CHACTERIZATION OF DRUG RESISTANCE MECHANISMS AGAINST ANTIFUNGAL AGENT VORICONAZOLE

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ABSTRACT

DRUG RESISTANCE MECHANISM AGAINST ANTIFUNGAL AGENT VORICONAZOLE

The rapid progress in medicine provides an increase in both average life span but also increases number of patients who need intensive care throughout the world. The morbidity and mortality of systemic fungal infections have become a serious problem because of suppressed immunity due to chemotherapy or intentional use of suppressants after tissue/organ transplantations. Voriconazole (Vfend, Pfizer) is one of antifungal agent against these fungal infections as a derivative of fluconazole. However some fungi types gain resistance against this agent. In this project, we use budding yeast *Saccharomyces cerevisiae* as a model organism to identify the genes that cause resistance Voriconazole by performing a genome wide screening. MRS3 and TRI1 genes were determined as resistant genes against lethal dose of Voriconazole. Their overexpression exhibited resistance to Voriconazole and also Ketoconazole in cross-resistance test. According to real-time PCR results, both MRS3 and TRI genes showed overexpression in IC_{50} of Voriconazole treatment when compare with their untreated status. Microaaray analysis indicated the expression change of our resistant genes is not more than 2 fold. In addition to expression analysis, functional analysis such as membrane dynamics tests have salt (NaCl, LiCl) and cationic drug (HygB,spermine,TMA) tolerance, membrane potential, volume/size measurements and cytoplasmic pH values were analyzed to reveal the mechanisms of resistance. Our results showed that no linkage between Variconozole resistance provided by MSR3 and TRI1 genes and functional analyses mentioned above.

ÖZET

MANTAR ÖNLEYİCİ İLAÇ VORİKONAZOLE KARŞI OLAN DİRENÇLİLİK MEKANİZMASININ KARAKTERİZASYONU

Kemoterapi gibi bağışıklık sisteminin değişik nedenlerle zayıflamasına ya da transplantasyonlar sonrasında doku reddine neden olabilen tepkisini azaltmak amacıyla bilerek zayıflatılmasına bağlı olarak gelişen sistemik mantar enfeksiyonlarının morbidite ve mortalitesi de önemli ölçüde artmaktadır. Vorikonazol, sistemik mantar enfeksiyonlarına karşı kullanılan bir tür mantar ilacıdır. Fakat birçok mantar türü bu tarz ilaçlara direnç kazandığı gibi Vorikonazole karşı dirençli mantar türleri de mevcuttur. Mantar ilaçlarına karşı gelişen dirençlilik mekanizmalarının aydınlatılması bu olumsuz gelişmenin önünü kesecek, yeni tedavi kombinasyonlarının uygulanabilmesinin yanı sıra yeni ilaçların geliştirilmesine katkıda bulunacaktır. Bu çalışmada *Saccharomyces cerevisiae* model organizması kullanılarak, BY4741 yabani tip maya suşu içerinde dirençlilik çalışmaları yapılmıştır. Genom düzeyinde yapılan taramalar sonucu bulunan MRS3 ve TRI1 genlerinin fazla ifadelenmesi, öldürücü doz ve daha yüksek dozlarda Vorikonazole karşı dirençlilik göstermiştir. Bu iki gen de çapraz dirençlilik testlerinde aynı azol grubunda bulunan Ketokonazole karşı dirençlilik göstermiştir. Bu genlerin öldürücü dozun yarısı kadar Vorikonazole muamelesinin ardından, eş-zamanlı PCR analizine göre ekspresyonunun ilaç uygulanmamış durumlarına göre fazla olduğu gözlemlenmiştir. Tüm genom mikroarray analizinde ise genlerin ifadelemesinin iki kat değişiklikten az olmasından ötürü, MRS3 ve TRI1 genlerine rastlanmamıştır. Fonksiyonel analiz olarak membran dinamikleri test çalışmaları yapılmıştır. Dirençli genlerin ve mutantlarının tuz ve katyonik ajanlar varlığında tepkisi test edilmiştir. Sonuçlara gore tuz ve katyonik ajanlara karşı gelişen dirençlilik mekanizmasının, Vorikonazole dirençlilik mekanizmasıyla alakası olmadığı anlaşılmıştır. Membran potansiyeli ve hücre büyüklüğü/hacmi ölçümlerinde de genin aşırı ifadelendiği hücrelerde ve mutant hücrelerinde farklı bir sonuca rastlanmamıştır. Ek olarak sitoplazmik pH ölçümünde de pHuolin plasmidi içeren mutant hücrelerin yabani tip maya suşuna gore pH değişimlerinin olmadığı gözlemlenmiştir

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CHAPTER 1

INTRODUCTION

1.1. Invasive Fungal Infections

In recent years, invasive fungal infections have increased significantly with the new interventions in preventive, symptomatic, and therapeutic medical area (Enoch, Ludlam, & Brown, 2006). The AIDS epidemic, anticancer chemo-therapy, haematopoietic stem cell and organ transplantation and new developments in antineoplastic and immunosuppressive agents, broad-spectrum antibiotic therapy, and prosthetic devices , and more aggressive surgery cause important morbidity and mortality in immune-suppressed patients because of invasive fungal infections (Letscher-Bru, 2003) Because of these complications due to invasive fungal infections, the importance of antifungal drugs has increased in terms of usage in modern medicine over the past 30 years. The number of systemic antifungal drugs is not so many. Even worse, many fungi have gained resistance to these drugs (Roemer & Krysan, 2014).

1.2. Antifungal Drugs

The therapeutic options for invasive fungal infections are quite limited when compared with the number of treatment options for bacterial infections. Three main classes of antifungal drugs are available: polyenes, azoles, and echinocandins used in medical area. Azoles and polynes from these three classes of antifungal agents had been introduced into the clinics until 1980 and then new class had been discovered: echinocandins (Figure 1.1.) (Butts & Krysan, 2012).

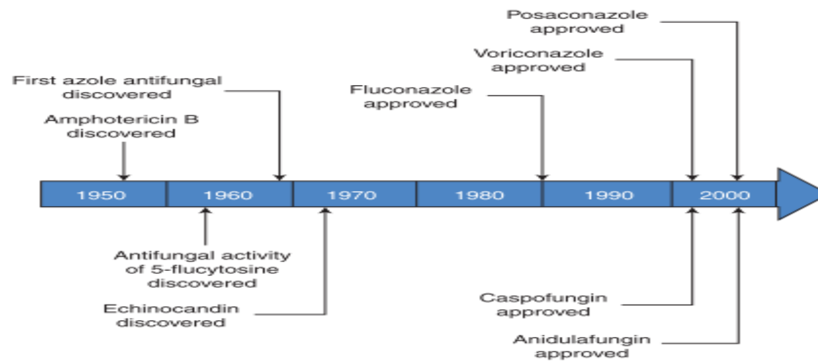

Figure 1.1. Time line showing key milestones of antifungal drug development (Source: Roemer & Krysan, 2014)

1.2.1. Polyenes

The oldest class of antifungal drugs; polyenes, has been used to treat systemic infections from the 1950s until the discovery of the azoles. Amphotericin B deoxycholate (Figure 1.2a) is the common antifungal agent among polyenes however the primary disadvantage of polyenes is the toxic effect so that its lipid-associated formulations had been developed. (Ghannoum & Rice, 1999)

1.2.1.2. Mechanism of Action

Amphotericin B is active against yeast and most filamentous fungi such as Candida species, Aspergillus species, Cryptococcus neoformans and dimorphic fungi except Candida lusitaniae, Candida guillermondii, Scedos- porium spp. and A. terreus (Enoch et al., 2006).

Target structure of Amphotericin B is plasma membrane. The interaction of AmB with ergosterol, a membrane sterol that is unique to fungi, results in the formation of aqueous porin channels.(Figure 1.2b) The configuration of pores composed of an annulus of eight amphotericin B molecules linked to ergesterol hydrophobically. The subsequent formation of pores causes changed permeability of membrane results in leakage of vital cytoplasmic components such as potassium ions and cause death of the organism at last (Ghannoum & Rice, 1999).

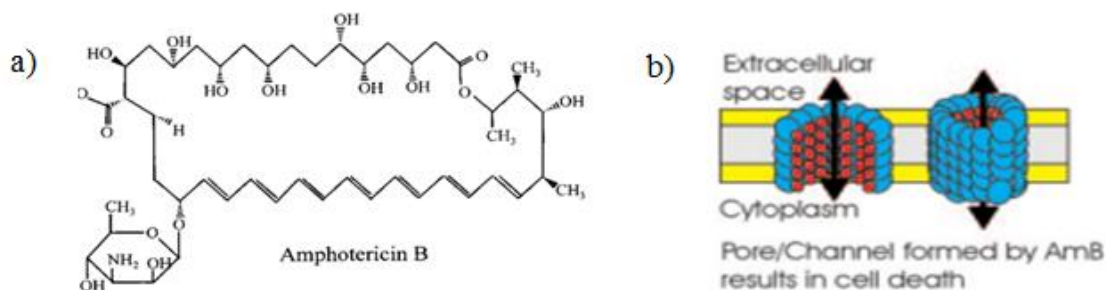

Figure 1.2. a. Structure of Amphotericin B, b. Mechanism of polyene action (Source:Mukherjee, Sheehan, Hitchcock, & Ghannoum, 2005)

1.2.2. Echinocandins

The echinocandins, which are lipopeptides, are newly discovered antifungal pharmaceutics. This antifungal agents are fungistatic toward Candida and Aspergillus species especially have important therapeutic for candidiasis but they have no fungicidal activity against zygomycetes or against Cryptococcus, Trichosporon, Scedosporium, and Fusarium species (Kanafani & Perfect, 2008).

The first example of echinocandins is Caspofungin (Figure 1.3a) which is a semi-synthetic water soluble lipopeptide. It is produced from a fermentation product of the fungus Glarea lozoyensis (Letscher-Bru, 2003)

1.2.2.1. Mechanism of Action

Caspofungin inhibits the synthesis of β(1,3)-D-glucan that is the most important component of the cell wall (Figure 1.3b). Mechanical strength and shape of the wall is determined by solid three dimensional structure of β(1,3)-D-glucan chains. Blocking of β(1,3)-D-glucan synthetase causes both fungistatic and fungicidal effects. Blockage of the cell wall synthesis reduces the fungal growth such as growth as pseudohyphae, thickened cell wall, and buds failing to separate from mother cells as a fungistatic effect. The most important fungicidal effect caused by the changes in the integrity of cell wall is becoming osmotically sensitive which leads to destruction of the fungal cell (Letscher-Bru, 2003).

