

Drug-resistant cassettes for the efficient transformation of *Candida guilliermondii* wild-type strains

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Introduction

Candida guilliermondii (teleomorph *Pichia guilliermondii*) is an ascomycetous yeast widely distributed in nature and can be a common constituent of the normal human microbial communities. However, this fungal species is also an agent of candidiasis and has been described as an emerging pathogen characterized by its propensity to develop resistance to antifungal agents during treatment (Pfaller *et al.*, 2006; Desnos-Ollivier *et al.*, 2008; Savini *et al.*, 2011). More precisely, recent studies specified that *C. guilliermondii* accounts for 1–3% of all candidemia and that most cases of *C. guilliermondii* infection are associated with oncology patients (Girmenia *et al.*, 2006; Pfaller *et al.*, 2006). Besides its clinical relevance, *C. guilliermondii* also remains an interesting biotechnological model for the industrial production of value-added metabolites (Leather, 2003). The extraordinary capacity of *C. guilliermondii* to metabolize C5 sugars from hemicellulosic plant byproducts is nowadays studied by various teams in order to produce biocom-

Abstract

Candida guilliermondii is an opportunistic emerging fungal agent of candidiasis often associated with oncology patients. This yeast also remains an interesting biotechnological model for the industrial production of value-added metabolites. The recent whole-genome sequencing of the *C. guilliermondii* ATCC 6260 reference strain provides an interesting resource for elucidating new molecular events supporting pathogenicity, antifungal resistance and for exploring the potential of yeast metabolic engineering. In the present study, we designed an efficient transformation system for *C. guilliermondii* wild-type strains using both nourseothricin- and hygromycin B-resistant markers. To demonstrate the potential of these drug-resistant cassettes, we carried out the disruption and the complementation of the *C. guilliermondii* *FCY1* gene (which encodes cytosine deaminase) known to be associated with flucytosine sensitivity in yeast. These two new dominant selectable markers represent powerful tools to study the function of a large pallet of genes in this yeast of clinical and biotechnological interest.

pounds such as xylitol (Mussatto *et al.*, 2005), riboflavin (Boretsky *et al.*, 2007), ethanol (Schirmer-Michel *et al.*, 2008) or γ -aminobutyric acid (Guo *et al.*, 2009).

The recent sequencing of the *C. guilliermondii* ATCC 6260 reference strain whole genome clearly reflects the increasing interest of the scientific community in this yeast (Butler *et al.*, 2009). All these aspects make *C. guilliermondii* an attractive model for elucidating new molecular events supporting pathogenicity, antifungal resistance and for exploring the potential of yeast metabolic engineering. The establishment of a convenient transformation system becomes indispensable for genetic manipulations in this haploid species. A small number of metabolic gene markers are already available to carry out the genetic transformation of *C. guilliermondii* laboratory auxotrophic strains (Boretsky *et al.*, 1999, 2007; Millerioux *et al.*, 2011). However, currently, the lack of dominant markers that confer resistance to various drugs precludes genetic studies in *C. guilliermondii* wild-type isolates.

As observed in *Candida albicans*, the *C. guilliermondii* CTG codon encodes serine rather than leucine (referred as 'CTG

clade'), which is not the case for more distantly related species from the 'whole-genome duplication' clade including *Saccharomyces cerevisiae* and *Candida glabrata* (Butler et al., 2009). Therefore, over the last decade, some effort was focused on the adaptation of drug-resistant genes (often with alternative leucine codons), initially used in *Escherichia coli* and *S. cerevisiae*, to favor their expression in species belonging to the CTG clade. Four *Candida* drug-resistant markers are currently available: the *IMH3* marker encoding inosine 5'-monophosphate dehydrogenase, which confers resistance to mycophenolic acid (Köhler et al., 1997; Staib et al., 2000; Beckerman et al., 2001; Gácsér et al., 2005), the *ble* marker encoding a glyoxalase, which confers resistance to the bleomycin/phleomycin/zeocin group (Tang et al., 2003; Dmytruk et al., 2006; Laplaza et al., 2006; Wang et al., 2006), the *SAT-1* marker encoding nourseothricin acetyltransferase, which confers resistance to nourseothricin (Roemer et al., 2003; Reuss et al., 2004; Shen et al., 2005), and the *HPH^{II}* marker encoding hygromycin phosphotransferase, which confers resistance to hygromycin B (Hara et al., 2000; Yehuda et al., 2001; Basso et al., 2010).

