

Review

Deus ex Candida genetics: overcoming the hurdles for the development of a molecular toolbox in the CTG clade

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Dominant selectable markers, reporter genes and regulatable systems remain powerful molecular tools for genetic and cell biology studies in fungi. Among *Saccharomycotina*, it is currently accepted that most species belonging to the genus *Candida* have adopted a specific codon usage, whereby the CTG codon encodes serine instead of leucine. This group is now widely referred to as the CTG clade. For a long time, this uncommon genetic code has precluded the use of the available *Saccharomyces* or bacterial markers and reporter systems for genetic studies in *Candida* species. Over the last 15 years, increasing effort has been made to adapt drug-resistance markers, fluorescent protein variants, luciferase and recombinase genes to favour their expression in species related to the yeast CTG clade. In addition to the growing set of *Candida* genome sequences, these codon-optimized molecular tools have progressively opened a window for the investigation of the conservation of gene function within *Candida* species. These technical advances will also facilitate future genetic studies in non-*albicans* *Candida* (NAC) species and will help both in elucidating the molecular events underlying pathogenicity and antifungal resistance and in exploring the potential of yeast metabolic engineering.

Introduction

Candida albicans and non-*albicans* *Candida* species (NAC) are ascomycetous yeasts which have been heavily studied over the last 20 years due to their clinical importance, their biotechnological interest and their biological control potential.

In humans, some of these fungal agents are responsible for candidiasis and have been described as emerging pathogens characterized by their propensity to develop resistance to antifungal agents during treatment. A modification of candidiasis epidemiology has occurred over the last two decades, in particular with an increase of NAC candidiasis (e.g. *Candida dubliniensis*, *Candida parapsilosis* and *Candida lusitanae*) and in the variation of the susceptibility of *Candida* species to antifungal agents. These changes are promoting research programmes aimed at identifying new molecular events supporting pathogenicity and antifungal resistance in these emerging yeasts. The interest in NAC species is notably strengthened by the growing set of available *Candida* genome sequences provided by the Broad Institute (Butler *et al.*, 2009).

Besides their clinical relevance, various *Candida* species (e.g. *Candida tropicalis*, *Candida maltosa*, *Candida famata*, *Candida rugosa*), and also *Pichia stipitis*, represent powerful biotechnological models for bioconversions or industrial production of value-added metabolites. The efficient ability

of certain *Candida* strains to metabolize plant by-products is being evaluated by a number of research teams in order to produce biocompounds such as antibiotics, vitamins, complex alkanes and biofuel. The trend towards this *Candida* ‘white biotechnology’ originates from the extraordinary capacities of a small number of *Candida* species to metabolize C₅ sugars from hemicellulosic waste (Akinterinwa *et al.*, 2008).

In addition, some *Candida* species have important potential for biological control. Some antagonistic yeasts from the CTG clade (e.g. *Candida guilliermondii* and *Candida oleophila*) are used for post-harvest biological control of spoilage fungi during storage of plant-derived products. However, the mechanisms by which these yeasts exert their biocontrol activity have not been fully elucidated (Sundh & Melin, 2011).

All these aspects make *Candida* species attractive models for exploring new molecular mechanisms relating to pathogenicity, antifungal resistance, metabolite bioproduction and biological control. The development of convenient transformation systems, selectable markers and reporter genes for the genetic manipulation of these yeast species has therefore become indispensable.

Since the late 1990s, it has been accepted that the majority of yeast species belonging to the genus *Candida* have

adopted a particular codon usage (Kawaguchi *et al.*, 1989; Ohama *et al.*, 1993; Sugita & Nakase, 1999). More recent phylogenetic studies based on whole-genome analysis indicate that the *Saccharomycotina* can be subdivided into two major groups: (i) species whose genomes have undergone a whole-genome duplication (referred to as the WGD clade), including *Candida glabrata* and yeasts from the genus *Saccharomyces*, and (ii) species that translate CTG as serine instead of leucine (referred to as the CTG clade), including *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. maltosa*, *C. famata*, *C. guilliermondii*, *C. lusitaniae*, *C. oleophila*, *C. rugosa* and *P. stipitis* (Fitzpatrick *et al.*, 2006, 2008, 2010; Butler *et al.*, 2009). For a long period, this particular codon usage has precluded the use of *Saccharomyces* or bacterial markers and reporter genes for genetic studies in *Candida* species. To address this problem, various research groups have concentrated on the adaptation of drug-resistance markers, fluorescent protein variants and luciferase genes to allow the functionality of the expressed proteins in species related to the yeast CTG clade. In the present review, we compile 15 years of studies attempting to modify selectable marker and reporter gene sequences to favour their use in *Candida* species.

Dominant selection markers

Dominant selection genes are commonly used for genetic transformation of a wide range of organisms. In *Candida* species, two major groups of selectable genes are now widely employed to carry out genetic modifications: metabolic markers and drug-resistance cassettes.

As early as the 1970s, the development of a procedure for metabolic-marker-mediated yeast transformation usually began by the generation and isolation of an auxotrophic recipient strain. Following the identification of the disrupted metabolic pathway by supplementation with the appropriate amino acid or base, a study of the genetic basis was undertaken by complementation and mapping (Kakar & Magee, 1982). Once identified and isolated, the gene encoding a functional enzyme that complemented the disrupted metabolic pathway was used as dominant marker in the corresponding auxotrophic recipient strain. For example, many *C. albicans* genetic approaches are based on the BWP17 triple auxotroph (*his1 arg4 ura3*) in which *HIS1*, *ARG4* and *URA3* wild-type genes can be used as selectable markers (Wilson *et al.*, 1999; Samaranayake & Hanes, 2011). These approaches were progressively facilitated by the finding and use of antimetabolites. For example, the introduction of the agent 5-fluoroorotate in 1984 (Boeke *et al.*, 1984) allowed the development of a number of *URA3*-mediated transformation systems in many fungal species. This compound, toxic for prototrophic cells, permits in most cases an easy counter-selection of *ura3* or *ura5* genotypes from a randomly mutagenized population. In the same way, recent advances have demonstrated the potential of 5-fluoroanthranilate for counter-selection of *trp1*, *trp3*, *trp4* or *trp5* genotypes in

yeast (Toyn *et al.*, 2000; Cheon *et al.*, 2003, 2009; Lebel *et al.*, 2006; Jones *et al.*, 2008).

The first demonstration of the necessity to substitute CTG codons in a marker gene for functionality in a *Candida* species was achieved using the *Saccharomyces cerevisiae* *URA3* metabolic marker, which was used to transform a *C. maltosa ura3* recipient strain (Sugiyama *et al.*, 1995). However, drug-resistance cassettes remain the category of selectable markers for which codon usage has been extensively investigated and modified to allow functionality in the CTG clade (Fig. 1a). Although the selection step requires expensive and harmful compounds, drug-resistance markers have the advantage of theoretically allowing the transformation of all strains (especially wild-type strains) of a drug-sensitive *Candida* species (Table 1). As a consequence, these types of dominant markers allow numerous molecular analyses to be performed, including targeted gene disruption (Fig. 1b), random gene disruption (Fig. 1c), the transfer of specific transgenes (Fig. 1d) and/or promoter exchanges (Fig. 1e).

Hygromycin B resistance markers

The *HPH* gene, encoding hygromycin phosphotransferase, which confers resistance to hygromycin B, was the first codon-modified drug-resistance cassette adapted for a *Candida* species, in 2000 (Fig. 1a; Hara *et al.*, 2000). The authors exchanged the nine CTG codons with CTC codons in the ORF of the bacterial *HPH* gene using site-directed mutagenesis. To favour its expression in the n-alkane-assimilating yeast *C. tropicalis* (also known for its clinical relevance), they placed this codon-modified sequence under the control of the transcription-regulating region of the *C. tropicalis* phosphoglycerate kinase (*PGK*) gene. In 2001, the same team adapted this *HPH* cassette for gene disruption strategy and validated its usability by creating a homozygous *ura3* mutant in *C. tropicalis* (Hara *et al.*, 2001). During the same time period, Yehuda *et al.* (2001) reported the direct transformation of the biocontrol yeast *C. oleophila* by the codon-modified *C. tropicalis*-adapted *HPH* cassette. This cassette was notably used to demonstrate the possibility of homologous recombination in this species. Interestingly, in spite of its potential in the CTG clade, the codon-modified *HPH* cassette (Hara *et al.*, 2000) was apparently forgotten, only to reappear 10 years later (Millerioux *et al.*, 2011; Basso *et al.*, 2010).

