FEMS Yeast Research, 2022, 22, 1–7
DOI: 10.1093/femsyr/foac046
Advance access publication date: 1 September 2022
Research Article

A novel *Candida glabrata* doxycycline-inducible system for *in vitro*/*in vivo* use

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One sentence summary: This study proposes a novel tetracycline-inducible system for gene expression in the fungal pathogen *Candida glabrata*.
Editor: Carol Munro

Abstract

Candida glabrata is an important pathogen causing superficial to invasive disease in human. Conditional expression systems are helpful in addressing the function of genes and especially when they can be applied to *in vivo* studies. Tetracycline-dependent regulation systems have been used in diverse fungi to turn-on (Tet-on) or turn-off (Tet-off) gene expression either *in vitro* but also *in vivo* in animal models. Up to now, only a Tet-off expression has been constructed for gene expression in *C. glabrata*. Here, we report a Tet-on gene expression system which can be used *in vitro* and *in vivo* in any *C. glabrata* genetic background. This system was used in a mice model of systemic infection to demonstrate that the general amino acid permease Gap1 is important for *C. glabrata* virulence.

Keywords: *Candida glabrata*, conditional gene expression, mice, virulence

Introduction

Conditional gene expression systems in fungi that enable reversible, inducible expression are essential research tools in biomedicine and biotechnology (Roney et al. 2016). Inducible expression systems can be used to probe gene function, as well as to establish regulatory relationships between different genes (Arita et al. 2021). Furthermore, they can track how gene expression is directly linked to cellular response, which is impossible in deletion mutants (Arita et al. 2021).

Different regulatable gene expression systems exist based on nutrients, such as galactose, methionine, and copper ions (Hovland et al. 1989, Labbe and Thiele 1999, Mao et al. 2002) as well as CRISPR-Cas9 promoter-guided systems (Uthayakumar et al. 2020). While some other systems use light for induction of expression (Kozma-Bognar et al. 2012), chemical substances can also be used that do not affect fungal physiology, such as β-estradiol (McIsaac et al. 2013a, b, 2014) or tetracycline (Belli et al. 1998). Tetracycline-inducible (Tet-on) systems enable graded and gratuitous modulation of target gene transcription in eukaryotic systems from yeast to transgenic animals (Roney et al. 2016). A Tet-on system requires two constituents: a reverse transactivator (rtTA), which is a transcription factor that activates expression of a target gene when bound to tetracycline, and a promoter containing one or more tetracycline operators or tetracycline responsive elements (TREs).

A total of two properties of the rtTA have been subjected to optimization: increasing doxycycline (DOX; a tetracycline derivative) affinity of the transactivator and reducing basal gene expression in the uninduced state (Roney et al. 2016, Urlinger et al. 2000, Wishart et al. 2005). In order to reduce leaky expression, the rtTA was combined with a tetracycline-responsive repressor (Belli et al. 1998, Lamartina et al. 2003, Rossi et al. 2000). The rtTA target promoter was also optimized (Agha-Mohammadi et al. 2004, Loew et al. 2010) and positive feedback control for rtTA transcription was introduced (Markusic et al. 2005). More recently, single amino acid substitutions were introduced in the *Saccharomyces cerevisiae* reverse tetracycline transactivator to reduce leaky expression while maintaining DOX affinity (Roney et al. 2016). Leaky expression is especially problematic when rtTA is heavily overexpressed (Roney et al. 2016). Many different Tet-promoters are used, they typically contain the TRE in multiple copies and are linked to well-studied core promoters (Loew et al. 2010, Nakayama et al. 2011). Ideally, a terminator of another gene is situated upstream of the TRE, which avoids activation of the target gene because of nearby promoter-like sequences (Nakayama et al. 1998).

