Université de Montréal

Studying cross-talk between different transcriptional pathways

controlling azole resistance in *Candida albicans*

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Résumé

Tac1p et Mrr1p sont des régulateurs transcriptionnels importants impliqués dans la résistance aux azoles chez la levure *Candida albicans*. Des mutations gain de fonction dans ces régulateurs sont responsables de la surexpression constitutive des gènes impliqués dans la résistance aux azoles chez plusieurs isolats cliniques de *C. albicans*. Ces deux régulateurs peuvent également être activés de façon transitoire par certains composés chimiques. En se basant sur des résultats qui suggèrent que ces deux voies pourraient interagir, nous avons étudié si Mrr1p est impliqué dans la voie transcriptionnelle médiée par Tac1p. Tout d'abord, nous avons étudié si Mrr1p fonctionne dans la régulation positive transitoire de *CDR1* et *TAC1* induite par la fluphénazine, ces deux gènes étant des cibles de Tac1p et codant pour des effecteurs importants dans la résistance aux azoles. Les résultats suggèrent que Mrr1p joue un rôle complexe dans ce processus et pourrait supprimer l'induction transitoire de *TAC1*. Deuxièmement, nous avons étudié le rôle de Mrr1p dans la régulation positive constitutive de *CDR1* et *TAC1* médiée par une forme hyper-active de Tac1p dans un isolat clinique résistant aux azoles bien caractérisé, appelé 5674. Nous avons constaté que Mrr1p contribue à la résistance de 5674 au fluconazole, mais la délétion de *MRR1* dans cette souche n'a aucun impact sur les niveaux d'ARNm de *CDR1* et *TAC1*. De plus, nous avons également montré que les transcrits de *TAC1* dans la souche 5674 ont une longue région 5' non-traduite, mais que Mrr1p n'est pas impliquée dans la production de l'ARNm *TAC1* longue. Étant donné que

certains de ces résultats sont préliminaires, d'autres expériences seront nécessaires pour confirmer ces résultats et pour aborder les questions restantes.

Abstract

Tac1p and Mrr1p are both important transcriptional regulators of azole resistance in the yeast *Candida albicans*. Gain-of-function mutations in these factors are responsible for the constitutive upregulation of azole resistance genes in several *C. albicans* clinical isolates. Both factors can also be transiently activated by specific chemical compounds. Based on the results suggesting that these two pathways may interact, we studied whether Mrr1p is involved in the Tac1p-mediated transcriptional pathway. First, we investigated whether Mrr1p functions in fluphenazine-induced transient upregulation of *CDR1* and *TAC1*, both of which are Tac1p target genes and encode important effectors of azole resistance. The results suggest that Mrr1p plays a complicated role in this process and might suppress the transient induction of *TAC1*. Second, we studied the role of Mrr1p in hyperactive Tac1p-mediated constitutive upregulation of *CDR1* and *TAC1* as well as the resulting azole resistance in a well-characterized azole-resistant clinical isolate 5674. We found that Mrr1p contributes to the resistance of isolate 5674 to fluconazole, but that the deletion of *MRR1* in isolate 5674 had no impact on the mRNA levels of *CDR1* and *TAC1*. Moreover, we have also shown that the *TAC1* transcripts in isolate 5674 have a long 5' untranslated region and that Mrr1p is not implicated in producing this long *TAC1* mRNA. Since some of the results are preliminary, further experiments will be needed to confirm these results and address remaining questions.

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5-FC	5-fluorocytosine
ABC	ATP-binding cassette
ATP	Adenosine triphosphate
bp	Base pair
C. albicans	*Candida albicans*
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
DNA	Deoxyribonucleic acid
DRE	Drug-responsive element
EDTA	Ethylenediaminetetraacetic acid
FPZ	Fluphenazine
GlcNAc	N-acetylglucosamine
GOF	Gain-of-function
HIV	Human immunodeficiency virus
LiAc	Lithium acetate
MFS	Major facilitator superfamily
mRNA	Messenger ribonucleic acid
nt	Nucleotide

OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
uORF	Upstream open reading frame
UTR	Untranslated region

Acknowledgements

First, I would like to thank my supervisor Dr. Martine Raymond for her careful guidance, patience and trust throughout my studies. I really appreciate the opportunity that she offered me to study in Montreal, a city far away from my hometown. I would also like to thank Sandra Weber for her help with my experiments. I am grateful to Aline Khayat, who provided me with useful advice on my research. Thanks to all the other members of the Raymond Lab, from whom I have also learned a lot. Last, I would like to thank my parents for their encouragement and constant support throughout my life.

1. Introduction

1.1. *Candida albicans*

Candida albicans is one of the major human fungal pathogens. It is usually a commensal yeast, which can be found on the skin and mucosal surfaces of most healthy individuals. However, it can sometimes cause superficial mucosal infections, including vaginal and oral thrush [1]. Besides, it is also capable of leading to severe systemic infections, especially in immunocompromised individuals, such as HIV-infected patients and patients undergoing intensive chemotherapy treatment [2].

C. albicans is polymorphic and able to grow in three different forms: yeast, pseudohyphae and true hyphae [3]. The morphological changes between different forms occur when favoring environmental cues exist. The yeast-to-hyphae transition, which is a critical determinant of virulence of *C. albicans*, has been widely studied [4, 5]. *C. albicans* undergoes hyphal formation in response to many environmental signals, including, but not limited to, neutral pH, the presence of serum, 5% CO_2, the presence of GlcNAc and lack of nitrogen source [6].

C. albicans is predominantly diploid and has eight pairs of chromosomes. It is distantly related to *Saccharomyces cerevisiae*, the well-studied budding yeast. Although the genome of *C. albicans* was sequenced more than ten years ago, there are still a large number of uncharacterized genes. Thus people tend to do comparative analyses with *S. cerevisiae* as well

as other relevant fungal species to speculate on the possible function of a *C. albicans* gene before performing specific verification experiments. The *C. albicans* genome shows a large extent of plasticity, including loss of heterozygosity, aneuploidy and gross chromosomal rearrangements, which are associated with the cellular responses of *C. albicans* to many different kinds of environmental stresses and may lead to adaptive changes (e.g. antifungal resistance) that are critical for its survival under distinct unfavorable conditions [7-10].

1.2. Antifungal drugs to treat *C. albicans* infections

Several antifungal drugs are currently available for the treatment of *C. albicans* infections, among which four classes of drugs are predominantly used: polyenes, pyrimidine analogues, echinocandins and azoles. They are distinct from each other in terms of formulations, modes-of-action, toxicities, pharmacokinetics and bioavailabilities [11].

The polyenes are natural amphipathic antimycotics, which were the first applicable antifungals for clinical use [12]. The polyenes bind to ergosterol, the major sterol of fungal cell membranes, causing expansion of the lipid bilayer and thereby forming pores in the cell membrane [13]. The change in the cell membrane structure facilitates the diffusion of many small molecules across the cell membrane, which in turn results in fungal death [13]. The three main polyenes are nystatin, natamycin and amphotericin B, among which amphotericin B and its lipid formulations are used to treat severe systemic fungal infections [11, 12].

The only representative of the pyrimidine analogues is 5-fluorocytosine (5-FC), which is soluble in water and thus exhibits good oral bioavailability [11]. In *C. albicans* cells, 5-FC is deaminated by cytosine deaminase to produce 5-fluorouracil, which is capable of inhibiting the biosynthesis of DNA and RNA and thereby exerts antifungal activities [14]. 5-FC is mainly used in combination with amphotericin B to treat systemic candidiasis and rarely used as monotherapy due to the high frequency of the formation of resistance [14, 15].

The echinocandins are the most recent antifungal agents. They are semisynthetic lipopeptide molecules, which inhibit β-1,3-D-glucan synthase and thereby block the biosynthesis of β-1,3-D-glucan, an essential component of the fungal cell wall [16, 17]. As a result, fungal cells undergo cell wall damage and eventually cell death [11]. Micafungin, caspofungin and anidulafungin are the three major echinocandins. They need to be administered intravenously as they show very limited oral bioavailability [11]. Besides, the echinocandins are primarily used to treat invasive *Candida* infections and have good efficacy and safety profiles [18]. Although cases of echinocandin resistance have been increasingly documented, the frequency of resistance is still relatively low among most *Candida* species [19].

Azoles were first developed several decades ago and are nowadays the most widely used antifungal drugs all over the world. They are synthetic five-membered heterocyclic compounds, which are capable of inhibiting the cytochrome P450 lanosterol 14α-demethylase that is essential for the biosynthesis of ergosterol and thereby block the production of

ergosterol and lead to the accumulation of the toxic sterol intermediate 14α-methylergosta-8, 24(28)-dien-3β,6α-diol, which is able to cause fungal cell growth arrest [20-22]. The azole drugs are categorized into two groups: the triazoles and the imidazoles. The triazole group comprises of itraconazole, fluconazole, posaconazole and voriconazole, which are used for the treatment of both superficial and systemic candidiasis; the imidazole group includes miconazole, ketoconazole and clotrimazole, which are predominantly used to treat skin and mucosal *Candida* infections [11, 23].

1.3. Azole resistance in *C. albicans*

Due to the extensive clinical application as well as the fungistatic nature of the azole drugs and the fast adaptation capability of *C. albicans*, azole resistance in *C. albicans* has become a growing issue in many countries. Hence, researchers have been trying to uncover the mechanisms by which *C. albicans* becomes resistant to azoles.

1.3.1. Azole resistance effectors in *C. albicans*

1.3.1.1. Cdr1p, Cdr2p and Mdr1p

There are a number of efflux pumps in *C. albicans*, but only three of them have so far been found to be involved in azole resistance: Cdr1p, Cdr2p and Mdr1p.

Cdr1p and Cdr2p belong to the ATP-binding cassette (ABC) transporter family and share high sequence homology [24]. In the wild-type *C. albicans* cells that are susceptible to azoles, the *CDR1* gene is usually expressed at a low basal level conferring a certain level of tolerance to azoles, but the *CDR2* gene is not detectably expressed [23, 25, 26]. The concomitant overexpression of *CDR1* and *CDR2* has been observed in many azole-resistant clinical isolates of *C. albicans*, in which the deletion of *CDR1* and *CDR2* leads to azole hypersusceptibility [27, 28]. In addition, it has been shown that Cdr1p plays a more important role than Cdr2p in conferring azole resistance [27, 29]. Finally, the expression of *CDR1* and *CDR2* can be transiently upregulated in the presence of specific inducing compounds, including steroids, rhodamine and fluphenazine [30].

Mdr1p belongs to a different class of efflux pumps, the major facilitator superfamily (MFS) transporters, which transport their substrates by using the electrochemical gradient of protons across the cell membrane [31]. Unlike Cdr1p and Cdr2p that are capable of transporting many different types of azole drugs, Mdr1p appears to specifically transport fluconazole out of *C. albicans* cells [23]. The *MDR1* gene is not expressed at a detectable level in wild-type fluconazole-susceptible *C. albicans* strains, but is overexpressed in many fluconazole-resistant clinical isolates [32-35]. When *C. albicans* cells are treated with benomyl or hydrogen peroxide, *MDR1* gets transiently overexpressed [36].

1.3.1.2. Erg11p

In *C. albicans*, the *ERG11* gene encodes the enzyme lanosterol 14α-demethylase, which is the direct target of azoles. Mutations in *ERG11* can sometimes lead to alterations in the structure of this enzyme, thereby preventing the binding of azoles while not affecting its normal enzymatic activity [32, 37-40]. In some azole-resistant *C. albicans* clinical isolates, the contribution of specific *ERG11* mutations to azole resistance has been confirmed by different methods [38, 39, 41-44]. Besides, it has been reported in many azole-resistant clinical isolates of *C. albicans* that elevated expression of *ERG11* is responsible for the resistance to azoles, as higher amounts of azoles are thus required to inhibit Erg11p sufficiently [32, 45].

1.3.1.3. Erg3p

The *ERG3* gene encodes a sterol $\Delta^{5,6}$-desaturase, which is also an essential component of the ergosterol biosynthetic pathway in *C. albicans* [46]. When Erg11p is inhibited by azoles, the non-toxic 14α-methylated sterol intermediate begins to accumulate in the cell and is subsequently converted by Erg3p to 14α-methylergosta-8,24(28)-dien-3β,6α-diol [21]. Inactivation of *ERG3* can block the accumulation of this toxic sterol intermediate and therefore leads to azole resistance, which has already been recorded in several azole-resistant clinical isolates of *C. albicans* [46-51].

1.3.1.4. Pdr16p

PDR16 encodes a phosphatidylinositol transfer protein in *C. albicans*. *PDR16* overexpression renders an azole-susceptible *C. albicans* strain less sensitive to azoles [52]. In an azole-resistant clinical isolate of *C. albicans* where *PDR16*, along with *CDR1* and *CDR2*, is constitutively upregulated, the deletion of *PDR16* reduces its resistance to azoles while introducing a copy of *PDR16* back into the *pdr16Δ/Δ* mutant resumes the azole resistance level [52].

1.3.1.5. Rta3p

RTA3, which encodes a putative lipid translocase, is also involved in azole resistance in *C. albicans*: the deletion of *RTA3* decreases the resistance of an azole-resistant *C. albicans* clinical isolate to fluconazole and *RTA3* overexpression in the azole-susceptible wild-type strain SC5314 renders it less susceptible to fluconazole [53].

1.3.2. Transcriptional regulation of azole resistance in *C. albicans*

The expression of the above azole resistance effectors is controlled by specific transcription factors. In this section, I will elaborate on the major transcriptional pathways involved in azole resistance in *C. albicans* and how they interact.

1.3.2.1. Zinc cluster transcription factors regulating azole resistance

Zinc cluster transcription factors are the class III zinc finger proteins containing a unique $Zn(II)_2 Cys_6$ motif in their DNA-binding domains and are fungal-specific proteins [54]. They typically bind as monomers, homodimers or heterodimers to CGG triplets that are positioned as direct, inverted or everted repeats in the target promoters [54-56]. Besides, zinc cluster transcription factors often positively regulate their own expression [54]. **Figure 1** shows the structure of a canonical zinc cluster transcription factor.
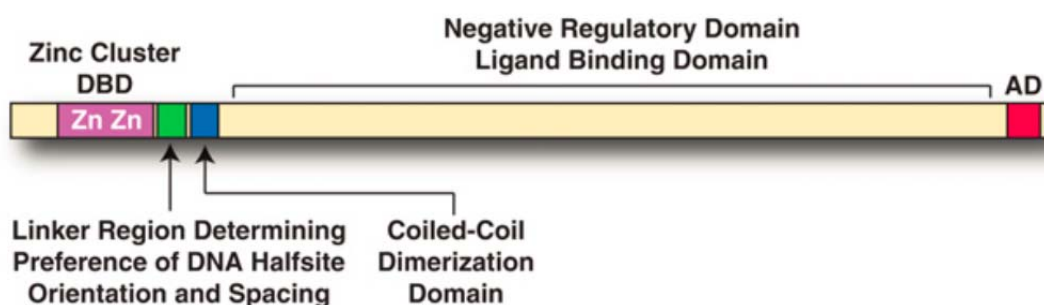
Figure 1. Structure of a typical zinc cluster transcription factor.
The zinc cluster DNA-binding domain (DBD) is commonly located at the N-terminus and the activation domain (AD) is at the C-terminus. This figure was taken from [57].