Basso *et al.* (2010) produced a synthetic *Escherichia coli* hygromycin B resistance gene with optimized *C. albicans* codons. In this synthetic sequence, the nine CTG codons were substituted by other leucine-encoding codons, and a series of codon modifications were added on the basis of the *C. albicans* codon usage table. They placed this sequence under the control of the *C. albicans* *TEF2* promoter and the *ACT1* terminator and demonstrated that this cassette could be used as dominant selectable marker in a set of *C. albicans* wild-type isolates. Millerioux *et al.* (2011) used the codon-modified *HPH* sequence

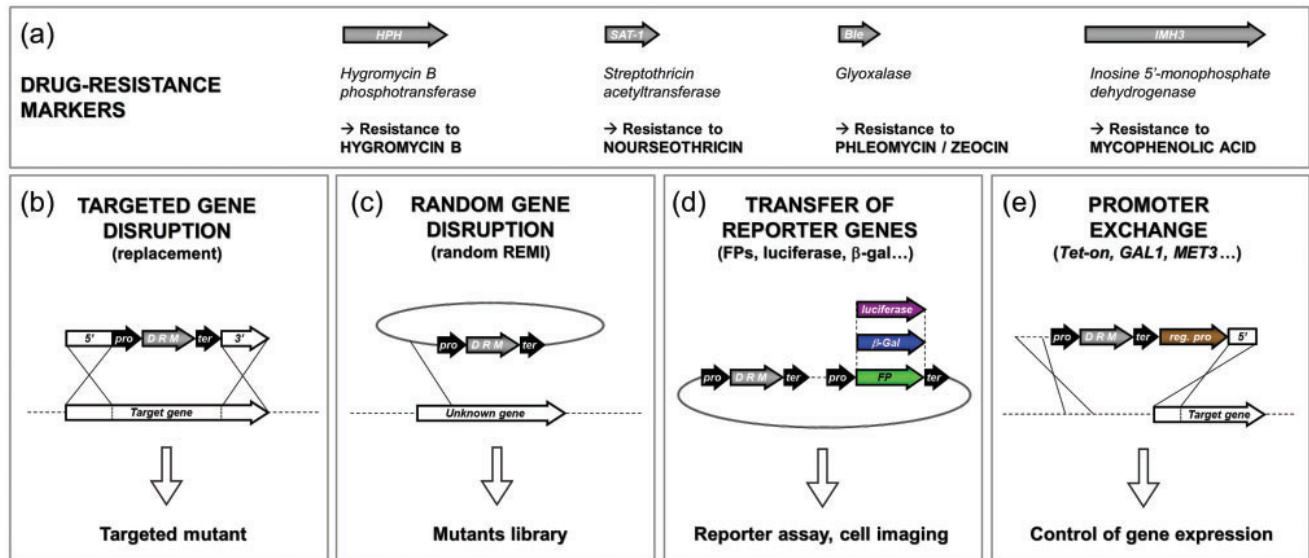


Fig. 1. Drug-resistance markers (DRM) available for transformation of wild-type strains from the CTG clade and various applications in yeast genetic studies. (a) Compilation of DRM and their corresponding selective antibiotics. (b) Use of DRM in targeted gene disruption by classical homologous recombination (see Basso *et al.*, 2010, as an example). (c) Application of DRM in random gene disruption using restriction-endonuclease-mediated integration (REMI) (Dmytruk *et al.*, 2006). (d) Use of DRM to transfer various reporter constructs (FP fusion, luciferase, β -galactosidase...). This strategy can be carried out thanks to autoreplicating plasmids (see Keppler-Ross *et al.*, 2008, as an example), PCR-amplified cassettes (see Milne *et al.*, 2011) or integrative vectors (see Staib *et al.*, 2000b). (e) Application of DRM in promoter exchange (i.e. exchanging the native promoter for a regulatable promoter; see Park & Morschhäuser, 2005, as an example). *pro*, promoter; *ter*, terminator; *reg. pro*, regulatable promoter; FP, fluorescent protein; β -gal, β -galactosidase.

(Hara *et al.*, 2000) to transform a series of *C. guilliermondii* wild-type strains. This codon-modified *HPH* marker allowed the selection of hygromycin B-resistant transformants, but only when the *HPH* sequence was placed under the control of the *C. guilliermondii* *PGK* promoter and terminator.

To date, the hygromycin-resistance gene is available for efficient transformation of wild-type strains of *C. tropicalis*, *C. oleophila*, *C. albicans* and *C. guilliermondii*. *Candida* species appear generally sensitive to hygromycin (used in rich media or buffered minimal media) at concentrations around 400–1000 $\mu\text{g ml}^{-1}$. Thus, this marker could be potentially adapted for use in various other species of the CTG clade, after appropriate changes to the transcription-regulating sequences.

Nourseothricin resistance markers

During the establishment of a global strategy allowing a large-scale identification of *C. albicans* essential genes, referred to as GRACE (gene replacement and conditional expression), Roemer *et al.* (2003) proposed the first codon-modified version of the *E. coli* *SAT-1* gene, encoding streptothricin acetyltransferase, which confers resistance to the antibiotic nourseothricin (Fig. 1a). The unique CTG codon was replaced by a CTT codon, and the modified *CaSAT-1* sequence was successfully expressed using the *C.*

albicans *ACT1* promoter and *PCK1* terminator. Reuss *et al.* (2004) developed a second version of this codon-optimized *SAT-1* by replacing the sole CTG codon by a CTC codon and placing this sequence under the control of the *C. albicans* *ACT1* promoter and the *URA3* terminator (referred to as *caSAT1*). In the same work, they also engineered a flipper variation of this *caSAT1* marker (coupled with a codon-modified recombinase system; see below) usable for multiple gene disruption. One year later, Shen *et al.* (2005) proposed a modified version of the *Streptomyces noursei* *nat1* gene to confer nourseothricin resistance in various *Candida* species. This synthetic gene, named *CaNAT1*, was produced by *de novo* synthesis using the *C. albicans* codon usage table and was placed under the control of transcription-regulating sequences of the *Ashbya gossypii* *TEF1* gene. These authors showed that the presence of this cassette in the genome of *C. albicans* does not impair growth or hyphal development of transformed strains. Furthermore, they demonstrated that *CaNAT1*, driven by the *A. gossypii* *TEF1* gene promoter and terminator, could be used to transform clinical isolates of *C. parapsilosis* and *C. lusitaniae* (Shen *et al.*, 2005). Ding & Butler (2007) adapted the *caSAT1* flipper cassette (Reuss *et al.*, 2004) to efficiently transform *C. parapsilosis* clinical isolates. However, they obtained nourseothricin-resistant isolates only after replacing the promoter driving *caSAT1* gene expression (*C. albicans* *ACT1* promoter) with the *C.*

Table 1. Compilation of drug-resistance markers adapted for *Candida* species

No. of CTG codons, number of CTG codons in the original nucleotide sequence; CTG repl., indicates if the CTG codons from the original nucleotide sequence have been replaced; Codon opt., indicates if a series of codon modifications guided by a codon usage table derived from highly expressed genes has been carried out; NS, not specified.