Candida glabrata is a human fungal commensal and pathogen that can cause both superficial and life-threatening infections in immunocompromised patients. It is closely related to *S. cerevisiae*, yet it is an obligate haploid and prefers nonhomologous end joining over homologous recombination to repair DNA (Gabaldon et al. 2013, Kurtzman and Robnett 1998, Roetzer et al. 2011). Unlike *S. cerevisiae*, the interest in *C. glabrata* stems only from the significant morbidity and mortality it causes in the clinic. However, *C. glabrata* pathogenesis is still not well-understood and only few specific genes or gene families have been linked to virulence (Kaur et al. 2007, Lopez-Fuentes et al. 2018, Schrevens et al. 2022). This is due to the limited existing *in vivo* studies on this organism (Ferrari et al. 2011a,b, Nakayama et al. 2011, Schrevens et al. 2022). A tetracycline-dependent gene expression system (Tet-off) has been described in *C. glabrata* that uses a tetracycline transactivator (tTA) coupled with TRE-containing promoters to decrease gene expression (Nakayama et al. 1998). This Tet-off system has been used to probe gene essentiality or gene function in *C. glabrata*

Received: July 4, 2022. **Revised:** August 17, 2022. **Accepted:** August 30, 2022

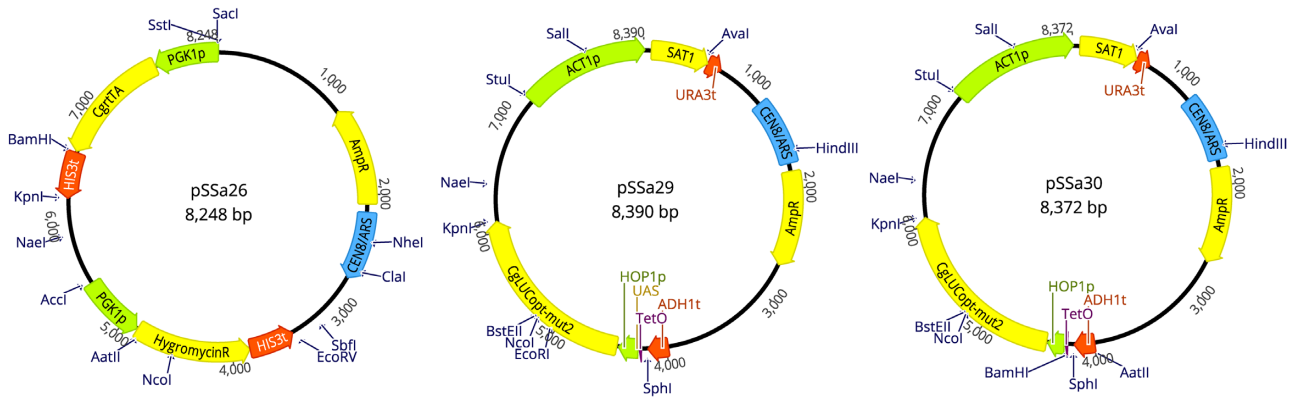

Figure 1. Plasmids used in this study. pSSa16 is used to express the *C. glabrata* codon-optimized reverse transactivator (CgrtTA). pSSa29 and pSSa30 were designed to express a codon-optimized luciferase under the control of the TetO operator, in the presence or absence of a UAS, respectively. Abbreviations: PGK1p: *S. cerevisiae* phosphoglycerate kinase promoter; HIS3t: imidazoleglycerol-phosphate dehydratase terminator; HOP1p: meiosis-specific gene promoter; TetO: tretracycline operator; UAS: upstream activating sequence; CgLUCtop-mut2: codon-optimized and mutant luciferase; SAT1: nourseothricin resistance gene; and ACT1p: actin I promoter.