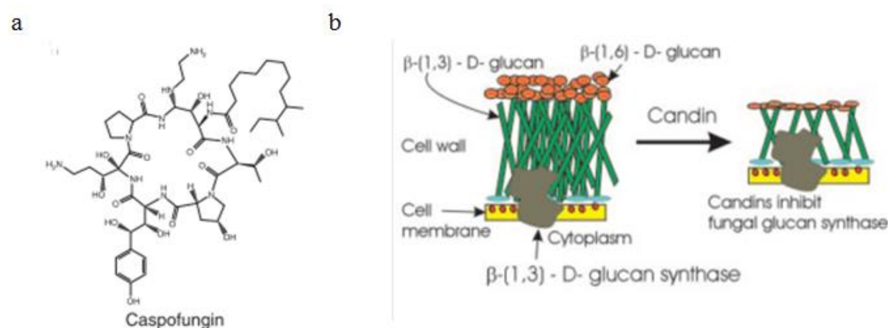

Figure 1.3. a. Structure of Caspofungin , b. Mechanism of echinocandin action (Source: Mukherjee et al., 2005).

1.2.3. Azoles

Azole class of antifungals was developed in the 1970s. Clotrimozole was the first azole but it has limited usage because of changeable concentrations in the blood. Later Miconazole was developed for the systemic infections. However its use was limited due to intravenously usage and it did not have more advantage than AmB. Ketoconazole is the first antifungal azole that can be taken orally and provide consistent blood intensity. It has an activity against Candida species except the C. glabrata and also has an affect against Coccidioides Histoplasma, Blastomyces, and dermatophytes. Another antifungal triazole is itraconazole which was firstly used in humans with oral and intravenous administration. Its in vitro activity is against most of Candida species, also Aspergillus spp. and dimorphic and dematiaceous fungi (Loeffler & Stevens, 2003; vanden Bossche, Marichal, Odds, Le Jeune, & Coene, 1992). The most widely used triazoles are fluconazole, posaconaze and voriconazole. (Figure 1.4a). Flucanozele is alternative triazole which has been used after chemotherapy or bone marrow transplantation to reduce the invasive candidiasis and also is used in the treatment of oropharyngeal and esophageal candidiasis in AIDS patients and also in neutropenic patients. Posaconazole actives against Aspergillus ,Cryptococcus and Coccidioides as well as Candida spp in vitro and in vivo (Loeffler & Stevens, 2003).

Voriconazole (Vfend, Pfizer) is a recently developed as a derivative of fluconazole and under investigating triazole . It was constructed by a fluoropyrimide grouping and the addition of a methyl group to the propanol backbone of fluconazole in place of one triazole moiety (McGinnis et al., 1997). Because of this excellent pharmacokinetics, it has effect against fluconazole-resistant Candida albicans, Candida

krusei and Candida glabrata apart from the effect on Aspergillus spp and several emerging moulds, including Fusarium spp. and Scedosporium spp. with both in vivo and in vitro activity (Nguyen & Yu, 1998). Therefore, it has been approved in Europe for the treatment of fluconazole-resistant invasive Candida infection and also is currently used as a best choice for Aspergillus and it is head-to-head clinical trial with Amphotericin B (Roemer & Krysan, 2014).

1.2.3.1. Mechanism of Action

Azoles have inhibitory function in ergesterol synthesis by binding to lanosterol demethylase which is a specific enzyme in ergosterol biosynthesis. Ergesterol is the most important sterol for membrane integrity as a bioregulator of membrane fluidity and asymmetry in the fungal plasma membrane. Lanosterol demethylase contains heme domain of which protein cocatalyzes cytochrome P-450-dependent 14α-demethylation of lanosterol (Hitchcock, Dickinson, Brown, Evans, & Adams, 1990). Azoles bind to its primary target site, heme domain, with a specific nitrogen atom in the azole ring to the iron atom of the heme domain. This interaction results in blocking 14α-demethylase function so ergesterol depletion and accumulation of sterol precursors such as lanosterol, 4,14-dim- ethylzymosterol, and 24-methylenedihydrolanosterol which are 14 α -methylated sterols occurs (Figure 1.4b). These changes in membrane content lead to altered structure and function of plasma membrane (Ghannoum & Rice, 1999). Fungal plasma lipids are also target for azoles. Azoles can be interact with the 3-ketosteroid reductase enzyme which has a role in methylsterol biosynthesis (Joseph-Horne & Hollomon, 1997).

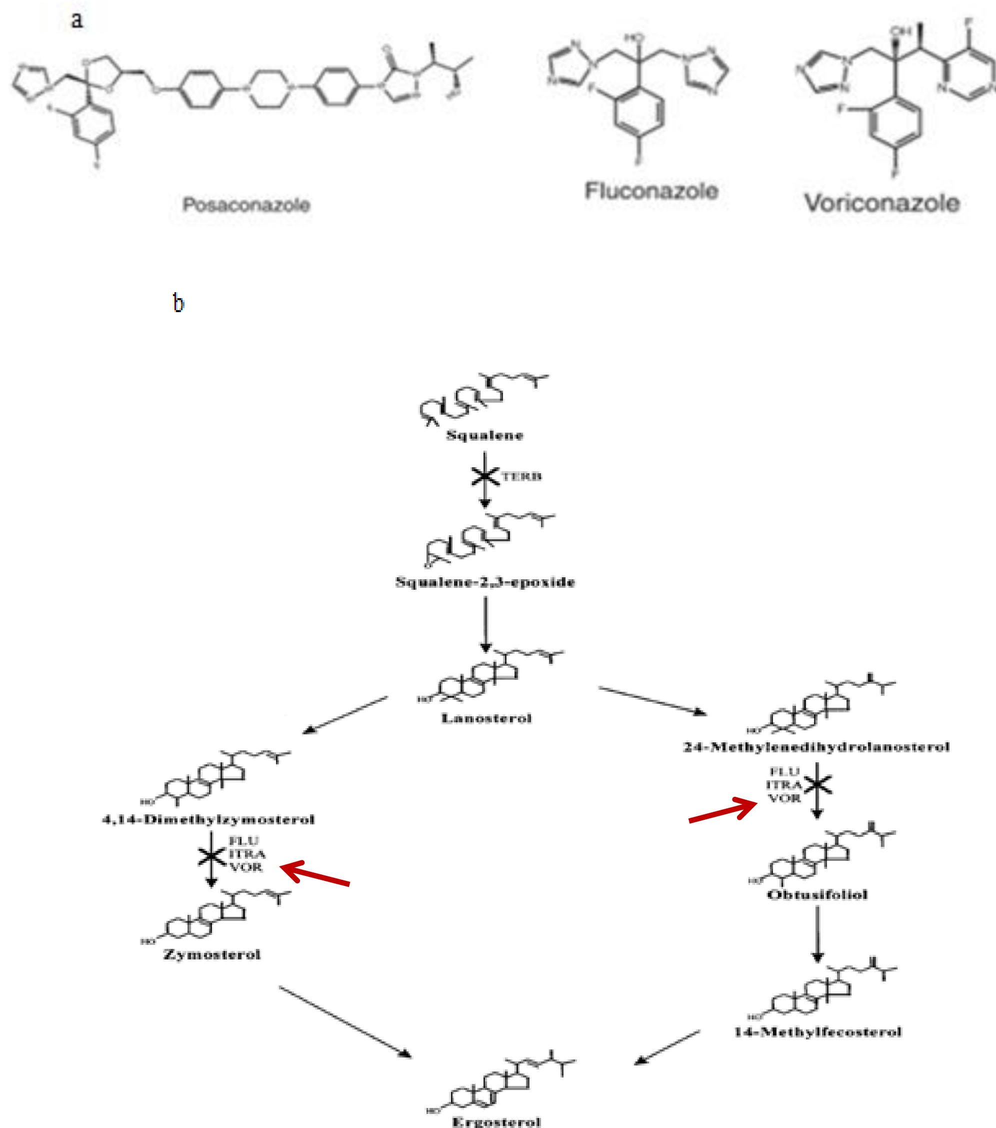

Figure 1.4. a. Structure of azole compounds (Posaconazole, Fluconazole and Voriconazole), b. Ergosterol biosynthetic pathway. Steps at which various antifungal agents exert their inhibitory activities are shown (Source: Ghannoum & Rice, 1999).

1.3. Antifungal Resistance

The number of drugs is not so many and due to resistance, new antifungal agents and new drug combinations should be developed. Recent studies show that fungal infections are resistant to azoles in 75 % of the cases, and to amphotericin-B in 34 % of the cases in AIDS and tuberculosis patients (Samie & Mashao, 2012).

Microbiological resistance means insensitivity of fungus to an antimicrobial drug by in vitro sensitivity test in its MIC of the drug and compared wih other isolates

of the same species. Resistance could be due to in vitro resistance which are primary(intrinsic) and secondary(acquired) resistance or clinical resistance. Primary resistance naturally found in some fungi that has never exposed to drug. Reversely, secondary resistance is gained after exposure of the organism to the antifungal agent and is usually dependent on altered gene expression (Rex et al., 1997). Clinical resistance means failure of a therapy for a certain indication and reversion of infection with an organism that is not associated with in vitro resistance. Changes in the host's immune system, such as neutropenia and poor bioavailability of the drug exposed can be given as examples for clinical failure (Rex et al., 1997).

1.3.1. Polyene Resistance

Changes in lipid composition of plasma membrane could be the reason for resistance to polyenes. For example defects in ERG3 gene which involves in ergesterol biosynthesis result in reduction of ergesterol and accumulation of other sterols in the fungal membrane. This situation leads to binding site deficiency for AmB (Kanafani & Perfect, 2008). The other reason for the resistance come up with the increase in the stability of the cell wall with the alteration in the β-1, 3 glucans content in the fungal cell wall. Thus, larger molecules such as AmB entrance to the plasma membrane are influenced by the cell wall (Loeffler & Stevens, 2003).

1.3.2. Echinocandin Resistance

FKS gene codes for the catalytic subunit and RHO 01 gene codes for the regulatory subunit of of β(1,3)-D-glucan synthase which is the principal target of echinocandin. Specific mutation on these genes in S. cerevisiae or C. albicans cause high level resistance to caspofungin, and all other echinocandins. Additionally, mutations in GNS1 gene, another cell wall synthesis gene whose enzyme has a role fatty acid elongation confers low level resistance. Further studies should be done to improve to figure out of these mechanisms of echinocandin resistance (Ghannoum & Rice, 1999; Letscher-Bru, 2003)

1.3.3. Azole Resistance

According to resistance mechanism, resistance to an azole can be lead to cross resistance to other azoles or can be azole specific. Four different resistance mechanisms shown in Figure 1.5 which are decreased drug concentration, up-regulation of target enzyme, target site alteration and development of bypass pathways have been described in Candida species.

Drug concentration is decreased due to change in efflux pumps activity. CDR genes and MDR genes are the transporter genes have role in efflux pumps in Candida species (vanden Bossche et al., 1992). Up regulation of CDR1, CDR2, and MDR1 has been confirmed in azole-resistant C. albicans. CgCDR1 and PDH1 transporter genes in C. glabrata and CdCDR1 and CdMDR1 transporters in *Candida dubliniensis* have also been demonstrated as a transporter genes active role in resistance. In fact, CDR gene up-regulation causes resistance to almost all azoles (Sanguinetti et al., 2005).

