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Overexpression of yeast YLR177w gene provides tolerance to antifungal drug tioconazole

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Abstract

Aim: To find genes that provide resistance to antifungal drug tioconazole by a genomic DNA screening approach.

Materials and Methods: Wild-type yeast (BY4741) cells were transformed with a high copy genomic DNA expression library and transformants that can grow on toxic levels of Tioconazole were determined. Library plasmids were isolated from the transformants and sequenced for the identification of potential tioconazole resistance gene(s).

Results: Yeast YLR177w gene was isolated as a potential tioconazole resistance gene. Its overexpression led cells to tolerate normally toxic levels of tioconazole. Deletion of the YLR177w gene from the yeast genome made cells more vulnerable to the drug.

Conclusion: Yeast YLR177w gene encodes a putative protein of unknown function. Our analyses suggest a potential function for the YLR177w gene for tioconazole resistance.

Introduction

The mortality of fungal infections has progressed rapidly in recent years, and the number of drugs that can be used in these severe infections has decreased due to drug resistance [1,2]. Determination of antifungal drug resistance mechanisms will help us to develop ways for the reversal of resistance and to combine the existing drugs more effectively for treatment purposes [3]. It may also benefit us to disclose the similarities and discrepancies with other drug resistance mechanisms, shedding light on common pathways and providing opportunities to design and develop new drugs [4]. Knowing the resistance mechanisms, it is possible to screen infectious fungi isolated from patients to assess whether they have pre-gained resistance to drugs of choice [5].

Tioconazole belongs to the imidazole group of antifungals and topical formulations of tioconazole are used for a wide spectrum of activity against many pathogenic fungi. Tioconazole inhibits 14-alpha demethylase, an enzyme in the cytochrome P-450 complex that converts lanosterol to ergosterol, an important constituent of the yeast cell membrane. Thus, tioconazole blocks ergosterol biosynthesis and results in altered membrane permeability, stability and function of membrane-dependent proteins/enzymes [6,7].

Azole resistance may emerge through different mechanisms including upregulation or alteration of the drug target, activation of efflux transporters, or other changes that diminish drug toxicity [8]. Amino acid changes in the drug binding sites are relatively common in azole-tolerance mechanisms in fungi. The most common changes are seen near the heme-binding region of ERG11 [9,10].

Upregulation of *ERG11* also occurs among azole-resistant isolates of *Candida* species [11] which contributes to resistance because an increase in the target protein levels requires more drugs for effective inhibition [12]. Apart from ERG11 status, modifications that lead to reduced ergosterol contents such as ERG3 mutations ($\Delta^{5,6}$-desaturase) can provide azole tolerance as well [13].

Increased drug efflux seems to be more important in azole resistance than *ERG11* mutations [14] and there is a crosstalk between the *ERG11* mutations and the activation of the transcription factor PDR1 [15]. The ATP binding cassette (ABC) transporters such as CDR1, CDR2, SNQ2 have also been implicated in azole resistance in different

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fungal pathogens [16]. ABC proteins play roles in drug efflux using ATP. Major facilitator superfamily transporters (MFS) function by drug: proton antiport and can transfer multiple substrates across the membrane and thus play roles in azole resistance [17]. The *MDR1* gene encodes an MFS transporter that leads to enhanced azole efflux and azole resistance when overexpressed [18].

Even though there are many studies on the mechanisms of drug resistance for other azole drugs in different fungal species, tioconazole resistance has not been elucidated well. There is only one study on the Web of Science database regarding the identification of tioconazole resistance genes. In this study, using a genetic screening study, mutations in the ACR1 gene were found to be important in tioconazole resistance in *Aspergillus nidulans* [19]. Thus, additional studies on the mechanisms of tioconazole resistance may have scientific and clinical importance.

In this work, we used baker's yeast as the model system to study the mechanisms of drug resistance for tioconazole. Yeast is a widely used organism for antifungal drug research because it is a member of fungi and has the widest spectrum of molecular tools that can be used for an organism. We screened a yeast genomic DNA library to identify the genes that provide tioconazole resistance when overexpressed. Our results showed that the yeast YLR177w gene, encoding a protein with no known functions, provides tioconazole resistance upon overexpression from a plasmid.