In the present work, we adapted two of the aforementioned selectable genes, nourseothricin and hygromycin B drug-resistant markers, to generate cassettes for their use in *C. guilliermondii* and we evaluated their applicability for the efficient genetic transformation of various wild-type strains.

Materials and methods

Strains and media

Four *C. guilliermondii* wild-type strains from the Centre National de Référence Mycologie et Antifongiques of the Institut Pasteur (ODL9-811, ODL9-846, ODL12-1131 and MYCO55-BD), the whole genome sequenced reference strain from the American Type Culture Collection (ATCC 6260) and the ATCC 6260 derived *ura5* mutant NP566U (Millerioux

et al., 2011) were used in our investigations. Yeast strains were routinely cultivated in liquid YPS medium (1% yeast extract, 2% peptone, 2% sucrose) at 30 °C under agitation (150 r.p.m.). Nourseothricin (Werner BioAgents, stock solution prepared in water at a concentration of 100 mg mL⁻¹) and hygromycin B (Sigma-Aldrich, stock solution prepared in water at a concentration of 25 mg mL⁻¹) were added when necessary at the indicated concentration (see figure legends). YNB (0.67% yeast nitrogen base with ammonium sulfate and without amino acids, 2% sucrose) was supplemented or not with flucytosine (Sigma-Aldrich, stock solution prepared in water at a concentration of 12.8 mg mL⁻¹) as required. Solid media were prepared with 2% agar.

PCR amplifications

For plasmid constructions, *SAT-1* and *HPH^{II}* coding sequences were amplified with Pfu (Promega). Yeast colony PCRs were performed as described in Akada et al. (2000) with GoTaq polymerase (Promega). The integration of the *SAT-1* marker into nourseothricin-resistant colonies was confirmed by PCR using primers SAT1S and SAT2R (Table 1). The integration of the *HPH^{II}* marker into hygromycin B-resistant colonies was confirmed by PCR using primers HYGS1 and HYGR2 (Table 1). The *FCY1*-disrupted strains were first screened by PCR with primers FCY11 and FCY12 (Table 1). The *FCY1* gene disruption was then authenticated following PCRs using primers SAT1S or SAT2R with primers located outside of the homology arms (not shown). The *FCY1*-complemented strains were screened by PCR using primers FCY11 and HYGR2. PCR conditions for amplification were those indicated by the supplier.

Plasmids construction

To construct plasmid pURA5-*SAT-1*, the 522 bp *SAT-1* coding sequence was amplified with primers SAT1S and

Table 1. Oligonucleotides

Primers	Sequence (5'–3')	Sources/references
SAT1S*	CTGAGACTCGAGATGAAAATTCGGTGATCCCTG	This study
SAT2R†	CTGAGACTGCAGGGCGTCATCCTGTGCTCCCGAG	This study
HYGS1*	CTGAGACTCGAGATGAAAAAGCCTGAACCTACCG	This study
HYGR2‡	CTGAGACTGCAGTTCCTTGCCCTCGGACGAGTG	This study
FCYS	GACTACAGTAAGGTGAACATCACC	Millerioux et al. (2011)
FCYR	GTCACCTCCATAGGGAGCACTGG	Millerioux et al. (2011)
FCY11	CAGTGAGCTCGGAACGAGGTTTCC	Millerioux et al. (2011)
FCY12	GCAAGCAGCAGACGCTACCAATGG	Millerioux et al. (2011)
PGKP5‡	GGTACCTCTAGAATCGATTGATCAGTCAGCGGAGGAGTCTCCGCAAGAGCC	This study
PGKT6§	TGATCAATCGATTCTAGAGGTACCGGCGAGTTGTGATGAAGATCTTG	This study

*The underlined sequence corresponds to the XhoI site.

