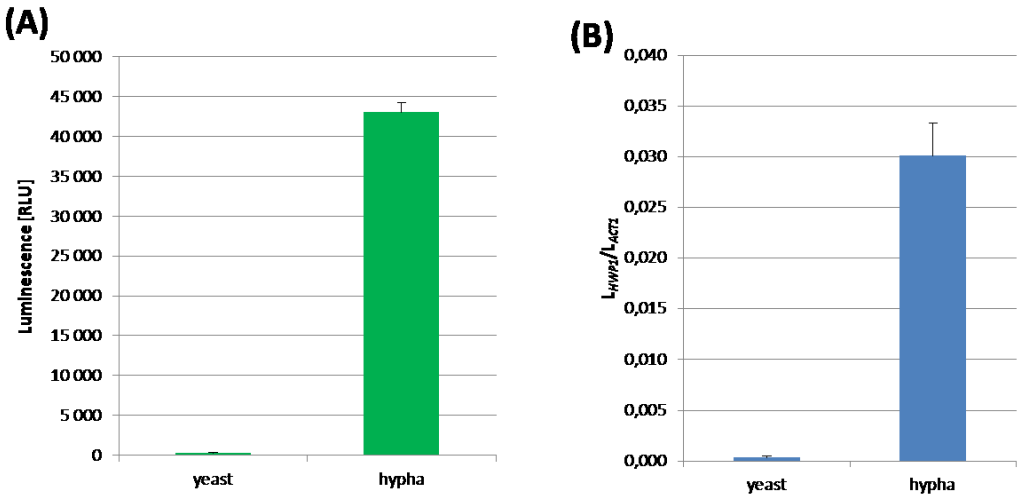


Figure III.3-2. Impact of hyphal induction on *HWP1p* expression
C. albicans cells form the strains HwKGU (*HWP1p-CBGluc*) and ipD-R (*ACT1p-CBRluc*) were grown at an initial OD_{600}=0,2 in either YPD at 30°C (yeast) or in YP+10 % serum at 37°C (hyphae). After 2 hours, the luminescence of both strains was measured by mixing 100 µl of cells with 100 µl go Chroma-Glo reagent. Unfiltered luminescence values are shown (A) Luminescence of *HWP1p*. (B) Relative *HWP1* expression (L_{HWP1}/L_{ACT1}). The error bars show the standard deviation for 2 independent transformants.

3.1.3. *UME6*

The *UME6* gene has been described as having a role in the cAMP-PKA pathway triggering yeast to hyphae regulation (Zeidler *et al.,* 2009). Banerjee *et al.* (2008) showed that *UME6* is induced during hyphal induction.

The activity of the *UME6* promoter was measured using the UmK7GU strain (*UME6-CBGluc*). Promoter activity during yeast growth (YPD medium, 30°C) was compared with hyphae (YP +10 % serum, 37°C). The luminescence of *UME6-CBGluc* after 2h of growth in yeast growth conditions was weak at 176±87 RLU (background noise at 46 RLU), while in hyphal growth conditions, luminescence increased to 1 501±100 RLU (Figure III.3-3A). As described in the previous paragraph, the *ACT1* promoter activity was used as a reference to calculate the relative expression level of *UME6* (L_{UME6}/L_{ACT1}). For this, the luminescence was measured for cultures of ipD-R and UmK7GU strains grown in the same conditions. *UME6* relative expression level was $3.2\pm1.7\times10^{-4}$ for yeast and $11.4\pm1.5\times10^{-4}$ for hyphae (Figure III.3-3B). Hence, the *UME6* relative expression level was 3.6 fold higher in hypha than in yeast. This increase of the relative *UME6* expression shows *UME6* promoter induction in hyphal growth condition, albeit not as strong as for *HWP1p.*

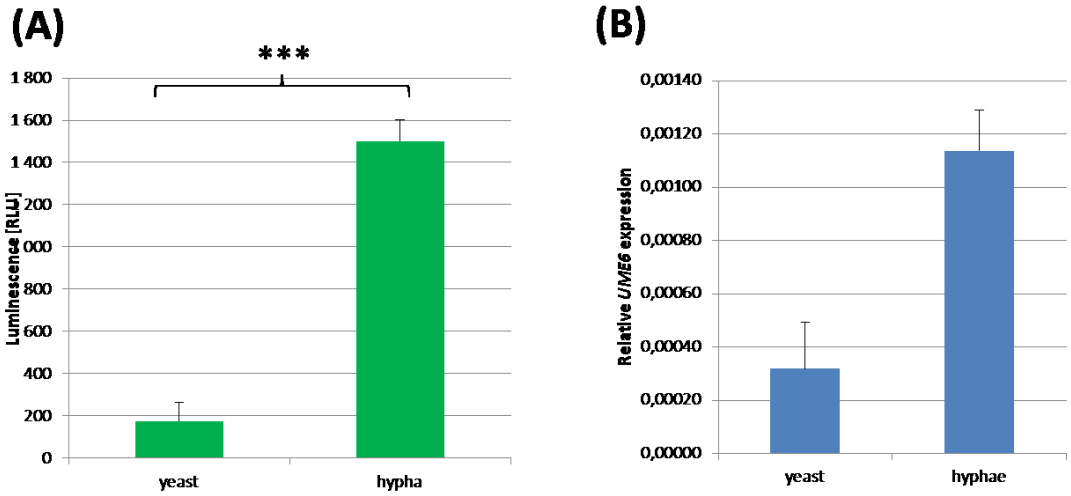


Figure III.3-3. Impact of hyphal induction on *UME6* expression (mixed culture experiment)
C. albicans cells form the strains UmK7GU (*UME6p-CBGluc*) and ipD-R (*ACT1p-CBRluc*) were grown at an initial OD_{600}=0,2 in either YPD at 30°C (yeast) or in YP+10 % serum at 37°C (hyphae). After 2 hours, the luminescence of both strains was measured by mixing 100 µl of cells with 100 µl Chroma-Glo reagent. Unfiltered luminescence values are shown (A) Luminescence of *UME6p*. (B) Relative *UME6* expression (L_{UME6}/L_{ACT1}). The error bars show the standard deviation for 4 transformants.

3.1.4. Early hyphal induction analysis for *HWP1* and *UME6*

The mixed culture measurements described above for *HWP1* and *UME6* promoters using the *ACT1* promoter activity as a reference point makes comparison between the relative activities of *HWP1* and *UME6* promoters possible. During these experiments, the activity of *HWP1p* was 156 fold higher than *UME6p* in yeast form and 6.8×10^{4} fold higher in the hyphal form $\left(\frac{L(HWP1)/L(ACT1)}{L(UME6)/L(ACT1)}\right)$. These results show that the promoter activity for the wall protein Hwp1 is much stronger than the promoter activity for the transcription factor Ume6.

Using HwK7GU and UmK7GU strains, expression of *HWP1* and *UME6* promoters was compared during the early hyphal induction. At t=0 min, cells from an overnight culture in YPD were diluted to an initial OD_{600}=0.2 in YP+10 % serum and incubated with shaking at 37°C. Probes were taken regularly and their luminescence was assessed as previously described. After 10 min of hyphal induction, a weak luminescence signal significantly higher than background noise was measured for both promoters. Between 10 and 20 min, luminescence decreased slightly for *UME6p* (0.8x) and increased for *HWP1p* (1.8x). Luminescence then increased strongly between t=20 min and t=60 min for both promoters: 15 fold for *UME6p* and 142 fold for *HWP1p* (Figure III.3-4). After 60 min hyphal induction, a slighter luminescence increase was observed (1.8x for *UME6p* and 2x for *HWP1p*).

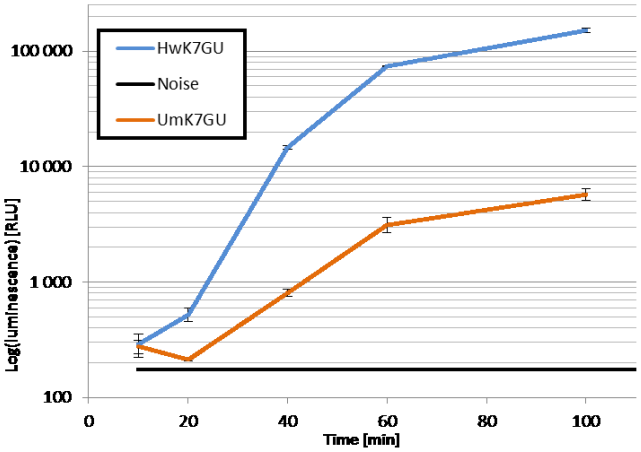


Figure III.3-4. Comparison between *HWP1* and *UME6* promoter activity in the early phase of hyphal induction
Cells from the strains an overnight culture in YPD of HwK7GU and UmK7GU strains were diluted to an initial OD_{600}=0.2 in YP+10 % serum and incubated with shaking at 37°C (t=0 min). From each culture, probes were taken regularly and their luminescence was measured after mixing 100 µl of cells with 100 µl Chroma-Glo reagent. The average for 2 HwK7GU clones and 3 UmK7GU clones is shown. The noise was measured in wells containing non-luminescent *C. albicans* CAI4 cells.

3.1.5. *TCC1*

Tcc1 is a protein interacting with Tup1 to repress hypha-specific genes. Its deletion leads to formation of hyphae in yeast growth conditions (Kaneko *et al.,* 2006). The *TCC1* promoter is bound by the Efg1 transcription factor in yeast form but shortly after hyphal induction Efg1 disappears from *TCC1* locus (Lassak *et al.,* 2011).

The *C. albicans* strain ipTGL (*TCC1p-CBGluc*) was used to compare the level of luminescence in yeast form and hyphal form. A slight increase in the *TCC1p-CBGluc* luminescence was observed during hyphal induction: $2.13x10^5$ to $2.59x10^5$ RLU after 2 h (Figure III.3-5A). The relative *TCC1* expression (L_{TCC1}/L_{ACT1}) expression was 0.39 for yeast form and decreased approximately two fold to 0.20 after 2 h of hyphal growth (Figure III.3-5B).