Materials and Methods

Design of the study

Wild type BY4741 yeast strain (*S. cerevisiae*, MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; EUROSCARF, Köhlerweg, Oberursel, Germany) was used in this study. First, the minimum inhibitory concentration (MIC) of Tioconazole was determined. Yeast cells transformed with the genomic DNA library were transferred to a medium containing a lethal dose of tioconazole. Plasmid DNA was obtained from growing colonies. The genes carried by the plasmids were determined by sequence analysis.

Yeast Growth, minimum lethal dose determination and gDNA library screening

Tioconazole (CAS No: 65899-73-2, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used in the experiments. The high-copy gDNA library on YEP13 plasmids (ATCC No.37323, Manassas, Virginia, United States) was transformed into BY4741 yeast cells by the standard lithium acetate (LiAc) procedure [20]. Experiments were performed on Yeast Nitrogen Base (YNB), 2% Glucose media containing appropriate amino acids and bases. Plates were held at 30°C for 3 days and photographed.

Minimum inhibitory concentration analysis

MIC analysis was performed using the Broth Microdilution Test (BMD), described in EUCAST EDef 7.3.2 [21] for the *S. cerevisiae* strain BY4741. Briefly, the stock solution (160 mg/ml) of tioconazole was prepared in 100% dimethyl sulfoxide (DMSO). Two-fold serial dilution was performed eleven times by using DMSO in Eppendorf tubes. Then, all samples were diluted again 100-fold using the Yeast Peptone Dextrose (YPD) medium (2% peptone, 2% glucose, 1% yeast extract, pH 6.5). A 100 µl aliquot of samples was transferred to 96-well plates. In sterile water, 1-5x10^5 CFU/mL yeast cell solution (inoculum) was prepared and transferred in equal volumes to 96-well plates containing different concentrations of the tioconazole. After the cell solution was added, the final concentrations of the tioconazole were between 0.8 and 800 mg/Liter, and the cell concentration was 0.5-2.5x10^5 CFU/ml. The MIC was determined spectrophotometrically at 530 nm after plates were incubated at 30°C for 24 hours. The MIC value was calculated as the lowest concentration causing at least a 50% reduction in cell growth.

Gradient plate preparation and spotting assay

For the gradient plates, 50 ml of media was poured into 120 mm x 120 mm Petri dishes and slanted at an angle of 45° until the media was solidified. The same amount of media containing the lethal dose of tioconazole was poured and the plate was held on the bench till it solidified. For spotting assays, suspensions of fresh cells (OD_{600}=2) were serially (ten-fold) diluted and pipetted onto the media. For each spot, 5 µl of OD_{600} 0.02 culture were pipetted. The plates were incubated at 30°C for 3 days.

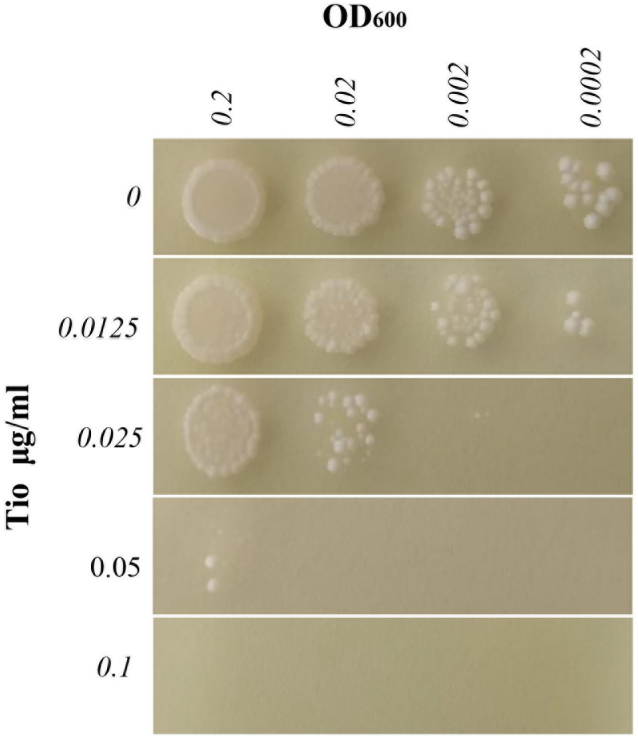

Figure 1. Spotting assay for BY4741 cells on agar plates with shown concentrations (0.0125 µg/ml - 0.1µg/ml) of tioconazole. The cells were diluted serially (OD_{600} 0.2 – 0.0002), and a 5-µl cell solution was pipetted to each spot. Tio represents tioconazole and OD_{600} represents the optic density of cells at 600 nm wavelength.