Gene name	Gene product	Species	No. of CTG codons	Name of adapted version	CTG repl.	Codon opt.	Promoter/terminator	Used in	Reference(s)
HPH	Hygromycin B phosphotransferase	<i>Escherichia coli</i>	9	HYG#	Yes	No	CtPGK/CtPGK	<i>C. tropicalis</i>	Hara <i>et al.</i> (2000, 2001)
				HYG#	Yes	No	CtPGK/CtPGK	<i>C. oleophila</i>	Yehuda <i>et al.</i> (2001)
				CaHygB	Yes	Yes	CaTEF2/CaACT1	<i>C. albicans</i>	Basso <i>et al.</i> (2010)
SAT-1	Streptothricin acetyltransferase	<i>Escherichia coli</i>	1	HPH# (HYG#)	Yes	No	CguiPGK/CguiPGK	<i>C. guilliermondii</i>	Millerioux <i>et al.</i> (2011)
				caSAT-1	Yes	No	CaACT1/CaPCK1	<i>C. albicans</i>	Roemer <i>et al.</i> (2003)
				caSAT1	Yes	No	CaACT1/CaURA3	<i>C. albicans</i>	Reuss <i>et al.</i> (2004)
								<i>C. parapsilosis</i>	Gácsér <i>et al.</i> (2007); Nguyen <i>et al.</i> (2009)
ble	Glyoxalase	<i>Streptomyces noursei</i>	8	SAT1	Yes	No	CguiPGK/CguiPGK	<i>C. guilliermondii</i>	Millerioux <i>et al.</i> (2011)
				CpSAT1	Yes	No	CpACT1/CaURA3	<i>C. parapsilosis</i>	Ding & Butler (2007)
				CaNAT1	Yes	Yes	AgTEF1/AgTEF1	<i>C. albicans</i>	Shen <i>et al.</i> (2005)
								<i>C. parapsilosis</i> <i>C. lusitanae</i> <i>C. rugosa</i>	Tang <i>et al.</i> (2003)
IMH3	Inosine 5'-monophosphate dehydrogenase	<i>Streptoalloteichus hindustanus</i>	5	zeo-n	Yes	No	ScGAL1/ScCYC1 ScTEF1/ ScCYC1 CrLIP3/NS		Wang <i>et al.</i> (2006)
				ble	Yes	No	ScGAP/ScCYC1	<i>C. farinosa</i>	Laplaza <i>et al.</i> (2006)
		<i>Staphylococcus aureus</i>	0	ble	Yes	Yes	PsTEF1/PsXYL2	<i>P. stipitis</i>	Dmytruk <i>et al.</i> (2006)
				Sa_ble	No	No	CjTEF1/NS	<i>C. famata</i>	Köhler <i>et al.</i> (1997)
		<i>Candida albicans</i>	0	IMH3	No	No	CaIMH3/CaIMH3	<i>C. albicans</i>	Beckerman <i>et al.</i> (2001)
				IMH3 ^r	No	No	CaIMH3/CaIMH3	<i>C. tropicalis</i>	Staib <i>et al.</i> (2000b); Wirsching <i>et al.</i> (2001)
				MPA ^R	No	No	CaIMH3/CaIMH3	<i>C. dubliniensis</i>	Gácsér <i>et al.</i> (2005); Kosa <i>et al.</i> (2007)
				IMH3 ^r	No	No	CaIMH3/CaIMH3	<i>C. parapsilosis</i>	Dmytruk <i>et al.</i> (2011)
		<i>Candida famata</i>	0	Dh_IMH3	No	No	CjIMH3/CjIMH3	<i>C. famata</i>	
					No	No			

parapsilosis *ACT1* promoter (generating the *CpSAT1* version). Millerioux *et al.* (2011) used the codon-modified sequence *caSAT1* (Reuss *et al.*, 2004) to transform *C. guilliermondii* wild-type strains. The expression of the *SAT1* marker was once again only obtained after replacing the *caSAT1* transcription-regulating sequences by the *C. guilliermondii* PGK promoter and terminator. Interestingly, Gácsér *et al.* (2007) and Nguyen *et al.* (2009) achieved the disruption of *C. parapsilosis* secreted lipase genes and fatty acid synthase genes, respectively, directly using the *caSAT1* flipper cassette (Reuss *et al.*, 2004), without changing the regulating sequences.

Growth inhibition of *Candida* species is observed using a nourseothricin concentration of approximately 100–200 $\mu\text{g ml}^{-1}$ (in rich media or buffered minimal media). Although quite expensive, nourseothricin and codon-adapted streptothricin acetyltransferase genes appear promising to transform a large variety of *C. albicans* wild-type isolates as well as other *Candida* species (Shen *et al.*, 2005; Gácsér *et al.*, 2007; Nguyen *et al.*, 2009). For this reason, *caSAT1* has recently been used as a dominant selectable marker to investigate the role of a small number of genes in *C. albicans* (Walia & Calderone, 2008; Melo *et al.*, 2008; Tsao *et al.*, 2009; Kur *et al.*, 2010).

Phleomycin/zeocin resistance markers

The third codon-modified drug-resistance cassette was originally developed in 2003 (Tang *et al.*, 2003). The authors modified the five CTG codons of the *Streptoalloteichus hindustanus ble* gene (*Sh ble*), encoding a glyoxalase, which confers resistance to the bleomycin/phleomycin/zeocin antibiotic group, to transform wild-type strains of *C. rugosa* (Fig. 1a). In these experiments, the codon-modified version of *Sh ble*, referred to as *zeo-n*, was successfully driven by the *GAL1* and *TEF1* promoters from *S. cerevisiae* or by the *LIP3* promoter from *C. rugosa*. Three years later, Wang *et al.* (2006) published a similar approach using a codon-modified sequence of *Sh ble*, harbouring the five modified CTG codons and driven by the *S. cerevisiae* GAP gene promoter and *CYC1* terminator to transform the yeast *Pichia farinosa*. In the same manner, Laplaza *et al.* (2006) developed another version of codon-optimized *Sh ble* by replacing the five CTG codons by TTG, allowing in turn the efficient transformation of strains of *Pichia stipitis* (formerly *Scheffersomyces stipitis*, a species belonging to the CTG clade). Interestingly, during the same year, Dmytruk *et al.* (2006) proposed a different strategy for the development of a zeocin-resistance cassette to transform *C. famata* wild-type strains. Instead of modifying CTG codons in the well-known *Sh ble* gene, they derived some benefit from the expanding set of available prokaryote gene sequences by hunting for another bacterial zeocin/phleomycin-resistance gene that does not contain CTG codons. They noticed that the methicillin-resistant *Staphylococcus aureus* (MRSA) 252 *ble* gene contains no CTG codons, and demonstrated that this MRSA sequence, under the control

of the *TEF1* promoter, can be used as dominant selectable marker in *C. famata*.

Although high frequencies of spontaneous resistant mutants can be observed in various *Candida* isolates (authors' unpublished observations), *Candida* species appear generally sensitive to zeocin and phleomycin (used in rich media) at concentrations of 100–400 $\mu\text{g ml}^{-1}$. Thus, the MRSA CTG codon-less *ble* gene, controlled by appropriate transcription-regulating sequences, could become a widely used drug-resistance marker in the CTG clade, as is already the case for filamentous fungi (for a review see Weld *et al.*, 2006).

Mycophenolic acid resistance markers

Although it is not strictly a codon-modified version of a resistance gene, a fourth drug-resistance marker currently available for *Candida* species can be cited to conclude this section. Köhler *et al.* (1997) demonstrated that plasmid-mediated overexpression of the *C. albicans* *IMH3* gene (encoding inosine 5'-monophosphate dehydrogenase) confers resistance to the drug mycophenolic acid in *C. albicans* (Fig. 1a). Not long after, this marker was also employed to design a stage-specific virulence and white-opaque switching reporter system in *C. albicans* (Staib *et al.*, 1999; Strauss *et al.*, 2001). Finally it was adapted as an *IMH3* flipper cassette (coupled with an engineered recombinase system; see below) for sequential gene disruption (Wirsching *et al.*, 2000; Morschhäuser *et al.*, 2005). Further studies showed that various mutations leading to amino acid substitutions are required in the *IMH3* marker copy to confer mycophenolic acid resistance (Beckerman *et al.*, 2001; Köhler *et al.*, 2005), potentially explaining the highly variable natural sensitivity/resistance toward this drug among *Candida* species/isolates (Beckerman *et al.*, 2001; Köhler *et al.*, 2005). Nevertheless, this dominant selectable marker has been used over the last decade in a small number of *C. albicans* genetic studies and has been successfully used to transform strains of *C. tropicalis* (Beckerman *et al.*, 2001), *C. dubliniensis* (Staib *et al.*, 2000b, 2001; Wirsching *et al.*, 2001), *C. parapsilosis* (Gácsér *et al.*, 2005; Kosa *et al.*, 2007) and *C. famata* (Dmytruk *et al.*, 2011).

Reporter genes

Fluorescent proteins

Fluorescent proteins (FPs, Fig. 2a) are powerful tools in cell biology since they are often used as transformation reporters (Fig. 2b) as well as for monitoring both protein localization/interaction (Fig. 2c) and promoter activity (Fig. 2d) in a wide range of fungal species. Over the last 15 years, various FP re-engineered sequences with optimized codons and improved brightness have been developed for expression in *Candida* species (Table 2).