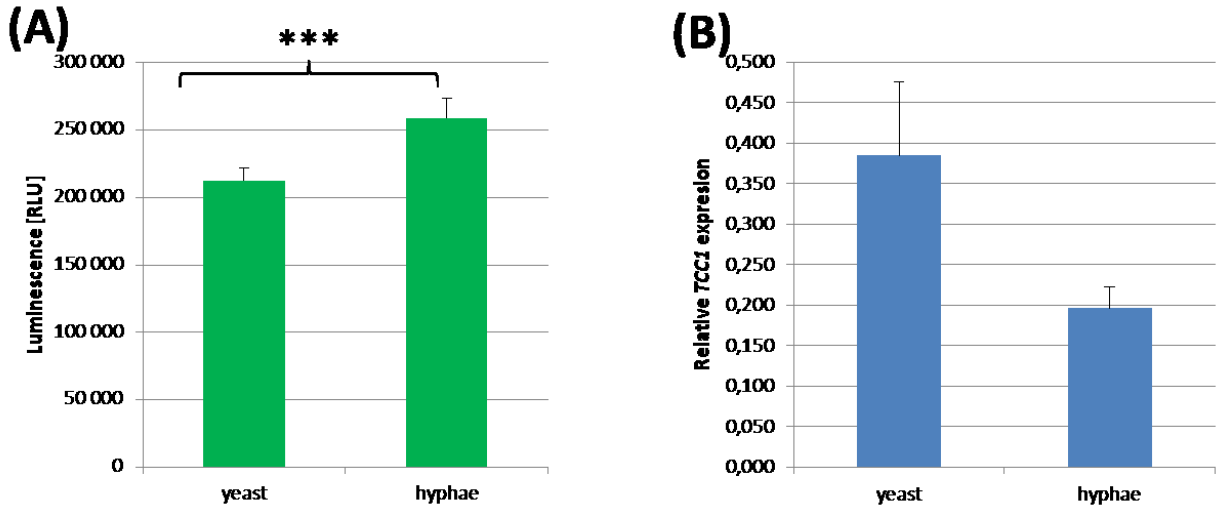
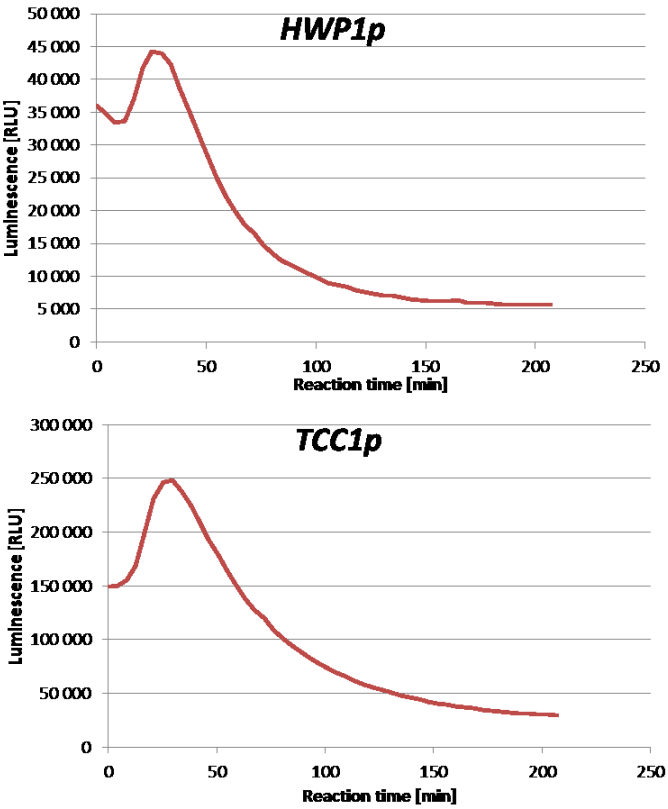


Figure III.3-5. *TCC1* promoter activity during yeast and hyphal growth
C. albicans cells form the strains ipTGL (*TCC1p-CBGluc*) and ipD-R (*ACT1p-CBRluc*) were grown at an initial OD_{600}=0,2 in either YPD at 30°C (yeast) or in YP+10 % serum at 37°C (hyphae). After 2 hours, the luminescence of both strains was measured by mixing 100 µl of cells with 100 µl Chroma-Glo reagent. Unfiltered luminescence values are shown (A) Luminescence of *TCC1p*. (B) Relative *TCC1* expression (L_{TCC1}/L_{ACT1}). The error bars show the standard deviation for 2 technical replicates of 3 different transformants.

3.1.6. Influence of the promoter on reaction kinetics of the click beetle luciferases

To asses if diferent promoters would impact the reaction kinetics of the click beetle luciferases, the kinetics of the luminescence signal was compared for three different promoters: *HWP1* (HwK7GU strain), *TCC1* (ipTGL strain) and *UME6* (UmK7GU strain). Because Hwp1 and Ume6 are exclusively hypha-associated proteins and because Tcc1 is also present during hyphal growth, luminescence kinetics were measured during hyphal growth (3 h, YP+10 % serum, 37°C). Kinetics of the reactions were identical and only the signal strength differed from one promoter to the other. The L_{max} values accurately reflected the different promoter activities (Figure III.3-6)



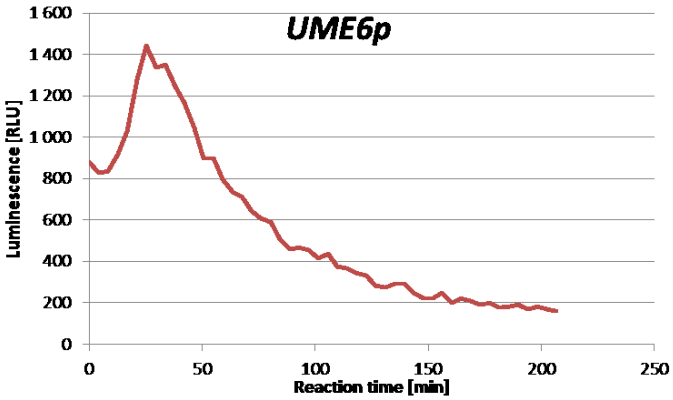


Figure 3-6. Promoter influence on click beetle luciferase reaction kinetics
Cells from an overnight culture in YPD of the *C. albicans* strains HwK7GU (*HWP1p-CBGluc*), ipTGL (*TCC1p-CBGluc*) and UmK7GU (*UME6p-CBGluc*) were grown in hyphal inducing conditions (3h; YP+10 % serum; 37°C; initial OD_{600}=0,2). Luminescence was measured over 200 min after mixing 100 µl of cells with 100 µl Chroma-Glo reagent.

3.2. *HWP1*, a reporter of hyphal growth

HWP1 is not expressed in yeast form but is strongly expressed upon hyphal induction (Loeb *et al.,* 1999). In hypha form, Hwp1 is one the most abundant proteins in *C. albicans* cell wall (Heilmann *et al*., 2011). *HWP1* has therefore been used as a reporter of hyphal growth for potential application such as high-throughput screening (Heintz-Buschart *et al.,* 2013).

The *HWP1-CBluc* constructs (Results 1.3.3) were used to compare the impact of different growth media on hyphal growth using the click beetle luciferases. Moreover, to evaluate the potential of *HWP1-CBluc* as a reporter in high throughput screening studies, the influence of the quorum sensing molecules farnesol and tyrosol on luminescence were measured.

3.2.1. Hyphal induction in different media

Hyphal growth can be induced by different environmental conditions, including the presence of serum, GlcNAc, low nitrogen concentration, or some specific medium like Lee's medium or mammalian tissue culture mediums (Sudbery, 2011). A specific hyphal induction medium might be inappropriate in certain circumstances or not suited to a specific experiment. The versatility of the luciferase reporter must therefore be tested in different hyphal induction media. Three different hyphal inducing media were tested in parallel and luminescence was measured using the HwK7GU strain (*HWP1p-CBGluc*; Results 1.3.3.1) at different time points. The first medium, YP+10 % serum is a strong inducer of hyphal growth due to the presence of horse serum (Barlow *et al.,* 1974). The second medium, Synthetic low-ammonium–dextrose (SLAD) contains low nitrogen concentration. The low nitrogen concentration induces hyphal growth in *C. albicans* (Csank *et al.,* 1998). The last medium tested, RPMI medium (Roswell Park Memorial Institute-1640), is a mammalian cell culture

media that will also induce hypha formation (laFleur *et al.,* 2006). As previously explained, *HWP1* promoter activity was used as a quantitative marker of hyphal growth in this experiment. Luminescence could already be seen at 8 min after hyphal induction, the first measurement point. At this time point, the measured luminescence was similar for the 3 media. After 17 min induction, the luminescence in YP+10 % serum was twice the signal observed in SLAD medium or RPMI. At the last time point (60 min), luminescence was $25x10^3$ RLU in YP+10 % serum, $2.5x10^3$ RLU in RPMI medium and $5.5x10^3$ RLU in SLAD medium (Figure III.3-7). These results show that the three hyphal induction mediums tested were compatible with luminescence measurements.

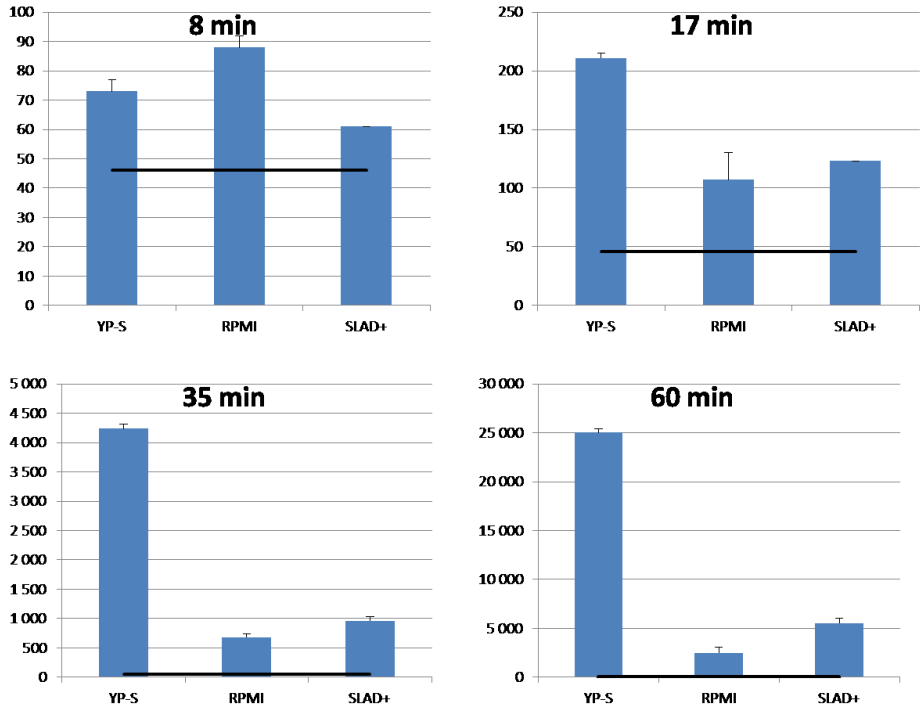


Figure III.3-7. Impact of media on the expression of *HWP1* during early hyphal induction
Cells from an overnight culture of the strain HwK7GU were washed and fresh cultures were set up in 3 hyphae inducing media: YP+10 % serum (YP-S), RMPI medium and SLAD- medium. Luminescence was assessed at different time points.

3.2.2. Effect of quorum sensing signalling molecules on *HWP1* expression

In *C. albicans*, the yeast-to-hypha transition is regulated by cell density (quorum sensing). Farnesol, a small organic compound intermediate in sterol biosynthesis secreted by *C. albicans,* plays a key role in this quorum sensing. The concentration of farnesol in a *C. albicans* culture is proportional with the cell density (Hornby *et al.,* 2001, Langford *et al.,* 2013). At high concentrations, Farnesol inhibits the formation of hyphae by repressing the cAMP/PKA signal transduction pathway (especially Cyr1; Lindsay *et al.,* 2012). On the other hand, tyrosol is a phenylethanoid structurally similar to tyrosine that promotes hypha-formation (Madhani, 2011; Chen *et al.,* 2004). The impact of both molecules on *C. albicans* hypha formation was examined using *HWP1* promoter activity as a marker of hyphal growth as described in the previous paragraph. The strains HwK7GU or HwK7RH were used for this purpose (*HWP1p-CBGluc* or *HWP1p-CBRluc;* Results 1.3.3.).