Plasmid isolation, cloning, and sequencing of the gene

Plasmids from a resistant colony were isolated from the yeast cells. To isolate the plasmids, overnight grown cells were exposed to 10U Lyticase and 20% sodium dodecyl sulfate (SDS), respectively, for disruption of the cell wall. Then, the plasmid isolation was performed by following the protocol of the Thermo Scientific GeneJET-Plasmid Miniprep Kit (#K0502, #K0503, Waltham, Massachusetts, United States). Afterward, plasmids were amplified in JM109 *E.coli* cells.

Gene cassettes included by plasmids were sequenced using a pair of plasmid-specific primers. The sequence data were compared with *Saccharomyces* Genome Database (SGD) and the YLR177w gene was identified in the expression cassette. The YLR177w gene was separately cloned into high-copy plasmid pAG425GPD-ccdB (Addgene, Watertown, Massachusetts, United States) by using the Gateway Cloning Technology® (Invitrogen, Waltham, Massachusetts, United States).

Results

Screening of genomic DNA library and identification of YLR177w gene

We determined the minimum lethal dose by spotting wild-type cells on YPD agar plates with several concentrations of tioconazole (Figure 1). The cells were not able to grow in 0.05 µg/ml tioconazole. Next, we determined the MIC value (at least 50% or more inhibition of growth) for tioconazole in the YPD medium following the EUCAST

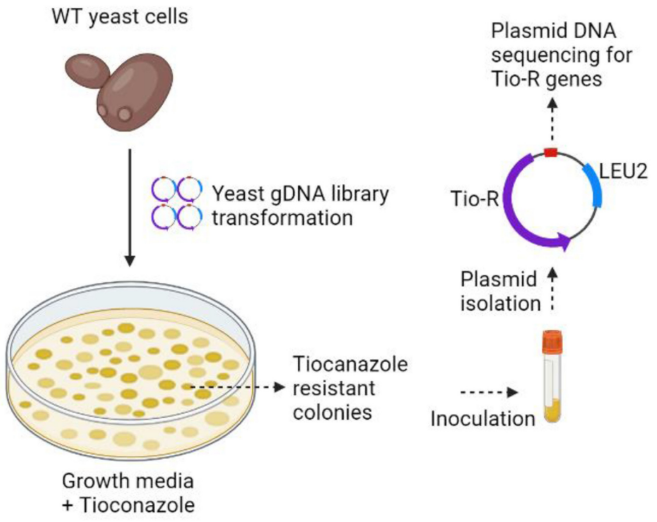

Figure 2. Flow chart for the experimental design. Tioconazole (Tio) and the *S. cerevisiae* BY4741 wild-type strain were used in the library screening. Transformed yeast cells were grown on a YNB agar medium containing a minimum lethal dose of tioconazole and supplemented with appropriate amino acids and bases. Tioconazole-resistant colonies were selected and inoculated in a liquid YNB medium with similar formulations without agar. Plasmid isolation was performed and plasmid DNAs were sequenced for Tioconazole resistance (Tio-R) genes.

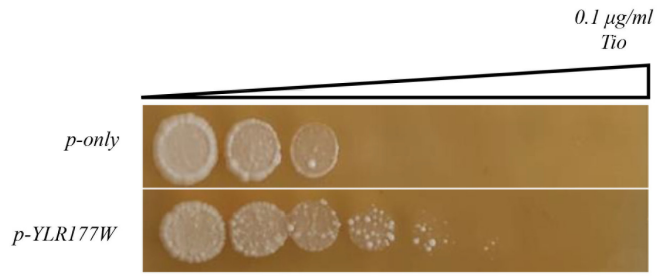

Figure 3. Spotting assay for overexpressing analysis. Wild-type cells overexpressing the YLR177w gene (p-YLR177w) were grown on plates containing a gradient (0 – 0.06 µg/ml) of Tio. Wild-type cells with sham plasmids were used as controls (p-only).