Cormack *et al.* (1997) published the first version of jellyfish (*Aequorea victoria*) green fluorescent protein (GFP) with *Candida*-optimized codons and enhanced brightness. This

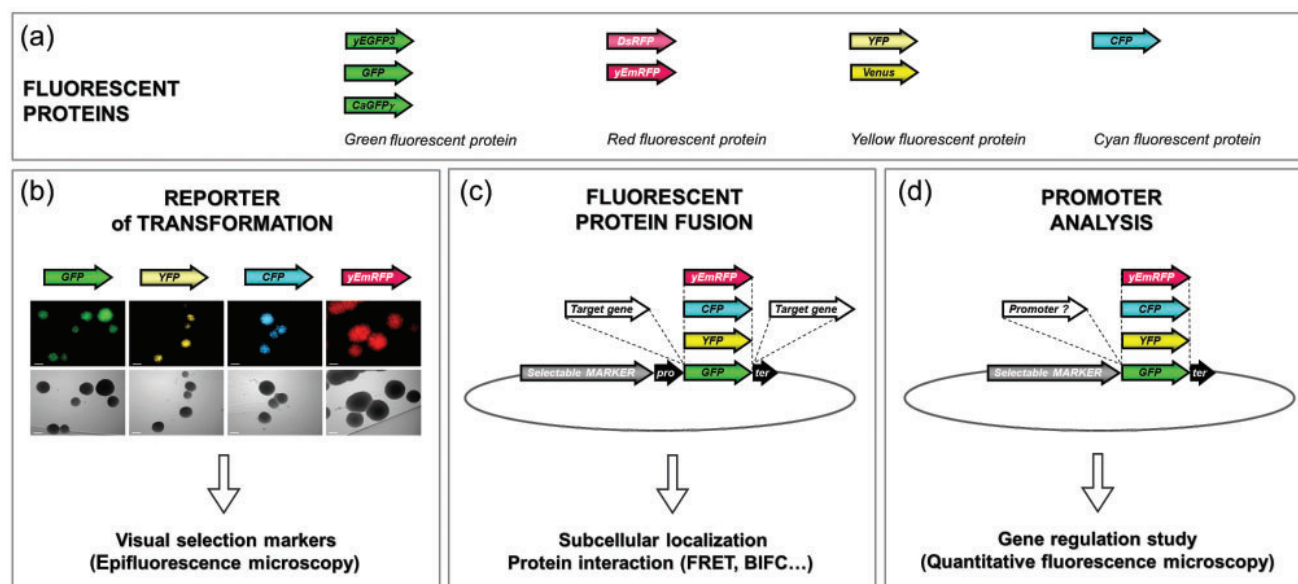


Fig. 2. Codon-optimized genes encoding FP variants adapted for use in the CTG clade. (a) Overview of FP genes. The *yEGFP3* version includes the chromophore fluorescence-increasing mutations (S65G and S72A) present in *GFPmut3* (Cormack *et al.*, 1997); the *GFP* version includes the chromophore fluorescence-increasing mutations S65T or S65A, V68L, S72A present in *GFPmut2* (Morschhäuser *et al.*, 1998); the *CaGFP_γ* variant includes mutations F64L, S65C, V163A and I167T (Zhang & Konopka, 2010). *DsRFP* corresponds to a codon-optimized version of mRFP (Gerami-Nejad *et al.*, 2009); *yEmRFP* is a codon-optimized version of the mCherry variant (Keppler-Ross *et al.*, 2008). YFP includes V68L, T203Y substitutions in *yEGFP3* (Gerami-Nejad *et al.*, 2001); the yeast Venus version is detailed by Reijnt *et al.* (2011). CFP includes F64L, Y66W, N146I, M153T, V163A and N164H substitutions in *yEGFP3* (Gerami-Nejad *et al.*, 2001). (b) Use of FPs as a series of four visual selection markers at the colony level (see Courdavault *et al.*, 2011). (c) Application of FPs in the generation of fusion proteins. This strategy can be carried out thanks to autoreplicating plasmids (see Keppler-Ross *et al.*, 2008, as an example), PCR-amplified cassettes (see Gerami-Nejad *et al.*, 2001, 2009) or integrative vectors (see Courdavault *et al.*, 2011). (d) Application of FPs to promoter analysis (see Morschhäuser *et al.*, 1998, as an example). *pro*, promoter; *ter*, terminator.

synthetic gene, named *yEGFP3*, included three types of modifications: (i) the unique CTG codon was replaced by a TTG codon; (ii) the two chromophore high-fluorescence mutations (S65G and S72A) present in *GFPmut3* (Cormack *et al.*, 1996) and (iii) a series of codon modifications guided by the codon usage table derived from highly expressed *C. albicans* genes. A short time later, Morschhäuser *et al.* (1998) published a study dealing with the expression of GFP variants in *C. albicans*. They demonstrated that GFPs that included the S65T or S65A, V68L, S72A chromophore mutations (corresponding to *GFPmut2*; Cormack *et al.*, 1996) displayed strong fluorescence emission compared to the wild-type and that the substitution of the CTG by a TTG codon was required for expression of the GFP in *C. albicans*.

A crucial step was then achieved three years later following the development of the yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) variants (Gerami-Nejad *et al.*, 2001). Using a site-directed mutagenesis approach, the authors modified various amino acid codons of the *yEGFP3* gene (Cormack *et al.*, 1997) to produce a YFP (V68L, T203Y substitutions) and a CFP (F64L, Y66W, N146I, M153T, V163A and N164H substitutions). These variants were originally engineered for use in mammalian cells (Heim &

Tsien, 1996). In contrast to the strategy detailed previously (Cormack *et al.*, 1997; Morschhäuser *et al.*, 1998), which expressed the GFP variant using autoreplicating plasmids, Gerami-Nejad *et al.* (2001) used a strategy that allowed GFP, CFP and YFP gene tagging through chromosomal integration of the FP fusion cassettes. This approach allowed the expression of the fusion protein to be visualized from their native promoters but was restricted to C-terminal fusions. Thanks to the generation of strains carrying various pairs of FP-tagged *C. albicans* proteins, these authors determined that YFP and CFP fusion proteins could be observed sequentially within a single yeast cell. These experiments represented pioneering work utilizing FP dual labelling in the CTG clade, although this strategy appeared unsuitable for N-terminal fusion and could be impaired for genes poorly expressed in the cell. In order to overcome these limitations, Gerami-Nejad *et al.* (2004) developed additional cassettes allowing N-terminal FP fusions. Furthermore, the presence of the *pMET3*, *pGAL1* or *pPCK1* regulatable promoters in this vector set (described in this study) facilitated controllable expression of the fusion proteins at their targeted locus.

Until 2008, the fourth colour from the repertoire of FPs, i.e. the monomeric *Discosoma* sp. red fluorescent protein

Table 2. Compilation of reporter genes adapted for *Candida* species

No. of CTG codons, number of CTG codons in the original nucleotide sequence; CTG repl., indicates if the CTG codons from the original nucleotide sequence have been replaced; Codon opt., indicates if a series of codon modifications guided by a codon usage table derived from highly expressed genes has been carried out; NS, not specified; NP, native promoter.