3.2.2.1. Farnesol

Cells of the *C. albicans* strain HwK7GU (*HWP1p-CBGluc*; Results 1.3.3.1.) were grown in hypha-inducing condition containing different concentrations of farnesol (YP+10 % serum, 37°C) for 3 h. As shown in Figure III.3-8, the luminescence of cells treated with 50 µM farnesol was 40-60 % of the untreated cells activity. In cells treated with 150 µM farnesol, *HWP1* promoter activity diminished to 3-35 % of the luminescence of untreated cells. Farnesol showed a dose-dependent effect on *HWP1* promoter activity.

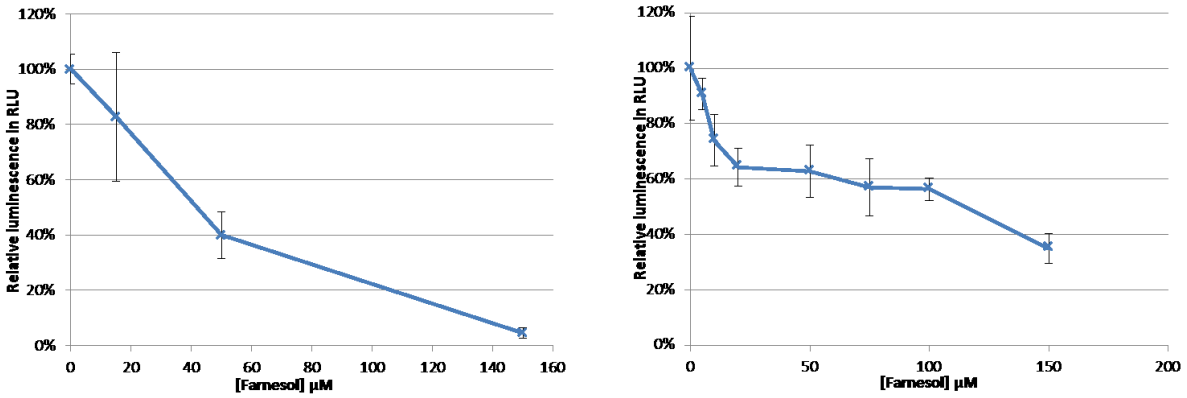


Figure III.3-8. Impact of Farnesol on *HWP1* promoter activity
Cells from the strain HwK7GU were inoculated in YP+10 % serum (OD_{600}=0,2). The cultures were next treated with farnesol or water at the indicated concentrations (maximal C_{final}=150 µM). Unfiltered luminescence was measured after 3h incubation by mixing 100 µl culture with 100 µl Chroma-Glo reagent. Results are expressed in percent of the untreated cell luminescence. The results of two distinct experiments are shown. The error bars show the standard deviation for two technical replicates.

3.2.2.2. Tyrosol

To measure the influence of tyrosol on *HWP1* promoter activity, cells of the HwK7RH strain (*HWP1p-CBRluc*; Results 1.3.3.2.) were placed in at OD_{600}=0.2, 37°C in YP- medium for an hour and treated with 100 µM tyrosol. Luminescence of treated cells was compared with a control sample treated with water. The luciferase activity of cells treated with tyrosol was 31% higher than untreated cells (Figure III.3-9).

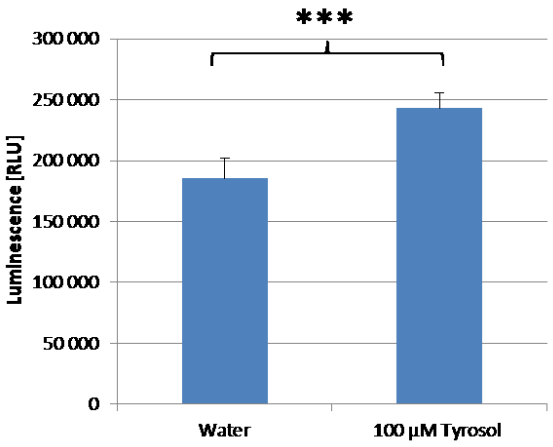


Figure III.3-9. Impact of tyrosol on *HWP1* expression
Cells from the strain HwK7RH were inoculated in YP- medium at OD_{600}=0.2. Cultures were either treated with Tyrosol or with water. After 1h incubation at 37°C, luminescence was measured by mixing 100 µl of culture with 100 µl Chroma-Glo reagent. *(In collaboration with Mario Kapitan)*

3.3. Dual colour measurements

The red and green click beetle luciferases have distinct emission spectra. Using optical filters and a mathematical correction, the signals can be separated (Results 2.1). This signal separation aims at the simultaneous measurement of the promoter activity of two genes. To verify the effectiveness of such a dual luciferase system, the luminescence activity of strain expressing a single luciferase was compared with a strain expressing at the same time a second luciferase. In a preliminary step, the culture of a strain expressing the red click beetle luciferase was mixed with a culture of a strain expressing the green luciferase. The light signal of this mix was compared with the activity of non-mixed single cultures. As the corrected signal was similar in mixed culture or in single cultures, the following step was the measurement of light activity of a strain with both luciferase gene integrated downstream of different promoters. The luciferase activity of a double transformant strain (HwAC or HwTc) was compared with single transformants.

3.3.1. Mix of two strains each expressing one luciferase gene

Before starting co-transformation experiments, the feasibility of dual colour reading was examined. For this, 3 strains were used: ipD-R (*ACT1p-CBRluc*), ipTGL (*TCC1p-CBGluc*) and HwK7GU (*HWP1p−CBGluc*). Cells from all strains were grown in identical condition, either in yeast (YPD, 30 °C) or hyphal growth conditions (YP+10 % serum, 37 °C). In order to test signal separation, 50 µl of ipD-R culture (red luciferase) was mixed before luminescence measurement with 50 µl ipTGL or HwK7GU (green luciferase). The luminescence of these mixed culture samples were compared with pure cultures (Figure III.3-10). The red and green click beetle luciferases signals were separated using optical filters and mathematical corrections as detailed in Results 2.1. The *ACT1p-CBRluc* activity read during yeast growth was $3\pm0.7x10^5$ RLU in average for single culture, and $2.5\pm0.7x10^5$ RLU when mixed with the *TCC1p-CBGluc* strain. The *ACT1p-CBRluc* activity for hyphal growth was measured at $7\pm0.8x10^5$ RLU in pure cultures and $6.5\pm1.1x10^5$ RLU in mixed cultures. The *TCC1p−CBGluc* activity was $1.0\pm0.1x10^5$ RLU for pure cultures and $1.1\pm0.1x10^5$ RLU for mixed cultures (yeast form). The *HWP1p-CBGluc* activity during hyphal growth was $23.4\pm1.3x10^3$ RLU for single cultures and $19.9\pm1.9x10^3$ RLU for mixed cultures. In conclusion, the results for individual cultures were nearly identical to mixed cultures measurements, with a variation of less than 5 % in average (calculated for each clone individually). This demonstrates the accuracy of the correction constants and filters used. Hence, the signal coming from red and green click beetle luciferases, mixed in the same sample, can be measured simultaneously.

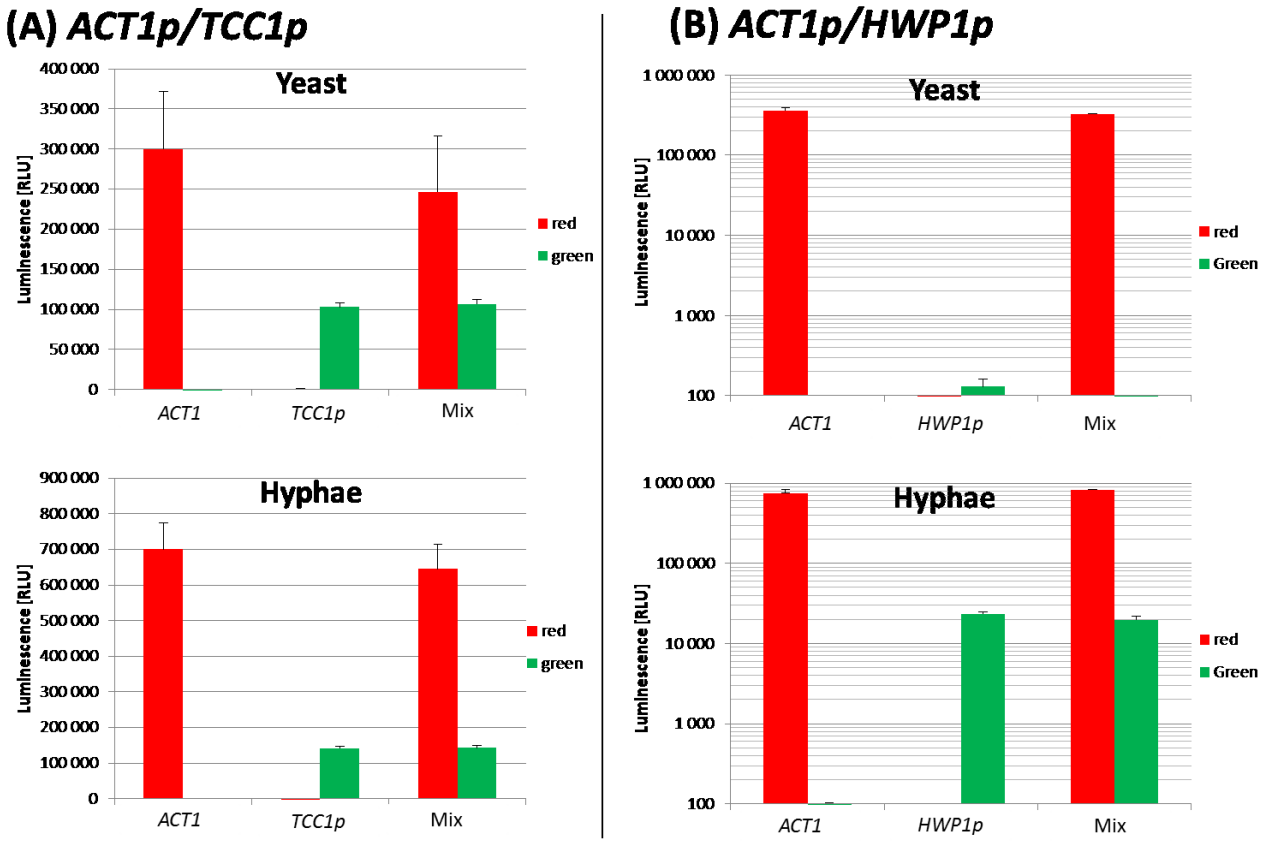


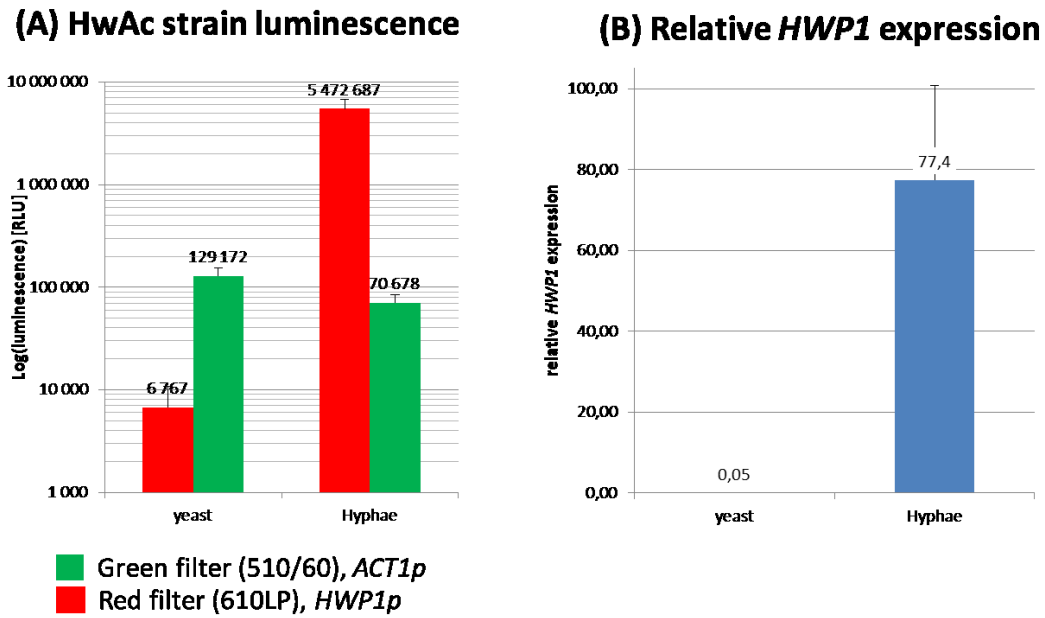
Figure III.3-10. Comparison of the luminescence of pure and mixed strains