Higher intracellular concentrations of some target proteins such as ERG11p or enzymes such as 14α-demethylase can be the other reason for reduced susceptibility. C.galabrata strain resistant to fluconazole and itraconazole according to overexpression of lanosterol demethylase. Upregulation of this enzyme may trigger much more ergesterol biosynthesis (vanden Bossche et al., 1992). In some Candida isolates, antifungal agent has no longer activity in ergosterol synthesis inhibition in the higher concentration of ERG11p. This overexpression of the target can be the reason of gene amplification, increased transcription rate or reduced degradation of the gene product. Also mutations in the gene at which proteins are target sites for antifungal agents prevent binding of azoles to enzymatic site. ERG11 mutation in C.krusei isolates can be given as an example of intrinsic resistance fluconazole due to target site alteration (Kanafani & Perfect, 2008).

Resistance mechanisms to azole compounds among Aspergillus species is not so common except fluconazole, however reduced intercellular concentration of itraconazole due to expression of efflux pumps and modification of the 14a- sterol demethylase enzyme are the two prevalent resistance mechanisms (Kanafani & Perfect, 2008).

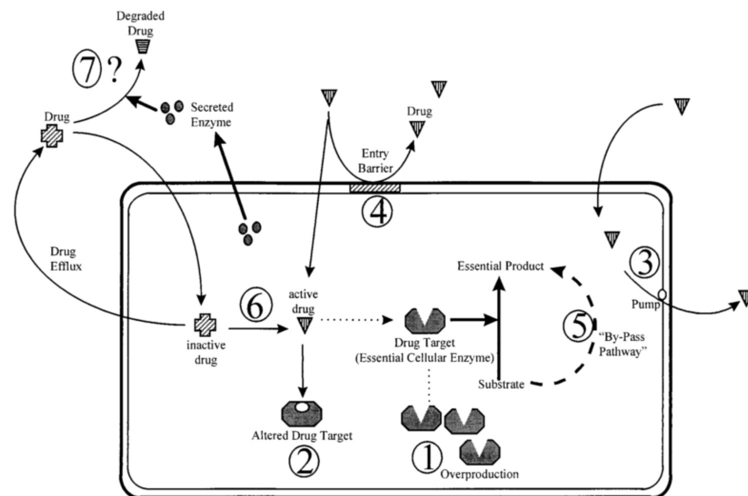

Figure 1.5. Resistance mechanisms that microorganisms might develop
(Source: Kanafani & Perfect, 2008)

1.4. Yeast as a Model for Drug Research

The budding yeast *Saccharomyces cerevisiae* whose genome was completely sequenced in 1996 is a preferred eukaryote as a model system. Yeast genome can be manipulated easily and their growth is technically easy as 90 minutes of life cycle as well as being economical. (Bharucha & Kumar, 2007; Sherman, 2002) . Accessibility to many functional genomic tools for this organism and also extensive database from SCD(Saccharomyces Genome Database) makes this organism valuable for genome wide analysis of biological, functional and chemical screenings (Menacho-Marquez & Murguia, 2007). In addition to accessibility to approximately 6400 open reading frames, 50% of the genes relevant to human diseases have yeast orthologous and 31% of the proteins in yeast genome have human orthologous. All these qualities make yeast perfect model organism for drug research (Menacho-Marquez & Murguia, 2007) .

The reason to choose S. cerevisiae as a model organism for this research instead of other fungi which are more related to antifungal drugs such as Candida or Aspergillus spp, yeast have stable haploid and diploid forms and it is nontoxic organism when compare with the Candida species that will be used later in the clinical part of the research.

1.5. Aim of the Study

In this study, we aimed to identify new resistance genes against Voriconazole by genome wide screening in S. cerevisiae. Also we tried to determine not only the resistant genes but also the other pathways that confer resistance to the cell, such as general stress-response genes, drug efflux pumps and metabolic genes. There are not so many studies to identify resistance for especially Voriconazole resistance mechanisms, so data from this study should be expanded. Drug resistance does not coming out because of single biochemical mechanism, and multiple mechanisms and interactions of many factors needed to be investigated. Elucidation of antifungal drug resistance mechanisms will help us to use these drugs more effectively, and discover potential targets for developing new drugs and inhibitors.

CHAPTER 2

MATERIAL AND METHODS

2.1. Determination of Lethal Dose

Wild type yeast strain BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was plated on YPD agar medium plates each includes different concentration of Voriconazole . The least concentration that the cells could not grow was determined. This lethal dose was used in all genomic screening experiments.

2.2.Genomic Library Screening

Commercialy obtained yeast genomic library (ATCC No.37323) which contains randomly restricted DNA fragments of the yeast genome in YEP13 plasmid, has leucin selection marker (LEU2), was transformed in BY4741 haploid yeast cells by using standart LiAc technique (Yeast Genetic Techniques-Cold Spring Harbour). Then these transformants grown on Yeast Nitrogen Based (Synthetic dextrose (SD), 2% Glucose) media lacks the leucine aminoacid and includes the lethal dose of Voriconazole. Resistant colonies that could able to grown on Voriconazole included plates were selected and re-inoculated overnight in Voriconazole included liquid media for confirmation of resistance.

All the experiments were performed by using Yeast Nitrogen Based media that either lacks the indicated corresponding aminoacid or not.

2.3. Bacterial Transformation, Plasmid Isolation and Sequencing

Plasmid isolation was performed from drug resistant colonies. Isolation was started with lyticase treatment from overnight growth yeast culture for 30 minutes and then plasmid isolation kit (Thermo-Molecular Biology-GeneJET Plasmid Miniprep Kit) was used. These isolated plasmids were amplified by JM109 E.coli competent cells via

heat shock procedure. Later, amplified plasmids were transformed back to BY4741 (haploid) wild type yeast cells. After 2-3 days incubation at 30^0C, transformed cells were tested by spot assay. Yeast-back strains were grown overnight and 1:5 dilutions was done in the morning. When yeast cells could be obtained at log phase after 3-5 hours incubation at 30^0C, yeast cells were washed twice by dH_2O and their OD_{600} values were arranged into 0.2. OD600 values were set to 0.2, 0.02, 0.002, 0.0002 by serial dilution with dH_2O Then 5µl of the serial diluted cells were spotted on agar plates which include different concentration of Voriconazole for the spot assays.

After confirmation of the resistance caused by plasmid DNA according to spot assay, gDNA cassettes in Yep13 plasmids were sequenced by using YEP13 primers and DNA sequencer ABI3130xl with ABI PRISM sequencing analysis v5.1 program.

2.4. Determination of Possible Resistant Gene

Genes from sequenced gene cassettes were determined by nucleotide-nucleotide BLAST program of NCBI. Before cloning the genes with possible resistance functions, one gene was determined from each gene cassette. For this determination each gene, was provided commercially from Thermo Scientific Open Biosystems Yeast ORF Collection (Figure 2.1) , was tested in Voriconazole included solid media. ORF plasmids of each candidate genes were transformed to yeast cells by LiAc technique (Yeast Genetic Techniques-Cold Spring Harbour) and grown and picked colonies were incubated 3 hours in the presence of liquid YNB-ura (-glucose, +2% raffinose) and then 3 hours in YNB-ura (-glucose, +2% galactose). Later, that cells were spotted onto YNB-ura (-glu, +gal) plates including different concentration of voriconazole.

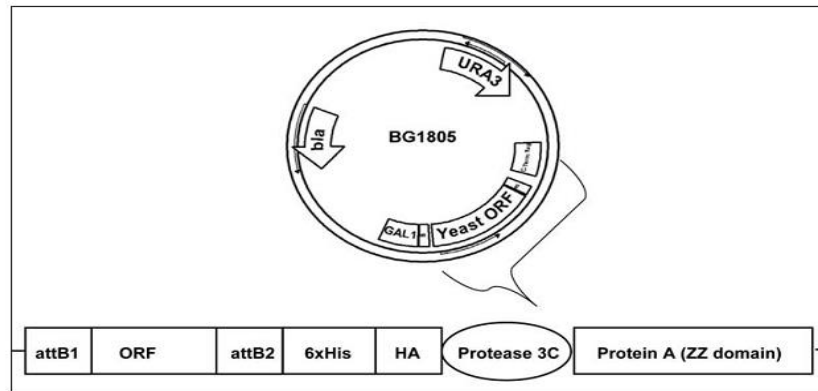

Figure 2.1. Schematic picture of BG1805 plasmid that provided from Thermo Scientific Open Biosystems Yeast ORF Collection (Source: Description, P. (n.d.). Thermo Scientific Open Biosystems Yeast ORF Collection, *3867)*.

2.5. Deletion Mutant Examination

Sensitivity of deletion mutants of determined drug resistant genes were observed by spot assay and growth curve test. Deletion mutant yeast cells were plated on YPD solid media including Voriconazole. Also these haploid knokout cells were tested in voriconazole including YNB+all media (+metionin, +leucine, +uracil, +histidine) instead of YPD.

2.6. Cloning

Possible resistant genes that were determined from spot assay were cloned into pAG425GPD-ccdB Gateway destination vector with leucine marker by using Gateway cloning system (Invitrogen) which bases on homologous recombination (Figure 2.2). For transformation part of the cloning procedure E.coli OMNI cells were used. Cloned plasmids were restricted by BSRGI enzyme and sequencing analysis was performed for confirmation.

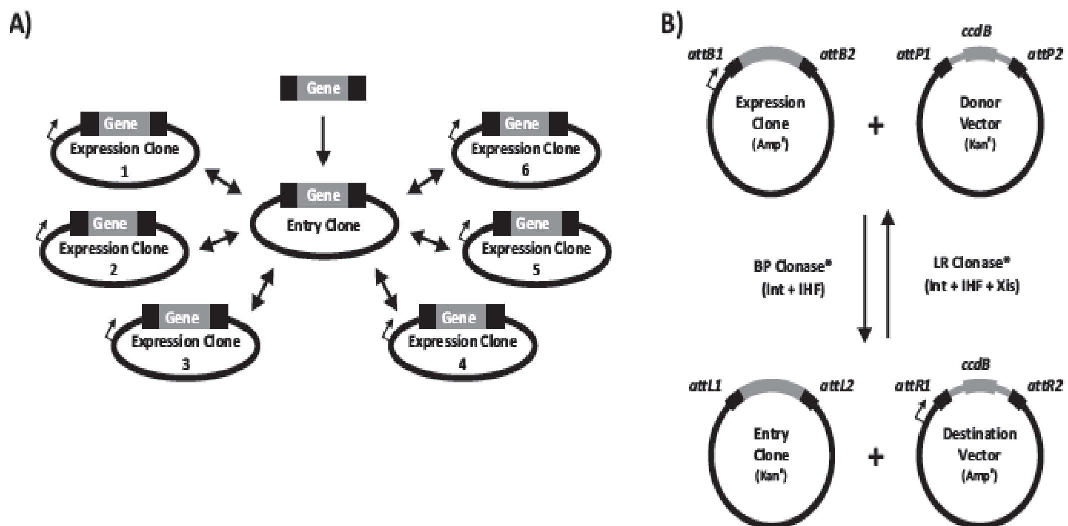

Figure 2.2. Gateway Cloning System; single step BP/LR combined Gateway reactions (Source: Liang, Peng, Baek, & Katzen, 2013)

2.7. Overexpression Assays

2.7.1. Spot Assay

Cloned genes were transformed into BY4741 (haploid) wild type cells and plated on selective media lacks leucine. Growth colonies were selected and spot assay was performed on selective media with toxic Voriconazole concentration to confirm the resistance. Each cloned genes also transformed into their related deletion mutant cells to confirm whether they provide resistance for drug sensitive cells.