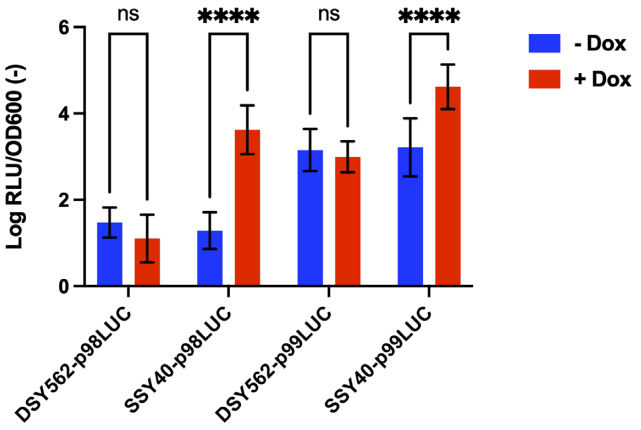

Figure 2. The DOX-inducible expression system is functional in *C. glabrata*. Both p98LUC (TetO-*HOP1* promoter containing a UAS in pSSa29) and p99LUC (TetO-*HOP1* promoter in pSSa30) conferred inducible luciferase expression upon DOX exposure (100 μg/ml) in the presence of CgrtTA (SSY40 background). Cells were grown until early exponential phase in the absence or presence of 100 μg/ml DOX, upon which luminescence and absorbance (at 600 nm) were measured. Results were obtained from at least three biological replicates. Unpaired *t*-tests were performed using Graph Prism 9.4.0. Significance symbols: ****: $P < .0001$; ns: not significant.

(Okamoto et al. 2022, Ueno et al. 2010). This system was also tested *in vivo*, in which it was shown that, when ergosterol biosynthesis is shut-down by *ERG9* down-regulation, *C. glabrata* can use host cholesterol for its survival (Nakayama et al. 2000).

In this study, a DOX-inducible expression system (Tet-on) was adapted to *C. glabrata*. This system can be used to assess virulence of specific genes within the same strain, excluding the effect of background mutations on the results. Using a DOX-inducible expression of the general amino acid permease *GAP1*, we showed that *GAP1* was critical for virulence during invasive infection in mice.

Materials and methods

Strains and growth media

All plasmid propagations were performed in *Escherichia coli* DH5α cells, which were grown in LB broth supplemented with ampicillin (0.1 mg/ml) when necessary. Liquid cultures were grown at 37°C under constant agitation and solid plates containing 0.7% Bacto Agar (Brunschwig, Switzerland) were incubated at 37°C for 16–20 hours.

Candida glabrata strains used in this work are listed in Table S1 (Supporting Information). *Candida glabrata* strains were grown in yeast peptone dextrose (YPD; 2% Bacto Peptone, 1% yeast extract, and 2% glucose) at 30°C under continuous shaking or synthetic complete (SC) medium [0.67% YNB without amino acids, 0.079% CSM complete (MP biomedicals) and 2% glucose] at 30°C under continuous agitation for liquid cultures.

Candida glabrata was transformed using electroporation and selection was carried out on 200 μg/ml nourseothricin and/or 300 μg/ml hygromycin B.

Growth tests were carried out in nitrogen starvation (NS) medium (0.17% YNB without amino acids and without ammonium sulphate, 2% glucose, 2% Bacto agar) supplemented with 5 mM -Citrulline (Sigma).

Construction of strains and plasmids

The reverse transactivator sequence was ordered as a synthetic gene (Eurofins Genomics, Germany) and cloned into the pVS29 backbone (Schrevens et al. 2022) using *SpeI* and *BamHI* restriction sites, thus resulting in pSSa26. Upon digestions with *NheI* (a restriction site situated in the CgCEN sequence of pSSa26), it was transformed into DSY562, resulting in strain SSY40.

The constructs in which p98 and p99 (Nakayama et al. 1998) drive luciferase expression were constructed upon amplification of each of the promoters using primers pTETO7-AatII-fwd and pTET9x-BglII-Rev to amplify the promoters from plasmids CGH98 and CGH99 (Nakayama et al. 1998) and pSSa13-BamHI-Fwd and pSSa13-AatII-Rev to amplify pSSa13 (Schrevens and Sanglard 2021) in order to incorporate the correct restriction sites. Resulting PCR products where digested with *BamHI* and *MluI* for pSSa13 and *BglII* and *MluI* for the promoters. The resulting fragments were then ligated and correct ligation was confirmed using sequencing, resulting in plasmids pSSa29 and pSSa30. These plasmids were transformed in *C. glabrata* after digestion with *HindIII* (a restriction site situated in the CgCEN sequence of pSSa29 and pSSa30) into SSY40 and DSY562, resulting in strains SSY42, SSY43, and SSY52. In order to create a construct for genome insertion to drive expression of a native *C. glabrata* gene, we constructed plasmids pSSa31