C. albicans cells with integrated luciferase downstream of the following promoters were used: *ACT1*, *TCC1* and *HWP1* (strains ipD-R, ipTGL and HwK7GU). *ACT1p* has been tagged with the red click beetle luciferase; *TCC1p and HWP1p* with the green luciferase. After an overnight culture, cells were grown for 2 h either in YPD at 30°C for yeast or in YP+ 10 % Serum at 37° for hyphae. 100 µl for pure samples or 50 µl of both strains for mixed samples were taken and added the 100 µl of Chroma-Glo reagent. Luminescence was measured and the filtered values corrected as specified in Results 2.1, the corrected L_{max} is shown. (A) Comparison between *ACT1p-CBRluc* and *TCC1p-CBGluc*. (B) Comparison between *ACT1p-CBRluc* and *HWP1p-CBGluc.* Error bars show standard deviation for two technical replicates of two (ipDR) or three (ipTGL and HwK7GU) separate transformants.

3.3.2. Double transformants: expression of both luciferase genes in the same cell

Strains with the red and green click beetle luciferase gene inserted genomically were constructed as described in Results 1.3.5. The selection marker *URA3* and *HIS1* were used to co-transform the *C. albicans* strain BWP17 with both luciferases.

To verify if the corrected luminescence signal from the strains with double luciferase integration were accurate, cells from the *C. albicans* HwAc strain (*HWP1p-CBRluc*, *ACT1p-CBGluc*) were grown either in YPD medium at 30°C (yeast) or in YP+10 % serum at 37°C (hyphae) and the activity of the *HWP1* promoter measured with this strain was compared with the HwK7RH strain. The activity of the *ACT1* promoter was measured through the green filter (510/60), while the activity of the *HWP1* promoter was quantified through the red filter (610LP). The luminescence signals were corrected as previously described in Results 2.1. For yeast growth conditions, green luminescence was strong with $129x10^3 \pm 24x10^3$ RLU while a weaker red luminescence was observed: $6.7x10^3 \pm 4x10^3$ RLU (Figure III.3-11A). Consequently, the luminescence ratio L_{HWP1}/L_{ACT1} was very low: 0.05±0.03. During hyphal growth, the green channel activity remained stable: $70x10^3 \pm 13x10^3$ RLU while the red luminescence increased strongly: $5.5x10^6 \pm 1.3x10^6$ RLU, approaching the saturation limit of the luminometer (Figure III.3-11A). The ratio L_{HWP1}/L_{ACT1} was 77.4 during hyphal growth. The L_{HWP1}/L_{ACT1} ratio was significantly higher during hyphal growth (77.4±23.2), when compared with yeast (0.05±0.03), showing a clear *HWP1p* induction (Figure III.3-11B). This matches the behaviour of *HWP1p* observed previously in those conditions (Results 3.1.2). Remarkably, the comparison of *HWP1* promoter activity in strains HwK7RH (single transformant) and HwAc (double transformant) showed that the luminescence measured in both strains was similar (Figure III.3-11C). The luminescence value differences between the two strains were in the range of measurement scattering (Figure III.3-11C).



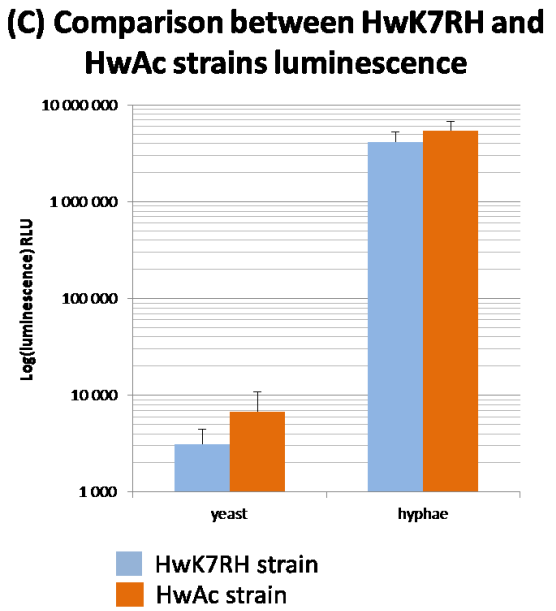


Figure III.3-11. Simultaneous dual colour measurement of strain HwAc (*HWP1p-CBRluc, ACT1p-CBGluc*)
Cells from the strains HwAc (*HWP1p-CBRluc*, *ACT1p-CBGluc*) or HwK7RH (*HWP1p-CBRluc*) were grown in yeast (A and C; overnight, YPD, 30°C) or hyphae growth condition (B and C; 3 h, YP+10 % serum, 37°C). Luminescence was measured and L_{max} values were corrected as described in Results 2.1. (A) HwAc strain luminescence, red and green filters. (B) *HWP1* relative activity (L_{HWP1}/L_{ACT1}). (C) Comparison of *HWP1p-CBRluc* activity between HwAc and HwK7RH strains, red filter. Error bars show the standard deviation of 8 independent clones.

A similar experiment was performed on the double transformant strain HwTc (*HWP1p-CBRluc*, *TCC1p-CBGluc*). The activity of the *TCC1* promoter was measured with the green filter (510/60) and the activity of the *HWP1* promoter was measured through the red filter (610LP). The Figure III.3-12 shows the luminescence in either yeast or hyphae growth conditions. Luminescence remained stable for the *TCC1* promoter and increased drastically for *HWP1* promoter (168 fold). In the yeast form, the *TCC1p* luminescence of the single transformant strain ipTGL was compared with the double transformant strain HwTc (Figure III.3-12B). The *TCC1p* activity in the two strains was nearly identical. As observed with HwAc strain, the difference in luminescence value can be explained by the scattering of the measurements. The accuracy of the click beetle luciferase for simultaneous measurement of two gene activity was thereby confirmed.

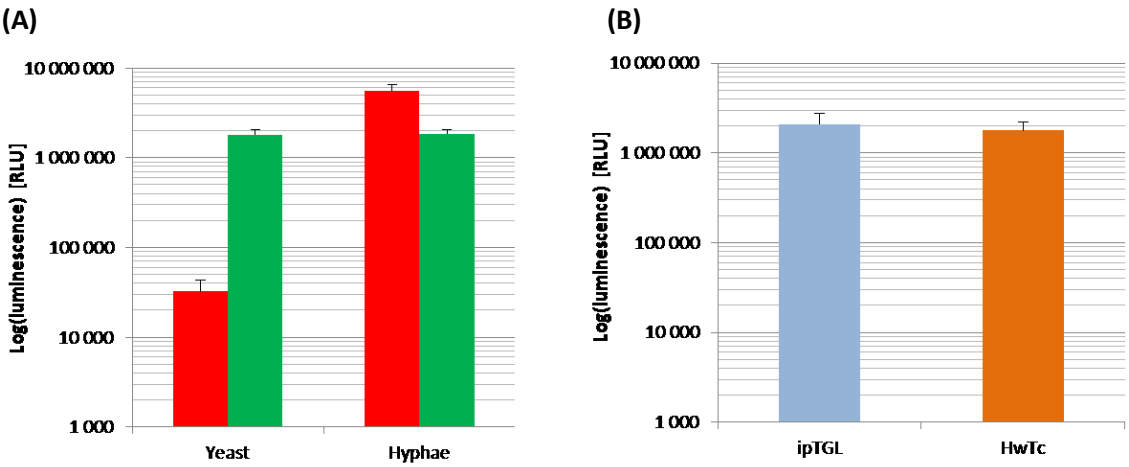


Figure III.3-12. Simultaneous Dual colour measurement of HwTc strain (*HWP1p-CBRluc, TCC1p-CBGluc*)
Cells from the strain HwTc (*HWP1p-CBRluc*, *TCC1p-CBGluc*) were grown in yeast (overnight, YPD, 30°C) or hyphal growth conditions (3 h, YP+10 % serum, 37°C). Luminescence was measured and L_{max} values corrected as described in Results 2.1. (A) HwTc strain luminescence, red and green filters. (B) Comparison of *TCC1p* activity in the ipTGL and HwTc strains, yeast form, green filter. Error bars show the standard deviation of 3 separate clones.

IV. Discussion

In this work, the click beetle luciferases were successfully adapted and used as a reporter protein in the human pathogenic fungus *C. albicans*. In the following, the protocol used and the efficiency of the click beetle luciferase as a reporter in *C. albicans* will be discussed in perspective with the existing reporters. The impact of click beetle luciferase on the study of the cAMP/PKA regulation pathway will then be examined. Finally, future prospective for the use of click beetle in *C. albicans* will be discussed.