Gene name	Gene product	Species	No. of CTG codons	Name of adapted version	CTG repl.	Codon opt.	Promoter/terminator	Used in	Reference(s)
GFP	Green fluorescent protein	<i>Aequorea victoria</i>	1	<i>yEGFP3</i>	Yes	Yes	<i>CaADH1/CaADH1</i>	<i>C. albicans</i>	Cormack <i>et al.</i> (1997)
							<i>CaMAL2/CaMAL2</i>		
							<i>PsADH2/PsADH2</i>	<i>P. stipitis</i>	Passoth <i>et al.</i> (2003)
							<i>CpGALI/CpGALI</i>	<i>C. parapsilosis</i>	Kosa <i>et al.</i> (2007)
							<i>CguiPGK/CguiPGK</i>	<i>C. guilliermondii</i>	Courdavault <i>et al.</i> (2011)
							<i>CaACT1/CaACT1</i>	<i>C. albicans</i>	Morschhäuser <i>et al.</i> (1998)
							<i>CaSAP2/CaACT1</i>		
							<i>CaSAP2/CaACT1</i>	<i>C. dubliniensis</i>	Saib <i>et al.</i> (2000b)
							<i>NP/ScURA3</i>	<i>C. albicans</i>	Zhang & Konopka (2010)
							<i>NP/CaADH1</i>	<i>C. albicans</i>	Gerami-Nejad <i>et al.</i> (2001)
RFP	Red fluorescent protein	<i>Discosoma</i> sp.	5	<i>yEmRFP</i>	Yes	Yes	<i>CguiPGK/CguiPGK</i>	<i>C. guilliermondii</i>	Courdavault <i>et al.</i> (2011)
							<i>NP/CaADH1</i>	<i>C. albicans</i>	Gerami-Nejad <i>et al.</i> (2001)
							<i>CguiPGK/CguiPGK</i>	<i>C. guilliermondii</i>	Courdavault <i>et al.</i> (2011)
							<i>NP/ScURA3</i>	<i>C. albicans</i>	Reijndt <i>et al.</i> (2011)
							<i>CaADH1/CaADH1</i>	<i>C. albicans</i>	Kepler-Ross <i>et al.</i> (2008)
LUC	Luciferase	<i>Renilla reniformis</i>	0	<i>DsRFP</i>	Yes	No	<i>CguiPGK/CguiPGK</i>	<i>C. guilliermondii</i>	Courdavault <i>et al.</i> (2011)
							<i>NP/CaADH1</i>	<i>C. albicans</i>	Gerami-Nejad <i>et al.</i> (2009)
							<i>CaWHI1/CaWHI1</i>	<i>C. albicans</i>	Srikantha <i>et al.</i> (1996)
							<i>CaEFla2/CaWHI1</i>		
							<i>CaGALI1/CaWHI1</i>		
		<i>Photinus pyralis</i>	9	<i>FLUC</i>	Yes	No	<i>CaOP4/CaWHI1</i>	<i>C. albicans</i>	Doyle <i>et al.</i> (2006a, b)
							<i>CaACT1/CaACT1</i>		
							<i>CaENO1/CaACT1</i>		
							<i>CaHWP1/CaACT1</i>		
							<i>CaACT1/CaPGA59</i>	<i>C. albicans</i>	Enjalbert <i>et al.</i> (2009)
LAC4	β -Galactosidase	<i>Kluyveromyces fragilis</i>	3	<i>gLUC59</i>	Yes	Yes	<i>CaEFT3/CaPGA59</i>		
							<i>CaTRX1/CaPGA59</i>		
							<i>CaTRR1/CaPGA59</i>		
							<i>CaIPF99/CaPGA59</i>		
							<i>CaHWP1/CaPGA59</i>		
lacZ	β -Galactosidase	<i>Streptococcus thermophilus</i>	2	<i>LAC4</i>	No	No	<i>CaACT1/CaACT1</i>	<i>C. albicans</i>	Leuker <i>et al.</i> (1992)
							<i>CmPGK/NS</i>	<i>C. maltosa</i>	Masuda <i>et al.</i> (1994)
							<i>CmPGK/CmPGK</i>	<i>C. tropicalis</i>	Hara <i>et al.</i> (2001)
							<i>CpGALI/CpGALI</i>	<i>C. parapsilosis</i>	Kosa <i>et al.</i> (2007)
							<i>CaMAL2/CaMAL2</i>	<i>C. albicans</i>	Uhl & Johnson (2001)

(mRFP), had still not been adapted for the expression in the CTG clade. Keppler-Ross *et al.* (2008) reasoned that the low expression level of the mRFP in *C. albicans* might be due to the particular codon usage encountered in many *Candida* species, particularly unread CTG codons and synonymous codons of yeast that are biased toward an A or T at the third position. Ninety-eight per cent of the mRFP codons were shown to contain either a C or a G at the third position. Thus, 218 of 237 codons were modified in the mCherry variant of mRFP (Shaner *et al.* 2004) to produce a yeast-enhanced mRFP (yEmRFP). Expressed through *C. albicans* auto-replicating sequence (ARS)-containing plasmids and driven by the *C. albicans ADH1* promoter, this enhanced yEmRFP was characterized by high fluorescence detectable at the blastospore and the hyphal stages.

Gerami-Nejad *et al.* (2009) also reported the construction of a codon-modified mRFP gene, named DsRFP. As with the previously developed yEmRFP, the codon-modified DsRFP signal was detectable in transformants at the colony level. These two later technical advances appeared crucial since they provided a second possibility for FP dual labelling for *Candida* cell biology by coupling expression of the yEmRFP and the already available yEGFP3 (Cormack *et al.*, 1997; Morschhäuser *et al.*, 1998). Finally, Reijntjes *et al.* (2011) demonstrated yEmRFP/yEGFP3 dual labelling in *C. albicans* following the co-expression of two eisosome-associated protein fusions. Furthermore, in the same study, the expression of the Venus YFP variant, containing the mutations F46L, F64L, S65G, V68L, Q69M, S72A, Q80R, M153T, V163A, S175G and T203Y compared to the wild-type *A. victoria* GFP, was carried out for the first time in a *Candida* species, adding a fifth FP variant usable in the CTG clade. More recently, Milne *et al.* (2011) developed a series of plasmids allowing the transfer of GFP, YFP, CFP and RFP fusion constructs into *C. albicans* wild-type strains utilizing the *CaNAT1* drug-resistance cassette (Shen *et al.*, 2005) as a selectable marker.

Interestingly, since the development of the first codon-optimized GFP for *C. albicans* (Cormack *et al.*, 1997; Morschhäuser *et al.*, 1998), FP fusion strategies have only been employed in a few other species belonging to the CTG clade, including *C. dubliniensis* (Staib *et al.*, 2000b), *P. stipitis* (Passoth *et al.*, 2003) and *C. parapsilosis* (Kosa *et al.*, 2007). More recently Courdavault *et al.* (2011) established a four-colour FP imaging protocol in *C. guilliermondii* (Fig. 3a) using both mCherry/GFP and YFP/CFP dual labelling (Fig. 3b–d). Additionally, a new GFP variant, CaGFP γ (including mutations F64L, S65C, V163A and I167T), with improved photostability is now available for expression in *Candida* species (Zhang & Konopka, 2010).

Luciferases

Luciferases are convenient and sensitive reporters for studying gene regulation in many organisms. To date, three types of luciferase are used in bacteria, animals and viruses (Fig. 4a). The firefly (*Photinus pyralis*) luciferase,

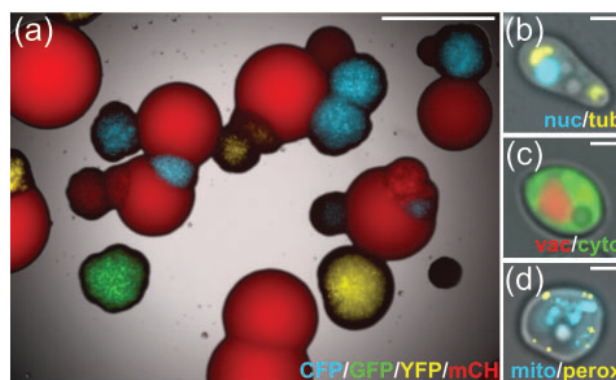


Fig. 3. Application of codon-optimized CFP, GFP, YFP and mCherry genes in a *Candida* species: *C. guilliermondii*. (a) Use of FPs as a series of four fluorescent visual selection markers. Cells from GFP-, YFP-, CFP- or mCherry-expressing strains were mixed in distilled water and plated. After 2 days of growth, the freshly formed colonies were observed by epifluorescence microscopy using individual filters. Bar, 1 mm. (b) Subcellular localization of CFP-Skn7p (nuclear, nuc) and YFP-Tub1p (mitotic spindle, tub) fusion proteins in the same cell. (c) Subcellular localization of Abg1p-mCherry (vacuolar, vac) and Ypd1p-GFP (nucleocytosol, cyto) fusion proteins in the same cell. (d) Subcellular localization of YFP-SKL (peroxisomal, perox) and tGcf1p-CFP (mitochondrial, mito) fusion proteins in the same cell. Bars in (b–d), 2 μ m. For further details, see Courdavault *et al.* (2011).

which converts the substrate luciferin to oxyluciferin in an ATP-dependent manner, remains the most commonly used luciferase due to various favourable pharmacokinetic parameters. The sea pansy (*Renilla reniformis*) luciferase and the copepod *Gaussia* (*Gaussia princeps*) luciferase, which both produce light emission from coelenterazine in an ATP-independent manner, remain less employed because they usually show limited biodistribution in models and oxidation in serum. Basically, the luminescence of these proteins can be used to analyse cell transformation (Fig. 4b) and promoter regulation/function (Fig. 4c) as previously described for FPs, but they can also be used for *in vivo* analysis (Fig. 4d). However, the adaptation and use of these reporter systems in *Candida* species (Fig. 4, Table 2) has been impeded by two main factors: the specific codon usage that modifies the sequence of the expressed heterologous proteins in *Candida* yeast cells and the restricted permeability of fungal cells, in particular hyphal forms, to the bioluminescent substrates (d'Enfert *et al.*, 2010).