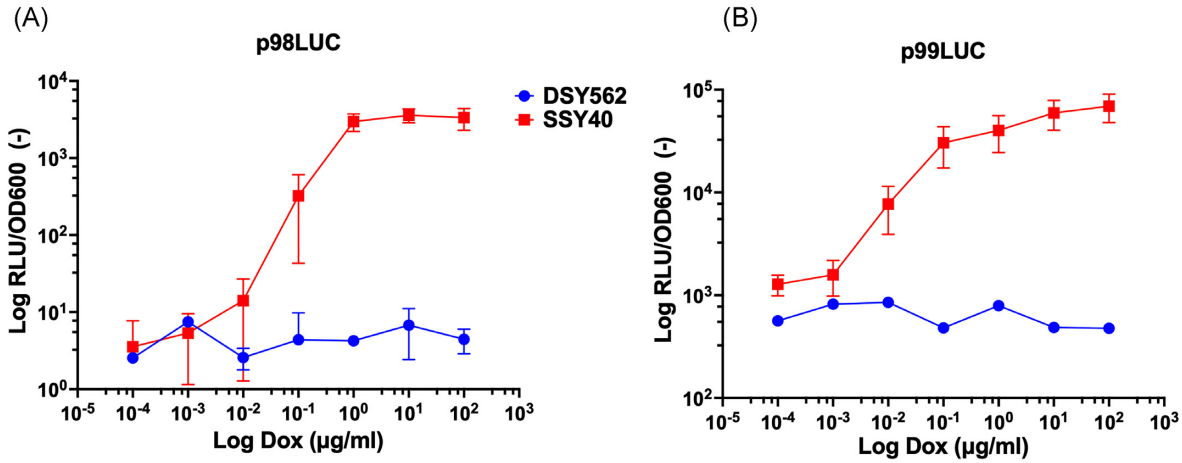

Figure 3. DOX response curves of *C. glabrata* cells containing either the DOX-inducible system under the control of the TetO-*HOP1* promoter in p98LUC (A) and p99LUC (B). Both promoters show an optimal expression from 1 to 10 µg/ml DOX, yet p99LUC is slightly more sensitive to DOX with clear induction at 0.01 µg/ml compared to 0.1 µg/ml for p98LUC. Results were obtained from at least two biological replicates.

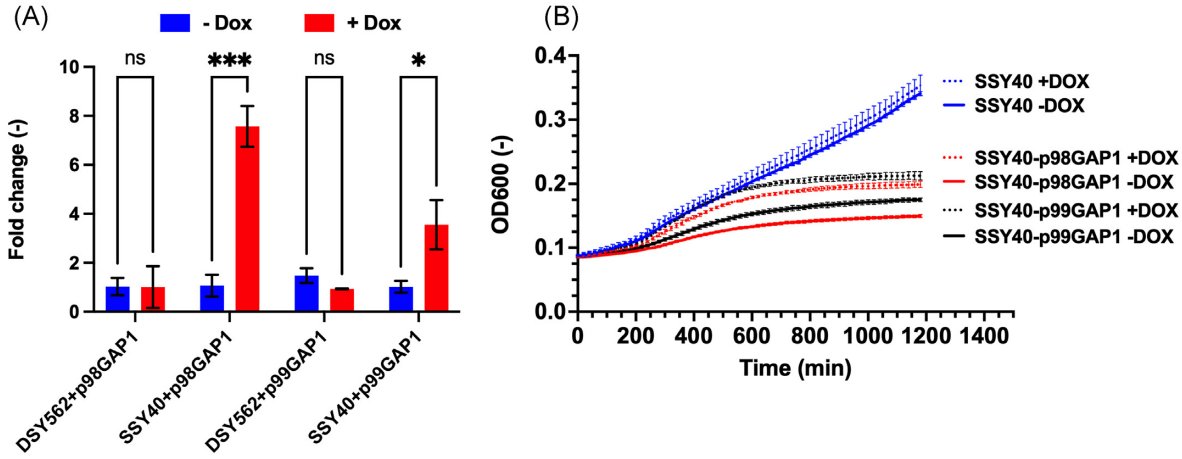

Figure 4. Expression of *GAP1* under the control of the DOX-inducible system. *GAP1* was induced by both p98 and p99, which affects growth on -citrulline as the sole nitrogen source. (A) qPCR of strains containing p98GAP1 or p99GAP1 with (SSY40) or without (DSY562) the CgrtTA in response to DOX. Expression of *GAP1* is induced only in the presence of the CgrtTA. Unpaired *t*-tests were performed using Graph Prism 9.4.0. Significance symbols: ***: $P \leq .001$; *: $P \leq .05$; ns: not significant. (B) Growth of *GAP1*-inducible strains on -citrulline as the sole nitrogen source. For both p98GAP1 and p99GAP1, growth is increased significantly in response to DOX.