2.7.2. Gradient Spot Assay

Overexpression tests also were done by gradient-spot assay. Gradient plate includes maximum toxic level of drug one side and minimum level of drug on the opposite side. In other words, the plate was prepared as the drug concentration was increased from one side to another. Differently from one-drug concentration spot assay with serial diluted spots from OD_{600}:0.2 , for gradient spot assay only OD_{600}:0.02 cells were spotted on the plate from one side to another

2.7.3. Growth Curve

For the confirmation of the spot assay, growth curve was performed. Transformants were grown overnight and after 3-5 hours from dilution, their OD_{600} was set up to 0.1 within YNB-leu media containing 150µg/ml Voriconazole and during 28 hours, the OD_{600} values were measured in every 3 hour.

2.8. Transcriptional Analysis of Resistant Genes

2.8.1. RNA Isolation and cDNA Synthesis

For RNA isolation, drug treated and non-treated log phase overexpression transformant cells and wild type BY4741 cells were used. The cells were exposed to half of toxic dose voriconazole for 2 hours and drug treatment was not use for control RNA. Total RNA was isolated by using RNA isolation kit (Thermo-Molecular Biology-GeneJET RNA Purification kit) following the manufacturer's protocol. Purity and concentration of isolated RNA was determined by nanodrop (ND-1000 Spectrophotometer). After DNaseI treatment, cDNA synthesis from these isolated RNA was performed by using cDNA synthesis kit (Thermo-First Strand cDNA Synthesis kit) as described by the manufacturer.

2.8.2. Real-Time PCR

Real-Time primers were designed acording to resistant genes and cDNA of possible resistant genes and BY4741-WT yeast cells as a template that were either exposed to voriconazole or not were used for real-time PCR analysis to check expression against voriconazole. SyBrGreen dye (SybRGreen/ROX enzyme mix-Thermo) and designed primers according to resistant genes and Actin primer as a control was used for reaction. Real-time PCR was done in Bio-Rad IQ5 program. Conditions for the reaction were pointed out in Table 2.1. The results were analyzed according to $\Delta\Delta Ct$ method.

Table 2.1. Real Time PCR reaction conditions

Step1			
	1 Cycle	95^0C (initial deneturation)	10:00 min
Step 2			
	30 Cycle	95^oC (denaturation)	00:15 min
		60^oC (annealing)	0:30 min
		72^oC (elongation)	0:30 min

2.8.3. Microarray Analysis

Isolated and DNase treated RNA from BY4741-WT strain that both voriconazole treated and without drug treatment was used for microarray analysis. Microarray Analysis was achieved by using 'Agilent-Yeast-One-Color' microarray and raw data was taken by Agilent Feature Extraction Software. At first preprocessing step was performed to remove the background values and normalization was done by quantile method. Later; Principal Component Analysis (PCA) was applied to these preprocessed data to check the similarity between Voriconazole treated and non-treated samples. According to clustering of these arrays, GO (Gene Ontology) Analysis was applied to specific region of this cluster in terms of both biological process and molecular function. In other words, 288 genes from this region was examined in order to understand what kind of properties of these genes are similar.

2.9. Functional Analysis of Resistant Genes

Functional analysis experiments were performed in Department of Membrane Transport, Institute of Physiology AS CR, as part of agreement with The Czech Academy of Sciences. Determination of membrane transport, pH measurement, spot assays to understand ion exchange and size-volume measurements were done in this institute.

2.9.1.Spot Assay

Possible resistant transformats and haploid deletion mutant cells were tested by spot assay in solid media with different concentration of chemicals. These chemicals are 1M and 1,3M NaCl, 0,3M and 0,5M LiCl, 100µg/ml and 150µg/ml Hygromycin B, 1mM spermine, 0,4M TMA and 0,7M TMA. Spot assay precedure that explained before was used. However 5 dilutions were done ans spots were put on solid media by replica plate.

2.9.2. Membrane Potential Measurement

Overexpression strains and mutant strains were measured so inoculation overnight of the cells was done with the media YPD for mutants and YNB-leu according to plasmid marker for overexpressions. Dilution was done 1:10 in the morning and in the afternoon when the OD was 0.5 preparation of the cells was started for membrane potential measurement. After washing procedure by dH_2O, yeast cells were re-suspended in MES-TEA buffer (pH was adjusted 6 by Triethanolamine) until final OD was 0,2 in 3 ml PMMA cuvettes. Before measurement, 12µl ($4x10^{-8}$ M) dye 3,3'-dipropylthiadicarbocyanine [diS-C_3(3)] was added to each cuvvette. Measurement was done by Photon Counting Spectrofluorometer (PCI). Parameters that used during measurement are indicated below.

Excitation wavelength: 531nm right emission wavelength: 590nm

Excitation dark: 141.500 emission dark: 21.58

2.9.3. Cytoplasmic pH Measurement

To examine the pH response of the cells without resistant genes, haploid deletion mutant cells was used for cytoplasmic pH measurement. pH changes was detected by pHI-U (URA marker, PROMadh1-PhLUORİN) plasmid.

2.9.3.1. pHI-U Transformation

pHoulorin plasmid was transformed into deletion mutant yeast cells by electroporation. Overnight inoculated yeast cells were diluted in the morning and when their OD was reached 0, 5 pHoulorin plasmid was transformed into deletion mutant yeast cells by electroporation. Overnight inoculated yeast cells were diluted in the morning and when their OD_{600} was reached 0.5, cells were centrifuged and washed with dH_2O and centrifuged again. Resuspention was done by 8ml with 25mM DTT. After 15 min incubation at room temperature while shaking gently, cells were precipitated by 4^0C centrifuge. Again washing procedure was applied by cold dH_2O. Yeast cells were resuspended twice respectively 5ml and 0,5ml cold TpEB which was prepared before with 10mM Tris-HCl (pH=7,5) , 0,1M $MgCl_2$ and 270mM saccharose. Each 100ul cell suspension was put in electroporation cuvettes (0,1cm) and also 0,3ul plasmid (pHI-U) was added into cells. 625V electroporation was applied for 24msec to plasmid and cell mixture for transformation. Then, 100ul cold H_2O was added in each cuvette. After 15 minute incubation at room temperature, yeast cell suspension was plated in YNB-ura solid media and incubate overnight at 30 ^0C. Growth colonies were picked and inoculate in agar medium. To confirm the cells could take the fluorescent plasmid, each strain was observed under Olympus fluorescent microscope with 100X image and also their 3D images (Nomarki images) were taken.

2.9.3.2. pH Measurement

Cells from picked colonies were growth in 10ml YNB-FpH to the OD600 =0,2. YNB-FpH media includes YNB-FpH, $(NH_4)_2SO_4$, glucose and auxotrophic supplements without uracil. This media was not autoclaved, sterilization was done by filtration. Calibration buffers found in laboratory whose pH are 5.69, 6.12, 6.46, 6.62, 6.81, 7.04, 7.31, 7.77 were prepared with Na_2HPO_4 and citric acid by using McIlvain's system. For calibration, BY4741 yeast cells were treated with digitonin. Calibration buffers and digitonin treated cell suspension was put in Elisa plate. Two blank was constructed with cells with empty vector not pHlourin. First blank included digitonin treated cells and PBS. The second blank vzorky had only growing cells with empty vector. Growing cell suspensions which will be measured also were put in Elisa-plate

in designed order. Incubation of cells with calibration buffers was done. Measurement of Elisa plate was performed in the fluorescent reader Synergy HT, Biotek. Intensity of fluorescence was 485nm and emission was 516nm. Results were designed according to Software Gen and Excel.

2.9.4. Size Measurement

Overexpression strains, mutant strains and empty vector pAG425 included mutant strains were cultured. When OD_{600} was reached 0.5, size and volume measurement was carried out by using CASY machine. At first background was measured with growth media. Later, 10 ml yeast cells were put in CASY cap with 10ml CASY buffer. CASY machine measure the samples 3 times and data was transformed to CASYexcell2.3.

2.10. Cross- Resistance Analysis

Resistant genes were compared in other drugs which are in the same group and different groups with Voriconazole to see if the resistance is specific or can be useful with different mechanism of action. Yeast cells with resistant genes were spotted on gradient plates which includes other drugs; Caspofungin, AND, AmB, Naftifin and Ketoconazole and incubated at 30^0C for 2-3 days. Then photos were taken to analyze the results.

2.11. Candida Homolog Analysis

2.11.1. Spot Assays

Homolog of S.cerevisiae resistant genes from C. albicans was found and they were amplified with PCR method by using specific primers. Gateway cloning system was used to clone amplified C.albicans genes into pAG425-GDP plasmids. These plasmids were again transformed into S. cerevisiae and spot assay were carried out in 150µg/ml Voriconazole included media.

2.11.2. Real- Time PCR

C.albicans strain were exposed to half of the minumum inhibitory concentration of Voriconazole and then RNA isolation was done and cDNA was constructed from both drug exposed and non-exposed cells. SyBrGreen dye (SybRGreen/ROX enzyme mix-Thermo) and designed primers according to resistant genes and Actin primer as a control was used for reaction. Real-time PCR was done in Bio-Rad IQ5 program. Conditions for the reaction were pointed out in Table1. The results were analyzed according to $\Delta\Delta Ct$ method.

2.12. Statistical Analysis

For real-time PCR and microarray analysis student's t-test was used for all statistical analysis. Also Benjamini-Hochberg correction was applied for microarray analysis.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Determination of the Toxic Concentration of Voriconazole

Lethal dose of Voriconazole for wild type BY4741 yeast strain was determined by testing cell growth on different concentration of Voriconazole included agar plates. First, drug concentration interval of 5µg/ml to 50 µg/ml according to literature was screened for two days and growth inhibition could not been observed (Figure 3.1.). Later, 75µg/ml and 100µg/ml Voriconazole were tested and no significant cell growth was observed (Figure 3.1.). 100 µg/ml was determined as a lethal dose of Voriconazole for BY4741 wild type strain.