In *C. albicans*, four zinc cluster transcription factors have been shown to cause azole resistance when activated by gain-of-function (GOF) mutations that have been selected in response to azole treatment: Tac1p, Mrr1p, Upc2p and Mrr2p. **Figure 2** shows the known inducing stimuli and the representative target genes of these zinc cluster transcription factors

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as well as the cross-talk between different transcriptional pathways.

Although these zinc cluster transcription factors can be activated by specific inducing stimuli (transient activation) or GOF mutations (constitutive activation) and thereby upregulate their target genes, the exact underlying molecular mechanisms are still poorly understood. It is believed that inducing compounds and GOF mutations are both able to alter the conformation of zinc cluster transcription factors, thereby enabling them to actively recruit specific transcription co-activators and the transcription machinery to the target genes. This is supported by a previous discovery that Pdr1p orthologues, which are also zinc cluster transcription factors controlling the expression of many drug resistance genes in *S. cerevisiae* and *Candida glabrata*, are directly bound by specific xenobiotics, leading to the interaction of their C-terminal activation domains with the Gal11p/MED15 subunit of the Mediator complex, which helps to recruit the transcription machinery to the target promoters, and as a result, the target genes of Pdr1p orthologues get upregulated [58].
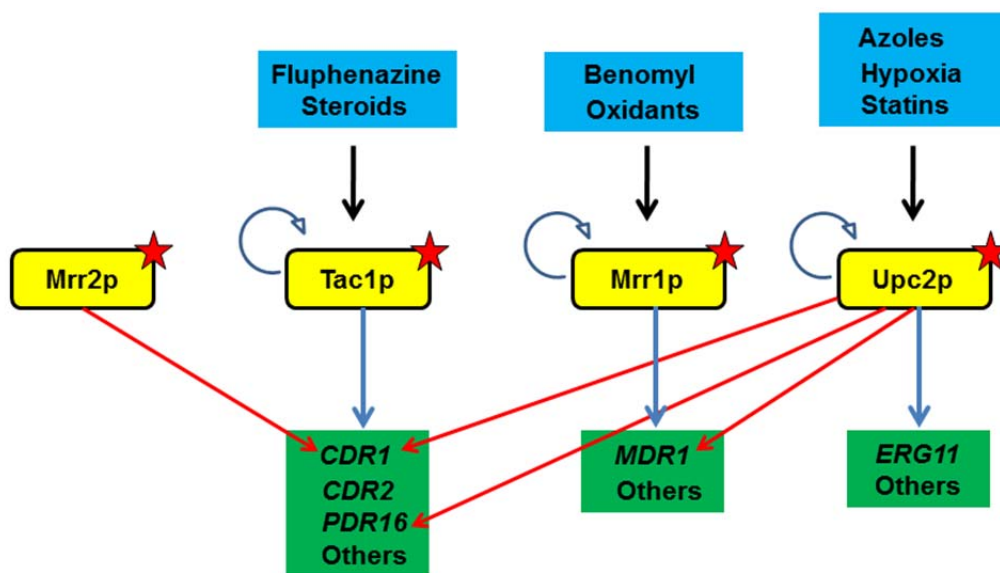
Figure 2. Major zinc cluster transcription factors mediating azole resistance in *C. albicans*.

Mrr2p, Tac1p, Mrr1p and Upc2p are the main identified zinc cluster transcription factors controlling azole resistance in *C. albicans*. In the green boxes are the major azole resistance effectors regulated by these zinc cluster transcription factors. In the blue boxes are some known inducing stimuli that can transiently activate these zinc cluster transcription factors, mimicking gain-of-function mutations in these factors, as denoted by the red stars. The circular arrows represent the known positive autoregulation of these factors. This figure is based on the information provided in [23] [59].

Tac1p plays a major role in fluphenazine-induced transient upregulation of *CDR1* and *CDR2* in *C. albicans* [25]. GOF mutations in *TAC1* lead to constitutive overexpression of *CDR1* and *CDR2* and many hyperactive *TAC1* alleles have been identified in a number of clinical isolates of *C. albicans* [7, 23, 25, 60]. As a typical zinc cluster transcription factor, Tac1p binds to the *cis*-acting drug-responsive element (DRE) located in the promoter regions of *CDR1* and *CDR2* which contains the CGG triplets [25]. Tac1p also binds to its own promoter and the binding is enriched in the region between the positions -1100 and -985 with

respect to the start codon, where multiple CGG triplets are present [61]. In addition, Tac1p binds to the promoters of *PDR16* and *RTA3* and regulates their expression [53, 62, 63].

Mrr1p is another important zinc cluster transcription factor contributing to azole resistance in *C. albicans*. When having a GOF mutation, Mrr1p significantly elevates the expression level of *MDR1*, resulting in fluconazole resistance; several GOF mutations in *MRR1* have been identified in fluconazole-resistant *C. albicans* strains [64, 65]. Besides, the binding of Mrr1p to the *MDR1* promoter has been confirmed by chromatin immunoprecipitation (ChIP) experiments, indicating that Mrr1p directly regulates *MDR1* [66].

The expression of *ERG11* is regulated largely by the zinc cluster transcription factor Upc2p, which also plays a part in the transcriptional regulation of the expression of many other genes required for ergosterol biosynthesis [67, 68]. Upc2p can also be constitutively activated by GOF mutations, leading to the overexpression of *ERG11* and the consequent azole resistance [60, 69-72]. The genome-wide location analysis of Upc2p has shown that Upc2p binds not only to the promoters of many ergosterol biosynthesis genes, but also to the *CDR1* and *MDR1* promoters [61]. Further experiments have showed that, under hypoxic or lovastatin-treatment conditions that activate Upc2p, Upc2p regulates the expression of *CDR1* and *MDR1* in a complex manner [61]. However, a GOF mutation in *UPC2* causes only a mild upregulation of *MDR1* and no detectable change in *CDR1* expression in an azole-resistant *C. albicans* clinical isolate [69].

Recently, a study on the artificial activation of the predicted zinc cluster transcription

factors in *C. albicans* has revealed that Mrr2p is a novel transcriptional regulator of *CDR1*, but not *CDR2*, and that the artificially activated Mrr2p is able to upregulate *CDR1* and give rise to fluconazole resistance independently of Tac1p [73]. Naturally occurring mutations in *MRR2* were identified in a series of fluconazole-resistant clinical isolates of *C. albicans* and some of them contribute to clinical azole resistance, as introducing the mutated *MRR2* allele into an azole-susceptible strain in which *MRR2* is deleted causes constitutive overexpression of *CDR1* as well as significantly decreased fluconazole susceptibility [74].

The zinc cluster transcription factor Fcr1p may also play a part in azole resistance in *C. albicans*. The deletion of *FCR1* in an azole-susceptible *C. albicans* strain decreases its sensitivity to several azole drugs, including fluconazole, ketoconazole and itraconazole, and the reintroduction of one copy of *FCR1* results in the phenotypic reversion, suggesting that Fcr1p negatively regulates azole resistance in *C. albicans* [75]. However, the contribution of Fcr1p to clinical azole resistance, if any, is still unknown.

1.3.2.2. Other transcription factors involved in the regulation of azole resistance

The bZIP transcription factor Cap1p is an important regulator of oxidative stress tolerance in *C. albicans* and it binds to the *MDR1* promoter [76-78]. A C-terminally truncated Cap1p, which is constitutively hyperactive, causes the constitutive overexpression of *MDR1* as well as increased fluconazole resistance partly independently of Mrr1p [66, 76]. Unlike the zinc

cluster transcription factors that are involved in azole resistance, no GOF mutations in *CAP1* have been documented to date in azole-resistant clinical isolates.

The transcription factor Ndt80p binds to the *CDR1* promoter and plays a positive role in miconazole-induced *CDR1* upregulation [79, 80]. The deletion of *NDT80* in a wild-type strain of *C. albicans* results in decreased azole tolerance [79]. Moreover, Ndt80p also regulates fluconazole-induced upregulation of *ERG11* and binds to the promoters of *CDR2*, *MDR1* and *ERG11*, suggesting that it may be involved in azole resistance in *C. albicans* [81]. However, Ndt80p is not essential for the fluconazole resistance mediated by hyperactive Tac1p, Mrr1p or Upc2p [82]. Although no hyperactive *NDT80* alleles have been discovered until now, it is possible that Ndt80p carrying a GOF mutation may upregulate the expression of some of the azole resistance effectors, thereby leading to azole resistance in *C. albicans*.

1.4. Rationale for this study

1.4.1. *TAC1* autoregulation

Our lab recently studied *TAC1* autoregulation in *C. albicans* and made some discoveries (Louhichi F, unpublished data). First, using luciferase reporter assays, our lab found that both the constitutive induction and the fluphenazine (50 μM) -induced transient activation of the *TAC1* promoter were abolished upon *TAC1* deletion, indicating that Tac1p regulates its own expression in a positive manner. Second, the binding of Tac1p to the *TAC1* promoter has been

demonstrated and mutating the 7 CGG triplets upstream in the *TAC1* promoter was shown to

abrogate this binding as well as *TAC1* upregulation in response to fluphenazine treatment or

mediated by a constitutively hyperactive Tac1p. The location and sequence of the Tac1p

binding sites in the *TAC1* promoter are shown in **Figure 3**. Third, it was shown that the

abrogation of *TAC1* autoregulation by mutating the CGG triplets of the *TAC1* promoter in an

azole-resistant strain harboring a GOF mutation in *TAC1* rendered this strain less resistant to

fluconazole, indicating that *TAC1* autoregulation contributes to fluconazole resistance. These

results clearly demonstrate that the *TAC1* promoter is a direct target of Tac1p.
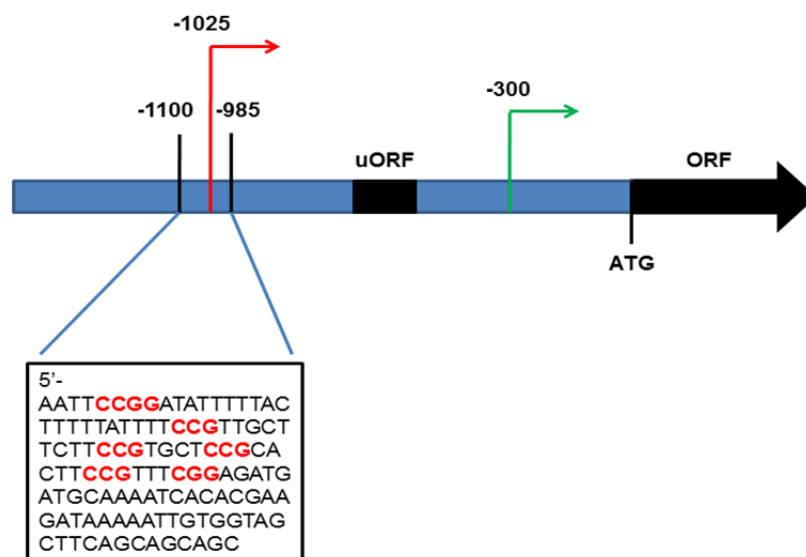
Figure 3. Schematic illustration of the structure of the *TAC1* gene.
The binding of Tac1p in the *TAC1* promoter is enriched in the region between the positions -1100 and -985 with respect to the ATG start codon. The sequence of this region is shown in the rectangle and the multiple CGG triplets (CCG for the complementary strand) are indicated in red letters. The black arrow denotes the open reading frame (ORF) of *TAC1* and the location of a putative upstream ORF (uORF) is also indicated. The red and green arrows indicate the locations of two known transcription start sites of *TAC1*.

1.4.2. Involvement of Mrr1p in fluphenazine-induced upregulation of *TAC1* and *CDR1* in strain SC5314

Over ten years ago, our lab found that two Tac1p target genes, *CDR1* and *PDR16*, could still be slightly induced by 100 μM of fluphenazine in the absence of Tac1p in strain SC5314 (**Figure 4**, lanes 3-6). This was surprising because Tac1p was believed to be the sole mediator of fluphenazine-induced transient *CDR1* upregulation in strain SC5314, as evidenced by the loss of *CDR1* induction by fluphenazine upon *TAC1* deletion [25]. However, in that article, the authors only used a relatively low concentration of fluphenazine (about 20 μM) to treat *C. albicans* cells for 20 min, which may explain why they did not discover the residual induction of *CDR1* by fluphenazine at higher concentrations in the absence of Tac1p. This finding by our lab suggested that another factor may be transiently activated by relatively high concentrations of fluphenazine, thereby causing the induction of *CDR1*, *PDR16* and potentially other Tac1p target genes independently of Tac1p. Using luciferase reporter assays, our lab found that the *TAC1* promoter could be induced by 150 μM of fluphenazine in the absence of Tac1p in strain SC5314, and that mutating all the 7 CGG triplets upstream in the *TAC1* promoter leads to the loss of *TAC1* induction by 150 μM of fluphenazine in the SC5314-derived *tac1Δ/Δ* mutant (**Figure 5**). These data suggested that this additional factor involved in the induction of several Tac1p target genes by fluphenazine is likely a zinc cluster transcription factor.
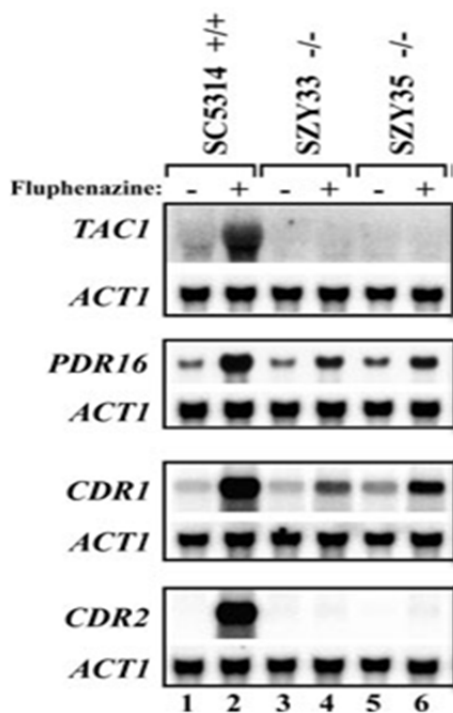
Figure 4. *CDR1* and *PDR16* can be residually induced by a relatively high concentration of fluphenazine in the absence of Tac1p in strain SC5314.