Since the luciferase gene of *R. reniformis* contains no CTG codon, Srikantha *et al.* (1996) expressed the luciferase gene in *C. albicans* and studied its use as a bioluminescent reporter system. Even though it was clearly demonstrated that the activity of this *R. reniformis* luciferase could be assayed in lysed or intact cells, the presumed low accessibility of the substrate coelenterazine to the cell cytoplasm appeared to be a major drawback for the

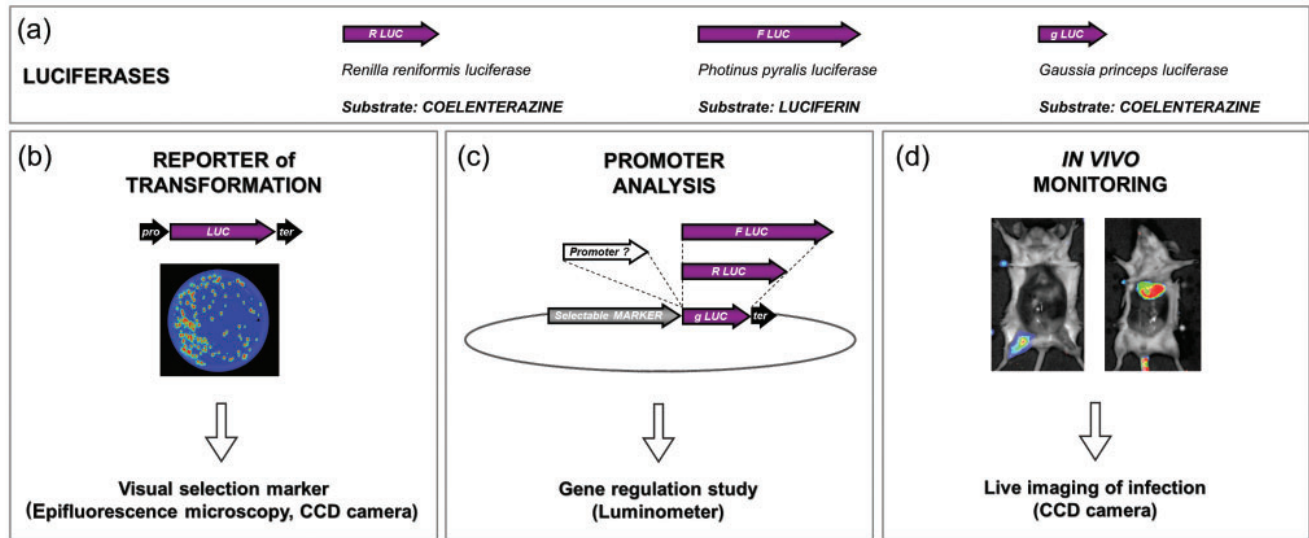


Fig. 4. Genes encoding luciferase (LUC) adapted for use in the CTG clade. (a) Compilation of *LUC* genes and their corresponding substrates. The *R. reniformis* *RLUC* does not contain CTG codons (Srikantha *et al.*, 1996); the *P. pyralis* *FLUC* version includes nine modified CTG codons (Doyle *et al.*, 2006a); the *gLUC* version corresponds to the codon-optimized *G. princeps* luciferase gene fused to the glycosylphosphatidylinositol-linked cell wall protein Pga59p (Enjalbert *et al.*, 2009). (b) Use of *LUC* as a reporter gene of cell transformation (see Doyle *et al.*, 2006a; Enjalbert *et al.*, 2009). (c) Application of *LUC* in promoter analysis (see Srikantha *et al.*, 1996, and Enjalbert *et al.*, 2009, as examples). (d) Use of *LUC* for live imaging of *Candida* infections (see Doyle *et al.*, 2006b, and Enjalbert *et al.*, 2009, as examples). *pro*, promoter; *ter*, terminator; CCD, charge-coupled device.

sensitivity of the system during *in vivo* experiments. Ten years later, Doyle *et al.* (2006a) modified the nine CTG codons of the firefly luciferase gene to TTG and attempted its expression in *C. albicans*, driven by the *C. albicans* *ENO1* gene promoter and *ACT1* terminator. In spite of the demonstration that this adapted cassette could be used in *C. albicans* as a selectable marker, the limiting factor once again appeared to be the substrates availability within the cells due to the low permeability of luciferin through the cell wall, precluding its use for *in vivo* experiments. However, a later study by Doyle *et al.* (2006b) reported the first use of a bioluminescent fungal pathogen in mouse infection models, using the previously described *P. pyralis* luciferase gene expressed by *C. albicans* cells. In these latter experiments, the luciferin had to be externally supplied to the *Candida* cells following injection of the substrate into the infected mice. Although this approach was clearly successful in the vaginal candidiasis model, this was not the case for the chronic systemic model of candidiasis, thus limiting the widespread use of such an approach (Doyle *et al.*, 2006b). To address the limitations of the available luciferase reporter systems (in particular luciferase/substrate accessibility), Enjalbert *et al.* (2009) developed a *C. albicans*-adapted luciferase reporter system consisting of the expression of a codon-optimized *G. princeps* luciferase gene fused to the gene encoding the recently characterized glycosylphosphatidylinositol-linked cell wall protein Pga59p (Moreno-Ruiz *et al.*, 2009). This construction circumvents the low cell permeability to

luciferase substrates by exposing the fusion proteins to the cell surface, which in turn allows efficient accessibility to external coelenterazine. The authors demonstrated that this luciferase reporter system shows high sensitivity compatible with *in vitro* gene expression studies and allows the progression of *C. albicans* infections in live animals to be monitored (except for haematogenously disseminated candidiasis). It is thus possible that this newly adapted luciferase will be useful in future *Candida* pathobiology studies.

β -Galactosidases

E. coli *lacZ*, encoding a β -galactosidase, has proved to be a highly versatile reporter gene in various models, including bacteria, animal cells and *S. cerevisiae*, and has been used to study many aspects of gene regulation, signal-transduction pathways and other cellular processes. Unfortunately, the coding sequence of the *E. coli* *lacZ* gene contains 51 CTG codons, which has for a long time prevented the adaptation of this reporter system to *Candida* species.

Leuker *et al.* (1992) developed the first β -galactosidase reporter system in *C. albicans* and *C. tropicalis* by expressing the *Kluyveromyces lactis* *LAC4* gene encoding a β -galactosidase (Table 2). Placed under the control of the *ACT1* promoter, the expression of this gene, which contains two CTG codons, gave rise to a detectable β -galactosidase activity that was demonstrated to be proportional to the number of integrated copies of the reporter

cassette. Since its development, this *K. lactis* *LAC4* reporter system has been used in a small number of gene expression studies (Delbrück & Ernst, 1993; Stoldt *et al.*, 1997; Leuker *et al.*, 1997). Although the adaptation of the *K. lactis* *LAC4* gene was also carried out in other *Candida* species (Masuda *et al.*, 1994; Hara *et al.*, 2001; Kosa *et al.*, 2007), the reliability of this system was called into question by Uhl & Johnson (2001). Using the same conditions, they provided firm evidence that no β -galactosidase activity could be detected using the previously described *K. lactis* *LAC4* reporter system. This notably could be explained by the fact that one of the two leucines encoded by a CTG codon in the *K. lactis* *LAC4* sequence occupies a conserved position requiring a hydrophobic residue in the β -galactosidase family, which could not be converted to serine without a loss of activity. Interestingly, in the same study, Uhl & Johnson (2001) successfully used the *lacZ* gene from the thermophilic bacterium *Streptococcus thermophilus*, which contains only one CTG codon in the extreme non-conserved C-terminus of the protein (Table 2). Furthermore, they demonstrated that the wild-type sequence of *S. thermophilus* *lacZ* or the codon-optimized version (in which the sole CTG has been replaced by TTA) resulted in equivalent activity in *C. albicans*. These latter results could explain why the *S. thermophilus* *lacZ* gene has been preferentially used in studies of signal-transduction pathways and gene regulation in *C. albicans* over the last decade (Li *et al.*, 2004; Ramsdale *et al.*, 2008).

Other genetic tools

Recombinases

Since their discovery in the mid-1980s, site-specific recombinases have become convenient molecular tools to achieve targeted genetic modifications in a wide variety of prokaryotic and eukaryotic genomes. To benefit from this strategy, two major recombinases have been engineered over the last 12 years (codon-optimized and with transcription-regulating sequences exchange) to enable their use in the CTG clade: the FLP/*FRT* and the Cre-*loxP* site-specific recombinases (Nunes-Duby *et al.*, 1998; Austin *et al.*, 1981; Sternberg & Hamilton, 1981) (Table 3).