1. Activity of click beetle luciferase in *C. albicans*

1.1. Codon optimisation and sensitivity

The click beetle genes on which the reporters used in this study are based had previously been optimised for mammalian cells (Almond *et al.,* 2002). The sequence of both genes encoding red and green click beetle luciferases was adapted in this work by modifying the codons for *C. albicans* specific codon usage, which included removal of the CUG codons (translated as serine in *C. albicans*; Results 1.1). The importance of the exchange of CUG codons was shown by Morschhäuser *et al*. (1998), the non CUG-adapted version of the GFP did not show any fluorescence in *C. albicans*. Both luciferases, encoded by multicopy plasmids, showed strong luminescence activity in transformants of *S. cerevisiae* and *C. albicans*. *C. albicans* strains were then constructed with both luciferase ORFs integrated genomically downstream of gene promoters, in order to test the validity of the click beetle luciferase as reporters. When the click beetle luciferase ORFs were genomically integrated downstream of the *ACT1* promoter, the measured luminescence signal was very strong. The observed sensibility threshold was of approximately 60 cells per well (Figure III.2.16). The low detection threshold makes the measurement of weakly expressed genes possible. The activity of the promoter of the transcription factor Ume6, for example, was detected using the click beetle green luciferase. Its luminescence level (≈1 500 RLU in hyphal form; Figure III.3.3) was far above the detection threshold (50-100 RLU; Figure III.2.3). Enjalbert *et al.* (2009) had described an innovative reporter system involving the *Gaussia princeps* (Gluc) luciferase. To solve the problem of the low permeability of *C. albicans* to coelenterazine (substrate of *Gaussia* luciferase), the Gluc protein was fused with a GPI-linked cell wall protein (Pga59). Because the protein fusion is displayed on the cell surface, the luciferase has access the substrate in the medium very efficiently and therefore produces a strong luminescence signal. So far, this surface display system was the most sensitive in *C. albicans*, reaching a sensitivity of a thousand cells (Enjalbert *et al.,* 2009). The threshold of ≈60 cells measured with the click beetle luciferases would make it a substantially more sensitive reporter; however, the two systems should be compared directly in the same experiment before drawing definitive conclusions. The Gluc-system was designed for *in vivo* studies of *C. albicans* infection in animal models and, because it involves secretion of the reporter, might not be applicable in certain situations. Doyle *et al.* (2006) used the firefly luciferase as reporter in *C. albicans*; however the firefly luciferase gene was not codon-optimised for *C. albicans*. The luciferase *Fluc* gene has a codon adaptation index of only 0.27 and its expression might therefore not be optimal in *C. albicans* (Brock *et al.,* 2012). The *CBRluc* and *CBGluc* used in this study both have a CAI≥0.9 % guaranteeing a strong expression. The firefly luciferase gene used by Doyle *et al.* (2006) and first described by DeWet *et al.*

(1987), contained a SKL sequence at the C-terminal end of the protein, which is responsible for protein targeting to the peroxisome. Cells expressing a luciferase including the SKL peroxisome targeting sequence grow significantly slower than cells expressing a version of the luciferase without it (Leskinen *et al.,* 2003). The targeting of luciferase in the peroxisome also diminishes significantly the luminescence signal, presumably because of different environmental conditions and decreased permeability to luciferin (Leskinen *et al.,* 2003). Both CBRluc and CBGluc used in this work do not contain the SKL peroxisome targeting sequence. Both the high codon adaptation and the lack of peroxisome targeting sequence may contribute greatly to the excellent sensitivity of click beetle luciferases in *C. albicans*.

The high sensitivity of click beetle luciferases can probably be related to the very high quantum yield of beetle luciferases. The quantum yield of beetle luciferase is 88 % against 6-7 % for the *Renilla* luciferase (Seliger *et al.,* 1960; Matthews *et al.,* 1977). The click beetle luciferase signal was able to accurately measure small activity levels (transcription factors) but also highly expressed proteins like Hwp1. This demonstrates the scaling capability of the click beetle luciferases gene reporters in *C. albicans*. Increased sensitivity can be achieved, if crude extract or permeabilised cells using flash lysis procedures are used (Results 2.2.3). Importantly, no differences could be observed in the scattering of the luminescent measurements using crude extracts or live cell assays (Figure III.2-12).

The wild-type strain CAI4 mixed with Chroma-Glo reagent produced the same amount of background light signal than an empty well (Figure III.2.3). This proves that in the condition tested in this work, the sensitivity is limited only by the background noise of the luminometer (Figure III.2.3). Enjalbert *et al.* (2009) identified auto-luminescence of coelenterazine as a limitation of their *Gaussia* luciferase system. In contrast, beetle luciferin is not subject to auto-oxidation and therefore does not produce background luminescence (Brock, 2012). When the click beetle luciferase was integrated downstream of inactive promoters, like *HWP1p* or *UME6p* during yeast growth, the level of luminescence was very low (<200 RLU for *UME6p-CBGluc*; Figure III.3.3). These minimal luminescence levels are probably due to a small basal activity of the promoters. The use of deep cooled sensors could push the detection limit further as the cooled modern photomultiplier tubes can detect single photons (Contag *et al.,* 2002). It is worth mentioning that a higher background luminescence was observed when microtiter plates were exposed to artificial neon light (data not shown). The white plastic plates, which yielded the highest luminescence signals, are particularly sensitive to this phenomenon.

Another luminescence system was recently developed for the yeast *S. cerevisiae* using the bacterial *lux* system (Sanseverino *et al.,* 2005). Interestingly, in this system the yeast is transformed with all the genes needed for luminescence, including the substrate; thus, the yeast cells were autoluminescent. This autoluminescent system, designed as an oestrogen-inducible bioluminescent reporter, potentially has several limitations for use as a reporter in *C. albicans*: long lag between expression and maximal luminescence (6 h), low substrate availability, poor signal intensity and the need to adapt every *lux* gene to *C. albicans* (Sanseverino *et al.,* 2005; Brock *et al.,* 2012). A system could nevertheless be engineered in this fashion for the click beetle luciferases in *C. albicans* (or *S. cerevisiae*) but at the present time, the synthesis pathway of beetle luciferin has not been established.

1.2. Comparisons with firefly luciferase

Two methods were used to insert the click beetle luciferase gene in the genome of *C. albicans*: vector based and PCR based (Results 3.3.1). Using any of these methods, a new target gene can be chosen and the click beetle luciferase reporter integrated at the locus of choice in the genome of *C. albicans*. The protocol developed in this work, using an equal mix of Chroma-Glo reagent and live cells was tested successfully with yeast and hyphal forms (Figure III.3.1). The luminescence measured with *ACT1p-CBRluc* transformants was twice as strong in hyphal growth conditions compared to yeast growth (Results 3.1.1). Swoboda *et al.* (1994) did not observe this substantial increase but described a slight increase of the expression of *ACT1p* after 2 h in YP+10 µl serum at 37°C. Doyle *et al.,* constructed a *C. albicans* strain with the firefly luciferase downstream of the strong *C. albicans ENO1* promoter. These authors observed a drastic diminution of luminescence in hyphae compared to yeast (Doyle *et al.,* 2006). Because the diminution of luminescence was not observed in crude extracts, Doyle and colleagues hypothesised that the permeability of *C. albicans* hyphae to luciferin was lower than that of yeast cells. This limitation was not observed with the click beetle luciferases in the conditions used in this study. It is worth mentioning that the media used for hyphal induction in both studies were different: RPMI+10 % serum or YP+10 % serum; however, this should not affect luciferin permeability of *C. albicans* cells. Enjalbert *et al.* (2009) also compared the luminescence of yeast and hypha forms but using the coelenterazine-based *Gaussia* luciferase. When the *Gaussia* luciferase gene was integrated downstream of *ACT1* promoter, the luminescence levels were equivalent in yeast and hypha, which contrasts with our results. It would be valuable to compare the luminescence level of crude extracts and live cells for yeast and hyphae with the ipD-R strain (*ACT1p-CBRluc*). Provided cells are grown at the same temperature and concentration, this experiment would allow a comparison of yeast and hypha permeabilities to luciferin.

1.3. Luminescence kinetics

The measurement of HwK7GU and UmK7GU strains (*HWP1p-CBGluc* and *UME6p-CBGluc*) shows luminescence after 10 min post hyphal induction. The time delay between gene activation and the increase of the click beetle luciferase reporter's signal is very short. It would be interesting to measure the luminescence activity of crude extract before in the first 10 min of the hyphal induction using the HwK7RH strain, which has a stronger activity than the HwK7GU strain. This quick signal increase is a strong advantage over fluorescent proteins when studying the dynamics of regulations; especially in the case of yeast-to-hypha transition, the changes in gene expression are rapid. When expressed, the fluorescent proteins must mature (folding and chromophore formation) before a signal can be detected; this maturation can take up to several hours (Gordon *et al.,* 2007). The diminution of the reporter signal upon downregulation of the gene expression remains to be evaluated for the click beetle luciferases in *C. albicans*. A long half-life, as in the case of GFP reporters (≈26 h in mammalian cells), is a significant drawback for a gene reporter (Corish *et al.,* 1999). In order to test the click beetle luciferase's signal, the *CBGluc* gene could be integrated downstream of the *YWP1* promoter. *YWP1* is a gene expressed during yeast growth only (Granger *et al.,* 2005). The diminution of luminescence could then be measured after hyphal induction in YP+10 % serum at 37 °C.

The kinetics of the luminescence signal was monitored after mixing the probes with Chroma-Glo reagent by measuring the luminescence over time. The half-life for *CBGluc* luminescence is 250 min

and 50 min for *CBRluc*. The half-life of the light signal associated with the green click beetle luciferase was considerably longer that the red click beetle luciferase. This result confirms similar observations made when expressing the luciferase in mammalian cells (Almond *et al.,* 2003). For comparison, the half-life for the *Renilla* luciferase is ≈60 sec, among other reasons because of the very low turnover of the enzyme (Matthews *et al.,* 1977). Because of this longer signal half-life, multiple samples can be measured simultaneously, whereas for the *Renilla* luciferase samples must be read one at a time. Because of the diminished handling requirements, the scattering of the luminescence measurements was lower for click beetle luciferases. Moreover, the use of live-cells rather than crude extracts also limits variation of the results. This was confirmed In *E. coli* by Tauriainen *et al.* (1999) with the firefly luciferase. The method used for assessing of *Renilla* luciferase activity (one measurement only, shortly after mixing the reagents) leads to significant data scattering, especially since the luminescence half-life of the *Renilla* luciferase is short. Measuring the luminescence over time and computing the L_{max} value might mitigate this issue.

The slow decrease of luminescence activity over time illustrated in Figure III.2.18 is probably due to the production by the luciferase of dehydroluciferyl-adenylate (L-AMP) or other reaction side products, which inhibit the luciferase activity (Marques *et al.,* 2009). The Chroma-Glo reagent contains coenzyme A (CoA) that helps circumvent this problem. Indeed, the luciferase can catalyse the reaction of L-AMP with CoA that produces dehydroluciferyl-CoA (L-CoA), a much less potent inhibitor (Fraga *et al.,* 2005). This side reaction should be kept in mind when using luciferin instead of Chroma-Glo reagent: CoA should be added to the samples for a stronger and more stable luminescence. The reaction kinetics were unaffected by cycloheximide, which is a known inhibitor of protein *de novo* synthesis in fungi. This and the lack of growth of *C. albicans* in the presence of Chroma-Glo reagent suggest that Chroma-Glo reagent blocks translation in *C. albicans*. The luminescence of a culture can therefore not be read over time when adding Chroma-Glo reagent; rather, probes must be taken regularly from the culture and mixed with Chroma-Glo reagent. Alternatively, luciferin could be used to measure luminescence over time. The regular addition of Chroma-Glo reagent in the reaction mix did not change the luminescence demonstrating that the Chroma-Glo reagent is in excess in the reaction, even with strong promoters like *ACT1p*.

1.4. Simultaneous dual luciferase measurement

The distinct wavelengths of the CBRluc and CBGluc luciferases make a simultaneous measurement of both luciferases possible. In this work, an experimental protocol was established for *C. albicans* using optical filters and subsequent signal calibration for an efficient separation of the red and green click beetle luciferase signals. The efficiency of the system was measured by comparing light signals from individual strains carrying either *CBRluc* or *CBGluc* integrated genomically with the signal from a mixture of these strains. The luminescence signal was identical in single or mixed strains demonstrating the effectiveness of the system (Figure III.3-10).