Strain SC5314 and two independent SC5314-derived *tac1Δ/Δ* mutants SZY33 and SZY35 were treated or not with 50 μg/ml (about 100 μM) of fluphenazine for 20 min. Total RNA extracts were then prepared and analyzed by Northern blotting with *TAC1*, *PDR16*, *CDR1* and *CDR2* probes, respectively. *ACT1* was used as a loading control. This figure was modified from [63].
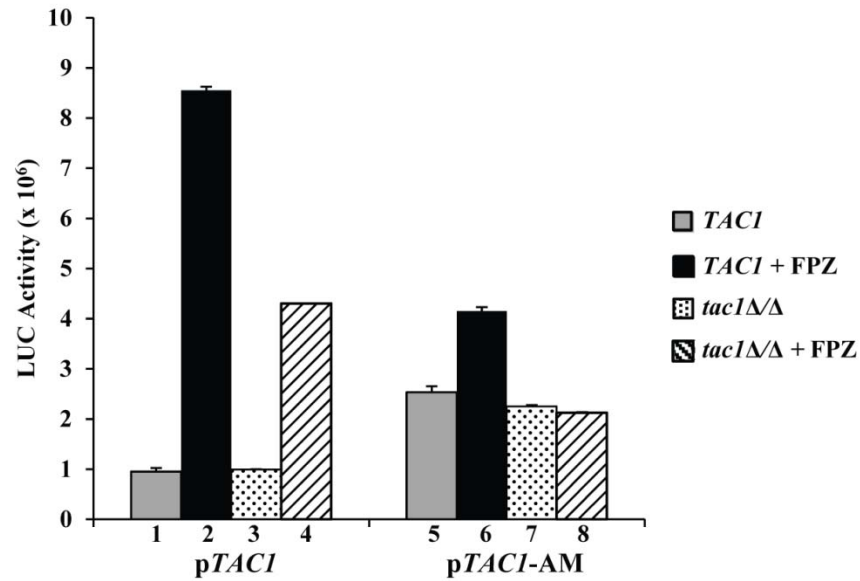
Figure 5. CGG triplets are important for the fluphenazine induction of the *TAC1* promoter in strain SC5314 and the SC5314-derived *tac1Δ/Δ* mutant.
Strain SC5314 and the SC5314-derived *tac1Δ/Δ* mutant carrying either the wild-type *TAC1* promoter-luciferase construct (p*TAC1*) or a mutated *TAC1* promoter-luciferase construct (p*TAC1*-AM with all the upstream 7 CGG triplets mutated) were treated or not with 150 μM of fluphenazine for 4 h. Protein extracts were prepared and luciferase assays were then conducted. The results represent the means ± standard deviations from three independent experiments, each performed in duplicate. These experiments and this figure were made by Louhichi F, a former PhD student in our lab (unpublished data).

Using ChIP-chip assays, our lab has found that Mrr1p binds to the promoters of *TAC1* and *CDR1* at the same location as Tac1p does, implying that Mrr1p may work together with Tac1p to regulate the expression of *TAC1* and *CDR1* [62, 66]. Our lab therefore sought to test whether Mrr1p is involved in the induction of *TAC1* and *CDR1* by fluphenazine in strain SC5314. Since relatively low and high concentrations of fluphenazine may transiently activate different transcriptional regulators, our lab therefore used 50 μM and 150 μM of fluphenazine to treat the cells before studying *TAC1* and *CDR1* induction by fluphenazine. Luciferase

reporter assays were used to study the activation of the *TAC1* and *CDR1* promoters. **Figure 6** shows the results previously obtained by our lab. First, in the absence of Tac1p, the *TAC1* promoter is hardly induced by 50 μM of fluphenazine (top panel, lane 7 and lane 9), but well induced by 150 μM of fluphenazine (top panel, lane 8 and lane 10) although the induction level is lower than that in strain SC5314. Second, upon *MRR1* deletion in strain SC5314, the induction of *TAC1* by 50 μM of fluphenazine is almost not affected (top panel, lane 17 and lane 19), but *TAC1* induction by 150 μM of fluphenazine is abolished (top panel, lane 18 and lane 20). As for *CDR1*, similar results were obtained (bottom panel) although they are less clear than the results for *TAC1*, as the induction levels of *CDR1* in response to fluphenazine treatment are lower than those for *TAC1*. Taken together, these data indicate that *MRR1* is involved in the transient induction of *TAC1* and *CDR1* by relatively high concentrations of fluphenazine. Moreover, they suggest that Tac1p may only be well activated by relatively low concentrations of fluphenazine and Mrr1p only by relatively high concentrations of fluphenazine.
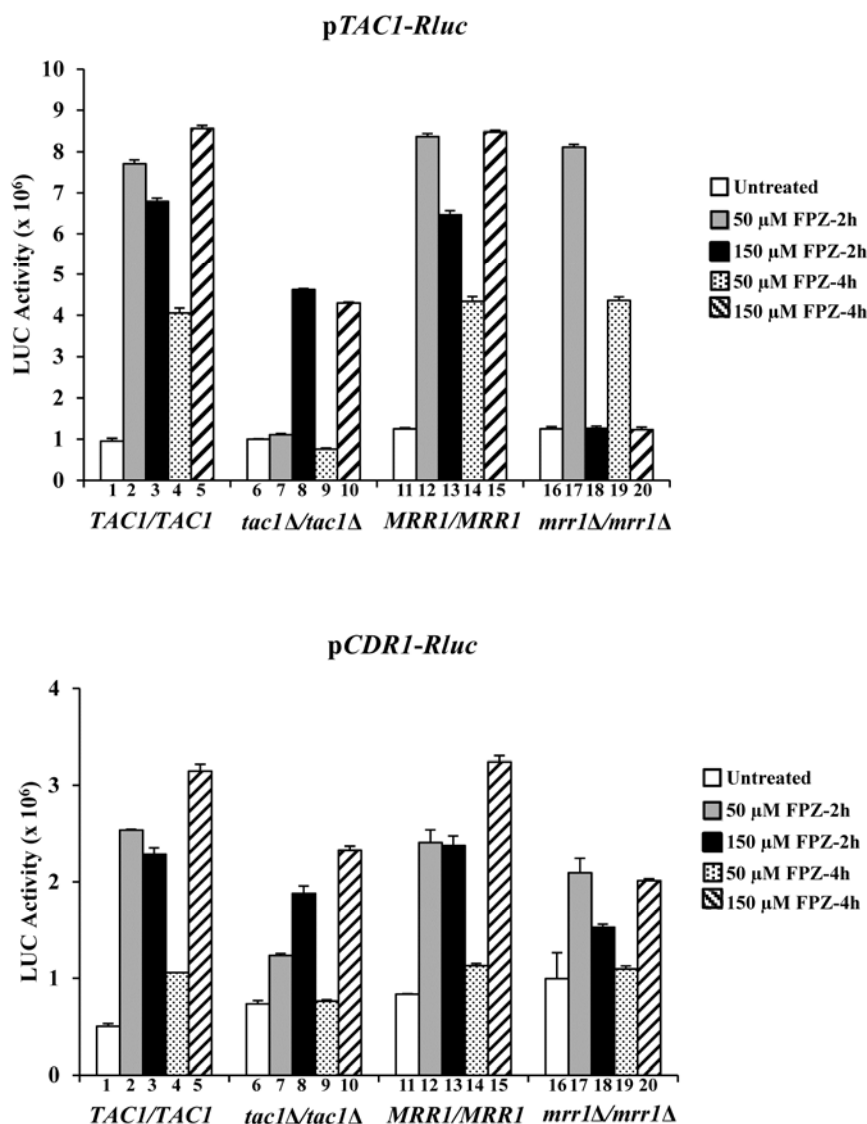
Figure 6. Activation of the *TAC1* and *CDR1* promoters by fluphenazine in strain SC5314 and the SC5314-derived *tac1Δ/Δ* and *mrr1Δ/Δ* mutants.

Strain SC5314 and the SC5314-derived *tac1Δ/Δ* and *mrr1Δ/Δ* mutants carrying either the *TAC1* promoter-luciferase construct or the *CDR1* promoter-luciferase construct were treated or not with either 50 μM or 150 μM of fluphenazine for 2 h and 4 h respectively. Protein extracts were prepared and luciferase assays were next performed. The results represent the means ± standard deviations from three independent experiments, each performed in duplicate. These experiments and this figure were made by Louhichi F, a former PhD student in our lab (unpublished data).

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1.5. Hypothesis and objectives of this study

First, in order to further verify the hypothesis that Mrr1p is positively involved in fluphenazine-induced transient upregulation of *TAC1* and *CDR1*, we specifically aimed to construct a SC5314-derived *tac1*Δ/Δ *mrr1*Δ/Δ mutant and compare it with the SC5314-derived *tac1*Δ/Δ mutant in terms of *TAC1* and *CDR1* induction levels in response to fluphenazine treatment.

As Tac1p and Mrr1p are both important transcription factors mediating azole resistance in *C. albicans*, it is therefore of great value to study the role of Mrr1p in Tac1p-mediated azole resistance. Based on the previous findings, I hypothesized that Mrr1p may positively regulate the azole resistance mediated by constitutively hyperactive Tac1p in clinical isolates of *C. albicans*. Thus my second objective was to investigate the involvement of Mrr1p in the constitutive overexpression of *TAC1* and *CDR1* and the consequent azole resistance caused by a GOF mutation in *TAC1*. The specific objective was to delete *MRR1* in a well-characterized azole-resistant clinical isolate of *C. albicans* harboring a GOF mutation (N972D) in *TAC1* and study the resulting mutant in terms of *TAC1* and *CDR1* mRNA levels and azole susceptibility.

2. Materials and methods

2.1. Yeast strains and growth media

The *C. albicans* strains used in this study are listed in **Table I**. All the strains were routinely grown in YPD media (1% yeast extract, 2% peptone, 2% dextrose) at 30°C with agitation at 250 rpm unless mentioned otherwise. To make solid YPD media, 2% agar was added to the liquid media.

Table I. *C. albicans* **strains used in this study**

Strain	Genotype/description	Parental strain	Reference
SC5314	Azole-susceptible wild-type strain		[83]
SZY35	*tac1*Δ::FRT/*tac1*Δ::FRT		[63]
SCMRR1M4A	*mrr1*Δ::FRT/*mrr1*Δ::FRT		[64]
JLY7	*tac1*Δ::FRT/*tac1*Δ::FRT	MRR1HOM1	This study
	*mrr1*Δ::FRT/*mrr1*Δ::FRT		
MRR1HET1	*tac1*Δ::FRT/*tac1*Δ::FRT	SZY35	This study
	MRR1/ *mrr1*Δ::*SAT1-FLIP*		
MRR1HET2	*tac1*Δ::FRT/*tac1*Δ::FRT	SZY35	This study
	MRR1/ *mrr1*Δ::*SAT1-FLIP*		

MRR1HET3	*tac1*Δ::FRT/*tac1*Δ::FRT	MRR1HET1	This study
	MRR1/ *mrr1*Δ::FRT		
MRR1HOM1	*tac1*Δ::FRT/*tac1*Δ::FRT	MRR1HET3	This study
	*mrr1*Δ::*SAT1-FLIP* / *mrr1*Δ::FRT		
SCTAC1LUC	*CDR4*/*cdr4*Δ::P$_{TAC1}$-RLUC	SC5314	Louhichi F, manuscript in preparation
SZY35TAC1LUC	*tac1*Δ::FRT/*tac1*Δ::FRT	SZY35	Louhichi F, manuscript in preparation
	CDR4/*cdr4*Δ::P$_{TAC1}$-RLUC		
MRR1TAC1LUC	*mrr1*Δ::FRT/*mrr1*Δ::FRT	SCMRR1M4A	Louhichi F, manuscript in preparation
	CDR4/*cdr4*Δ::P$_{TAC1}$-RLUC		
JLY7TAC1LUC	*tac1*Δ::FRT/*tac1*Δ::FRT	JLY7	This study
	*mrr1*Δ::FRT/*mrr1*Δ::FRT		
	CDR4/*cdr4*Δ::P$_{TAC1}$-RLUC		
5457	Azole-susceptible clinical isolate		[52]
5674	Azole-resistant clinical isolate	5457	[52]
JLY1	*MRR1*/ *mrr1*Δ::*SAT1-FLIP*	5674	This study
JLY2	*MRR1*/ *mrr1*Δ::*SAT1-FLIP*	5674	This study
JLY3	*MRR1*/ *mrr1*Δ::FRT	JLY1	This study
JLY4	*MRR1*/ *mrr1*Δ::FRT	JLY2	This study
JLY5	*mrr1*Δ::FRT/*mrr1*Δ::*SAT1-FLIP*	JLY3	This study

JLY6	*mrr1*Δ::FRT/*mrr1*Δ::*SAT1-FLIP*	JLY4	This study
SZY31	*tac1*Δ::FRT/*tac1*Δ::FRT		[63]
Gu4	Azole-susceptible clinical isolate		[33]
Gu5	Azole-resistant clinical isolate	Gu4	[33]

2.2. Plasmid construction and extraction

The plasmid containing the *SAT1* flipper cassette and the plasmid containing the *MRR1*

deletion cassette were kindly provided by Dr. Joachim Morschhäuser [64, 84]. The *MRR1*

deletion cassette of the latter plasmid contains the *SAP2* promoter upstream of the *FLP* gene;

the *SAT1* flipper cassette of the former plasmid was used to construct a new *MRR1* deletion

cassette and contains the *MAL2* promoter upstream of the *FLP* gene (see **Figure 7** and **Figure**

15). The luciferase reporter construct containing the *TAC1* promoter region was previously

prepared by our lab. Basically, the *TAC1* promoter region from -1500 to -1 with respect to the

ATG start codon was inserted into the plasmid pC4-Rluc-SAT1 which was also previously

constructed by our lab and integrates at the *CDR4* locus through homologous recombination.

The *Escherichia coli* host strains containing the above plasmids were routinely grown at 37°C

for 12 to 16 h in Luria-Bertani (LB) media supplemented with 100 μg/ml ampicillin (Sigma)

before plasmid extraction. The plasmids were isolated with the use of the QIAprep Spin

Miniprep Kit (Qiagen). The extracted plasmids containing the *MRR1* deletion cassette were

next digested with the restriction enzymes ApaI and SacI in order to release the cassette. Similarly, using the restriction enzymes KpnI and SacI, the *TAC1* promoter-containing luciferase reporter constructs were released from the plasmids.

2.3. *C. albicans* transformation and verification of transformants

The transformation of different *C. albicans* strains was carried out primarily following the lithium acetate (LiAc) method with minor modifications [85]. The overnight culture of *C. albicans* was diluted in 25 ml of YPD media to an OD_{600} of 0.05 and the diluted culture was incubated until its OD_{600} reached 0.3 to 0.4. The culture was then centrifuged at 3,000 rpm for 5 min and the cell pellets were resuspended in 6.25 ml of LiAc solution and centrifuged again at 3,000 rpm for 5 min. The cell pellets were resuspended in 125 μl of LiAc solution and 100 μl of the resuspended cell pellets were mixed with 5 μg of the purified *MRR1* deletion cassette or the *TAC1* promoter-containing luciferase reporter construct as well as 5 μl of pre-boiled salmon sperm DNA (20 μg/μl), in order to form the transformation mixture, which was incubated at 30°C for 30 min without shaking. 600 μl of PEG/LiAc solution was then added to the transformation mixture, which was then incubated overnight at 30°C on an overhead motor. On the next day, a 30 min heat shock at 44°C was applied to the cells. After that, the cells were resuspended in 2 ml of YPD media and incubated for 4 h with gentle agitation. Finally, the cells were harvested, resuspended in 100 μl of YPD media and spread on YPD agar plates

containing 200 µg/ml nourseothricin (WERNER BioAgents). The YPD plates were incubated at 30°C for 2 to 3 days before the transformants were obtained and analyzed by PCR.