The first recombinase gene specifically engineered for expression in a *Candida* species was that encoding the FLP recombinase, originally discovered in *S. cerevisiae* (Vetter *et al.*, 1983). Staib *et al.* (1999) proposed the first codon-modified version of the *S. cerevisiae* *FLP* gene (referred to as *caFLP*), in which the three CTG codons were replaced by TTG codons. *caFLP* was driven by the *SAP2* (encoding a virulence associated secreted aspartyl proteinase) promoter and *ACT1* terminator to monitor, in an infection-stage-specific fashion, the excision of the *IMH3* mycophenolic acid resistance gene flanked by direct repeats of the minimal FLP recombination target sequence (*FRT*). In the same year, the *SAP2* promoter-driven *caFLP*, coupled with the *URA3* metabolic marker to produce a *C. albicans* *URA3* flipper cassette, was used to generate homozygous *C.*

albicans mutants disrupted for the *CDR4* gene (encoding an ABC transporter) and the *MDR1* gene (encoding a membrane transport protein of the major facilitator superfamily), indicating the capacity of this recombinase system to carry out multiple gene disruption in *C. albicans* (Morschhäuser *et al.*, 1999). Staib *et al.* (2000a) then developed a second version of the codon-modified *FLP* gene, referred as *ecaFLP*, including the four amino acid exchanges described by Buchholz *et al.* (1998) in the *caFLP* sequence (P2S, L33S, Y108N and S294P). These modifications were shown to confer an enhanced activity upon the enzyme.

Michel *et al.* (2002) showed that placing *ecaFLP* under the control of the *SAP2* inducible promoter (more precisely the *C. albicans* *URA3* flipper cassette) allowed the development of a strategy for the identification of essential genes in *C. albicans*. As a continuation of this pioneering work demonstrating the potential of the codon-modified *FLP* gene, two studies proposed an alternative inducible promoter, *MAL2* (replacing the *SAP2* promoter), to drive the recombinase gene (Sánchez-Martínez & Pérez-Martín, 2002; Reuss *et al.*, 2004). In these approaches, *FLP* expression could be induced by growth of the transformants in maltose-containing medium. Sánchez-Martínez & Pérez-Martín (2002) first developed a strategy permitting the integration/excision of foreign DNA using a *MAL2* promoter-driven *caFLP*. In addition, Reuss *et al.* (2004) developed the well-known *caSAT1* flipper cassette consisting of the *MAL2* promoter-driven *ecaFLP* coupled with the *caSAT1* marker and flanked by direct repeats of the *FRT* sequence. After the selection of nourseothricin-resistant transformants (insertion of the *caSAT1* flipper sequence), FLP-mediated excision of the *ecaFLP-caSAT1* cassette from the genome could be achieved by culturing the transformants in maltose-containing medium. More recently, Park & Morschhäuser (2005) developed a tetracycline-inducible version of the *ecaFLP* system (see next section). The *caSAT1* flipper cassette of Reuss *et al.* (2004) was adapted by Ding & Butler (2007) to efficiently transform *C. parapsilosis* clinical isolates. The authors were unable to use the *caSAT1* flipper construct directly. To obtain maltose-induced excision of the cassette, they therefore replaced the promoter driving expression of the *caFLP* gene by the *C. parapsilosis* *MAL2* promoter (*CpFLP*). This *CpSAT1* flipper cassette was then used to sequentially generate a *ura3/ura3* auxotrophic strain and to disrupt the *BCR1* gene (involved in biofilm formation). In contrast to Ding & Butler (2007), Gácsér *et al.* (2007) and Nguyen *et al.* (2009) successfully achieved sequential disruptions with FLP-mediated excision of the cassette in *C. parapsilosis*, using the *caSAT1* flipper cassette directly (without changing the regulatory sequences).

The second type of recombinase, Cre, was adapted for use in the CTG clade in 2005. Dennison *et al.* (2005) produced the first version of a synthetic codon-modified *cre* gene in which all the 18 CTG codons were replaced by TTG and driven by the inducible *C. albicans* *MET3* promoter. To

Table 3. Compilation of recombinases and other regulatable systems adapted for *Candida* species

No. of CTG codons, number of CTG codons in the original nucleotide sequence; CTG repl., indicates if the CTG codons from the original nucleotide sequence have been replaced; Codon opt., indicates if a series of codon modifications guided by a codon usage table derived from highly expressed genes has been carried out; NS, not specified.

Gene name	Gene product	Species	No. of CTG codons	Name of adapted version	CTG repl.	Codon opt.	Promoter/terminator	Used in	Reference(s)
<i>FLP</i>	Recombinase	<i>Saccharomyces cerevisiae</i>	3	<i>caFLP</i>	Yes	No	<i>CaSAP2/CaACT1</i>	<i>C. albicans</i>	Staib <i>et al.</i> (1999); Morschhäuser <i>et al.</i> (1999)
				<i>ecaFLP*</i>	Yes	No	<i>CaSAP2/CaACT1</i> <i>CaMAL2/CaACT1</i> <i>CaSAP2/CaACT1</i>	<i>C. albicans</i> <i>C. parapsilosis</i>	Staib <i>et al.</i> (2000a); Reuss <i>et al.</i> (2004) Gácer <i>et al.</i> (2007); Nguyen <i>et al.</i> (2009)
<i>cre</i>	Recombinase	P1 bacteriophage	18	<i>CpFLP cre</i>	Yes	No	<i>CpMAL2/CpACT1</i> <i>CaMET3/NS</i>	<i>C. parapsilosis</i> <i>C. albicans</i>	Ding & Butler (2007) Dennison <i>et al.</i> (2005)
<i>tetR</i>	Tetracycline-dependent transactivator	<i>Escherichia coli</i>	1 (3) 1 (1)	<i>Cre</i> <i>tTA†</i> <i>carTAT‡</i>	Yes	No	<i>PsXYL1/PsXYL1</i> <i>CaENO1/CaWHI1</i> <i>CaADH1/CaACT1</i> <i>CaOP4/CaACT1</i>	<i>P. stipitis</i> <i>C. albicans</i> <i>C. albicans</i>	Laplaza <i>et al.</i> (2006) Nakayama <i>et al.</i> (2000) Park & Morschhäuser (2005)

*This version includes the four amino acid exchanges P2S, L33S, Y108N and S294P described by Buchholz *et al.* (1998).

†*tTA* consists of the codon-modified *E. coli tetR* gene fused to the codon-modified nucleotide sequence encoding the *S. cerevisiae* Hap4 activation domain. The *tetR* gene contains one CTG codon and the nucleotide sequence encoding the ScHap4 activation domain contains three CTG codons.

‡*carTAT* is composed of the reverse codon-modified *E. coli tetR* gene (*carTetR*) fused to the codon-modified nucleotide sequence encoding the *S. cerevisiae* Gal4 activation domain. The *carTetR* gene contains one CTG codon and the nucleotide sequence encoding the ScGal4 activation domain contains one CTG codon. The *carTetR* gene product contains the five amino acid exchanges (S12G, E19G, A56P, D149E and H179R) described by Urlinger *et al.* (2000).

demonstrate its potential in *C. albicans*, they carried out the sequential disruption of the *ADE2* and *MET15* genes. One year later, Laplaza *et al.* (2006) engineered a second version of a codon-modified *cre* gene by changing the 18 CTG codons to various *Candida* frequently used codons. Since their development, the codon-modified FLP and Cre systems have been rarely employed in *Candida* species. Nevertheless, because FLP- or Cre-mediated multiple gene disruption strategies do not always require the handling of toxic compounds, unlike the widely used *URA3* blaster systems (utilizing the harmful 5-fluoroorotate), their use may well become more widespread in future.

Tetracycline-regulatable systems

The TetR/*tetO* tetracycline-regulatable system has emerged as a powerful system to control gene expression in prokaryotic and eukaryotic cells. Originally, the archetypal *E. coli* TetR/*tetO* system was composed of two modules: the TetR transactivator and the *tetO* operator from the promoter of tetracycline-resistance genes. In the absence of tetracycline, TetR binds the *tetO* sequence and represses the expression of fused tetracycline-resistance genes. When added, tetracycline binds the TetR repressor and provokes its dissociation from the *tetO* operator, inducing the expression of tetracycline-resistance genes (Gossen *et al.*, 1993) (Table 3).