†The underlined sequence corresponds to the PstI site.

‡The underlined sequence corresponds to the KpnI, XbaI, ClaI, BclI and SalI sites.

§The underlined sequence corresponds to the KpnI, XbaI, ClaI and BclI sites.

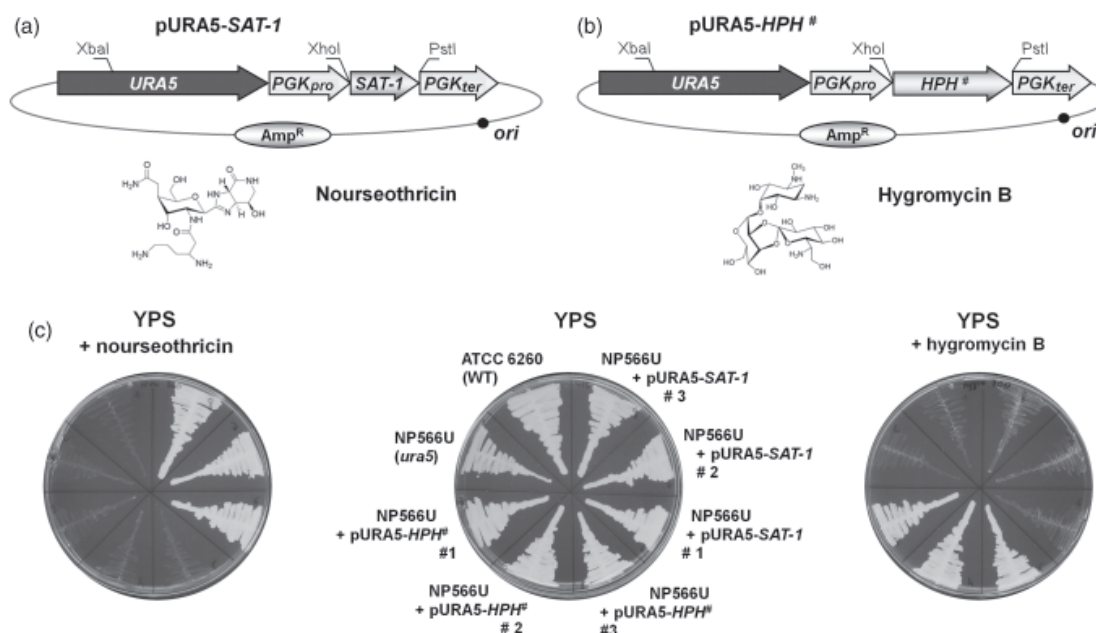


Fig. 1. Transformation of the NP566U strain with pURA5-SAT-1 or pURA5-HPH[#]. (a) Schematic representation of pURA5-SAT-1. The XbaI unique site was used for linearization before transformation. (b) Schematic representation of pURA5-HPH[#]. (c) Nourseothricin and hygromycin B sensitivity of transformants. Nourseothricin and hygromycin B resistances tests were carried out for each strain by streaking ATCC 6260, NP566U and three representative clones from each transformation onto YPS supplemented or not with nourseothricin (150 µg mL⁻¹) or hygromycin B (500 µg mL⁻¹).