Specifically, the integration of the *TAC1* promoter-containing luciferase reporter construct at the *CDR4* locus was verified using two pairs of primers: for the first pair, the forward primer (MR2683) is located upstream from the ORF of *CDR4* and the reverse primer (MR2005) in the luciferase reporter construct, and the expected fragment size is 5792 bp; for the second pair, the forward primer (MR1392) is located in the luciferase reporter construct and the reverse primer (MR2684) downstream from the ORF of *CDR4*, and the expected fragment size is 3726 bp.

2.4. Luciferase assays

The luciferase assay protocol was previously established by our lab and some adjustments were made for this study. Overnight cultures were diluted in 10 ml of YPD media to an OD_{600} of 0.2 and the diluted cultures were incubated until their OD_{600} reached 0.7. Next the cells were treated or not with a certain concentration of fluphenazine (Sigma) for either 2 or 4 h at 30°C with shaking. The cells were harvested at 4°C, washed with 1 ml of cold sterile water and then washed with 1 ml of cold luciferase buffer (0.5 M NaCl; 0.1 M K_2HPO_4, pH 6.7; 1 mM EDTA, pH 8.0; 1 mM PMSF, freshly added to the buffer and 0.6 mM sodium azide). The washed cell pellets were resuspended in 200 µl of cold luciferase buffer and transferred to a

screw-cap tube. 200 μl of acid-washed glass beads (Sigma) were added to the tube and the cells were disrupted at 4°C for 2 to 3 min with the use of a minibead beater (BioSpec Products). The tube was then centrifuged at 4°C and 13,000 rpm for 20 min and the supernatant was taken out. The protein extracts were quantified using the Bradford assays and the quality was tested by SDS-PAGE followed by Coomassie staining. 15 μg of each protein extract was added in duplicate to a 96-well microtiter plate and 150 μl of luciferase buffer containing 0.5 μM of the Renilla luciferase substrate coelenterazine H (Sigma) was then added to each well. After 5 to 10 min, the plate was read using the Synergy Neo microplate reader (BioTek Instruments).

2.5. Total RNA extraction

Overnight cultures were diluted in 50 ml of YPD media to an OD_{600} of 0.1 and the diluted cultures were incubated until their OD_{600} reached 1.0. The cells were harvested and washed with sterile water and the total RNA was prepared using the hot phenol method [86]. For fluphenazine treatment, when the OD_{600} of the diluted cultures reached 1.0, 10 ml of each culture was incubated with a certain concentration of fluphenazine for a certain period of time before the harvesting and washing of the cells for total RNA extraction. The concentration of the isolated total RNA was measured by spectrophotometry.

2.6. Northern blot analysis

Northern blotting was performed as described previously [52]. 25 μg of each RNA sample was tested. The *CDR1* and *TAC1* probes for Northern blotting were prepared as previously published [52, 63].

2.7. RT-PCR and RT-qPCR

2 μg of the total RNA was firstly treated with the TURBO DNase (Thermo Fisher Scientific) to eliminate DNA contamination. Next, first-strand cDNA for each RNA sample was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For the RT-PCR experiments, specific primers were used for the amplification of the obtained cDNA. The SYBR® Green method was used for the RT- qPCR experiments as previously described by our lab [61]. In this study, a 10 μl reaction mixture was employed, containing the Fast SYBR® Green Master Mix (Applied Biosystems), first-strand cDNA and the specific forward and reverse primers. The PCR program was set as follows: 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing & extension at 60°C for 20 sec. All the RT-qPCR experiments were conducted using the ABI PRISM 7900HT (Applied Biosystems). At the end of each test, a dissociation curve was generated to exclude the possibility of unspecific amplifications. The 18S rRNA gene was used as the endogenous control for normalization. The relative quantification of the target genes was performed by

using the most common comparative C_T method ($2^{-\Delta\Delta CT}$ method) [87]. All the primers used are

listed in **Table II**.

Table II. Primers used in this study

Primer	Sequence (5'-3')
Verification of *MRR1* deletion	
MR2700	GGGTTGTCAAATTTCCTGTCGG
MR2699	CCCCAATACACCGTGAAATAGG
Verification of the integration of the luciferase reporter construct at the *CDR4* locus	
MR2683	CATTACCCATTCACAACGTGCTTC
MR2005	TCTCATATGAAAATTTCGGTGATCCCTGAG
MR1392	CGTTTGTGTCTCTAATCGTATGC
MR2684	GTTGGGGATCTGATTTGACCG
Construction of the *MRR1* deletion cassette	
MR2780	TCAGATATCGAATTATTAATCTAATTTATTAAAAATGTCAAT TGCCACCACCCCTATAGAGAGCTCCACCGCGGTGGCGGC CGCT

MR2781	TACATCTATACATATAGAACATATAATTAACATAAGAGCTG
	CCAATTCACCAGAATCAATGGTACCGGGCCCCCCCTCGA
	GGAA
MR2782	TATTCCAAAAATTGTTTTTGTTTATGCCCTTCTTTTTTTTTC
	TTGACGGATAAATCAGTATCAGATATCGAATTATTAAT
MR2783	GCGCAATTTCTTAAATTGAAAAGAATGAAAATGGAAAAA
	ACCGTTAAACGATATACTACATACATCTATACATATAGAAC

RT-PCR

| MR2869 | TTTGATTGTGACAATAGTGT |
| MR2870 | CAATGGACTAAAACCAGAGG |

RT-qPCR

*CDR1*_Forward	ATTCTAAGATGTCGTCGCAAGATG
*CDR1*_Reverse	AGTTCTGGCTAAATTCTGAATGTTTTC
*TAC1*_Forward	TGGCAATGTATTTAGCAGATGAGG
*TAC1*_Reverse	TGCTTGAACTGAGGTGAATTTTG
*TAC1*_long_Forward	CATGTGTGATTTATCCAGTCCAAGT
*TAC1*_long_Reverse	GCTAAGAGAAGGTAGAACCGTCAT
18S rRNA_Forward	CACGACGGAGTTTCACAAGA
18S rRNA_Reverse	CGATGGAAGTTTGAGGCAAT

2.8. Fluconazole susceptibility tests

Liquid microtiter plate assays were performed as previously published [63]. Fluconazole (Sigma) was dissolved in water for the preparation of stock solutions (5 mg/ml). In this study, the fluconazole concentrations tested were 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.20 μg/ml in **Figure 17** and 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.1, 0.05, 0.025 μg/ml in **Figure 21**. Cell growth was measured by spectrophotometry at OD_{620} after incubation at 30°C for 48 h.

2.9. Genomic DNA isolation

C. albicans strains were grown overnight in 10 ml of YPD media with shaking. The cells were harvested at room temperature and washed with sterile water. Genomic DNA was extracted using the glass beads method as described previously [88]. During the isolation process, RNase A was used to eliminate RNA contamination in the genomic DNA extracts. The amount of the extracted genomic DNA was measured by spectrophotometry.

3. Results

3.1. Determination of the role of Mrr1p in fluphenazine-induced transient upregulation of *TAC1* and *CDR1*

3.1.1. Construction of the SC5314-derived *tac1*Δ/Δ *mrr1*Δ/Δ mutant

As explained in the Introduction section, our lab recently studied the change in the induction levels of *TAC1* and *CDR1* upon the deletion of either *TAC1* or *MRR1* in strain SC5314. In order to further verify the role of Mrr1p in the transient induction of *TAC1* and *CDR1* by fluphenazine, we sought to study how the residual induction levels of *TAC1* and *CDR1* would change in the absence of both Tac1p and Mrr1p in strain SC5314 (i.e. whether the residual induction in the SC5314-derived *tac1*Δ/Δ mutant would disappear upon *MRR1* deletion). To construct the SC5314-derived *tac1*Δ/Δ *mrr1*Δ/Δ mutant, I deleted *MRR1* in the SC5314-derived *tac1*Δ/Δ mutant SZY35. The *SAT1*-flipping strategy was used for *MRR1* deletion as it allows the recycling of the selection marker [84]. The structure of the *MRR1* deletion cassette is shown in **Figure 7**, and **Figure 8** shows how the *SAT1*-flipping strategy should work. Basically, the SC5314-derived *tac1*Δ/Δ mutant was transformed with the *MRR1* deletion cassette, which then replaced one *MRR1* allele through homologous recombination. Next the *SAT1* flipper cassette was excised by its FLP-FRT system to recycle the selection marker [34]. With the use of this method, the other *MRR1* allele was also deleted. In this way, both *MRR1* alleles in the SC5314-derived *tac1*Δ/Δ mutant were replaced by the FRT sequence.

To verify the deletion of *MRR1*, the genomic DNA was prepared from the transformants and analyzed by PCR using the primers flanking the coding sequence of *MRR1*. **Figure 9A** shows the PCR result for some critical intermediate *mrr1Δ* mutants: two heterozygous mutants in which the first *MRR1* allele was replaced by the *SAT1* flipper cassette (lane 2 and lane 3) and one homozygous mutant in which the second *MRR1* allele was also replaced (lane 4). Finally, six *tac1Δ/Δ mrr1Δ/Δ* mutants were obtained (**Figure 9B**, lanes 2-7), one of which was named JLY7 (**Figure 9B**, lane 4) and used for subsequent experiments.
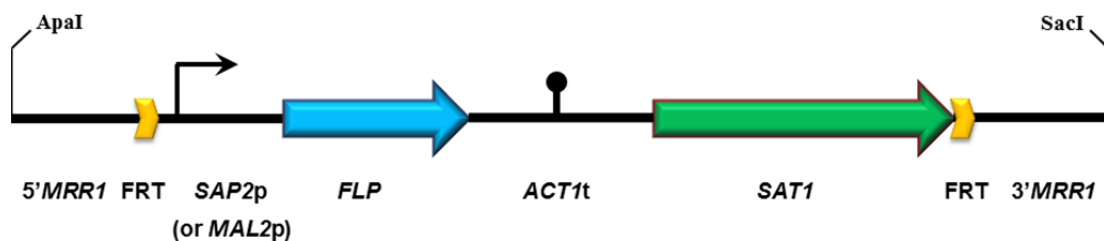
Figure 7. Structure of the *MRR1* deletion cassette.
The *MRR1* deletion cassette contains the upstream and downstream homology regions of *MRR1* (5'*MRR1* and 3'*MRR1*) and the complete *SAT1* flipper cassette. The *SAT1* flipper cassette is mainly composed of the selection marker *SAT1* conferring resistance to nourseothricin and the *FLP* gene encoding a site-specific recombinase, which can recognize the FRT sequence at both ends of the cassette and thereby excise the cassette. *SAP2*p and *MAL2*p denote the *SAP2* promoter and the *MAL2* promoter, respectively, which are used to control the expression of *FLP*. *ACT1*t denotes the terminator of *ACT1*. The locations of the restriction enzymes ApaI and SacI that are used to release the deletion cassette from the plasmid are also indicated.
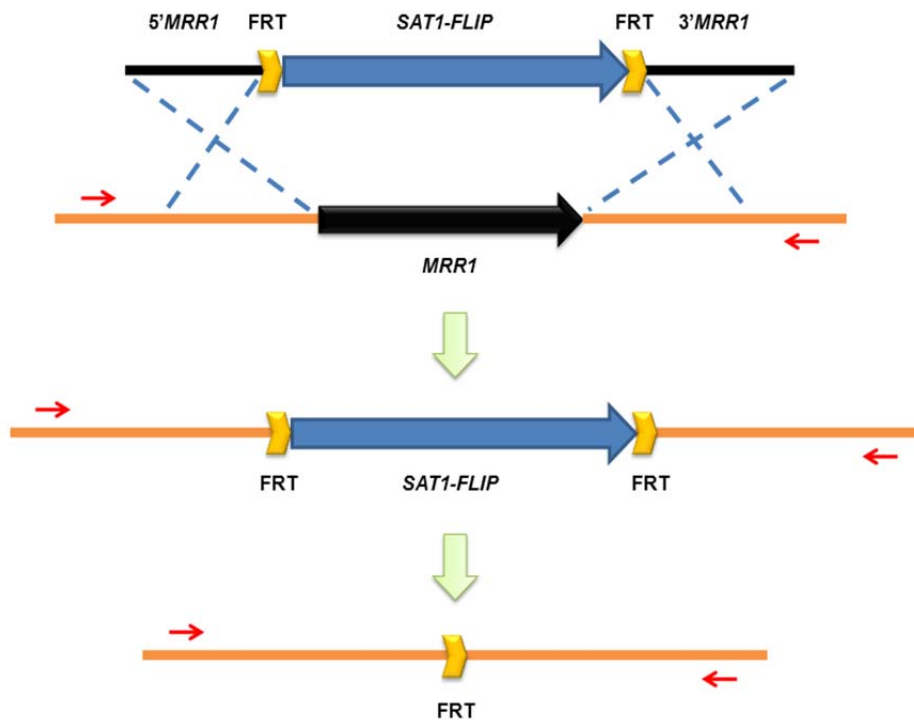
Figure 8. Schematic illustration of the deletion of *MRR1* using the *SAT1*-flipping strategy. *SAT1-FLIP* denotes the *SAT1* flipper cassette. Red arrows indicate the locations and directions of the primers MR2700 (forward primer) and MR2699 (reverse primer) used to analyze the transformants by PCR. The amplicon size for the intact *MRR1* allele is 4039 bp. For the FRT sequence, the amplicon size is 852 bp. The amplicon size for the *SAT1* flipper cassette is either 5547 bp or 4955 bp, depending on the *MRR1* deletion cassette used (see Figures 9, 15 and 16).
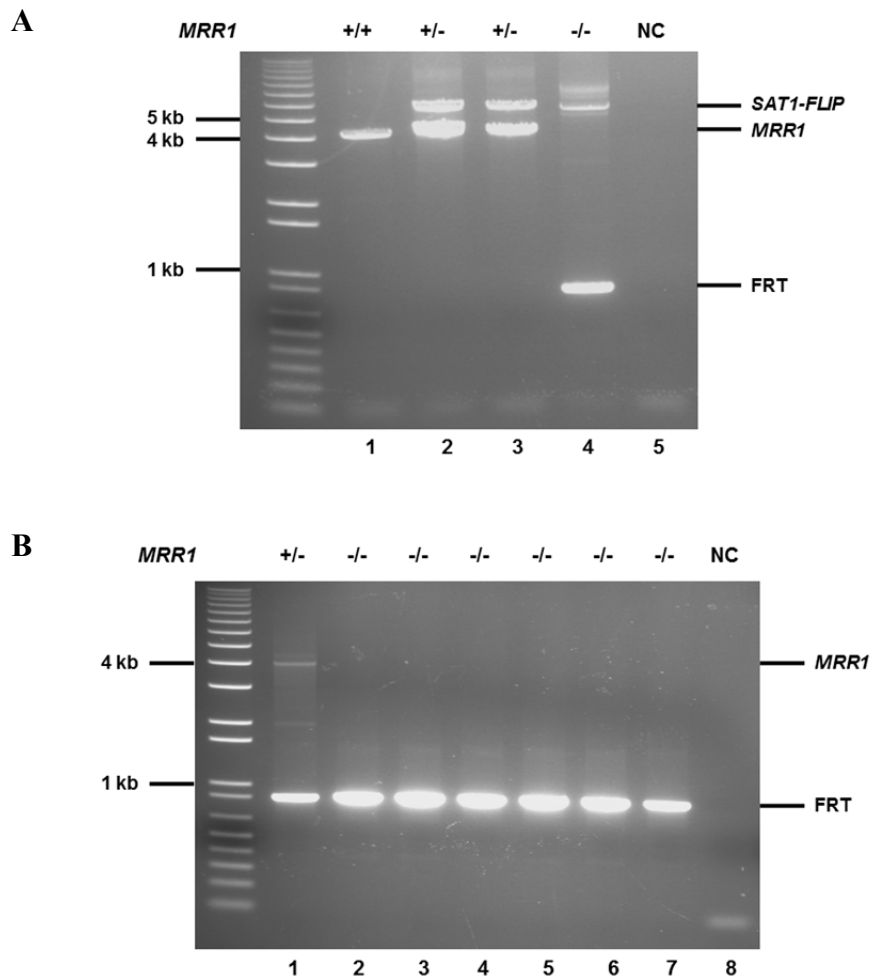
Figure 9. PCR verification of the deletion of *MRR1* in the SC5314-derived *tac1*Δ/Δ mutant.