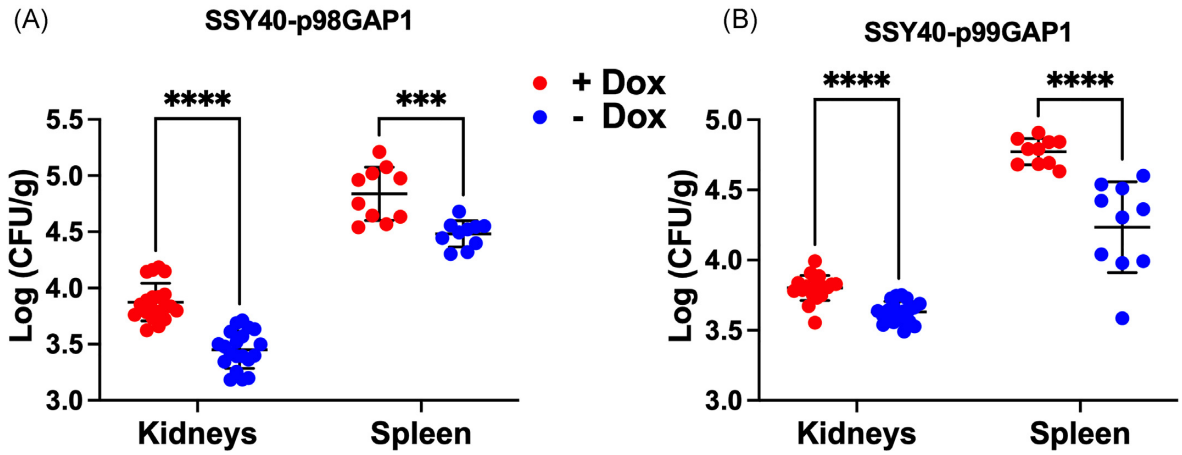

Figure 5. The *C. glabrata* DOX-inducible system can be used during invasive infection in mice. *GAP1* expression was under the control of the p98 and p99 promoters in SSY40 (SSY40-p98GAP1 and SSY40-p99GAP1, respectively). A total of 10 mice per group were injected in the lateral tail vein with *C. glabrata* cells and treated intraperitoneally daily with DOX (50 mg/kg) or PBS. On day 5 mice were sacrificed and CFUs were counted. Mann–Whitney tests were performed using Graph Prism 9.4.0. Significance symbols: ****: $P < .0001$; ***: $P < .001$.