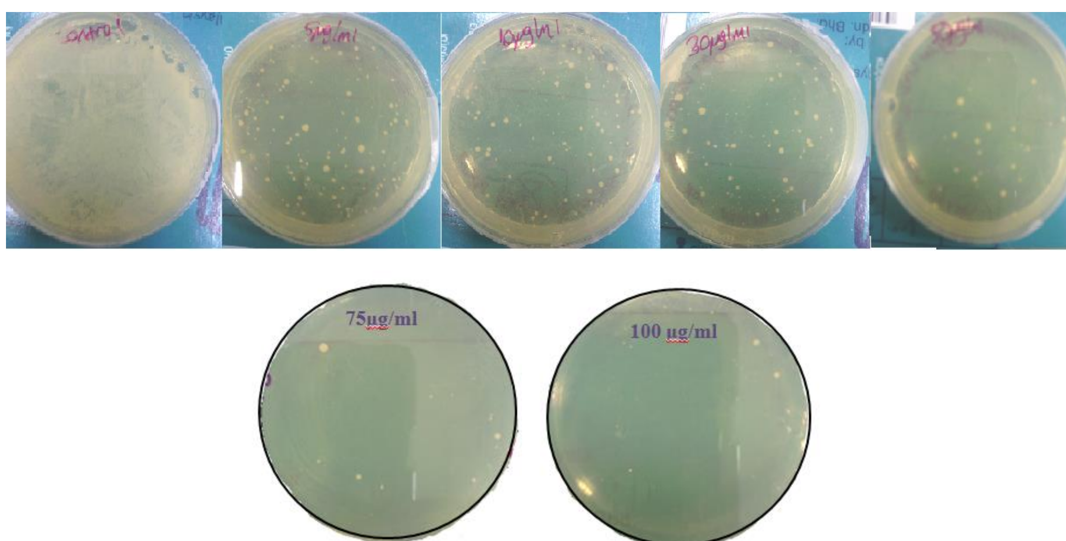

Figure 3.1. Lethal dose determination on YPD agar plates (0 µg/ml, 5 µg/ml, 10µg/ml, 30 µg/ml, 50 µg/ml and 75 µg/ml, 100 µg/ml Voriconazole)

3.2. Identification of the Drug Resistant Genes

3.2.1 Determination of Resistant Colonies by Genomic Library Screening

The genome-wide screening was achieved by using *S.cerevisiae* yeast gDNA library which was constructed by cutting with Sau3A enzyme and then ligating into YEp13 plasmid which was restricted by BamHI enzyme (Nasmyth & Reed, 1980). These plasmids with DNA fragments was transformed to BY4741 yeast cells and then grown on YNB-leu media including the lethal dose 125µg/ml Voriconazole to choose the resistant colonies (Figure 3.2.). Resistant colonies were chosen and re-inoculated overnight in liquid media including 150 µg/ml Voriconazole for confirmation of resistance.

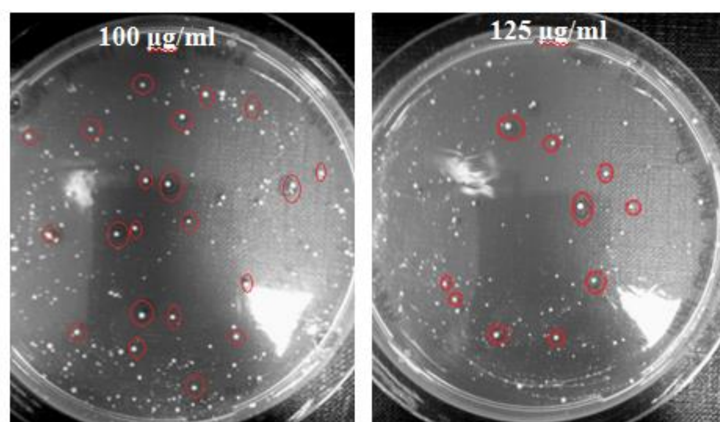

Figure 3.2. BY4741 wild type cell colonies transformed with genomic library and showed resistance to 100µg/ml and 125µg/ml Voriconazole

3.2.2. Resistance Against 150µM Voriconazole

Only seven cultures among all re-inoculated colonies could growth in 150 µM Voriconazole. Those colonies were grown on selective media and yeast plasmid isolation was done from these colonies. Isolated plasmids were amplified in JM109 E.coli competent cells to obtain required amount of plasmid DNA for sequencing.

To confirm the resistance caused by plasmid DNA; amplified plasmids were transformed back to BY4741 wild type yeast cells. According to spot assay three yeast colonies exhibited resistance to 150 µg/ml Voriconazole. (Figure 3.3)

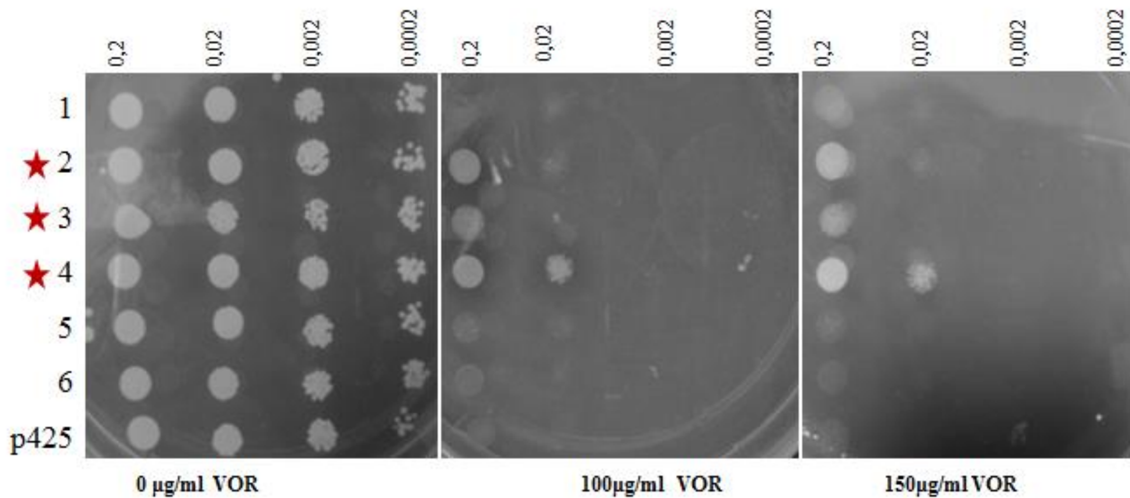

Figure 3.3. Spot assay results from expected resistant colonies that have yeast back plasmids.

3.2.3. Sequencing Results of the 150µM Voriconazole Resistant Colonies

Plasmids of final resistant colonies were sequenced and gene cassettes that include candidate genes was determined. Two of resistance colonies gave the same gene cassette. (Figure 3.4.). The genome cassettes were evaluated via BLAST analysis of Saccharomyces Genome Database (SGD). Plasmids from genomic library may include more than one gene because of random restriction. According to results, resistant cassettes include two genes. Candidate genes were selected from each cassette for transcriptional and functional analysis. (Table 3.1.)

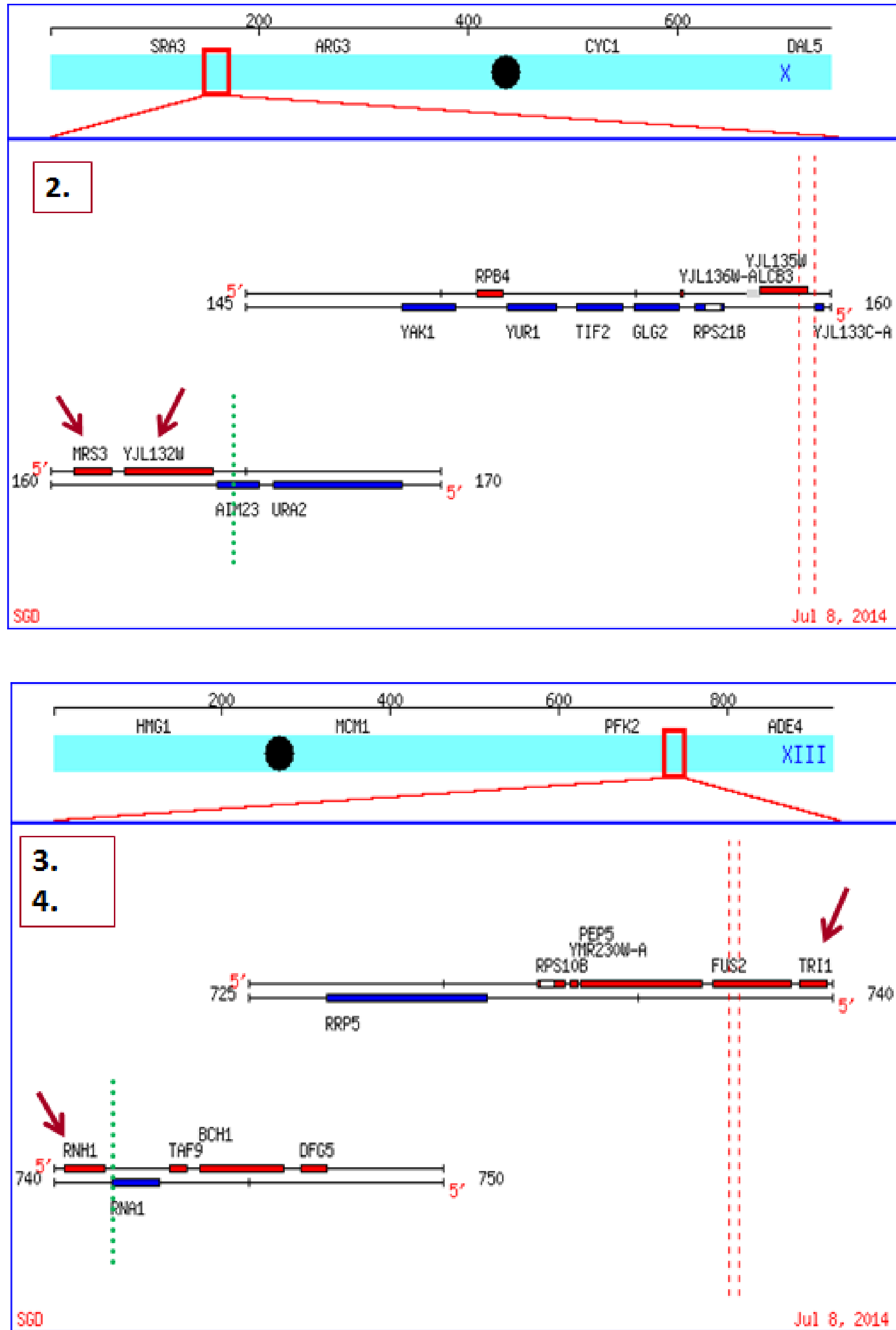

Figure 3.4. The genomic expression cassettes that show resistance against 150µg/ml Voriconazole. The arrows indicate the genes that determined to be cloned primarily, Double red line refers to sequenced region; Green line refers to border of the gene cassette.

Table 3.1. Display of genes that showed resistance to 150μM Voriconazole and function of these genes.

SYSTEMATIC NAME	GENE NAME	DESCRIPTION	FUNCTION
YMR233W	TRI1	-	Non-essential sumoylated protein of unknown function; similar to components of human SWI/SNF complex including SMRD3; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm, nucleus and nucleolus; TRI1 has a paralog, UAF30, that arose from the whole genome duplication
YJL132W	-	-	Putative protein of unknown function; localizes to the membrane fraction; possible Zap1p-regulated target gene induced by zinc deficiency; YJL132W is a non-essential gene
YJL133W	MRS3	Mitochondrial RNA Splicing	Iron transporter, mediates Fe2+ transport across inner mito membrane; mitochondrial carrier family member; active under low-iron conditions; may transport other cations; MRS3 has a paralog, MRS4, that arose from the whole genome duplication
YMR234W	RNH1	RNase H	Ribonuclease H1; able to bind double-stranded RNAs and RNA-DNA hybrids; associates with RNAse polymerase I

3.3. Determination of Possible Resistant Gene

To understand which gene provides resistance, each cloned gene was tested in the presence of Voriconazole. Candidate genes were transformed into wild type BY4741 yeast cells and induced by galactose since the plasmids had gal promoter. Next, cells were spotted onto YNB-ura (-glu, +gal) gradient plate including 150 μg/ml Voriconazole. According to spot results, MRS3 from one gene cassette and TRI1 gene from other gene cassette was determined as resistant genes because of their significant growth when compared with the empty plasmid carrying(pAG426) cells. (Figure 3.5.)