The first tetracycline-regulatable system usable in *Candida* species was adapted by Nakayama *et al.* (2000) (Fig. 5a). This system, referred to as Tet-off, consists of the codon-modified *E. coli* TetR fused to the activation domain of the Hap4 transcriptional activator (tTA) and the TR promoter, including a yeast minimal promoter element coupled with a tetracycline operator sequence (*tetO*). The four CTG codons of the gene encoding the fusion transactivator tTA were replaced by other leucine codons. In this regulatable system, in the absence of tetracycline, the tTA transactivator binds the *tetO* sequence, allowing the active expression of the target gene. The addition of tetracycline inhibits the binding of tTA to *tetO*, thus turning off the target gene expression (Fig. 5a). This Tet-off system has become a versatile tool to investigate the function of essential genes (which cannot be deleted from the genome; e.g. Yang *et al.*, 2006) as well as virulence-associated genes (Saville *et al.*, 2003). In this way, Roemer *et al.* (2003) used this latter development to design the *C. albicans* GRACE strategy.

Park & Morschhäuser (2005) proposed a second tetracycline-regulatable system referred to as the Tet-on system, consisting of a reverse tetracycline-controlled transactivator (*cartTA*) and the P_{tet} promoter (Fig. 5b). The codon-modified *cartTA* gene was composed of the *carTetR* nucleotide sequence fused to the sequence encoding the *S. cerevisiae* Gal4 activation domain (displaying a CTG to TTG codon replacement). The *carTetR* sequence was generated following codon modifications in the *TetR* gene introducing the five amino acid exchanges (S12G, E19G,

A56P, D149E and H179R) described by Urlinger *et al.* (2000) to invert the effect of tetracycline on the expressed transactivator *cartTA*. The P_{tet} promoter includes a minimal promoter element from the *C. albicans* *OP4* gene fused to the *tetO* tetracycline operator sequence. In this latter strategy, the expression of a target gene can be accurately positively regulated by the addition of tetracycline to engineered cells (Fig. 5b). Since its construction, this regulatable system has facilitated a number of investigations into the role of specific genes on the behaviour of *C. albicans* (Bernardo *et al.*, 2008; Saville *et al.*, 2006; Staib *et al.*, 2008; Carlisle *et al.*, 2009; Becker *et al.*, 2010).

Concluding remarks

Candida species are attractive models for exploring new molecular mechanisms relating to pathogenicity, antifungal resistance, metabolite bioproduction and biological control. As a consequence, and predominantly since the 1990s, the establishment of convenient transformation systems as well as the development of selectable markers and reporter genes has become indispensable to carry out genetic manipulations in these yeast species. As underlined in this paper, the establishment of such molecular tools was for a long time impeded due to the codon usage in the CTG clade. However, as soon as PCR-mediated mutagenesis techniques became available at the end of the 1990s, the *Candida* researcher community (particularly *C. albicans* researchers) progressively adapted *Saccharomyces* genetic tools for use in *Candida* species. Recently, this tendency was once again illustrated by the adaptation of the *Saccharomyces* two-hybrid system for use in *C. albicans* (Stynen *et al.*, 2010). After 15 years of research, the *Candida* clade toolbox is now considered to be reasonably well developed. Some research groups currently propose global strategies employing a wide range of dominant markers, reporter genes and other protein tags for use in particular *Candida* species, as is the case for the PCR-amplified functional analysis (PFA) cassette series for *C. albicans* (Gola *et al.*, 2003; Schaub *et al.*, 2006; Reijns *et al.*, 2011; Milne *et al.*, 2011).

As highlighted in the present review, three major genetic modifications have to be carried out for the adaptation of selectable markers, reporter genes or regulatable systems for their expression in the CTG clade. First, the substitution of all CTG codons of the expressed sequence by other leucine-encoding codons appears indispensable in all studies, with the exception of CTG codons located at the extreme 3' end of the sequence and encoding a non-conserved leucine residue (Uhl & Johnson, 2001). Secondly, in the case of a complete synthetic sequence, a series of codon modifications guided by a codon usage table derived from highly expressed genes in the target species can act positively on the expression of the sequence (Cormack *et al.*, 1997; Shen *et al.*, 2005). For example, the nucleotides G or C should be omitted at the third position

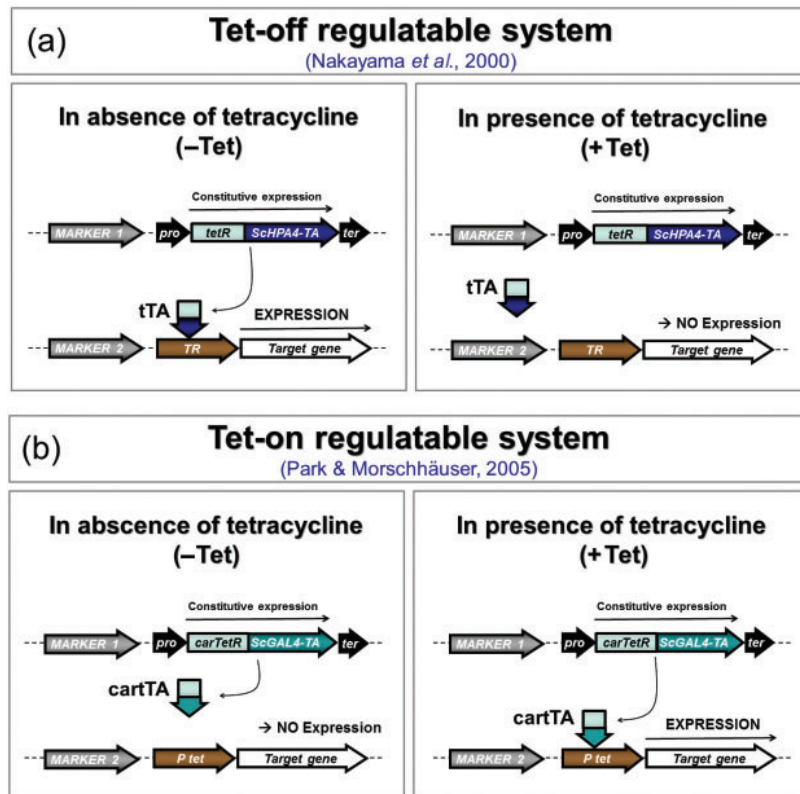


Fig. 5. Tetracycline-regulatable systems adapted for genetic studies in the CTG clade. (a) The Tet-off system consists of the *tTA* transactivator and the *TR* promoter. The *tTA* gene is composed of the codon-modified *E. coli TetR* fused to the codon-modified nucleotide sequence encoding the *S. cerevisiae* Hap4 activation domain (*ScHPA4-TA*). In the absence of tetracycline (-Tet), the *tTA* transactivator binds the *TR* sequence, allowing the active expression of the target gene. The addition of tetracycline (+Tet) inhibits the binding of *tTA* with *TR*, thus downregulating the expression of the target gene. (b) The Tet-on system consists of a reverse tetracycline-controlled transactivator (*cartTA*) and the *P_{tet}* promoter. The codon-modified *cartTA* gene is composed of the *carTetR* nucleotide sequence fused to the codon-modified sequence encoding the *S. cerevisiae* Gal4 activation domain (*ScGAL4-TA*). The *carTetR* sequence was generated following codon modifications in the *TetR* gene introducing amino acid exchanges to invert the effect of tetracycline on the expressed transactivator *cartTA*. In this instance, the addition of tetracycline results in the active expression of the target gene and the absence of tetracycline inhibits the expression of the target gene.

of each codon for expression in *C. albicans* (Keppler-Ross *et al.*, 2008). Finally, the suitability of *Candida* transcription-regulating 5' and 3' regions used to drive the expression of a heterologous codon-optimized sequence in various species of the CTG clade remains controversial. As an example, although the *C. albicans ACT1* promoter was shown by different teams to be able to drive the expression of various codon-modified sequences in a series of *Candida* species (Gácsér *et al.*, 2007; Nguyen *et al.*, 2009), this same promoter proved ineffective for the expression of heterologous genes in the hands of other researchers (Ding & Butler, 2007; Millerioux *et al.*, 2011).

In conclusion, it is possible that the range of *Candida*-adapted molecular tools will increase thanks to two major aspects. First, the progressive reduction in the price of selection drugs (hygromycin B, nourseothricin, zeocin...), substrates (luciferin, coelenterazine...) and automated gene synthesis will favour the development of genetic tools that will become commonplace in research laboratories for a number of *Candida* species. Furthermore, the growing set of sequenced *Candida* genomes (Butler *et al.*, 2009) and the availability of databases of metabolic pathways (Rossignol *et al.*, 2008; Skrzypek *et al.*, 2010) will facilitate the isolation of homologous constitutive or regulatable promoters and terminators for use in specific *Candida* species. In a wider perspective, the increasing number of sequenced genomes

(specifically prokaryotic genomes) will help in identifying species/isolates harbouring interesting CTG codon-less coding sequences.

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