and pSSa32 (Figure S1, Supporting Information). First, the *SAT1* marker was amplified from pSSa13 using primers SAT1-FWD1 and SAT1-REV-F and p98 and p99 were amplified from CGH98 and CGH99 (Nakayama et al. 1998) using primers p9xo-FWD-F and p9xo-REV1. A fusion PCR with these PCR products was carried out using primers SAT1-FWD2-EcoRI and p9xo-REV2-KpnI, which was then cloned into pDS1706 using *Eco*RI and *Kpn*I restriction sites. The resulting plasmids, pSSa31 and pSSa32, where next used to create SAT1-pTET-*GAP1* fusion constructs both for p98 and p99 to replace the genomic *GAP1* promoter by a tetracycline-regulatable promoter [see Figure S2 (Supporting Information) for schematic view]. First, SAT1-pTET as a 2.2-kb fragment was amplified from pSSa31 or pSSa32 using primers pACT1-FWD-fusionGAP1 and p9xo-REV-fusionGAP1, while the overlapping regions with *GAP1* (537 bp from ATG) and its promoter (526 bp upstream of the first 500 bp of the *GAP1* promoter) were amplified from DSY562 DNA using primers GAP1-FWD-f2NEW and GAP1-REV-f2, and pGAP1-FWD-f1 and pGAP1-REV-f1NEW, respectively. The three resulting fragments were fused using nested primers Gap1-fusionprimer2-FWD and GAP1-fusionprimer2-Rev, resulting in the *SAT1* marker flanked with regions overlapping with *GAP1* and its promoter. The p98 and p99-containing PCR products were transformed into SSY40 using electroporation (Kannan et al. 2019) together with a reconstituted RNA–protein complex (RNP) containing a synthetic guide RNA directed to the *GAP1* promoter [gGAPp, Table S3 (Supporting Information), obtained from Integrated DNA Technologies, Inc., Coralville, IO, USA]. The resulting strains were designated as DSY5705 and DSY5706. Constructed plasmids and their characteristics are summarized in Table S2 (Supporting Information). Plasmids pSSa26, pSSa29, pSSa30, pSSa31, and pSSa32 can be obtained from Addgene under numbers 186784, 179503, 179502, 179504, and 179 505, respectively. Primers are listed in Table S3 (Supporting Information).

Luciferase tests

Candida glabrata cells were grown overnight in SC medium and subsequently 10-fold diluted in SC medium with or without 100 μl/ml DOX and further grown until early exponential phase. A volume of 90 μl of the resulting culture were transferred to a black, half area 96-well plate (costar) and 10 μl of -luciferin (16 mg/ml; Biosynth AG, St-Gallen, Switzerland) was automatically injected into each well followed by 3 s of orbital shaking before luminescence was measured in a luminometer (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The optical density at 600 nm (OD600) of 100 μl of the early exponential culture was also measured in the same spectrophotometer in order to normalize luminescence by OD600. In order to test the response to DOX of the two promoters, the same assay was carried out using different concentrations of DOX ranging from 0.00001 to 100 μg/ml.

Growth tests

Candida glabrata cells were pregrown overnight in YPD medium at 30°C under shaking conditions and subsequently washed with 5 ml PBS before being diluted to an OD600 of 0.1 in NS medium containing 5 mM -citrulline with or without 100 μg/ml DOX in a 200 μl 96-well plate. This plate was next measured every hour by absorbance (600 nm) for 20 hours in a microplate reader at 30°C under regular shaking.DOX at a concentration of 100 μg/ml did not exert growth inhibition compared to control conditions in YPD medium (Figure S3, Supporting Information).

qPCRs

Candida glabrata cells were pregrown in SC overnight and diluted to OD600 (0.1) in SC with or without 100 μg/ml DOX and grown until early exponential phase. RNA was extracted as earlier reported (Vale-Silva et al. 2016). And qPCRs were carried out using 0.2 μM of each primer and 0.2 μM of the TaqMan probe using the iTaq Universal Probe kit (Biorad) according to manufacturer's instructions. Sequences of primers and probes are listed in Table S3 (Supporting Information). Fold change expression was calculated using the ΔΔCt method and normalized to *RDN58* as the housekeeping gene. qPCRs were carried out with a Quantstudio 3 device.

Invasive infection of mice

All animal experiments were carried out according to the approval of the Institutional Animal Use Committee, Affaires Veterinaires du Canton de Vaud, Switzerland (authorization VD1734.5a) at the University Hospital Center of Lausanne. Animals were housed in ventilated cages with *ad libitum* access to food and water. Female BALB/c mice, 6-week-old (Charles River, France), were injected with 10e7 *C. glabrata* cells in the lateral tail vein. DOX (50 mg/kg) was injected once daily intraperitoneally and on day 5 mice were sacrificed using CO_2. The kidneys and the spleen were extracted, homogenized, and plated for colony forming unit (CFU) counting.