SAT2R (Table 1) using the pSFS2 plasmid (Reuss *et al.*, 2004) as a template. This fragment was digested at its extremities by XhoI and PstI and cloned between XhoI and PstI sites of pURA5-GFP (unpublished plasmid) after releasing of the *yeGFP* coding sequence, resulting in pURA5-SAT-1 (Fig. 1a). To obtain plasmid pURA5-HPH[#], the 1023 bp *HPH[#]* coding sequence was amplified with primers HYGS1 and HYGR2 (Table 1) using the pRS-HYG[#] plasmid (Hara *et al.*, 2000) as a template. This fragment was digested at its extremities by XhoI and PstI and cloned between XhoI and PstI sites of pURA5-GFP after the release of the *yeGFP* coding sequence, resulting in pURA5-HPH[#] (Fig. 1b). To construct plasmid pG-SAT-1 and pG-HPH[#], *SAT-1* and *HPH[#]* (each bordered by *C. guilliermondii* phosphoglycerate kinase transcription-regulating sequences) were amplified with primers PGKP5 and PGKT6 (Table 1) and each cloned into pGEMT easy vector (Promega) (Fig. 2). To obtain plasmid pG-5'*FCY1*-PGK_{pro}-*SAT-1*-PGK_{ter}-3'*FCY1*, pG-*FCY1* (Millerioux *et al.*, 2011) was digested with NarI, which is located at the center of the *FCY1* coding sequence (Fig. 3b). The resulting digested plasmid was ligated to the *SAT-1* fragment (bordered by *C. guilliermondii* phosphoglycerate kinase transcription-regulating sequences) released after the digestion of the pG-SAT-1 plasmid by ClaI. The 5'*FCY1*-PGK_{pro}-*SAT-1*-PGK_{ter}-3'*FCY1* disruption cassette was generated using primers FCYS and FCYR (Table 1) and pG-5'*FCY1*-PGK_{pro}-*SAT-1*-PGK_{ter}-3'*FCY1* as a template. For

constructing the complementation vector p-*FCY1*-*HPH[#]* (Fig. 4b), pG-*FCY1* was digested with SpeI and ligated to the *HPH[#]* fragment (bordered by *C. guilliermondii* phosphoglycerate kinase transcription-regulating sequences) released after the digestion of the pG-*HPH[#]* plasmid by XbaI.

Yeast transformation

Transformation of *C. guilliermondii* cells was performed using a lithium acetate procedure as described previously (Boretsky *et al.*, 2007). After heat shock, cells were pelleted (3500 g, 10 min) and suspended in 3 mL YPS, incubated for 5 h at 30 °C under agitation (150 r.p.m.), centrifuged (3500 g, 5 min), resuspended in 100 µL 1 M sucrose, plated onto selective medium and incubated at 30 °C for 3–5 days.

Results and discussion

Sensitivity of *C. guilliermondii* strains to nourseothricin and hygromycin B

We initiated this work by testing the sensitivity of *C. guilliermondii* strains toward nourseothricin and hygromycin B. Complete inhibition of the growth of all strains (ODL9-811, ODL9-846, ODL12-1131, MYCO55-BD and ATCC 6260) was observed (after 4 days of culture) on YPS plates containing 150 µg mL⁻¹ nourseothricin or 500 µg mL⁻¹ hygromycin B.