C. albicans strains were constructed, which carry both red and green click beetle luciferases ORF integrated genomically. The luminescence signal of these strains was compared with the equivalent single transformants strain. For *HWP1p*, both single transformant strain (HwK7RH; *HWP1p-CBRluc*) and double transformant strain (HwAc; *HWP1p-CBRluc*, *Act1p-CBGluc*) showed a clear activation of the *HWP1* promoter upon hyphal growth (Figure III.3-11B). Moreover, the *HWP1p-CBRluc* luminescence measured in both strains was similar, within the limits of sample-to-sample variation. Likewise, for *TCC1p*, the single transformant parental strain (ipTGL; *TCC1p-CBGluc*) and the double

luciferase strain (HwTc; *HWP1p-CBRluc*, *ACT1p-CBGluc*) had similar luminescence levels (Figure III.3-12B). The click beetle luciferases can thus be used in *C. albicans* to effectively monitor the activity of two genes simultaneously.

Signal separation with high quality optical filters is completed by a careful post-measurement calibration that takes into account the luciferase and measurement system specificity (filters and luminometer; Results 2.1.3). Because of the possibility of measuring the activity of two gene activities at the same time, the *ACT1* gene can be used as a reference. Alternatively, for a detailed comparison of two genes, the luciferase ORFs can be integrated downstream of both promoters and their activity compared directly and tested in a range of conditions.

To measure the activity of two genes simultaneously, a system had previously been employed in commercial test kits (Dual-Glo Luciferase Assay System, Promega). This Dual-Glo luciferase system uses both a coelenterazine based luciferase (*Renilla* or *Gaussia* luciferases) and a beetle luciferin-based luciferase (firefly or click beetle luciferases). Because of the use of coelenterazine, cells must be broken in order to use this system in *C. albicans*. Following cell lysis, a first reagent containing beetle luciferin is added to the sample. After measurement of the firefly luciferase activity (or CBluc), the second reagent is added. This second reagent stops the first reaction and contains the substrate for the second (coelenterazine). Aside from being experimentally cumbersome, which significantly impairs the accurateness and practicality of the system, it has several drawbacks. First, the firefly/click beetle luminescence can only be read at one time point, which limits the accurateness of the results, as discussed before (Discussion 1.3). Second, because the second luciferase is coelenterazine-based, cells must be broken and the signal's peak is very short (Lassak *et al.,* 2011; Results 2.4). Finally, as two different luciferases are used it is unlikely that their activity will be exactly similar in identical environment and temperature conditions. On the other hand, the CBRluc and CBGluc luciferases are structurally and chemically almost identical and use the same substrate. Consequently, when using click beetle luciferases, the luminescence of both colours can be conveniently monitored at the same time.

2. Luciferases as reporters of the cAMP/PKA pathway

The click beetle luciferase genes were integrated downstream of promoters of several relevant components of the cAMP/PKA pathway: *HWP1* (cell wall protein abundantly expressed in hyphal form only), *UME6* (transcription factor expressed only in hyphal form) and *TCC1* (part of a global repression system, represses hypha-specific genes).

Using the *HWP1p-CBluc* strains, a negligible luminescence signal was observed in the yeast form, while a very powerful signal was measured during hyphal growth (184 fold stronger than yeast, Results 3.1.2). These results are in accordance with the observation by Staab *et al*. (1996) that Hwp1 was solely expressed during hyphal growth. The strength of the luminescence signal during hyphal growth is also consistent with the report by Heilmann *et al.* (2011) that Hwp1 was the most abundant protein in the cell wall of *C. albicans* hyphae. The click beetle luciferases reported accurately the activity of the *HWP1* promoter. The comparison of *HWP1* and *ACT1* expression levels during hyphal growth (HwAc strain) shows that *HWP1* expression is 77 time stronger than *ACT1* expression, which demonstrates the very high level of *HWP1* promoter activity during hyphal growth (Results 3.4.2). These results are in contradiction with the observation of Doyle *et al.* (2006), who reported a level of expression similar to *ACT1* during hyphal growth. In addition, these authors observed an increase in *HWP1* expression after 1 h post hyphal induction, while this was measured at 10 min with the click

beetle luciferase (Results 3.1.4). Doyle *et al.* (2006) observed a ratio of ≈10 between yeast and hyphal growth conditions luminescence, which is much lower than the 816 ratio measured here (Results 3.1.2). The *HWP1* promoter activity is highly dependent on the hyphal inducing condition, but the media used are comparable. Doyle *et al.* (2006) also reported a high background activity for wild-type strains not expressing the luciferase (≈20 fold lower than *HWP1* activity during hyphal induction). In contrast, using the click beetle luciferase, the activity of non-luminescent strains was negligible (Figure III.2-3; $5.5x10^4$ fold lower than *HWP1* activity during hyphal induction). These contrasting observations are probably due to the optimisation of the luciferase ORF sequences discussed earlier (Discussion 1.1). The detection system, charge-coupled device against photomultiplier tube, and the use of Chroma-Glo reagent instead of pure luciferin might also make a difference.

Using the UmK7GU (*UME6-K7GU*) and ipD-R (*ACT1-CBRluc*) strains, the relative expression of *UME6* was measured during hyphal growth. The relative *UME6* expression level (L_{UME6}/L_{ACT1}) was 26 fold lower than *HWP1*; this is consistent with the role of Ume6 as a transcription factor (Results 3.1.2 and 3.1.3; Banerjee *et al.*, 2008). The activity of the *UME6* promoter was also strongly induced during hyphal growth; the luminescence signal was 4 times higher in hypha than in yeast. Barnejee *et al.* (2008) reported a similar induction (Results 3.1.3). The activity of *HWP1* and *UME6* promoters was monitored during early hyphal induction by measuring the luminescence signal in HwK7GU (*HWP1p-CBGluc*) and UmK7GU (*UME6p-CBGluc*) strains. A luminescence signal was observed after 10 min. The induction of *HWP1p* was much higher than *UME6p* in the first 40 minutes (51x for *HWP1* and 3x for *UME6*). Between 40 min and 100 min, the induction of *HWP1* remained higher but the difference was less drastic: 10x for *HWP1p* and 7x for *UME6p*. A slight diminution in *UME6* activity at 20 min was measured, this experiment should be repeated in order to verify the reproducibility of this phenomenon. Barnejee *et al.* (2008) reported that Ume6 is up-regulated at 15 min post hyphal induction only, which is coherent with the aforementioned measurements (Barnejee *et al.,* 2008). To gain insight in the early hyphal induction mechanisms, the activity could be monitored during the first 10 min of hypha-induction for both *UME6*, *HWP1* and *TCC1*. The use of HwK7RH strain and a corrected version of UmK7GU strain would insure an optimal sensitivity (Results 1.3). Barnejee *et al.* (2008) suggested that Nrg1 (repressed under hyphal growth, associated with Tup1 global repressor) and Ume6 are engaged in a negative regulation loop and that the increase in *UME6p* activity is subsequent to *NRG1* down-regulation. To learn more about the regulation of these genes and the time-frame of their respective regulations it would be interesting to construct *C. albicans* strains with the click beetle luciferase gene downstream of the *NRG1* promoter. In particular, double transformants strains with *UME6* and *NRG1* (or *UME6* and *EFG1*) would allow a close monitoring over time of the regulation pathways during early hyphal induction and hyphal maintenance. Mutant strains of the cAMP/PKA pathway could be used in conjunction with the luciferase reporters.

The luminescence of the ipTGL strain (*TCC1p-CBGluc*) was measured during yeast and hyphal growth. A slight but significant up-regulation of the *TCC1* promoter was observed upon hyphal growth (1.2x; Results 3.1.5). However, using the *ACT1p* expression as a reference, the relative *TCC1* expression level (L_{TCC1}/L_{ACT1}) was lower in hyphae than in yeast (0.5x). Lassak *et al.* (2011) observed a similar diminution of relative *TCC1* expression during hyphal induction. It is however worth mentioning that the absolute level of *TCC1p* activity increased slightly during hyphal induction (Figure III.3.5A). This result could be further confirmed by monitoring the *TCC1p* activity over time during hyphal induction in a similar manner as for *HWP1* and *UME6* promoters.

The decisive advantage of click beetle luciferase reporters is that once the strains are constructed, the comparison of the activity in a variety of conditions is straightforward. It would for example be interesting to measure the activity of *HWP1* promoter during hyphal induction in hypoxic

conditions. Although the luciferase needs oxygen to catalyse the oxidation of luciferin, samples can be taken from anoxic cultures, mixed directly with the Chroma-Glo reagent and kept on ice. Once the samples are collected, they can be loaded on microtiter plates and the luminescence measured in normal conditions (30 °C, with oxygen). Alternatively, samples could be mixed with Chroma-Glo lysis buffer and frozen in liquid nitrogen until measurement time.

A strain with *EFG1p-CBluc* fusion genomically integrated would be an essential tool to understand the cAMP/PKA pathway. In particular, such a strain would shed light on the regulation during hyphal induction, where *EFG1* is temporarily down-regulated (Lassak *et al.,* 2011; Stoldt *et al.,* 1997). Promoter dissection as realised by Lassak *et al.* for the *EFG1* promoter is also possible with click beetle luciferases, with the advantage of the ease of use and accuracy of the measurements.

3. Future perspectives and potential applications of the click beetle luciferases in *C. albicans*

In the pharmaceutical industry, a common way to find new compounds to treat infections is to test the impact of large libraries of compounds on cell viability or a specific physiological processes. Recently, research has turned towards compounds able to attenuate virulence factors (Jiang, 2002). In *C. albicans* the filamentation is a key virulence factor because of its role in pathogenicity and the associated expression of virulence factors (Introduction 1. and 2.; Lo *et al.,* 1997). As mentioned above, Hwp1 expression is characteristic of hyphal growth (Staab *et al.,* 1996, 1999; Results 3.1.2). A fusion of *HWP1–lacZ* constructed by Hogan *et al.* (2004) was used by Heintz-Buschart and colleagues (2013) for high throughput screening. The *HWP1-lacZ* strain was efficient for high throughput screening in *C. albicans* and the authors managed to isolate molecules inhibiting *C. albicans* hyphal growth. Given the advantages of click beetle luciferase over β-galactosidase, we tested the potential of CBRluc/CBGluc for high throughput screening. The facilities for real high-throughput screening of large compounds libraries were not available for this work, but the proof of principle was provided by the quorum-sensing molecules farnesol and tyrosol. A significant diminution in *HWP1*-induced luminescence was observed when cells were treated with farnesol (Results 3.3.1). However, the measured effect was not as strong as reported by Heintz-Buschart *et al.* (2013). YP+10 % serum was used in this work instead of SLAD medium by these authors. The concentration at which farnesol inhibits filamentation has been known to be subject to high variation depending on the conditions of the experiment (Mosel *et al.,* 2005). In particular, albumin, present in serum, can drastically increase the concentration of farnesol needed to inhibit filamentation (Mosel *et al.,* 2005). The results observed in this experiment could in the future be confirmed by repeating the measurements using SLAD medium and fresh E,E-farnesol. The capacity of tyrosol, another quorum sensing molecule, to increase filamentation was also tested. *C. albicans* cells in the presence of tyrosol displayed a stronger luminescence by 31 % than the control. Both of these experiments show the potential of *HWP1p-CBluc* and more generally of click beetle luciferase for high throughput screening.

The fusion of the *ACT1* promoter and GPI-anchored *G. princeps* luciferase (*ACT1p-gLUC59*) was successfully used by Enjalbert *et al.* (2009) as a reporter of *C. albicans* cells viability. In the same manner, the HwAc strain (*HWP1-CBRluc* and *ACT1-CBGluc*) could be used to monitor cell viability. With this double integration strain, the capacity of treated cells to filament can be measured at the same time as their viability, thereby avoiding the need to assess it in a separate experiment. This system would allow the simultaneous testing of two essential parameters, enabling a quick selection of potential new antifungal drug candidates.