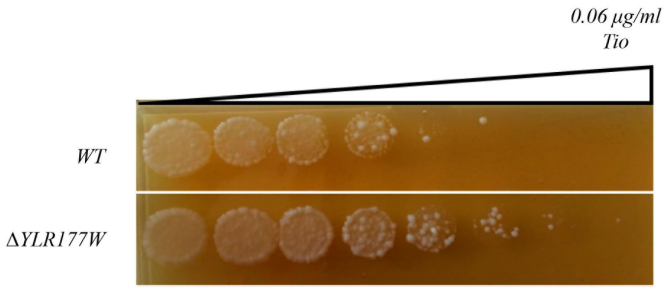

Figure 4. Spotting assay showing the growth rates of the wild-type (WT) and a deletion mutant of the YLR177w gene (ΔYLR177w).

broth microdilution protocol. Tioconazole had a MIC value of 0.025 µg/ml. In general, our MIC analysis and spotting assay were consistent with each other. Then, we transformed the gDNA library into wild-type yeast cells and spread them onto plates containing selective media and a minimum lethal dose of the drug (0.1 µg/ml tioconazole). We picked a survived colony; isolated the plasmids, amplified and transformed them back to the wild-type cells to confirm the resistance. We confirmed that the drug resistance phenotype of the transformed cells was not a false positive event, instead, cells gained resistance to the drug because of the plasmid that they harbored. This plasmid was sequenced and the expression cassette information was obtained via Basic Local Alignment Search Tool (BLAST) analysis from Saccharomyces Genome Database (SGD). The library plasmid that transformed the initial cells contained the full-length YLR177w gene. Therefore, we wanted to clone the YLR177w gene into a new plasmid to be sure of the results and to use it in the next steps.

Cloning and expression of YLR177w gene

We cloned the YLR177w gene independently to a new over-expression vector (pAG425), transformed it into fresh wild-type cells, and tested its overexpression on gradient plates. We observed that cells over-expressing the *YLR177w* gene were able to resist tioconazole when compared to the control cells carrying empty plasmids (p-only)

(Figure 3).

As the next step, we analyzed the deletion mutants of the *YLR177w* gene for their tioconazole resistance. Contrary to what we expected, the deletion mutants (ΔYLR177w) were not drug-sensitive (Figure 4), which suggests that the YLR177w gene provides tioconazole resistance only when it is overexpressed.

Discussion

In this study, we screened a yeast gDNA library in wild-type yeast cells to find genes that provide resistance to the antifungal drug tioconazole. Through the analyses of the transformants, we obtained a plasmid that harbored the full-length YLR177w gene in the expression cassette. Subsequent analyses showed that overexpression of the YLR177w gene leads to tioconazole resistance in yeast cells, however, its deletion did not perturb the drug sensitivity of the cells. YLR177w gene has no known function and we linked this gene with a potential role in drug metabolism for the first time.

The inevitable outcome of the extensive use of azoles as antifungal drugs is the development of drug resistance. There are many host and fungal factors associated with antifungal drug resistance mechanisms [22]. Recent studies with clinical isolates of Candida showed that in over 37% of the cases ERG11 gene contains mutations [23-25]. Overexpression of ERG11 or drug efflux pumps such as CDRs and MDRs are usually not common under culture conditions of clinical isolates [11].

The YLR177w gene encodes a protein that is localized to the cytoplasm [26], but its function is not known. It is phosphorylated in a Dbf2-Mob1-dependent manner. The Dbf2-Mob1 complex plays role in exiting mitosis [27], and the biochemical consequences of phosphorylation by Dbf2-Mob1 are not known. Unfortunately, there is no additional information about the functions of YLR177w. On the other hand, the YLR177w gene has a paralog PSP1 (YDR505c) with partially known functions in DNA replication. Overexpression of the PSP1 gene suppressed the mutations in the POL1 gene (catalytic subunit of DNA polymerase alpha), POL3 gene (DNA polymerase delta) and CDC6 gene [28]. However, none of these interactions have connections with the known mechanisms of azole resistance.

The biochemical interactions of YLR177w suggest a possible role in DNA synthesis, mitotic cell division and tioconazole resistance. The nature of this relationship should be further investigated to reveal the mechanisms. Our analyses had limitations for the follow-up analyses of the cells overexpressing the YLR177w gene. We only overexpressed the YLR177w gene and checked the growth rates of the cells treated with tioconazole. Global analyses such as total transcriptomic profiling or similar analyses should be performed to highlight the possible biochemical paths that YLR177w acts thru for tioconazole resistance.

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