Genomic DNA of the strains was used as PCR templates and the primers MR2700 and MR2699 were used for DNA amplification. The expected fragment sizes for *SAT1-FLIP*, *MRR1* and FRT are 5547 bp, 4039 bp and 852 bp, respectively. NC denotes the negative control, for which water was used as the PCR template.

(A) Two heterozygous *mrr1*Δ mutants MRR1HET1 (lane 2) and MRR1HET2 (lane 3) were first obtained. A homozygous *mrr1*Δ mutant MRR1HOM1 (lane 4) was next constructed from strain MRR1HET1. Strain SC5314 (lane 1) was used as a positive control.

(B) The *SAT1* flipper cassette was excised from the homozygous *mrr1*Δ mutant MRR1HOM1 and six *tac1*Δ/Δ *mrr1*Δ/Δ mutants (lanes 2-7) were finally obtained, in which both *MRR1* alleles had been replaced by the FRT sequence. One of these mutants was named JLY7 (lane 4) and tested subsequently. A heterozygous *mrr1*Δ mutant MRR1HET3 (lane 1) was used as a positive control.

3.1.2. Measurement of the *CDR1* mRNA levels in the *tac1Δ/Δ mrr1Δ/Δ* mutant upon fluphenazine treatment

To determine the optimal conditions of fluphenazine treatment for the residual induction of *CDR1* in the SC5314-derived *tac1Δ/Δ* mutant, a time-course study was carried out using 100 μM and 150 μM of fluphenazine based on the previous studies by our lab. Northern blot analysis was performed using the *CDR1* probe. For the 100 μM fluphenazine group, 20 min treatment leads to the highest expression level of *CDR1*; as for the 150 μM fluphenazine group, *CDR1* is the best expressed after treatment for 5 or 10 min (**Figure 10**). Although the untreated control was not tested and the residual induction of *CDR1* was therefore not shown in the experiments, these fluphenazine conditions were still selected to study the induction of *CDR1* in the *tac1Δ/Δ mrr1Δ/Δ* mutant and other relevant strains in consideration of the highest expression levels of *CDR1* under these conditions. Northern blotting experiments were performed to determine the *CDR1* mRNA levels under these selected fluphenazine conditions. As can be seen in **Figure 11**, under both fluphenazine conditions, no residual induction of *CDR1* could be clearly observed in the SC5314-derived *tac1Δ/Δ* mutant (or in the *tac1Δ/Δ mrr1Δ/Δ* mutant) although the fluphenazine treatment had functioned properly as judged by the induction of *CDR1* in strain SC5314. This result seems to be inconsistent with our previous finding that *CDR1* can still be induced by 100 μM of fluphenazine in the absence of Tac1p in strain SC5314 [63]. Since the residual induction of *CDR1* by relatively high concentrations of fluphenazine was not observed in the SC5314-derived *tac1Δ/Δ* mutant or

tac1Δ/Δ mrr1Δ/Δ mutant, it was therefore impossible to evaluate the role of Mrr1p in fluphenazine-induced *CDR1* upregulation in the SC5314-derived *tac1Δ/Δ* mutant. To address the question by using another approach, a luciferase reporter assay was subsequently used.
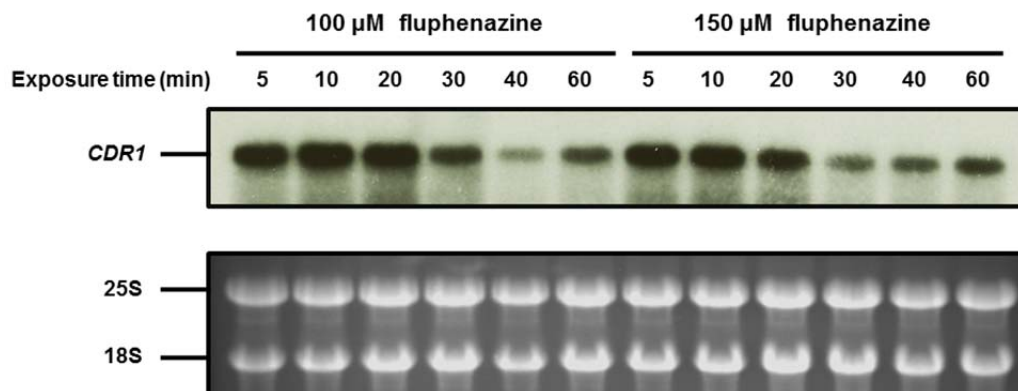
Figure 10. Determination of the optimum fluphenazine conditions for the induction of *CDR1* in the SC5314-derived *tac1Δ/Δ* mutant.
The SC5314-derived *tac1Δ/Δ* mutant SZY35 was exposed to either 100 μM or 150 μM of fluphenazine for the period of time shown at the top and total RNA extracts were then prepared and analyzed by Northern blotting with the *CDR1* probe. The 18S and 25S rRNAs were used as loading controls.
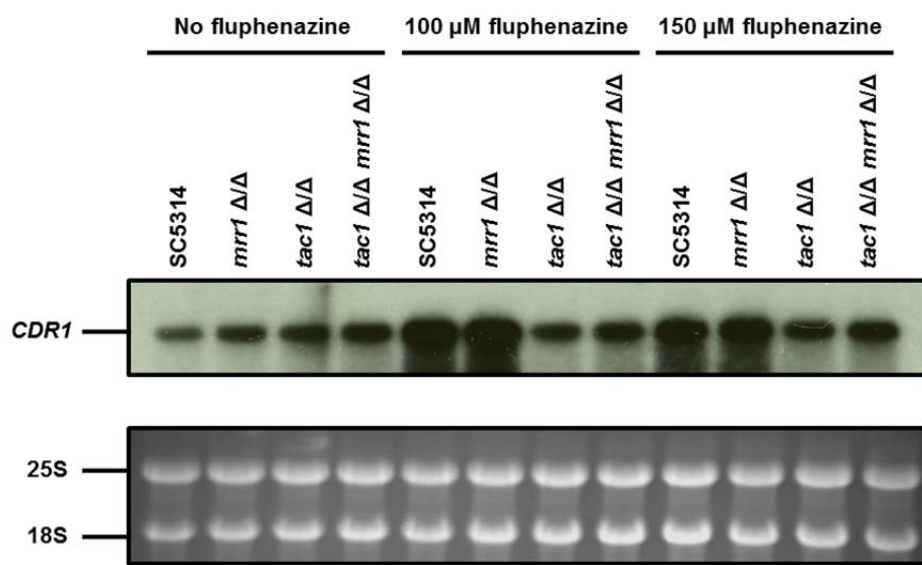
Figure 11. Northern blot analysis of the *CDR1* expression in strain SC5314 and the SC5314-derived mutants.
Strains SC5314, SCMRR1M4A, SZY35 and JLY7 were treated or not with either 100 μM of fluphenazine for 20 min or 150 μM of fluphenazine for 10 min. Total RNA extracts were then prepared and analyzed by Northern blotting. The 18S and 25S rRNAs were used as loading controls.

3.1.3. Comparison of the *TAC1* promoter activities in the *tac1Δ/Δ mrr1Δ/Δ* mutant and the related strains upon fluphenazine treatment

As explained in the Introduction section, our previous luciferase assay results (**Figure 6**) clearly show that the induction of *TAC1* by fluphenazine is more significant than that of *CDR1* in strain SC5314, the *tac1Δ/Δ* mutant and the *mrr1Δ/Δ* mutant. Also, the residual induction of *CDR1* by fluphenazine in the *tac1Δ/Δ* mutant was not clearly shown through our most recent Northern blotting experiments, complicating our study of *CDR1* induction. Therefore, we decided to first study the induction of *TAC1* by fluphenazine in the *tac1Δ/Δ mrr1Δ/Δ* mutant.

To this end, a luciferase reporter construct that contains the *TAC1* promoter region was introduced into the genome of the mutant, and the integration of the luciferase reporter construct was verified by PCR (see Materials and Methods section for further information). The luciferase activities therefore represent the activation levels of the *TAC1* promoter.

The *TAC1* promoter activity in the *tac1Δ/Δ mrr1Δ/Δ* mutant was tested upon fluphenazine treatment. In order to compare with other related stains carrying the *TAC1* promoter-luciferase construct and reproduce our previous results, I used the same fluphenazine conditions as our lab had previously used (**Figure 6**). The preliminary data from one experiment performed in duplicate are shown in **Figure 12**. First, the *TAC1* promoter appears to be induced by 50 μM of fluphenazine in the SC5314-derived *tac1Δ/Δ* mutant, but the seeming induction is not significant and needs to be further confirmed. Second, the *TAC1* promoter activity is higher in the SC5314-derived *tac1Δ/Δ mrr1Δ/Δ* mutant than in the SC5314-derived *tac1Δ/Δ* mutant under both fluphenazine conditions. Third, the deletion of *MRR1* in strain SC5314 does not seem to impair the transient upregulation of *TAC1* by 150 μM of fluphenazine. Although it appears that some of these preliminary results are not in line with our previous results (see **Figure 6**), the residual induction of *TAC1* by 150 μM of fluphenazine in the *tac1Δ/Δ* mutant has been confirmed. Moreover, since it was not clear whether Mrr1p is positively involved in the residual induction of *TAC1* by fluphenazine, further experiments were therefore to be performed subsequently.
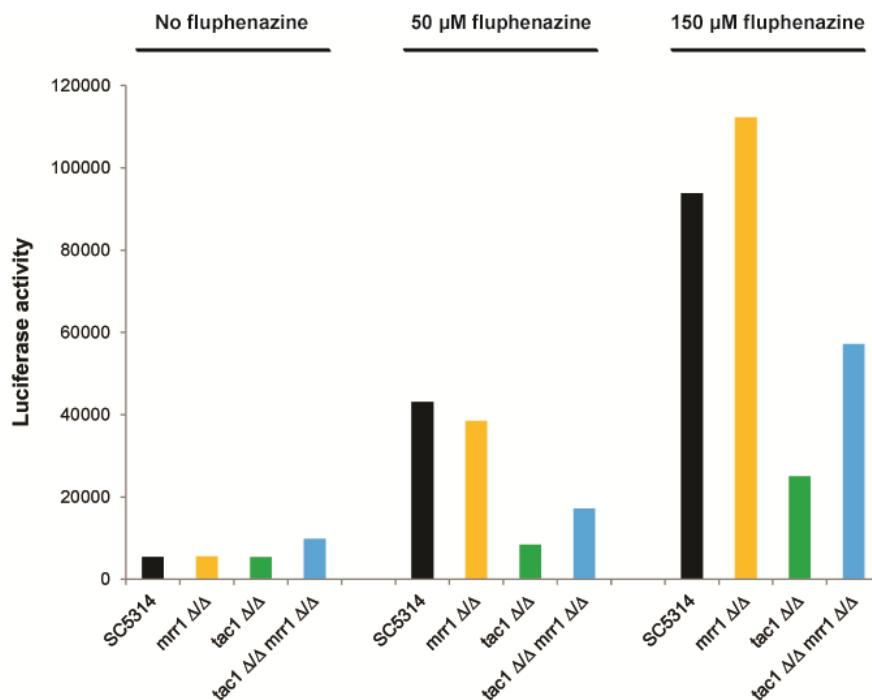
Figure 12. Activation of the *TAC1* promoter by fluphenazine in different strains.
Strains SCTAC1LUC, SZY35TAC1LUC, MRR1TAC1LUC and JLY7TAC1LUC were treated or not with either 50 μM of fluphenazine for 2 h or 150 μM of fluphenazine for 4 h. Protein extracts were prepared and luciferase assays were next performed. The luciferase activity values represent the means from one experiment performed in duplicate.

Although the above data are very preliminary, the difference between my result and our previous result was still surprising. One possibility was the variation within the fluphenazine quality or purity. Hence, many different concentrations of fluphenazine were used to treat the strains and study the induction of *TAC1*. A range of fluphenazine concentrations lower than 150 μM were first tested and the preliminary data from one experiment performed in duplicate are shown in **Figure 13**. For the SC5314-derived *tac1*Δ/Δ mutant, the *TAC1* promoter is residually activated only by relatively high concentrations (≥100 μM) of fluphenazine, which is in accord with our previous findings; for the SC5314-derived *mrr1*Δ/Δ mutant, the *TAC1*

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promoter is activated by different concentrations of fluphenazine and the activation level increases steadily as the fluphenazine concentration is increased, but it should be noted that for the 2 h treatment group, the activation level seems to begin to decrease at 150 μM of fluphenazine, suggesting that higher concentrations of fluphenazine may impair the optimal activation of the *TAC1* promoter. Besides, for the 4 h treatment group, the *TAC1* promoter in the *mrr1Δ/Δ* mutant seems to be less induced by ≤125 μM of fluphenazine than for the 2 h treatment group, suggesting that prolonged fluphenazine treatment may not be beneficial to the induction of *TAC1*, at least in the *mrr1Δ/Δ* mutant. Therefore, 2 h treatment with relatively high concentrations of fluphenazine was used in subsequent experiments.

Strain SC5314, the SC5314-derived *tac1Δ/Δ* mutant, *mrr1Δ/Δ* mutant and *tac1Δ/Δ mrr1Δ/Δ* mutant carrying the *TAC1* promoter-luciferase construct were simultaneously studied and the fluphenazine concentrations used range from 100 μM to 250 μM. **Figure 14** shows that for the SC5314-derived *tac1Δ/Δ* mutant, the residual activation of the *TAC1* promoter appears to be observed only at ≤175 μM of fluphenazine; for the SC5314-derived *mrr1Δ/Δ* mutant, the *TAC1* promoter is still induced by all these concentrations of fluphenazine although the induction levels vary; in the *tac1Δ/Δ mrr1Δ/Δ* mutant, the *TAC1* promoter activity is always higher than that in the SC5314-derived *tac1Δ/Δ* mutant, which further raises the possibility that Mrr1p may suppress fluphenazine-induced upregulation of *TAC1* in the SC5314-derived *tac1Δ/Δ* mutant. Interestingly, all the four strains exhibit a very similar trend in the activation of the *TAC1* promoter, suggesting that the induction of *TAC1* by relatively

high concentrations of fluphenazine may be at least partially independent of Mrr1p and Tac1p.