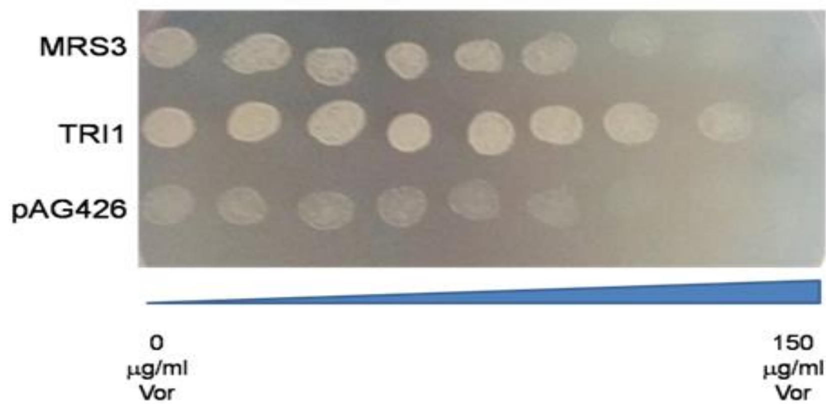

Figure 3.5. Gradient spot assay results that show the resistance in the presence of Voriconazole.

3.4. Sensitivity Assay

To show the sensitivity of the yeast cells without selected resistant genes, deletion mutants and wild type strain growth were tested by performing gradient spot assay on 150 μg/ml Voriconazole containing plates. Although growth of mutant cells was expected less than wild type, their growth was almost the same (Figure 3.6.)

Figure 3.6. Sensitivity test for mutant forms of candidate genes

3.5. Cloning of the Selected Genes

Voriconazole resistant candidate genes were cloned into pAG425GPD-ccdB plasmid (with selective Leu2 marker) via Gateway technology. Simple schematic explanation of both BP and LR steps was indicated below (Figure 3.7.) Cloning results were confirmed by both restriction with BSRGI enzyme (Fermentas) and sequencing analysis. Empty plasmid pAG425GDP-ccdb gives 4 bands (4949bp, 2582bp, 1286bp,

402bp) when restricted with BSRGI enzyme. If the gene integrated into plasmid, band pattern expected after BsrGI restriction is at 4949bp, 2582 bp and the bp of the gene of interest, if the gene is not restricted with BsrGI. Cloning of our candidate genes was successful according to BsrGI restriction pattern of the cloned genes (Figure 3.8.) and also to sequence analysis confirmed the successful cloning.

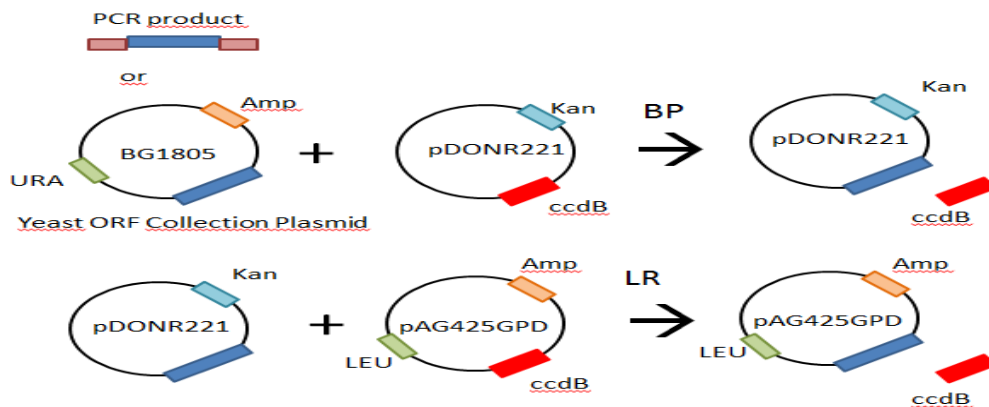

Figure 3.7. Gateway cloning steps, Amp: Amphicillin, Kan: Kanamycin, LEU : leucine, ccdB: toxic gene

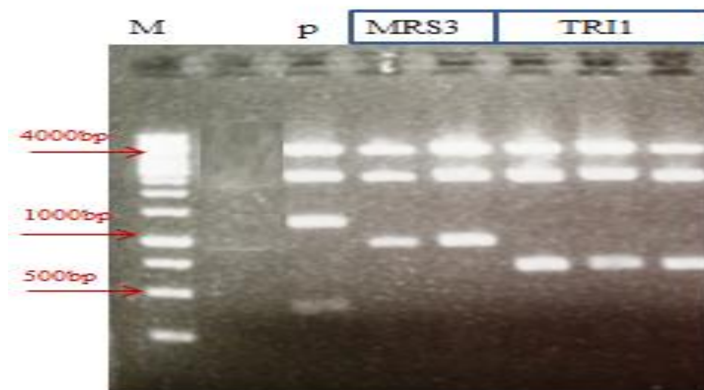

Figure 3.8. The BsrGI restriction pattern of the cloned genes. MRS3 (942bp), TRI1 (678bp), p indicates pAG425GDP-ccdb plasmid restriction results and M shows 1kb DNA marker.

3.6. Overexpression Assays

3.6.1. Spot Assays

After confirmation of cloning by restriction and sequencing, cloned genes were transformed back into BY4741 wild type strain. Their resistance was examined on 75µg/ml and 150 µg/ml Voriconazole containing media by spot assays. Growth of yeast transformants carrying MRS3 and TRI1 genes were compared with control samples. MRS3 and TRI1 expressions were slightly different when compared with pAG425. Also expression of yeast back plasmids was higher in Voriconazole (Figure 3.9.a).

Gradient spot assay was also carried out to check the resistance on decreasing Voriconazole concentration. High copy expression MRS3 gene resulted in better growth in Voriconazole with reference to empty plasmid carrying cells (Figure 3.9.b). There was no significant difference in TRI1 gene expression. (Data not shown)

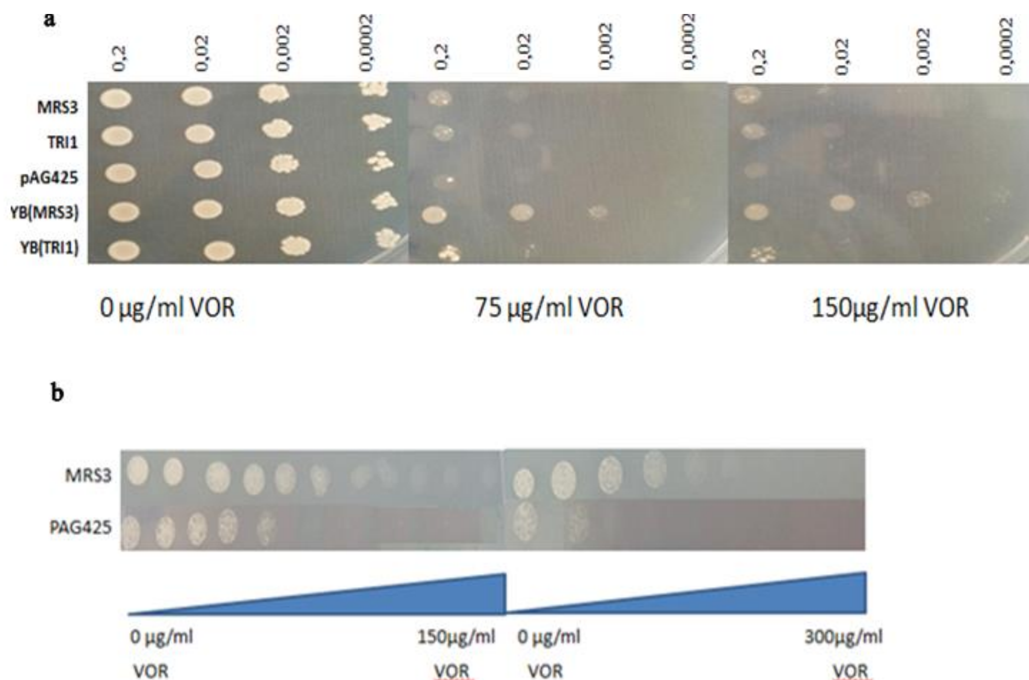

Figure 3.9. a. Spot Assays of cloned genes and yeast back plasmids on 75µg/ml and 150 µg/ml Voriconazole b. The Gradient-spot Assays of the MRS3 gene and empty plasmid on 150µg/ml and 300µg/ml Voriconazole.

3.6.2. Growth Assay

Spot assay results were tried to be confirmed by growth curve analyses. Only small a difference between MRS3 gene overexpression and plasmid-only sample was observed in growth curve analyses (Figure 3.10.)

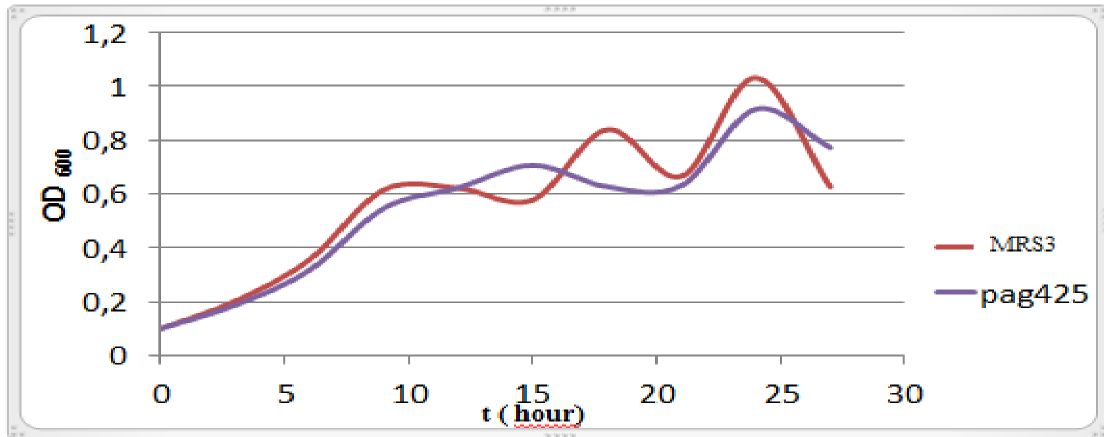

Figure 3.10. Growth curve assay of MRS3 and only plasmid transformants

3.7. Transcriptional Analysis

3.7.1. Real-Time PCR

For Real-Time PCR analysis half of the minimum inhibitory concentration was (75 µg/ml) used. Then RNA was isolated from cells that were either treated with 75µg/ml Voriconazole or not. For internal control, actin gene of *Saccharomyces cerevisiae* was used. Expression of genes from Voriconazole treated and non-treated cells were compared based on actin expression. Percentage of MRS3 and TRI1 expression levels were indicated in Figure 3.11. by comparing expression of these genes in wild type . Up-regulation was determined in MRS3 and downregulation was observed in TRI1 overexpression strains when treated with Voriconazole for 2 hours.