Results

Construction of strains and plasmids

First, a codon-optimized synthetic gene was designed based on the improved reverse transactivator constructed by Roney et al. (2016) and was linked to the *S. cerevisiae GAL4* activating domain with a SV40 nuclear localization signal (NLS), which was also used for the tetracycline repressible system in *C. glabrata* (Nakayama et al. 1998). This resulted in the *C. glabrata* reverse transactivator (CgrtTA). CgrtTA was next cloned into a plasmid able to replicate episomally or be integrated into the genome into the CEN/ARS locus (pSSA26; Fig. 1) using hygromycin resistance selection. The two most efficient Tet promoters developed for *C. glabrata* tetracycline repressible expression, p98 and p99, which differ only in the presence of an upstream repressor sequence in p98 compared to p99 (Nakayama et al. 1998), were cloned into pSSA13 to drive the expression of the *C. glabrata* codon-adapted red shifted luciferase. The resulting plasmids (pSSA29 and pSSA30; Fig. 1) can be integrated as well as used episomally using nourseothricin resistance selection (Schrevens and Sanglard 2021). Thus, both CgrtTA expression and Tet promoters can be transferred simultaneously to any *C. glabrata* genetic background.

Luciferase expression is significantly induced in the presence of DOX

The red-shifted firefly luciferase that was codon-adapted to *C. glabrata* (Schrevens and Sanglard 2021) was used as a reporter gene to assess efficacy of the Tet-on system. Cells were pregrown in complete medium overnight, diluted in fresh medium with or without 100 μg/ml DOX and then cultured until early exponential phase. Both luminescence, upon addition of D-luciferin, and absorbance (600 nm) were measured in order to normalize luminescence for the optical density of the culture. As shown in Fig. 2, addition of DOX resulted in significantly higher luminescence for both p98LUC (luciferase driven by TetO-*HOP1* promoter containing a UAS in pSSa29) and p99LUC (luciferase driven by by a TetO-*HOP1* promoter in pSSa30) in the presence of the reverse transactivator (strain SSY40). In absence of CgrtTA (strain DSY562),

no significant increase in luminescence was observed. A higher background luminescence was observed for the p99LUC construct compared to p98LUC. p99LUC also led to a higher absolute luminescence compared to p98LUC, however, the latter construct showed a larger difference between induced and noninduced expression.

In order to test the dose response to DOX, concentrations ranging from 0.00001 to 100 μg/ml were tested in the luminescence assay. Clear induction was detected at 0.1 μg/ml for p98LUC and 0.01 μg/ml for p99LUC (Fig. 3A and B, respectively). Interestingly, similar to Fig. 2, the background expression for p98LUC was significantly lower compared to p99LUC.

Inducible expression of general amino acid permease 1 (GAP1) affects virulence

Uptake, metabolism, and specific regulation of nitrogen was shown to be of high importance for *C. glabrata* virulence (Schrevens et al. 2022). Nitrogen is one of the most important essential nutrient sources, and probably available in limited amounts extracellularly inside a host (Zhang et al. 2018). Different pathways, transporters, and receptors responsible for a tightly controlled use of this nutrient have been well studied in *S. cerevisiae* (Zhang et al. 2018). *Saccharomyces cerevisiae* contains 24 amino acid transporters, typically consisting of 12 transmembrane domains and a cytoplasmically oriented C- and N-terminus (Cain and Kaiser 2011, Jack et al. 2000). The general amino acid permease Gap1 is well-studied in *S. cerevisiae* and was shown to have a broad substrate specificity and high substrate affinity (Jauniaux and Grenson 1990). Gap1 was shown to be important for virulence in *C. glabrata* during urinary tract infection (Schrevens et al. 2022). Due to its potentially central role in the uptake and regulation of amino acid metabolism, similarly to *S. cerevisiae*, Gap1 may also functions during invasive infection. During invasive infection, *C. glabrata* is phagocytosed by macrophages (Kasper et al. 2015) and an important nitrogen source inside the phagolysosome could be amino acids, which was shown during *C. albicans* infection (Vylkova and Lorenz 2014). Therefore, we decided to use *GAP1* to determine whether the *C. glabrata* DOX-inducible system can be used to assess virulence inside a host. The p98 and p99 promoters were inserted into the genome of *C. glabrata* (strain SSY40 and DSY562) to replace the native *GAP1* promoter. To verify that *GAP1*-expression could be induced, *C. glabrata* cells were grown to early exponential phase in the absence or presence of 100 μg/ml DOX and RNA was extracted for qPCR. Figure 4(A) shows that *GAP1*-expression is induced about 8-fold when driven by the p98 promoter and about 4-fold in case of the p99 promoter. This difference is similar to the luminescence assay, in which p98 resulted in stronger induction, yet p99 resulted in higher expression. Additionally, we tested whether growth on -citrulline as the sole carbon source was increased upon induction with DOX. Both the p98-GAP1 and the p99-GAP1 constructs grew slower compared to the strain in which *GAP1* is expressed from its native promoter (SSY40), yet significantly faster compared to the noninduced conditions (Fig. 4B). As in the luminescence assay and the qPCR, p99GAP1 grows slightly faster compared to p98GAP1 upon DOX addition.