These data, although obtained in a single experiment performed in duplicate, also suggest that

other factor(s) may also mediate fluphenazine-induced transient activation of *TAC1*.
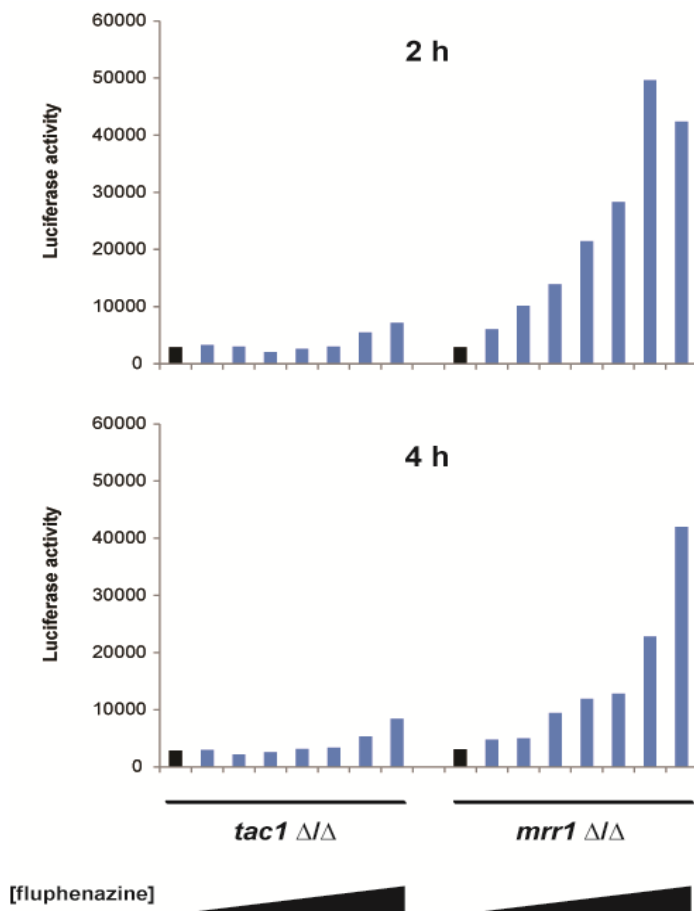
Figure 13. Activation of the *TAC1* promoter by fluphenazine in the SC5314-derived *tac1*Δ/Δ and *mrr1*Δ/Δ mutants.
Strains SZY35TAC1LUC and MRR1TAC1LUC were treated or not with relatively low concentrations of fluphenazine for either 2 h (top) or 4 h (bottom); the concentrations used were 12.5, 25, 50, 75, 100, 125 and 150 μM. Protein extracts were prepared and luciferase assays were conducted. The luciferase activity values represent the means from a single experiment performed in duplicate. The black column for each group represents the no-fluphenazine control.

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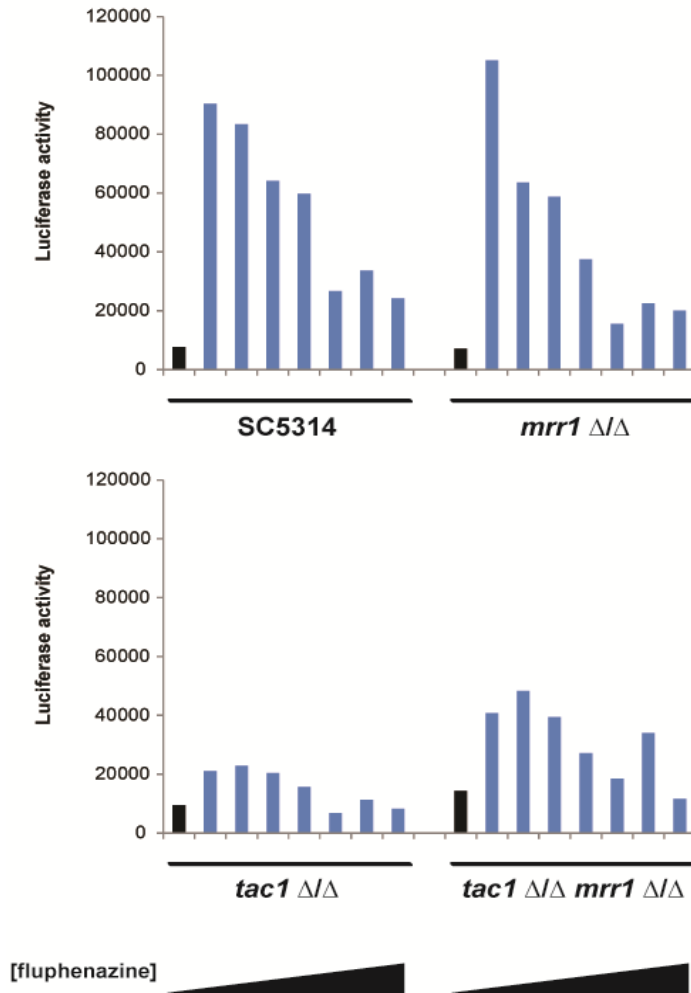
Figure 14. Activation of the *TAC1* promoter by fluphenazine in strain SC5314 and the SC5314-derived *tac1*Δ/Δ, *mrr1*Δ/Δ and *tac1*Δ/Δ *mrr1*Δ/Δ mutants.

Strains SCTAC1LUC, SZY35TAC1LUC, MRR1TAC1LUC and JLY7TAC1LUC were exposed or not to relatively high concentrations of fluphenazine for 2 h; the concentrations tested were 100, 125, 150, 175, 200, 225 and 250 µM. Protein extracts were prepared and luciferase assays were performed. The luciferase activity values represent the means from one experiment performed in duplicate. For each group, the black column represents the no-fluphenazine control.

3.2. Mrr1p plays a role in azole resistance but is not involved in the constitutive upregulation of *TAC1* and *CDR1* in isolate 5674

As demonstrated in the Introduction section, our previous luciferase assay results indicated that Mrr1p plays a positive role in the transient induction of *TAC1* and *CDR1* by fluphenazine, although my preliminary data do not support this finding. Since Mrr1p binds to the promoters of *TAC1* and *CDR1* at the same location as Tac1p does and the exact molecular mechanism by which fluphenazine transiently induces *TAC1* and *CDR1* expression is unknown, it was therefore interesting to study whether Mrr1p is involved in the constitutive overexpression of *TAC1* and *CDR1* as well as the resulting azole resistance mediated by constitutively hyperactive Tac1p.

Previously, our lab identified a GOF mutation (N972D) in *TAC1* in an azole-resistant *C. albicans* clinical isolate 5674, where Tac1p acts as the pivotal regulator of azole resistance and the deletion of *TAC1* abolishes the constitutive upregulation of several important Tac1p target genes, including *CDR1*, *CDR2*, *TAC1*, *PDR16* and *RTA3*, thereby abrogating azole resistance [52, 53, 63]. Besides, there is no mutation in *MRR1* in isolate 5674, as indicated by whole genome sequencing of isolate 5674 (unpublished data). Since the clinical isolate 5674 had been well characterized, we therefore used this strain to study whether Mrr1p plays a part in Tac1p-mediated azole resistance.

3.2.1. Deletion of *MRR1* in isolate 5674 by homologous recombination

To investigate whether Mrr1p contributes to Tac1p-mediated azole resistance, we sought to delete *MRR1* in isolate 5674. This time, instead of directly using the *MRR1* deletion cassette from Dr. Joachim Morschhäuser, a PCR-based strategy to construct a new *MRR1* deletion cassette was employed (**Figure 15**). Basically, the 120 bp upstream and downstream homology regions of *MRR1* were added to the original *SAT1* flipper cassette through two rounds of PCR. Again, the *SAT1*-flipping strategy was used for *MRR1* deletion in isolate 5674 (see **Figure 8**). Using this new cassette, the first *MRR1* allele was deleted and two independent heterozygous *mrr1Δ* mutants JLY1 and JLY2 were obtained (**Figure 16**, lane 2 and lane 5). After the excision of the *SAT1* flipper cassette, two independent heterozygous *mrr1Δ* mutants JLY3 and JLY4, in which one *MRR1* allele had been replaced by the FRT sequence, were obtained (**Figure 16**, lane 3 and lane 6). The new *MRR1* deletion cassette was then used to delete the second *MRR1* allele. However, after several attempts, no homozygous *mrr1Δ* mutant was obtained. Subsequently, using the previous *MRR1* deletion cassette provided by Dr. Joachim Morschhäuser, two independent *mrr1Δ/Δ* mutants JLY5 and JLY6 were obtained (**Figure 16**, lane 4 and lane 7).
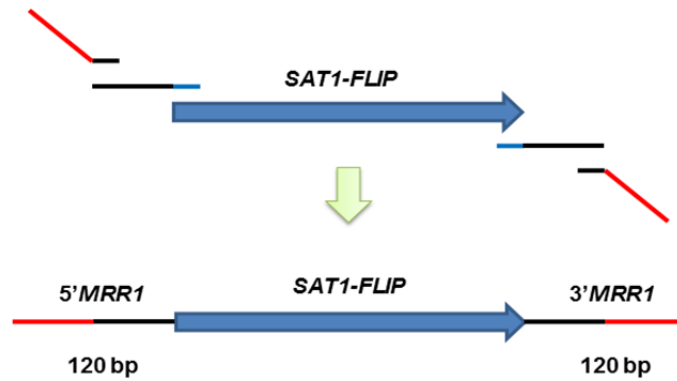
Figure 15. Schematic illustration of constructing the *MRR1* deletion cassette.
The upstream and downstream homology regions of *MRR1* were added to the *SAT1* flipper
cassette after two rounds of PCR (the primers MR2780 and MR2781 used for the first round
and MR2782 and MR2783 for the second round). The resulting *MRR1* deletion cassette is
4457 bp in length with a 120 bp left homology arm (5'*MRR1*) and a 120 bp right homology
arm (3'*MRR1*), which are long enough for efficient homologous recombination.
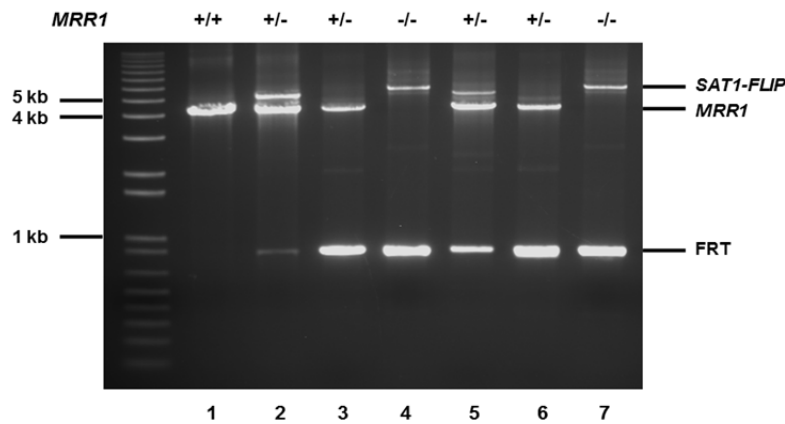
Figure 16. PCR verification of the deletion of *MRR1* in isolate 5674.
Genomic DNA of the strains was used as PCR templates and the primers MR2700 and
MR2699 were used for DNA amplification. The expected fragment sizes for *MRR1* and FRT
are 4039 bp and 852 bp, respectively. For *SAT1-FLIP*, the expected fragment size is 4955 bp
in strains JLY1 (lane 2) and JLY2 (lane 5), and 5547 bp in strains JLY5 (lane 4) and JLY6
(lane 7). Isolate 5674 (lane 1) was used as a positive control. Note that the FRT sequence was
detected in strains JLY1 (lane 2) and JLY2 (lane 5) because the *MAL2* promoter in the *SAT1*
flipper cassette is leaky and the FLP recombinase can therefore be weakly expressed in the
absence of the inducer maltose.

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3.2.2. *MRR1* deletion in isolate 5674 leads to reduced fluconazole resistance

To assess the impact of *MRR1* deletion on azole sensitivity, a liquid microtiter plate assay with

isolate 5674, two heterozygous *mrr1*Δ mutants JLY3 and JLY4 and the two *mrr1*Δ/Δ mutants

JLY5 and JLY6 was performed. As can be observed in **Figure 17A**, the two heterozygous

*mrr1*Δ mutants are more sensitive to 100 μM of fluconazole than the parental strain 5674, and

the deletion of the remaining *MRR1* allele in the heterozygous *mrr1*Δ mutants causes a further

increase in the susceptibility to 100 μM of fluconazole. The two *mrr1*Δ/Δ mutants are about

3-fold less resistant to 100 μM of fluconazole than isolate 5674, this difference being

statistically significant (**Figure 17B**). These data demonstrate that Mrr1p contributes to the

resistance of isolate 5674 to fluconazole.
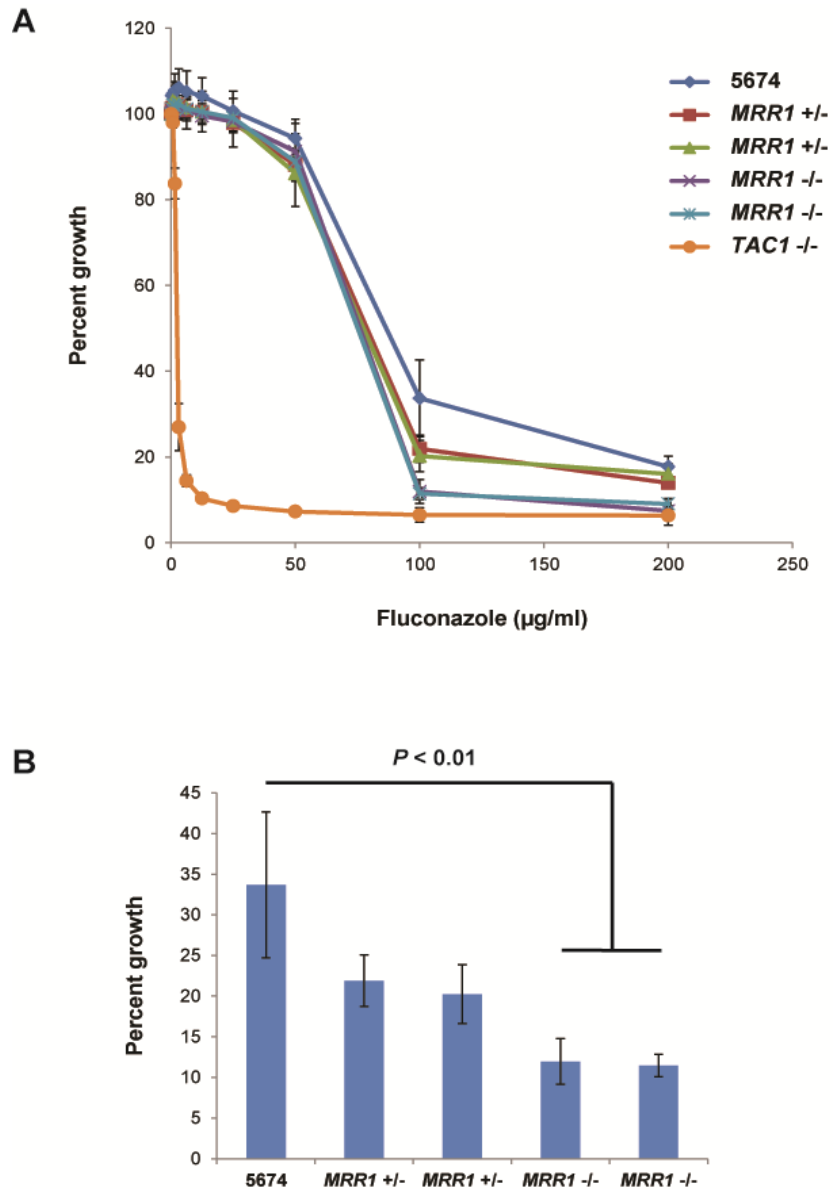
Figure 17. The deletion of *MRR1* in isolate 5674 results in decreased fluconazole resistance.