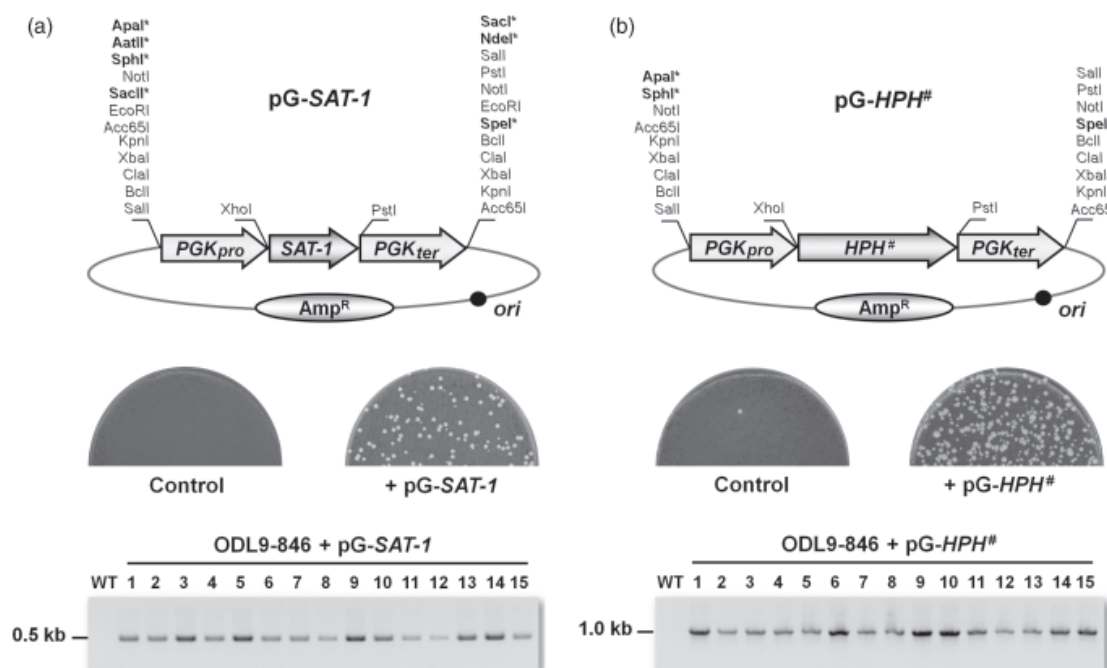


Fig. 2. (a) Selection and characterization of nourseothricin-resistant colonies after the transformation of *Candida guilliermondii* with plasmid pG-SAT-1. A representation of pG-SAT-1 (*single site restriction enzymes) and representative plates of selection for nourseothricin resistance 4 days (30 °C) after the transformation of *C. guilliermondii* cells (ODL9-846) with sterile water (control) or 5 µg of linearized pG-SAT-1 are shown. The Apal unique site was used for linearization before transformation. Yeast colony PCR on a subset of 15 randomly selected clones was performed using specific primers (SAT1S and SAT2R) to amplify the SAT-1 sequence. (b) Selection and characterization of hygromycin B-resistant colonies after transformation with plasmid pG-HPH#. A representation of pG-HPH# and representative plates of selection for hygromycin B resistance 4 days after the transformation of *C. guilliermondii* cells (ODL9-846) with sterile water (control) or 5 µg of linearized pG-HPH# are shown. The Apal unique site was used for linearization before transformation. Yeast colony PCR on a subset of 15 randomly selected clones was performed using specific primers (HYGS1 and HYGR2) to amplify the HPH# sequence. Single site restriction enzymes are marked in bold with an asterisk.

Expression of codon-optimized SAT-1 and HPH# genes in *C. guilliermondii*

In a first series of experiments, we failed to obtain any nourseothricin- and hygromycin B-resistant transformants when we attempted to transform *C. guilliermondii* strains with 0.2, 1 or 5 µg of pSFS2 plasmid (Reuss *et al.*, 2004) and pRS-HYG# plasmid (Hara *et al.*, 2000), respectively. The SAT-1 coding sequence is under the control of the *C. albicans* ACT1 promoter and the *C. albicans* URA3 terminator in plasmid pSFS2 (Reuss *et al.*, 2004), whereas the HPH# coding sequence is bordered by the *Candida tropicalis* phosphoglycerate kinase promoter and terminator (Hara *et al.*, 2000). It is thus likely that these heterologous transcription-regulating sequences preclude the expression of SAT-1 and HPH# in *C. guilliermondii*.

For this reason, we placed SAT-1 and HPH# under the control of *C. guilliermondii* transcription-regulating sequences. We constructed two plasmids composed of (1) the pGEM-T backbone vector; (2) the recently developed *C. guilliermondii* URA5 selection marker (Millerioux *et al.*, 2011); (3) a 626-bp sequence corresponding to the