We next injected strains containing either the p98-GAP1 or the p99-GAP1 promoters in the bloodstream of mice. Half of the mice were injected peritoneally with DOX (50 mg/kg) on a daily basis in order to induce *GAP1* expression, while the other half were injected with PBS. Mice were sacrificed after 5 days, and the kidneys and the spleen were extracted in order to determine CFUs. CFUs were significantly lower in the absence of DOX in all organs and for both constructs (Fig. 5), showing that high *GAP1* expression is required for virulence of *C. glabrata* during invasive infection.

Discussion

We developed here a Tet-on expression system for the human opportunistic fungal pathogen *C. glabrata*. This system is based on the improved reverse tetracycline transactivator (rtTA) in *S. cerevisiae* from Roney et al. (2016). In their work, background gene expression was reduced to almost undetectable levels, which is however not the case in our system (Roney et al. 2016). This difference could be attributed to the use of different promoters and more specifically to the promoters used in the *C. glabrata* tetracycline repressible system (Nakayama et al. 1998). Both p98- and p99 promoters are based on the *S. cerevisiae HOP1* promoter with the presence of the upstream repression sequence in p98 and the absence of this sequence in p99. The promoters for Tet-on and Tet-off systems in *S. cerevisiae* have been subjected to several improvements (Belli et al. 1998, Cuperus et al. 2015, Gari et al. 1997, Murphy et al. 2007, Roney et al. 2016, Urlinger et al. 2000), and it is possible that these promoters could be also suited for *C. glabrata*.

To test background expression in the absence of DOX, Roney et al. (2016) used a Tet-on system based on a modified rtTA and enhanced GFP as a gene expression reporter, which was quantified by flow cytometry. We used here the same type of modified rtTA but adapted to *C. glabrata* and luciferase as a reporter system, which results in high sensitivity when measured by luminescence in whole cells. Compared to a GFP reporter system, the luciferase reporter results in detectable reporter expression even if gene expression is at low levels (in the absence of DOX), which may contribute to the differences between the Tet-on system used in the two species. Additionally, leaky expression is especially problematic for a tight control of Tet promoter when the rtTA is strongly overexpressed (Roney et al. 2016). Since the relatively strong *S. cerevisiae PGK1* promoter was used for CgrtTA expression in *C. glabrata*, this may have contributed to a less stringent control of the Tet promoter in absence of DOX.

The Tet-on system presented here was used in an animal model to probe the effect of a given gene (*GAP1*) on *C. glabrata* virulence. We performed the comparison between parallel experiments with two states of cells with low (-DOX) and high gene expression (+DOX). Other experimental conditions are possible, in which gene expression may be first maintained high by DOX addition and next reduced by removing DOX. This allows to modulate gene expression at different time points during the course of the infection in mice and may reveal the function of a given gene during the different steps in pathogenesis.

In conclusion, our work established a Tet-on system for *in vitro* and *in vivo* gene expression approaches in any in *C. glabrata* genetic background. This system may also be used to overexpress *C. glabrata* genes under controlled conditions to screen genes for novel functions as was carried out in *C. albicans* with the Tet-on overexpression collection (Chauvel et al. 2012).

Supplementary data

Supplementary data are available at *FEMSYR* online.

Acknowledgments

Authors are thankful to Danielle Brandalise for excellent technical assistance. This work was supported by a grant (31003A_172958) of the Swiss National Research Foundation to DS.

Conflict of interest. None declared

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