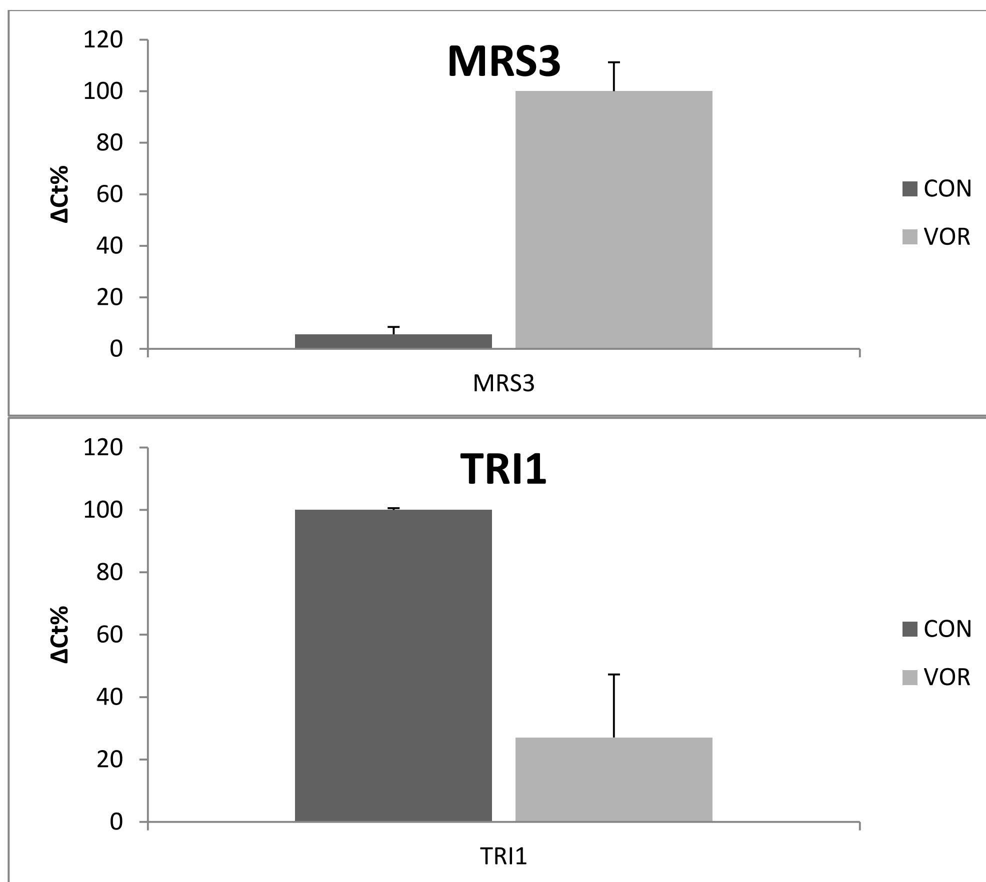

Figure 3.11. Real-Time Results

3.7.2. Microarray Analysis

Microarray analysis was performed by using Agilent-One-Color-Yeast Expression array. 766 genes were differentially regulated in Voriconazole exposed strain. 421 of these genes were up and 345 of them down regulated (2-fold or higher). The biological functions of the genes that were affected from Voriconazole treatment were identified (data not shown). According to microarray results, MRS3 and TRI1 were not in the up or down regulation gene list because the results only show at least 2 fold changes. GO biological analysis showed that Voriconazole increased the expression of many genes which were related to cellular development, reproduction, sporulation, cell wall assembly and thiamine metabolic process. In addition, some genes involved in sterol, alcohol and hyroxy compounds metabolism and also cell wall organization were down-regulated in the presence of Voriconazole. Biological processes of Up-genes and down-genes were represented in Table 3

Table 3.2. GO Biological Analysis Results

GO Biological Process Term for UP-genes
fungal-type cell wall assembly
meiotic cell cycle process
ascospore wall assembly
spore wall assembly
spore wall biogenesis
ascospore wall biogenesis
cellular developmental process
developmental process
cell wall assembly
ascospore formation
cell development
sporulation resulting in formation of a cellular spore
anatomical structure morphogenesis
cell differentiation
anatomical structure development
meiotic cell cycle
single-organism developmental process
cellular component assembly involved in morphogenesis
anatomical structure formation involved in morphogenesis
sporulation
cellular component morphogenesis
sexual sporulation
sexual sporulation resulting in formation of a cellular spore
developmental process involved in reproduction
single organism reproductive process
reproductive process in single-celled organism
reproduction
fungal-type cell wall biogenesis
thiamine metabolic process
alditol metabolic process
thiamine-containing compound metabolic process
sexual reproduction
multi-organism reproductive process
reproductive process

GO Biological Process Term for DOWN-genes
alcohol biosynthetic process
lipid metabolic process
lipid biosynthetic process
small molecule metabolic process
small molecule biosynthetic process
ergosterol biosynthetic process
phytosteroid biosynthetic process
cellular alcohol biosynthetic process
cellular lipid biosynthetic process
cellular lipid metabolic process
steroid metabolic process
sterol metabolic process
ergosterol metabolic process
phytosteroid metabolic process
organic hydroxy compound biosynthetic process
cellular alcohol metabolic process
steroid biosynthetic process
sterol biosynthetic process
alcohol metabolic process
single-organism biosynthetic process
organonitrogen compound biosynthetic process
organic hydroxy compound metabolic process
external encapsulating structure organization
cell wall organization

3.8. Functional Analysis

3.8.1. Spot Assays

To check the response of candidate genes in the presence of salts NaCl and LiCl both mutant strains and transformants were spotted on salt containing selective media. (Figure 3.12.). According to spot assays, there was no significant change in the overexpression of the genes when compared with plasmid-only transformant. Also deletion mutant cells do did not have any sensitivity to salt.

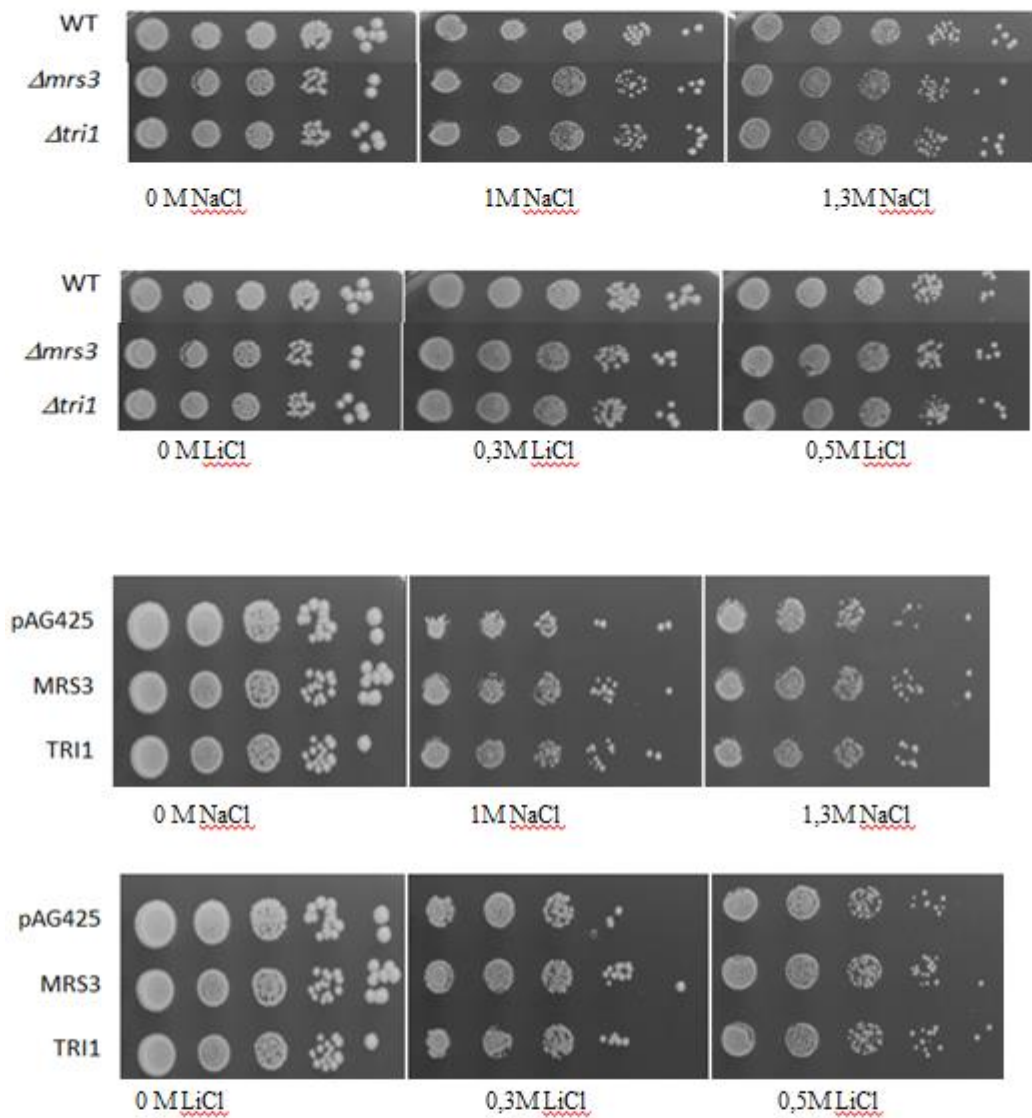

Figure 3.12. Spot assay in different concentration of NaCl and LiCl

Activity of the candidate genes were tested in the presence of cationic drugs; hygromycin B, spermine and tetramethylammonium(TMA), both mutants and overexpression transformants were tested. (Figure 3.13.) Again no difference was observed with respect to both sensitivity and resistance.