C. guilliermondii phosphoglycerate kinase 1 (PGK) gene promoter (PGK_{pro}); (4) a codon-optimized SAT-1 or HPH# coding sequence (SAT-1 in pURA5-SAT-1 and HPH# in pURA5-HPH#); and (5) a 659-bp sequence corresponding to the *C. guilliermondii* PGK terminator (PGK_{ter}) (Fig. 1). To demonstrate the capacity of these constructs to confer nourseothricin or hygromycin B resistance in *C. guilliermondii*, we transformed to prototrophy the NP566U strain (an *ura5* mutant derived from ATCC 6260, Millerioux *et al.*, 2011) with 500 ng of linearized pURA5-SAT-1 or pURA5-HPH#. More than 90% of the clones selected on YNB medium after transformation with pURA5-SAT-1 were nourseothricin resistant and similar percentages of the clones selected on YNB medium after transformation with pURA5-HPH# were hygromycin B resistant (Fig. 1). These data indicate that PGK transcription-regulating sequences allow a constitutive expression of SAT-1 and HPH# that confers nourseothricin and hygromycin B resistance to *C. guilliermondii* cells, respectively. Furthermore, these results show that SAT-1 does not confer hygromycin B resistance and HPH# does not confer nourseothricin resistance in this species.

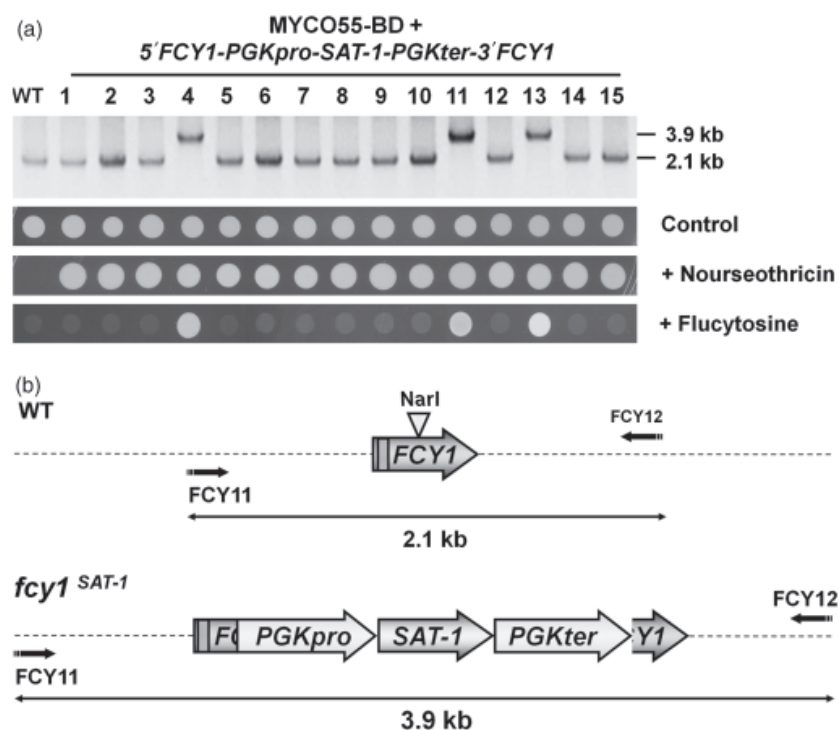


Fig. 3. Disruption of the *Candida guilliermondii* *FCY1* gene using the *SAT-1* marker. (a) For each strain derived from the MYCO55-BD transformation with the 5'FCY1-PGK_{pro}-SAT-1-PGK_{ter}-3'FCY1 cassette, the *FCY1* locus was analyzed by yeast colony PCR using FCY11 and FCY12 primers. Five microliters of washed cells (10^5 cells) were spotted onto YPS supplemented or not with $150 \mu\text{g mL}^{-1}$ nourseothricin. Flucytosine resistance was shown by spotting cells onto YNB supplemented with $8 \mu\text{g mL}^{-1}$ flucytosine. (b) Schematic representation of the resident loci (the hatched box corresponds to an intron identified in the *FCY1* coding sequence).