(A) The fluconazole susceptibility of strains 5674, JLY3 (*MRR1*+/-), JLY4 (*MRR1*+/-), JLY5 (*MRR1*-/-), JLY6 (*MRR1*-/-) and the negative control strain SZY31 (*TAC1*-/-) was determined using liquid microtiter plate assays. The data are presented as the relative growth of the cells in fluconazole-containing YPD media as compared with the growth of the same strain in fluconazole-free YPD media (100%). The results represent the means ± standard deviations from four independent experiments, each performed in duplicate.

(B) The data at 100 μg/ml of fluconazole are shown. Statistical analysis was performed using one-tailed Student's t-test.

3.2.3. Comparison of the mRNA levels of *TAC1* and *CDR1* in isolate 5674 and the 5674-derived *mrr1Δ/Δ* mutants

To investigate whether the reduced azole resistance in isolate 5674 upon *MRR1* deletion correlates with a decrease in the expression levels of the Tac1p target genes, the mRNA levels of *TAC1* and *CDR1* in isolate 5674 and the 5674-derived *mrr1Δ/Δ* mutants were determined. These two Tac1p target genes both encode critical effectors of azole resistance in isolate 5674 and their mRNA levels may be reflective of the expression of other Tac1p target genes that are also involved in azole resistance. Northern blotting experiments for isolate 5674 and the *mrr1Δ/Δ* mutants using the *TAC1* and *CDR1* probes reveal no obvious difference between these strains (**Figure 18**). To validate these results, RT-qPCR experiments were subsequently conducted for isolate 5674 and the two *mrr1Δ/Δ* mutants. Again, there was no significant difference between isolate 5674 and the two *mrr1Δ/Δ* mutants (**Figure 19**). Taken together, these data indicate that Mrr1p is not involved in the Tac1p-dependent constitutive transcriptional upregulation of *TAC1* or *CDR1* in isolate 5674. They also suggest that Mrr1p does not take part in Tac1p-mediated azole resistance in isolate 5674.
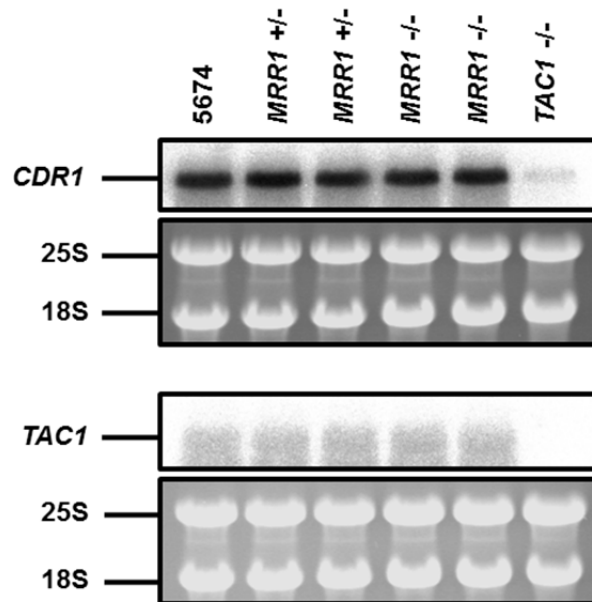
Figure 18. Northern blot analysis of the expression of *CDR1* and *TAC1* in isolate 5674 and the 5674-derived *mrr1Δ* mutants.

Total RNA extracts for strains 5674, JLY3 (*MRR1*+/-), JLY4 (*MRR1*+/-), JLY5 (*MRR1*-/-), JLY6 (*MRR1*-/-) and the negative control strain SZY31 (*TAC1*-/-) were prepared. Northern blotting experiments were then performed using the *CDR1* and *TAC1* probes. The 18S and 25S rRNAs were used as loading controls.
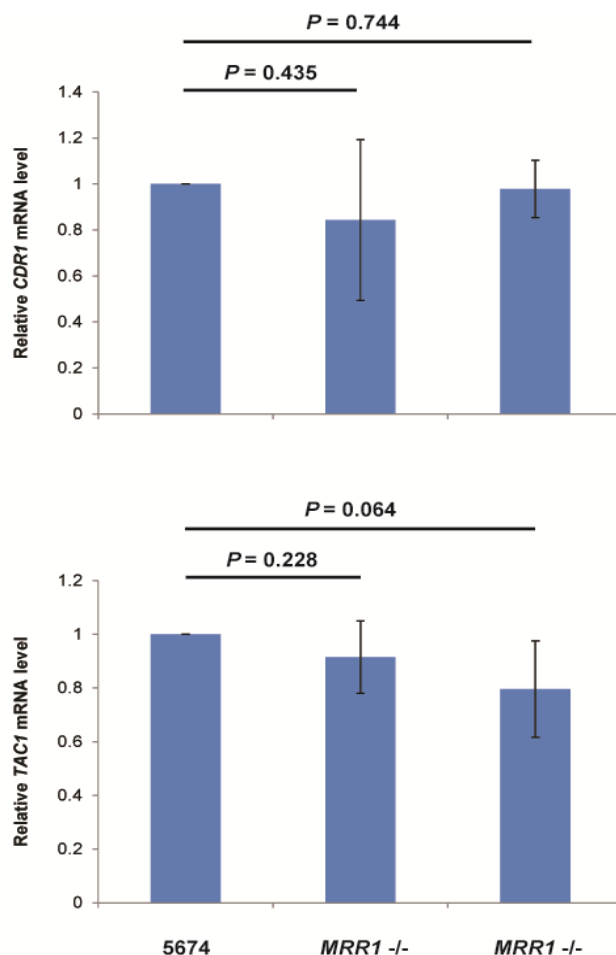
Figure 19. Measurement of the mRNA levels of *CDR1* and *TAC1* in isolate 5674 and the 5674-derived homozygous *mrr1Δ* mutants.
Total RNA extracts for strains 5674, JLY5 (*MRR1*-/-) and JLY6 (*MRR1*-/-) were prepared and subsequently used for the RT-qPCR analysis of *CDR1* and *TAC1* mRNA levels. The data represent the means ± standard deviations from at least four independent experiments, each performed in triplicate. Statistical analyses were performed using two-tailed Student's t-test.

3.2.4. Detection of the long 5' UTR of the *TAC1* transcript in isolate 5674 and the 5674-derived *mrr1Δ/Δ* mutants

As explained in the Introduction section, our lab previously investigated the binding sites of Tac1p in the *TAC1* promoter and found that its binding is enriched between the positions -1100

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and -985 with respect to the start codon; this region contains 7 CGG triplets whose mutation abolishes *TAC1* binding and autoregulation in isolate 5674. In the wild-type strain SC5314, *TAC1* is expressed at a basal level in the absence of fluphenazine exposure and the *TAC1* transcripts have a 5' untranslated region (UTR) of 300 nt in length [63, 89, 90]. Interestingly, in the azole-resistant *C. albicans* clinical isolate Gu5 which also harbors a GOF mutation in *TAC1*, the *TAC1* transcripts have a 1025 nt 5' UTR, which is much longer than that in strains SC5314 and Gu4 which are both azole-susceptible [91]. The above data suggest that the binding of hyperactive Tac1p to the upstream CGG-rich motif of the *TAC1* promoter may recruit the transcription machinery, thereby producing long *TAC1* transcripts. The locations of these two transcription start sites in the *TAC1* promoter are shown in **Figure 3**.

Two questions were therefore raised: (1) Do the *TAC1* transcripts in isolate 5674 also have a long 5' UTR? (2) If it is the case, is Mrr1p involved in the production of the long *TAC1* mRNA mediated by hyperactive Tac1p in isolate 5674? It should be noted that, in the previous RT-qPCR experiments using isolate 5674 and the two homozygous *mrr1Δ* mutants, the *TAC1* primers were specific to the coding sequence of the *TAC1* gene, so the *TAC1* transcripts with different lengths of 5' UTR were all detected at the same time, making it difficult to determine the levels of only the long 5' UTR-containing *TAC1* transcripts.

To answer the above questions, total RNA of isolate 5674 and the two independent *mrr1Δ/Δ* mutants was isolated and RT-PCR experiments using primers that are specific to the longer 5' UTR of *TAC1* were performed. As shown in **Figure 20A**, isolate 5674 and the two

independent *mrr1Δ/Δ* mutants have the *TAC1* transcripts with a long 5' UTR; by contrast, the long *TAC1* transcripts cannot be detected in isolate 5457, which is the azole-susceptible parental strain of isolate 5674. Moreover, the long *TAC1* transcripts were also observed in isolate Gu5, which is consistent with the previous finding [91]. However, the amplification efficiency for isolate Gu5 is much lower than for isolate 5674 and the two *mrr1Δ/Δ* mutants. This may be because the primers were designed according to the *TAC1* promoter sequence in isolate 5674 and there may be polymorphism in the *TAC1* promoter region among different *C. albicans* strains. Another possibility would be that the level of the long 5' UTR-containing *TAC1* mRNA in isolate Gu5 may be much lower than in isolate 5674, which could be tested by RT-qPCR experiments (see **Chapter 3.2.5**). The RT-PCR result reinforces the previous hypothesis that constitutively activated Tac1p may be associated with the production of the long 5' UTR-containing *TAC1* mRNA.

3.2.5. Comparison of the long 5' UTR-containing *TAC1* mRNA levels in isolate 5674 and the 5674-derived *mrr1Δ/Δ* mutants

To investigate the role of Mrr1p in producing the long 5' UTR-containing *TAC1* mRNA in isolate 5674, the expression levels of the long *TAC1* transcripts in isolate 5674 and the 5674-derived *mrr1Δ/Δ* mutants were determined by RT-qPCR. The primers used are specific to the longer 5' UTR of *TAC1* of isolate 5674. As can be seen in **Figure 20B**, there is no significant difference between isolate 5674 and the two independent *mrr1Δ/Δ* mutants,

indicating that the proportion of the long 5' UTR-containing *TAC1* transcripts in total *TAC1* transcripts does not change upon *MRR1* deletion in isolate 5674.
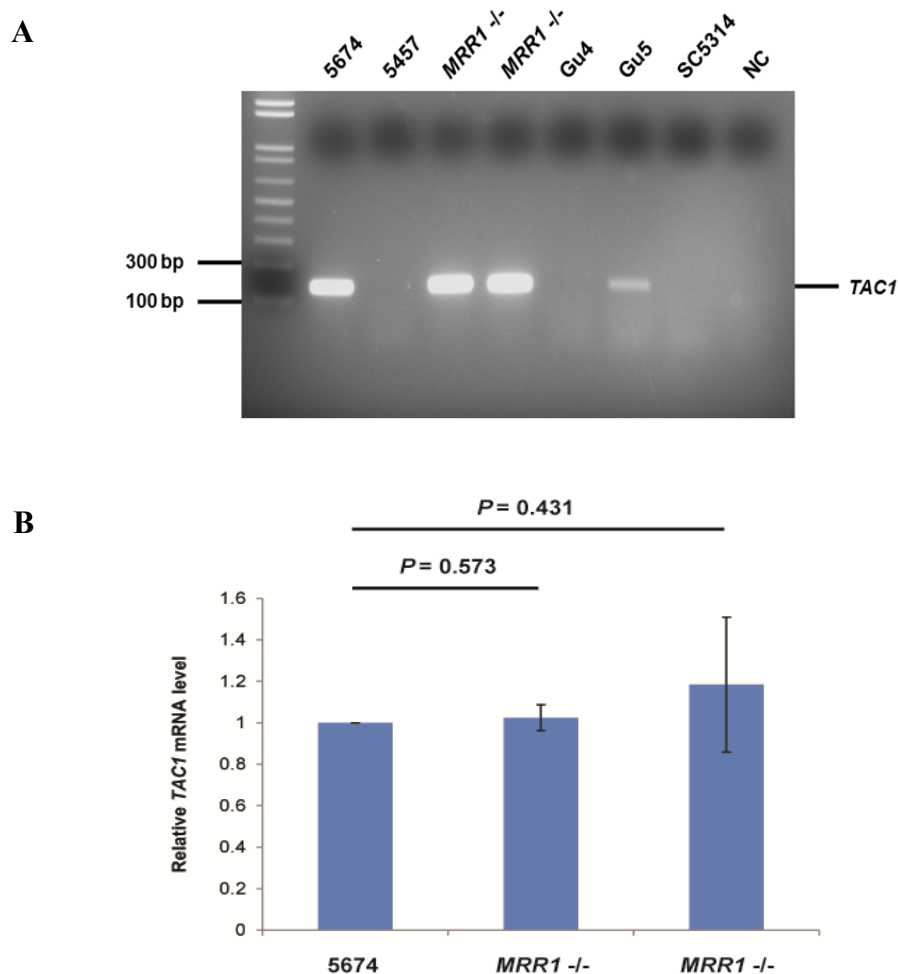
Figure 20. Measurement of the long 5' UTR-containing *TAC1* transcript levels in isolate 5674 and the 5674-derived homozygous *mrr1Δ* mutants.
Total RNA extracts for strains 5674, 5457, JLY5 (*MRR1-/-*), JLY6 (*MRR1-/-*), Gu4, Gu5 and SC5314 were prepared for the RT-PCR (A) and RT-qPCR (B) analyses.
(A) The primers MR2869 and MR2870 that are specific to the long 5' UTR of *TAC1* were used. The expected fragment size is 140 bp. Isolate Gu5 was used as a positive control. NC denotes the negative control, for which water was used as the PCR template.
(B) The RT-qPCR results represent the means ± standard deviations from three independent experiments, each performed in duplicate. Statistical analyses were performed using two-tailed Student's t-test.