Using the Axioskop 40 microscope (Zeiss) combined with Axiocam camera (Zeiss), we were unable to observe a signal for individual *C. albicans* cells expressing the click beetle luciferase (*ACT1-CBGluc*). Being able to observe variation between individual cells for the expression of a gene would however be a precious asset. Recently, Pierce *et al.* (2012) showed that individual *C. albicans* cells have different Efg1 expression level, putting forward the importance of single cell variation in the yeast-to-hypha transition regulation. This might explain in part the ability of *C. albicans* to form hyphae in a range of conditions and to survive in the host. In the future, the luminescence of individual cells could possibly be tested with a more sensitive microscope designed for luminescence (less photon loss during the data gathering) and a deep-cooled CCD to improve the signal/noise ratio of the sensor.

For pathogens, the use of animal models is essential to understand the behaviour of a micro-organism in conditions mimicking more or less accurately the human body. Luminescent strains have proved extremely useful for animal experiments; they avoid the sacrifice of the animals and permit a regular assessment of the disease's progression (Dumetz *et al.,* 2011). The firefly luciferase and a modified version of the *Gaussia* luciferase were used for this purpose in *C. albicans* (Doyle *et al.,* 2006; Enjalbert *et al.,* 2009). Of the two, the more efficient system was the system described by Enjalbert *et al.* to cope with the poor permeability of *C. albicans* cells to coelenterazine, the authors fused the *Gaussia* luciferase gene with a gene coding for a GPI anchored cell wall protein. The resulting fusion was displayed at the cell surface, allowing free access to the substrate. The resulting reporter was efficient for measuring the progress of *C. albicans* vulvo-vaginal and oropharyngeal infections and was used successfully for investigating new antifungal treatments (Enjalbert *et al.,* 2009; Pietrella *et al.,* 2010, 2012). However, this system was unsuccessful to monitor deep seated infections that occur during disseminated candidiasis. As discussed by these authors and by Brock *et al.* (2012), this could be due to a high background luminescence due to luciferin auto-oxidation, or to poor availability of coelenterazine in the organs. The firefly luciferase was also tested in animal models but was only partially successful in monitoring the deep seated infections because of weak luminescence signals especially in hyphae (Doyle *et al.,* 2006). The use of click beetle luciferases could solve some of these issues. The *Candida* adapted click beetle luciferase could be used in vivo employing the ipD-R strain (*ACT1p-CBRluc*). Potentially, this system would have several advantages. The beetle luciferin availability in the organs is better than for coelenterazine, which might help measurements for disseminated candidiasis. Unlike coelenterazine, beetle luciferin is not subject to auto-oxidation and the background luminescence should be lower. As previously mentioned, the presence of a peroxisome targeting sequence and the weak expression that penalised the firefly luciferase as a reporter have been solved (Doyle 2006; Leskinen *et al.,* 2003; Results 1.1). For studies where the role of hyphae in animal infection is of interest, the HwK7RH strain would be optimal because of the strength of the luminescence signal. Besides the benefits of *Candida*-adapted click beetle luciferases discussed above, the spectrum of emission of the red click beetle luciferase is well-suited for *in vivo* experiments because part of the emission spectrum is above 600 nm, where tissue absorption is less problematic (Brock *et al.,* 2012; Rice *et al.,* 2001; Results 1.2.3). This is especially important for deep-seated infections and might help alleviate the difficulties reported with in vivo experiments using luminescence to study disseminated candidiasis.

The advantages and drawbacks of currently used luminescent/fluorescent reporter proteins in *C. albicans* are summarised in Figure IV-1

Reporter	Advantages	drawbacks	References
GFP	Strong signal Protein localisation possible Does not require a substrate	Time lag between expression and signal GFP is very stable in *C. Albicans* Poor signal/background noise ratio Poor scaling between weak & strong promoters Requires Oxygen	Cormack *et al.,* 1997 Morschhäuser *et al.,* 1998
FbFP	As GFP Florescent in anoxic conditions	As GFP, but does not require oxygen Signal is weaker than GFP	Tielker *et al., 2009*
Rluc	Good signal/background noise ratio	Very short signal half-life in *C. albicans* Uses coelenterazine: Cell lysis needed in *C. albicans* Poor availability in animal tissues *in vivo* Auto-oxidation Not suited for high throughput studies	Srikantha *et al.,* 1996
gLUC59	Good signal/background noise ratio Surface display, no need for cell lysis Good sensitivity	Surface display, not suited to all cases Uses coelenterazine: Poor availability in animal tissues *in vivo* Auto-oxidation	Enjalbert *et al.,* 2009
Fluc	Excellent signal/background noise ratio Uses beetle luciferin: Live cells assays possible No Auto-oxidation	Poor luminescence in hyphae Average sensitivity Non codon-adapted for *C. albicans* Available only in one colour Contains SKL peroxisome targeting sequence	Doyle *et al.,* 2006
CBluc	Excellent signal/background noise ratio Excellent sensitivity codon-adapted for *C. albicans* Contains peroxisome targeting sequence Uses beetle luciferin: Live cells assays possible No Auto-oxidation Suited to high-throughput studies Available in red and green	Luminescence is not detectable in single cells Not tested *in vivo*	This work

<u>Figure IV-1</u>: Comparison of the different light-based reporters available in *C. albicans*

The advantages of the light-based reporters available in *C. albicans* are shown, with their advantages and drawbacks.

V. References

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VI. List of abbreviations

°C degree Celsius
5-FOA 5-fluoroorotic acid
Amp ampicilin
Bp base pair
BSA bovine serum albumin
C. albicans Candida albicans
CBluc Click beetle luciferase
CBGluc Click beetle green luciferase
CBRluc Click beetle red luciferase
CFU colony forming units
cAMP cyclic adenosine monophosphate
DNA deoxyribonucleic acid
DTT dithiothreitol
E. coli Escherichia coli
EDTA ethylenediamine tetraacetic acid
FbFP flavin mononucleotide-based fluorescent protein
Fluc firefly luciferase
GFP green fluorescent protein
GlcNAc N-acetylglucosamine
gLUC59 PGA59-gLUC fusion
gLUC *Gaussia princeps* luciferase
h hour
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV human immunodeficiency virus
IPTG isopropyl β-D-1-thiogalactopyranoside
Kbp kilo base pair
Kan Kanamycin
LiAc lithium acetate
L_{max} maximal luminescence
LT CD4+ thymus lymphocytes cluster of differentiation 4 positive
log logarithmic
MAPK mitogen-activated protein kinase
min minute
ml milliliter
mM milimolaire
M molaire
NaAc sodium acetate
ng nanogram
nm nanometer
OD_{600} optical density at 600 nm
ORF open reading frame
PCR polymerase chain reaction

PEG polyethylenglycol
PKA protein kinase A
qs quantum satis (quantity sufficient)
RNA ribonucleic acid
RLU relative luminescence unit
Rluc *Renilla reniformis* luciferase
RT room temperature
S. cerevisiae Saccharomyces cerevisiae
SDS sodium dodecyl sulphate
SLAD synthetic low-ammonium–dextrose
TAE tris-Acetat-EDTA
Tris trishydroxymethylaminomethane
V_f final volume
WT wild-type
X-gal 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
YNB yeast nitrogen base
YP yeast extract-Pepton
YPD yeast extract-Pepton-Dextrose

VII. Annexes

(A) Click beetle red luciferase genes

Upper strand: *CBRluc* (Almond *et al.,* 2003)
Lower strand: *CaCBRluc* (This work)

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...ca.a...t.ga.a..a..t..a........a.....t.....t.....t..t..t........a...tca..t..a.

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.a.....c..a..t..t..t...t.......a..at.g..t........t.....a.....t..t..t...tca..t...

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..............ta.a..............t..t..t..t........t........t..t........t..t..atc

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.........a........t..a..t.....t..t..at.......t.....t..a.....ttca..a.....c..t..c
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```
aacgttgaagctaccaaggaggccatcgacgacgacggctggttgcattctggtgattttggatattacgacgaagatga
..t..........t..a..a..t..t..t..t.....t..........a........c..t.....t..t........

gcatttttacgtcgtggatcgttacaaggagctgatcaaatacaagggtagccaggttgctccagctgagttggaggaga
a.....c.....t..t..ca.a..t..a..at.............a...tca..a.............a.....a..a.

ttctgttgaaaaatccatgcattcgcgatgtcgctgtggtcggcattcctgatctggaggccggcgaactgccttctgct
..t................t...a.a.....t.....t..t..t.....a...t....a..t..t...t....a..a...

ttcgttgtcaagcagcctggtacagaaattaccgccaaagaagtgtatgattacctggctgaacgtgtgagccatactaa
........t..a..a..a.....t........t..t........t..c.....tt........a.a..ttca.....a..

gtacttgcgtggcggcgtgcgttttgttgactccatccctcgtaacgtaacaggcaaaattacccgcaaggagctgttga
a......a.a..t..t..ta.a..c.....t..a..t..aa.a..t..t..t..t........ta.a..a..at......

aacaattgttggtgaaggccggcggttag
.............t..a.....t.....a
```

(B) Click beetle green luciferase genes

Upper strand: *CBG68luc* (Almond *et al.,* 2003)
Lower strand: *CaCBGluc* (This work)

```
atggtgaaacgcgaaaagaacgtgatctacggcccagaaccactgcatccactggaagacctcaccgctggtgagatgct
.....t..ga.a.....a.....t.....t..t.........t........t.......tt.g..t........a...t.

cttccgagcactgcgtaaacatagtcacctccctcaagcactcgtggacgtcgtgggagacgagagcctctcctacaaag
g...a....tt..a.a......tca..tt.g..a.....tt.g..t..t..t..t..t..t..atcat.g..t.......

aatttttcgaagctactgtgctgttggcccaaagcctccataattgtgggtacaaaatgaacgatgtggtgagcatttgt
....c..............tt.......t...tcat.g...........t...........t.....t..ttca......

gctgagaataacactcgcttctttattcctgtaatcgctgcttggtacatcggcatgattgtcgcccctgtgaatgaatc
.....a..c......a.a.....c.....a..t..t...........t..t..t.....c..t..t..a..t........

ttacatcccagatgagctgtgtaaggttatgggtattagcaaacctcaaatcgtctttactaccaaaaacatcttgaata
a..t..t........at.......a............tca.....a........t..c.....a........t.....c.

aggtcttggaagtccagtctcgtactaacttcatcaaacgcatcattattctggataccgtcgaaaacatccacggctgt
.a..t........t..a..aa.a.....t.....t..ga.a..t......t.......t..t........t..t..t...

gagagcctccctaacttcatctctcgttacagcgatggtaatatcgctaatttcaagcccttgcattttgatccagtcga
..atcat.g..a..t.....t..aa.a..ttca...........t...........a..a........c........t..

gcaagtggccgctattttgtgctcctccggcaccactggtttgcctaaaggtgtcatgcagactcaccagaatatctgtg
a.....t..t...........t..a..a..t..t...........a........t.....a.....t..a..c..t....