Direct transformation of *C. guilliermondii* using *SAT-1* and *HPH*[#] dominant markers

With the goal to facilitate molecular manipulations, we constructed two plasmids, pG-SAT-1 and pG-HPH[#], harboring the PGK_{pro}-SAT-1-PGK_{ter} or PGK_{pro}-HPH[#]-PGK_{ter} cassettes bordered by two multiple cloning sites (Fig. 2). To test whether the two cassettes could be used directly as selectable markers in *C. guilliermondii* strains, we transformed the ODL9-846 strain with 0.2, 1 or 5 μg of linearized pG-SAT-1 or pG-HPH[#]. No resistant clones were selected when the ODL9-846 strain was transformed with 0.2 or 1 μg of pG-SAT-1 or pG-HPH[#]. However, 50–200 nourseothricin- and hygromycin B-resistant clones were obtained when the ODL9-846 strain was transformed with 5 μg of pG-SAT-1 and pG-HPH[#], respectively (Fig. 2).

To verify that nourseothricin or hygromycin B resistance derived from the integration of the cassettes, we performed yeast colony PCR on a subset of 15 randomly selected clones using specific primers to amplify *SAT-1* or *HPH*[#] sequences. As shown in Fig. 2, all screened nourseothricin- or hygromycin B-resistant colonies displayed PCR products conformable with the presence of the *SAT-1* or the *HPH*[#] sequence, respectively. These results suggest that *SAT-1* and *HPH*[#] markers were incorporated into the genome of the ODL9-846 strain and provide evidence that PGK_{pro}-SAT-1-PGK_{ter} or PGK_{pro}-HPH[#]-PGK_{ter} cassettes could be used directly as dominant selection markers in *C. guilliermondii* wild-type strains.

Disruption of the *FCY1* gene: an example of gene knockout in *C. guilliermondii* wild-type strains using the *SAT-1* marker

To demonstrate the potential of these new drug-resistant markers in *C. guilliermondii*, we attempted, as an example, the disruption and subsequently the complementation of a gene that allows for easy mutant phenotype screening. We chose the *FCY1* gene encoding cytosine deaminase (involved in the pyrimidine salvage pathway) because the *fcy1* deletion in *C. guilliermondii* leads to strong flucytosine resistance (Millerioux *et al.*, 2011).

The *SAT-1* marker was first chosen to disrupt the *FCY1* gene in the five *C. guilliermondii* wild-type strains. For this purpose, a 5'FCY1-PGK_{pro}-SAT-1-PGK_{ter}-3'FCY1 fragment with 2000 bp of the 5' and 3' *FCY1* homologous arms was generated by PCR and used to transform the *C. guilliermondii* strains. Transformants derived from each host strain (selected onto $150 \mu\text{g mL}^{-1}$ nourseothricin-containing YPS plates) were subjected to flucytosine sensitivity testing and to PCR analysis. For all the screened clones, the flucytosine resistance phenotype was concomitant with homologous integration of the 5'FCY1-PGK_{pro}-SAT-1-PGK_{ter}-3'FCY1 cassette at the *FCY1* locus (genotype *fcy1*^{SAT-1}). Interestingly, PCR and phenotypic analyses showed that the *FCY1* gene had been replaced by homologous recombination in all the five transformed *C. guilliermondii* strains with variable targeting frequencies (Table 2). The best percentage of homologous targeting was obtained with the MYCO55-BD