3.3. Mrr1p may also be implicated in fluconazole tolerance in strain SC5314

Some of the Mrr1p target genes encode putative oxidoreductases that might contribute to coping with the oxidative stress induced by the fluconazole exposure [64]. Since *MRR1* deletion in isolate 5674 causes only a slight decrease in fluconazole resistance and the expression levels of *CDR1* and *TAC1* do not change upon *MRR1* deletion in isolate 5674, it is possible that the decreased resistance to fluconazole is due to the reduced expression of some Mrr1p target genes that are regulated by wild-type Mrr1p and associated with fluconazole resistance or tolerance. To test this possibility, we next sought to investigate whether Mrr1p also functions in fluconazole tolerance in strain SC5314, where there is no GOF mutation in *TAC1* and *CDR1* is expressed at a basal level [63].

Again, liquid microtiter plate assays were performed for strain SC5314 and the SC5314-derived *mrr1Δ/Δ* mutant. Although they were obtained in one experiment performed in duplicate, these data seem to indicate that the SC5314-derived *mrr1Δ/Δ* mutant is slightly more susceptible to fluconazole than strain SC5314, suggesting that Mrr1p may play a part in the tolerance of strain SC5314 to fluconazole (**Figure 21**). It is therefore tempting to speculate that Mrr1p might function independently of Tac1p in the regulation of fluconazole resistance in isolate 5674.
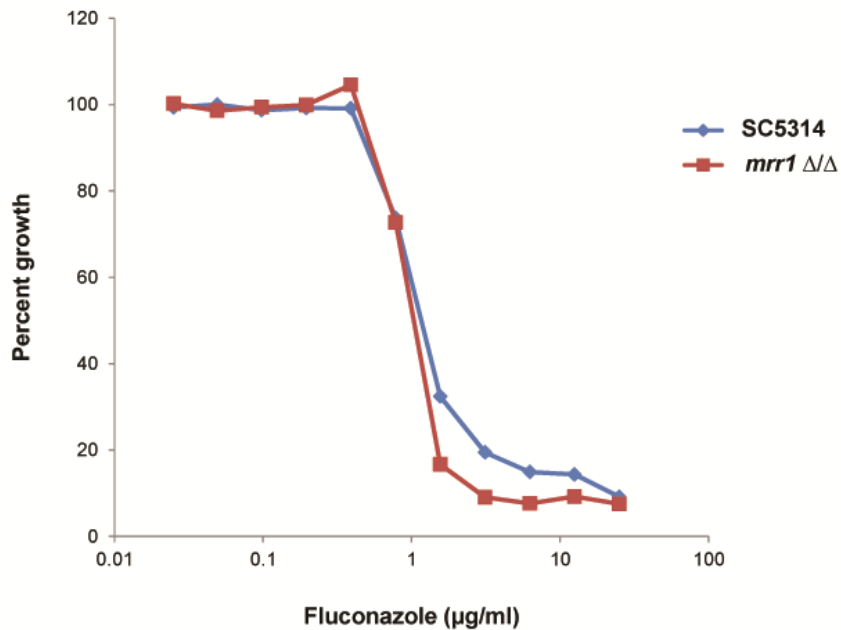
Figure 21. Determination of the role of Mrr1p in fluconazole tolerance in strain SC5314.
The fluconazole susceptibility of strain SC5314 and the SC5314-derived *mrr1Δ/Δ* mutant was determined using liquid microtiter plate assays. The results are presented as the percent growth of the cells in fluconazole-containing YPD media as compared with the growth of the same strain in fluconazole-free YPD media (100%). The data represent the means from a single experiment performed in duplicate.

4. Discussion

Azole resistance in *C. albicans* constitutes a serious clinical problem all over the world and many efforts have been made in recent years in order to reveal the underlying molecular mechanisms. Mrr1p- and Tac1p-mediated azole resistance is prevalent in many clinical isolates of *C. albicans* and has been extensively studied. However, little is known about *whether* these two transcriptional pathways interact in controlling azole resistance. Our lab recently got some preliminary evidence of a possible involvement of Mrr1p in fluphenazine-induced transient upregulation of *TAC1* and *CDR1*, which encouraged us to further study the cross-talk between the Mrr1p- and Tac1p-regulated transcriptional pathways.

In this study, we continued to investigate the role of Mrr1p in fluphenazine-induced transient upregulation of *TAC1* and *CDR1*. I first sought to reproduce our previous data of the residual induction of *CDR1* by fluphenazine in a SC5314-derived *tac1Δ/Δ* mutant using Northern blotting analyses. However, I did not manage to do so, even though the optimum fluphenazine conditions for *CDR1* induction in the *tac1Δ/Δ* mutant were used. Although very unlikely, it is possible that the SC5314-derived *tac1Δ/Δ* mutant that I used is genetically different from the original one due to an occurrence of certain mutations during cell passaging, so that it may get much less responsive to fluphenazine treatment, or the factor(s) responsible for the residual induction of *CDR1* by fluphenazine may consequently become incapable of upregulating *CDR1*. Notably, we have also performed a luciferase assay to study

fluphenazine-induced transient activation of the *CDR1* promoter in the SC5314-derived

tac1Δ/Δ mutant. It has been found that the *CDR1* promoter is activated by 150 μM of

fluphenazine in the absence of Tac1p in strain SC5314, although the induction level is low

(data not shown). This is in line with our previous result shown in **Figure 6** and these results

confirm the existence of the residual induction of *CDR1* by fluphenazine in the absence of

Tac1p in strain SC5314, which I was not able to clearly show through Northern blotting

analyses, possibly because Northern blotting is much less sensitive and quantitative than

luciferase assays.

As the Tac1p-independent fluphenazine induction of *TAC1* is more noticeable than that

of *CDR1* (see **Figure 6**), we therefore chose to first study whether the induction level of *TAC1*

would significantly decrease upon *MRR1* deletion in the SC5314-derived *tac1Δ/Δ* mutant.

Surprisingly, the SC5314-derived *tac1Δ/Δ mrr1Δ/Δ* mutant exhibited an unexpected feature

that its *TAC1* promoter activity is higher than that in the SC5314-derived *tac1Δ/Δ* mutant

under all the fluphenazine conditions tested, suggesting that Mrr1p might transcriptionally

inhibit fluphenazine-induced *TAC1* upregulation in strain SC5314. However, these results are

preliminary and further experiments are required for making firm conclusions. Besides, with

the use of luciferase assays, we can also study the residual induction of *CDR1* by fluphenazine

in the SC5314-derived *tac1Δ/Δ mrr1Δ/Δ* mutant and the related strains in the future.

It is well known that fluphenazine is able to induce the upregulation of many Tac1p

target genes, but the exact molecular mechanism is still unknown *until now*. Zinc cluster

transcription factors are functionally and architecturally akin to metazoan nuclear receptors and the activities of a number of zinc cluster transcription factors in *S. cerevisiae* are indeed regulated by specific small molecules, such as nutrients and environmental chemicals [57]. As mentioned in the Introduction section, in *S. cerevisiae*, the zinc cluster transcription factor Pdr1p can bind to ketoconazole, cycloheximide and rifampicin, thereby leading to the overexpression of its target gene *PDR5* that encodes a drug efflux pump [58]. These facts therefore raise the possibility that fluphenazine may bind directly to Tac1p and alter its conformation, thereby rendering it hyperactive. To test whether fluphenazine binds to Tac1p, we could conduct cold competition assays as previously published [58].

Fluphenazine belongs to the phenothiazine family commonly used in the treatment of psychoses, such as paranoia and schizophrenia. Although structurally distinct from azoles, fluphenazine is also a substrate for Cdr1p and Cdr2p in *C. albicans* [24]. Moreover, fluphenazine is an antagonist of calmodulin, which is a highly conserved calcium-binding protein among eukaryotes and plays a role in the growth of some fungi [92]. It was previously found that relatively low concentrations of fluphenazine exhibit only a weak antifungal effect, but can significantly strengthen the antifungal activities of fluconazole and ketoconazole in *C. albicans* [93, 94]. In accordance with the above findings, we have observed that the growth of the wild-type *C. albicans* strain SC5314 was suppressed in the presence of relatively high concentrations of fluphenazine (data not shown). Besides, the fluphenazine treatment of *C. albicans* results in the induction of several putative stress response genes, including *SAS3* and

GRP2 [95]. Taken together, the fact that fluphenazine can be harmful to *C. albicans* and bring about complex molecular changes within the cell due to its antifungal characteristics especially at relatively high concentrations inevitably complicates our investigation of whether and how Mrr1p functions in fluphenazine-induced transient upregulation of *TAC1*, *CDR1* and other Tac1p target genes involved in azole resistance.

Since it is likely that Mrr1p does not play a positive role in the Tac1p-independent induction of the *TAC1* promoter by relatively high concentrations of fluphenazine in strain SC5314, what could be the factor responsible for this process? A good candidate is the zinc cluster transcription factor Mrr2p. As explained in the Introduction section, Mrr2p can upregulate *CDR1* in a Tac1p-independent manner. It is thus possible that Mrr2p may also regulate the expression of *TAC1* and be positively involved in the transient induction of *TAC1* by fluphenazine independently of Tac1p. To test this possibility, we could first delete *MRR2* in the SC5314-derived *tac1*Δ/Δ mutant and then study whether the induction of the *TAC1* promoter by relatively high concentrations of fluphenazine would disappear upon *MRR2* deletion.

A new *MRR1* deletion cassette was constructed through two rounds of PCR and used to delete *MRR1* in the clinical isolate 5674. However, the design of the primers was based on the *MRR1* sequence of the wild-type strain SC5314 instead of isolate 5674, as it was the only *MRR1* sequence available at that time. Thus, the reason why the second allele of *MRR1* could not be deleted using the new deletion cassette is probably because there is polymorphism in

the non-coding upstream and downstream regions of *MRR1*, which has been confirmed by whole genome sequencing of isolate 5674. Although the *MRR1* deletion cassette from Dr. Joachim Morschhäuser also contains the upstream and downstream sequences of *MRR1* of strain SC5314, these homology regions are about 300 bp in length and should therefore work more effectively than the 120 bp homology arms of the new deletion cassette, so the problem of *MRR1* polymorphism was successfully overcome.

Mrr1p indeed contributes to azole resistance in the clinical isolate 5674, but very likely not through Tac1p-mediated mechanisms because the mRNA levels of *CDR1* and *TAC1* do not change upon *MRR1* deletion. However, we cannot exclude the possibility that Mrr1p may positively regulate the expression of other Tac1p target genes which are less important in the maintenance of azole resistance than *CDR1* and *TAC1* in isolate 5674. Besides, it is also possible that the decrease in azole resistance caused by *MRR1* deletion in isolate 5674 may be due to the changed expression of some of the Mrr1p target genes which are also involved in azole resistance or tolerance, as implied by our observation that the deletion of *MRR1* may also lead to reduced fluconazole tolerance in the wild-type strain SC5314. Here it is important to mention that Morschhäuser *et al*. previously found that *MRR1* deletion in strain SC5314 did not affect its sensitivity to fluconazole, but they did not show the detailed data (e.g. the percent growth of strain SC5314 and the *mrr1Δ/Δ* mutant at all the concentrations of fluconazole tested) [64]. Therefore, it is possible that there might be a tiny difference between strain SC5314 and the *mrr1Δ/Δ* mutant which they did not recognize. Since our results are

preliminary, one would need to repeat the experiment to ascertain that Mrr1p is involved in azole tolerance in strain SC5314.

Although there is no GOF mutation in *MRR1* in strains SC5314 and 5674, their wild-type *MRR1* alleles may also regulate the expression of specific genes that help to cope with the azole stress. To test the above possibilities, we could compare the gene expression profiles of isolate 5674 and the 5674-derived *mrr1Δ/Δ* mutant so that all the transcriptional changes upon *MRR1* deletion in isolate 5674 would be determined. In this way, we may uncover the exact role of Mrr1p in regulating azole resistance in isolate 5674.

Another interesting finding in this study is that the *TAC1* transcripts in isolate 5674 have a very long 5' UTR. This is not surprising as it was previously reported that in another azole-resistant clinical isolate that carries a GOF mutation in *TAC1*, the *TAC1* transcripts also have a long 5' UTR, and our lab has recently found that Tac1p binds to its own promoter at the CGG triplets about 1000 bp upstream of the start codon [91] [Louhichi F, unpublished data]. In strain SC5314, the 5' UTR of the *TAC1* transcripts is only 300 nt in length, suggesting that the basal level of *TAC1* transcription in strain SC5314 may be controlled by other transcriptional regulators independently of Tac1p. This is supported by our previous data and the results in **Figure 12** showing that *TAC1* deletion does not affect the basal activity of the *TAC1* promoter in strain SC5314. In eukaryotic cells, 5' UTR often plays an important role in controlling translational efficiency, either in a positive or negative manner; it can sometimes decrease the translational efficiency either by forming secondary structures that affect

translation initiation, or by using uORF to prevent the translation of the main ORF [96]. A good example in *C. albicans* is *UME6*, which encodes a critical transcriptional regulator of hyphal growth [97]. The 5' UTR of *UME6*, which contains two putative uORFs, is 3041 nt in length and it suppresses the translation of the *UME6* transcripts, resulting in reduced Ume6p levels [89, 98]. Interestingly, by performing *in silico* analyses, we found that the 1025 nt 5' UTR of *TAC1* also contains a putative uORF (see **Figure 3**), which raises the possibility that the longer 5' UTR may affect the translation of the longer *TAC1* transcripts.

In consideration of the important function of 5' UTR and the fact that Mrr1p also binds to the upstream CGG-rich motif of the *TAC1* promoter, we thus investigated whether Mrr1p contributes to the production of the long *TAC1* transcripts in isolate 5674. Although we have found that Mrr1p is not implicated in this process, it is still appealing to study whether transcriptional regulators other than Tac1p are involved. Besides, we could perform the primer extension assays to test whether isolate 5674, the 5674-derived *mrr1*Δ/Δ mutants and isolate Gu5 produce the *TAC1* transcripts with other different lengths (e.g. 300 nt) of 5' UTR. It may also be studied in the future whether the long 5' UTR of the *TAC1* transcripts plays a role in the translational regulation of *TAC1* and azole resistance, although there are no good approaches at the moment.

Since constitutively activated Tac1p is able to upregulate *CDR1* and *TAC1* without the requirement of Mrr1p in isolate 5674, will the transcription levels of *CDR1* and *TAC1* be elevated if a *MRR1* allele with a GOF mutation is introduced into the genome of isolate 5674?

It is possible that constitutively hyperactive Mrr1p may cooperate with constitutively hyperactive Tac1p to further upregulate some of the Tac1p target genes in either a synergistic or an additive fashion in isolate 5674. To test this hypothesis, we could replace one wild-type *MRR1* allele of isolate 5674 with a *MRR1* allele carrying a GOF mutation and test whether the mRNA levels of *CDR1* and *TAC1* would hence be increased. We could also study whether the proportion of the long *TAC1* transcripts in total *TAC1* transcripts would change upon the introduction of a *MRR1* allele carrying a GOF mutation into the genome of isolate 5674. Through these studies, we may gain deeper insights into the interactions between different transcriptional regulators of azole resistance in *C. albicans*.

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