tgcgtttgatccacgctctcgaccctcgtgtgggtactcaattgatccctggcgtgactgtgctggtgtatctgcctttc
.ta.a.....t..t...t.a..t..aa.a..t..............t..a..t..t.....tt....t...t....a...

tttcacgcctttggtttctctattaccctgggctatttcatggtcggcttgcgtgtcatcatgtttcgtcgcttcgacca
..c..t..t..c........a.....tt....t...........t..t...a.a..t..t.....ca.aa.a.....t..

agaagccttcttgaaggctattcaagactacgaggtgcgttccgtgatcaacgtcccttcagtcattttgttcctgagca
......t........a...........t..t..a..ta.a..a..t..t.....t..a.....t.........t..tca.
```

```
aatctcctttggttgacaagtatgatctgagcagcttgcgtgagctgtgctgtggcgctgctcctttggccaaagaagtg
....a..a........t.........t..tcatca...a.a..at....t.....t........a.....t........c

gccgaggtcgctgctaagcgtctgaacctccctggtatccgctgcggttttggtttgactgagagcacttctgctaacat
..t..a..t........aa.at....tt.g..a.....ta.a..t.....c..........atca.....a.....t..

ccatagcttgcgagacgagtttaagtctggtagcctgggtcgcgtgactcctcttatggctgcaaagatcgccgaccgtg
t...tca...a....t..a..c..a..a...tcat.....a.a..t.....at.g........t..a..t..t..ta.a.

agaccggcaaagcactgggcccaaatcaagtcggtgaattgtgtattaagggccctatggtctctaaaggctacgtgaac
.a..t..t.....tt....t..........t.....................t..a.....t..a.....t..t..t...

aatgtggaggccactaaagaagccattgatgatgatggctggctccatagcggcgacttcggttactatgatgaggacga
.....t..a..t..a........t.....c..c..c..t...t.g...tca..t..t........t........a..t..

acacttctatgtggtcgatcgctacaaagaattgattaagtacaaaggctctcaagtcgcaccagccgaactggaagaaa
...t........t..t...a.a.........................t..a.....t..t.....t...t.........

ttttgctgaagaacccttgtatccgcgacgtggccgtcgtgggtatcccagacttggaagctggcgagttgcctagcgcc
.....t....a..t..a.....ta.a..t..t..t..t..t.....t.....t..........t..a.....atca..t

tttgtggtgaaacaacccggcaaggagatcactgctaaggaggtctacgactatttggccgagcgcgtgtctcacaccaa
..c..t..t..g.....a..t..a..a..t........a..a..t.....t........t..aa.a..t..a..t..a..

atatctgcgtggcggcgtccgcttcgtcgattctattccacgcaacgttaccggtaagatcactcgtaaagagttgctga
g...t..a.a..t..t..ta.a.....t.....a......a.a..t.....t.....a..t...a.a.....a..at...

agcaactcctcgaaaaagctggcggctag
.a...t.at.g..........t..t..a
```

Figure S1. Alignment between the *C. albicans*-adapted and original click beetle luciferases ORFs sequences
The sequences genes encoding the click beetle red and green luciferases as published by *Almond et al*., 2003 were aligned with the *C. albicans*-adapted click beetle luciferases presented in Results 1.1. Sequences were aligned with Clone Manager (Sci-Ed), the bases differing between sequences are shown. (A) Gene encoding click beetle red luciferase; Upper strand: CBRluc (Accession number: AY258591; Almond *et al.*, 2003); Lower strand: CaCBRluc (This work). (B) Gene encoding click beetle green luciferase; Upper strand: CBG68luc (accession number: AY258593; Almond *et al.*, 2003), Lower strand: CaCBGluc (This work)

(A) Codon index for *C. albicans*, Candida Genome Database

```
UUU 30.1( 89902)  UCU 19.9( 59287)  UAU 25.8( 77027)  UGU  8.9( 26651)
UUC 14.1( 41994)  UCC  8.7( 26096)  UAC  9.7( 28880)  UGC  1.9(  5700)
UUA 38.9(116049)  UCA 27.0( 80576)  UAA  1.1(  3276)  UGA  0.3(  1019)
UUG 34.0(101379)  UCG  6.9( 20617)  UAG  0.6(  1797)  UGG  9.7( 28969)

CUU 10.4( 30999)  CCU 13.0( 38715)  CAU 15.6( 46627)  CGU  6.1( 18089)
CUC  2.8(  8297)  CCC  4.5( 13513)  CAC  5.6( 16075)  CGC  0.9(  2716)
CUA  6.0( 17757)  CCA 23.9( 71430)  CAA 37.2(110878)  CGA  5.2( 15521)
CUG  4.2( 12575)  CCG  3.1(  9325)  CAG  7.6( 22803)  CGG  1.3(  3768)

AUU 40.0(119355)  ACU 26.0( 77494)  AAU 48.3(144112)  AGU 17.8( 53230)
AUC 13.0( 38657)  ACC 11.7( 35024)  AAC 18.6( 55425)  AGC  5.0( 14955)
```

```
AUA 18.1( 54113)  ACA 19.5( 58294)  AAA 53.9(160765)  AGA 21.1( 62927)
AUG 17.8( 53092)  ACG  4.1( 12209)  AAG 19.5( 58175)  AGG  3.2(  9394)

GUU 26.2( 78121)  GCU 21.6( 64347)  GAU 45.6(136115)  GGU 24.1( 71867)
GUC  7.9( 23518)  GCC 10.0( 29911)  GAC 13.2( 39337)  GGC  4.4( 13184)
GUA  9.7( 28990)  GCA 16.2( 48339)  GAA 51.1(152586)  GGA 14.5( 43380)
GUG 10.4( 30930)  GCG  2.4(  7070)  GAG 13.2( 39354)  GGG  7.5( 22390)
```

(B) Codon index for *C. albicans*, Kazusa DNA research Institute Codon Usage Database

```
UUU 29.8( 18958)  UCU 22.0( 13984)  UAU 25.4( 16146)  UGU  9.4(  5964)
UUC 15.6(  9899)  UCC  9.7(  6145)  UAC 10.4(  6614)  UGC  1.8(  1135)
UUA 36.1( 22928)  UCA 26.4( 16751)  UAA  1.0(   632)  UGA  0.3(   180)
UUG 34.6( 21993)  UCG  6.8(  4341)  UAG  0.5(   336)  UGG 10.9(  6942)

CUU 10.2(  6456)  CCU 13.4(  8495)  CAU 14.7(  9373)  CGU  6.0(  3791)
CUC  2.6(  1636)  CCC  4.2(  2665)  CAC  5.6(  3578)  CGC  0.8(   523)
CUA  4.4(  2782)  CCA 26.3( 16709)  CAA 35.7( 22696)  CGA  4.1(  2604)
CUG  3.5(  2201)  CCG  2.7(  1721)  CAG  6.5(  4163)  CGG  1.0(   604)

AUU 40.5( 25761)  ACU 30.6( 19438)  AAU 42.7( 27162)  AGU 17.5( 11094)
AUC 13.5(  8590)  ACC 13.5(  8567)  AAC 18.2( 11560)  AGC  4.6(  2955)
AUA 14.4(  9127)  ACA 18.8( 11928)  AAA 49.0( 31114)  AGA 21.7( 13817)
AUG 18.2( 11591)  ACG  3.9(  2501)  AAG 18.3( 11660)  AGG  2.9(  1834)

GUU 30.1( 19155)  GCU 27.4( 17393)  GAU 43.7( 27797)  GGU 29.2( 18556)
GUC  9.1(  5773)  GCC 11.7(  7453)  GAC 13.4(  8545)  GGC  4.4(  2818)
GUA  8.6(  5460)  GCA 16.0( 10162)  GAA 49.9( 31701)  GGA 13.7(  8710)
GUG 10.4(  6612)  GCG  2.1(  1346)  GAG 11.9(  7547)  GGG  7.8(  4945)
```

Figure S2. Codon usage tables of *C. albicans*

The codon abundance in the genome of *C. albicans* is shown in the format: triplet, triplet frequency [per thousand], (number). (A) Codon index for *C. albicans*, Candida Genome Database (http://www.candidagenome.org/). (B) Codon index for *C. albicans*, Kazusa DNA research Institute Codon Usage Database (http://www.kazusa.or.jp/codon/)

VIII. Summary

The human fungal pathogen *Candida albicans* causes life-threatening infections in immunocompromised patients and is a major source of nosocomial illnesses. At present, virulence factors of *C. albicans* are intensely studied with the goal of identifying novel targets for antifungal drugs. The gene reporters currently employed to investigate the virulence factors of *C. albicans* have disadvantages including low sensitivity, high background signal or the necessity for cell lysis. In this work, we describe the use of the click beetle red and green luciferases (CBRluc and CBGluc) as versatile reporter proteins that produce light by catalysing the oxidation of beetle luciferin in the presence of oxygen and ATP.

The open reading frames for red and green click beetle luciferase were codon-adapted for *C. albicans* and were initially expressed in *Saccharomyces cerevisiae* and *C. albicans* strains using episomal plasmids. In both yeast species, CBRluc and CBGluc were produced and generated strong luminescence. In addition, the luciferases genes were chromosomally integrated into the *C. albicans* genome downstream of various promoters. All transformant strains were luminescent in the appropriate inducing conditions. In *C. albicans*, CBRluc and CBGluc luminescence spectra were verified and their distinct emission peaks could be measured separately using optical filters. Strong luminescence activity was observed in live cells of transformant strains, which could be improved further by a quick freeze/thaw cycle. Using the strong *ACT1* promoter, in optimised conditions, the click beetle luciferase sensitivity in live cells was measured to a threshold of 50 cells.

Genes implicated in the main signal transduction pathway regulating hyphal morphogenesis in *C. albicans* (cAMP/PKA pathway) were chosen as integration targets for *CBR/CBGluc*. During hyphal induction, *ACT1* promoter activity increased two fold, while *UME6* and *HWP1* promoter activities rose drastically; the activity of the *TCC1* promoter remained stable. The reactivity of the CBluc reporters was demonstrated by the detection of *UME6*- and *HWP1*-related (hypha-specific) signals only 10 min after hyphal induction. *HWP1-CBluc* activity, employed as a marker of hyphal growth, was used to measure the impact of different media on hypha formation. Farnesol, a quorum sensing molecule that promotes hypha formation significantly increased *HWP1-CBluc* related luminescence, while the hyphal inhibitor tyrosol diminished it. The capacity to accurately measure red and green signals at the same time was verified using strains carrying both luciferases under the control of two different promoters. After filtration and correction for spectra crosstalk, the luminescence of strains reflected precisely the activity of each promoter

The click beetle luciferases compare favourably against other gene reporters, since no cell lysis is required for their detection and the background signal is negligible. Furthermore, as shown with the *HWP1-CBluc fusion,* CBluc can be used in high throughput screening for novel antifungal compounds.

Acknowledgments

" Rien n'est précaire comme vivre
Rien comme être n'est passager
C'est un peu fondre comme le givre
Et pour le vent être léger
J'arrive où je suis étranger

Un jour tu passes la frontière
D'où viens-tu mais où vas-tu donc
Demain qu'importe et qu'importe hier
Le coeur change avec le chardon
Tout est sans rime ni pardon

Les arbres sont beaux en automne
Mais l'enfant qu'est-il devenu
Je me regarde et je m'étonne
De ce voyageur inconnu
De son visage et ses pieds nus "
Louis Aragon

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