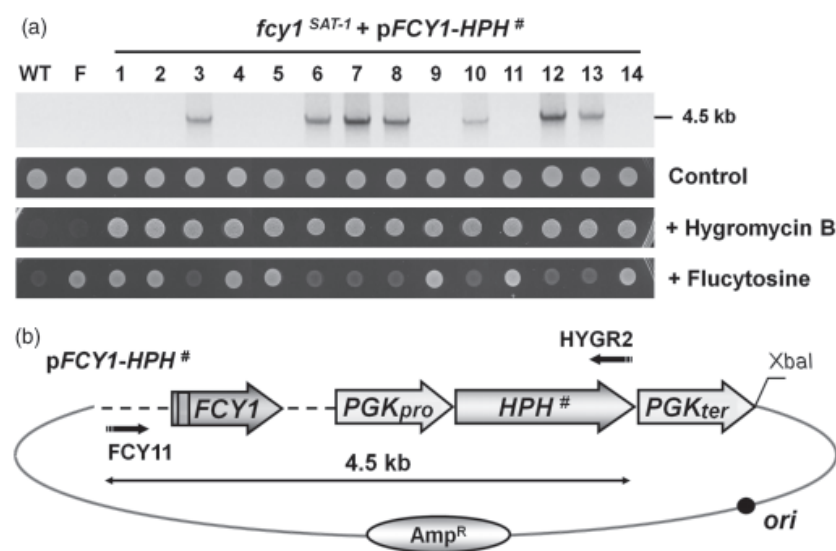


Fig. 4. Complementation of the *fcy1*^{SAT-1}-disrupted allele using the *HPH*[#] marker. (a) For each strain derived from the transformation of a representative MYCO55-BD *fcy1*^{SAT-1} mutant with p-*FCY1-HPH*[#], the integration of the complementation plasmid was analyzed by yeast colony PCR using FCY11 and HYGR2 primers. Five microliters of washed cells (10^5 cells) were spotted onto YPS supplemented or not with 500 $\mu\text{g mL}^{-1}$ hygromycin B. Flucytosine resistance was shown by spotting cells onto YNB supplemented with 8 $\mu\text{g mL}^{-1}$ flucytosine. F: MYCO55-BD *fcy1*^{SAT-1} parental strain used for transformation. (b) Schematic representation of the complementation plasmid. The XbaI unique site was used for linearization before transformation.

Table 2. Homologous targeting frequencies of *Candida guilliermondii* strains transformed with the 5'*FCY1*-*PGK*_{pro}-*SAT-1*-*PGK*_{ter}-3'*FCY1* disruption cassette

Strains	No. of transformants	No. of flucytosine-resistant clones	Homologous targeting frequency (%)
ODL9-811	191	6	3.1
ODL9-846	137	4	2.9
ODL12-1131	42	2	4.8
MYCO55-BD	15	3	20
ATCC 6260	82	3	3.7

strain (flucytosine sensitivity testing and PCR analysis are given as an example in Fig. 3). For the remaining nourseothricin-resistant and flucytosine-sensitive transformants, it is highly likely that they were derived from gene replacement at the *PGK* locus or ectopic integration (not studied).

The use of the *HPH*[#] marker for complementation of the *fcy1* null allele

In order to validate the capacity of the second available drug-resistant marker, i.e. *HPH*[#] to carry out the complementation of the *fcy1*^{SAT-1}-disrupted allele, we constructed a plasmid that contained both the *FCY1* wild-type allele and the *PGK*_{pro}-*HPH*[#]-*PGK*_{ter} cassette (p-*FCY1-HPH*[#], Fig. 4). This complementation vector was linearized and was used to transform two *fcy1*^{SAT-1} mutants (one derived from ODL9-811 and the other derived from MYCO55-BD). Transformants derived from both host strains (selected onto 500 $\mu\text{g mL}^{-1}$ hygromycin B-containing YPS plates) were subjected to flucytosine sensitivity testing and to PCR analysis. For all screened clones, the restored flucytosine sensitivity phenotype (30–50% of screened transformants) was concomitant with the integration of the complementation plasmid (Fig. 4).

Conclusion

In summary, the present study describes the development of an efficient transformation system for *C. guilliermondii* wild-type strains based on two new drug-resistant markers. The hygromycin B-resistant markers were already used as the selection gene in various yeast and filamentous species (for a review, see Weld *et al.*, 2006). Nevertheless, nourseothricin-resistant markers are rarely used for the transformation of fungi (for a review, see Basso *et al.*, 2010). Although our results suggest, as revealed in other previous studies (Boretzky *et al.*, 2007; Millerioux *et al.*, 2011), that transformed DNA is predominantly randomly integrated in *C. guilliermondii*, this work firmly demonstrates the potential of these two new selection markers to perform gene disruption/complementation in wild-type strains. We conclude that they represent powerful tools to study the function of a large pallet of genes, especially involved in antifungal resistance, pathogenicity, and for metabolic engineering in this emerging yeast.

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