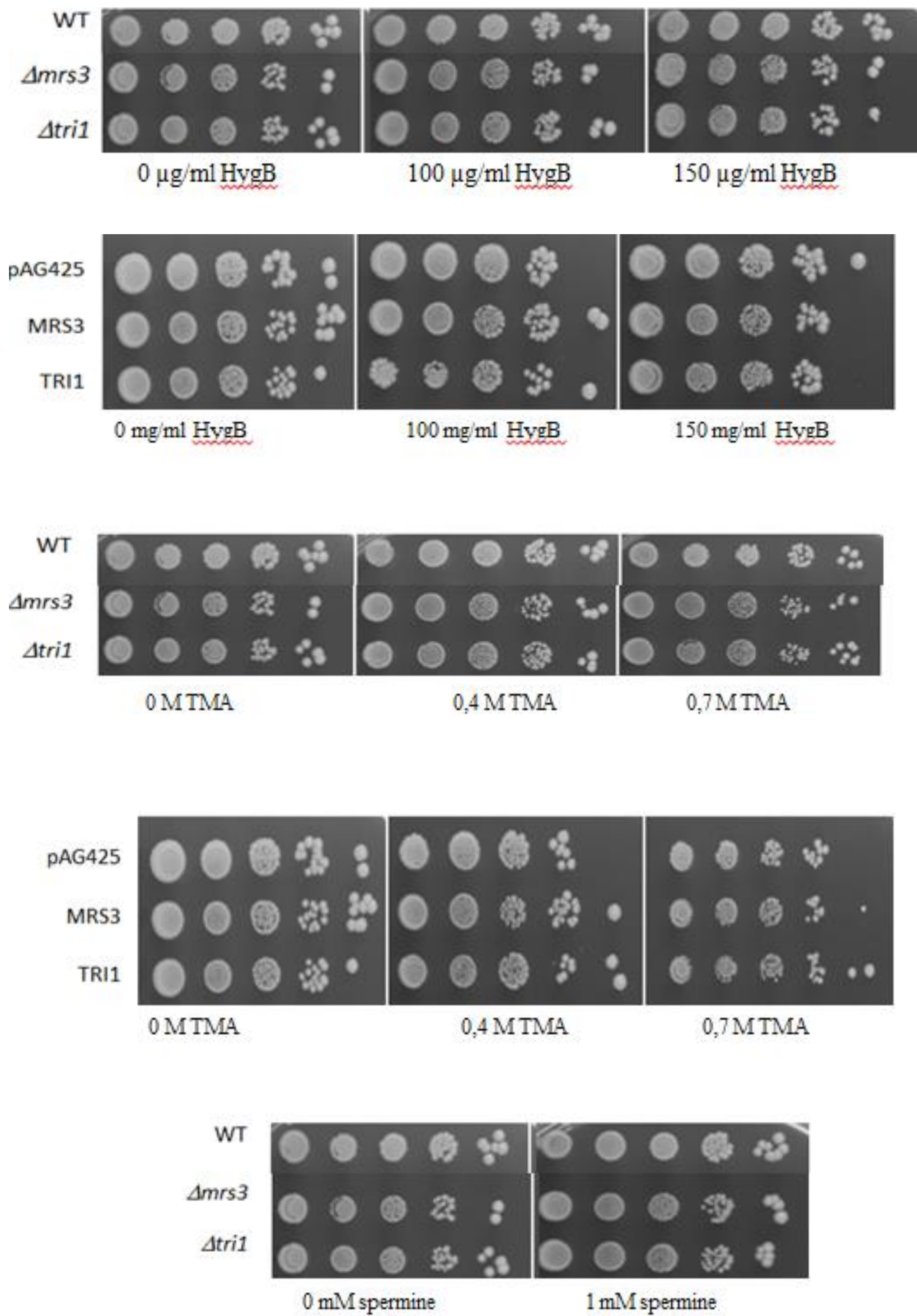

Figure 3.13.Spot assay for Hygromycin B; TMA and spermine resistance

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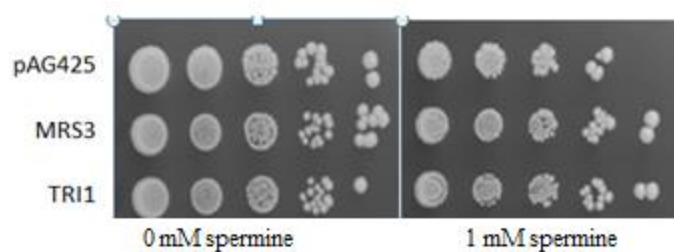

Figure 3.13 (cont)

3.8.2. Membrane Potential Measurement

Membrane potential changes of both mutant cells and overexpression transformants were determined by measuring the transport of 3,3'-dipropylthiadicarbocyanine [diS-C_3(3)] probe across the cell membrane. By help of Photon Counting Spectrofluorometer, wavelength of the dye was quantified for each strain. Results were not different from each other suggesting that mutant cells had normal membrane potential. (Figure 3.14.)

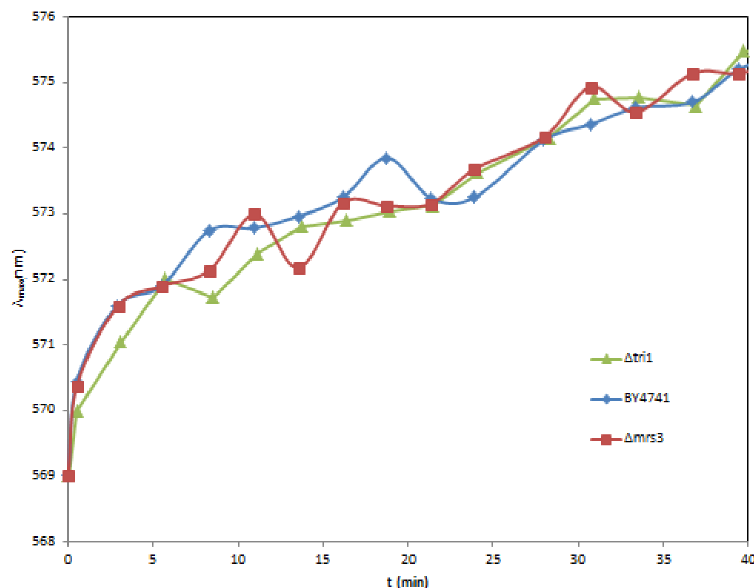

Figure 3.14. Membrane Potential Measurement of mutant type and overexpression plasmid included yeast cells.

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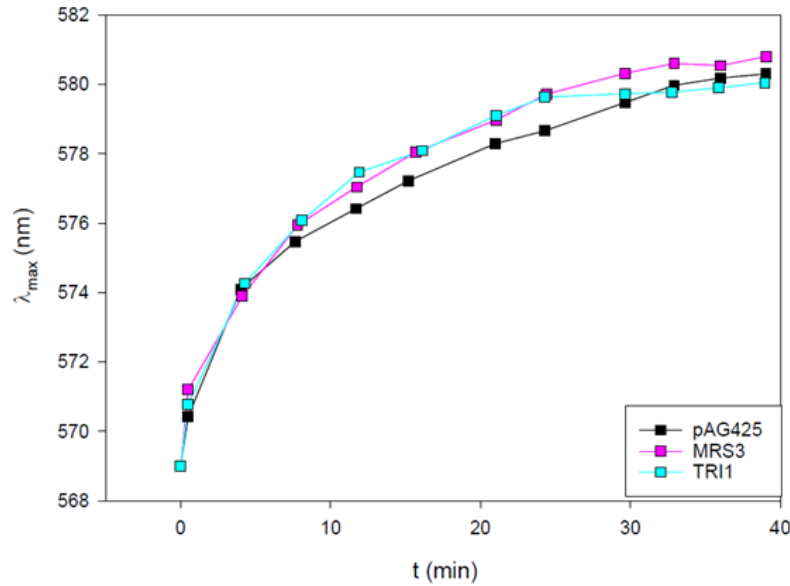

Figure 3.14. (cont)

3.8.3. Intracellular pH Measurement

pHI-U plasmid was transformed into haploid deletion mutant cells to check the pH response of the cells. To confirm the transformation and fluorescence, 100X image of cells were examined under fluorescent microscope. Nomarki images were also taken to check whether shape of the vacuoles was normal (Figure 3.15.). All transformants could take the plasmid and their Nomarki images were normal, vacuole shape changes did not occur both in the absence of candidate genes and in the presence of phoulorin plasmid. Later pH measurement was performed by fluorescent reader. Results were analyzed by excel and graph indicates Δmrs3 and Δtri1 yeast strains had almost same pH value as wild type (Figure 3.16.).

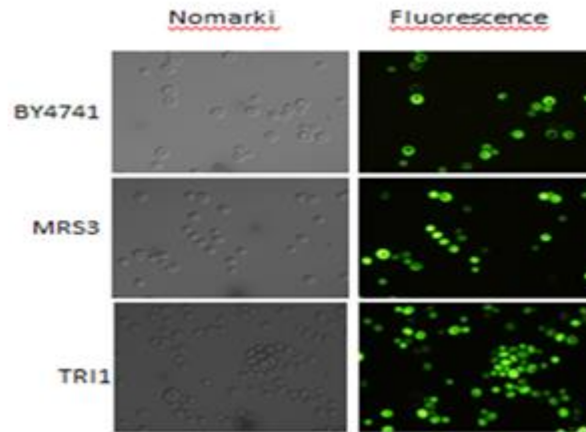

Figure 3.15. Nomarki anf fluorescent images of phoulorin containing cells.

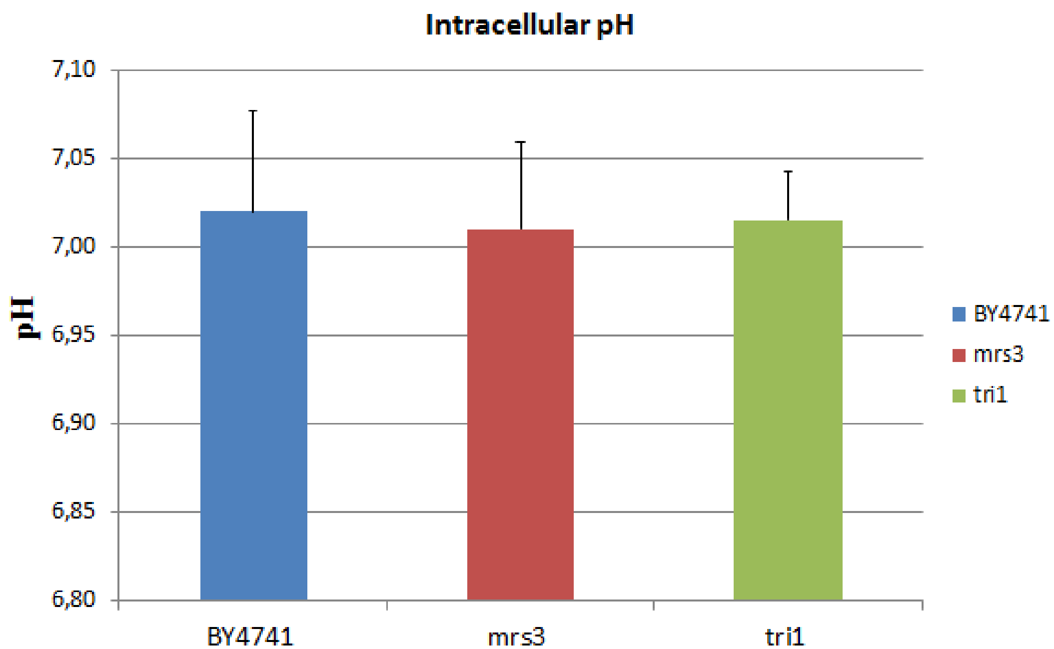

Figure 3.16. Intracellular pH measurement of phoulorin containing haploid mutant and wild type cells.

3.8.4. Size and Volume Measurement

Size and volume measurement was performed by CASY size measurement method. Both overexpression plasmids included and mutant strains were observed, wild stype strain was used as control. According to results, no difference was observed between transformants, mutants and wild type strain. (Figure 3.17)

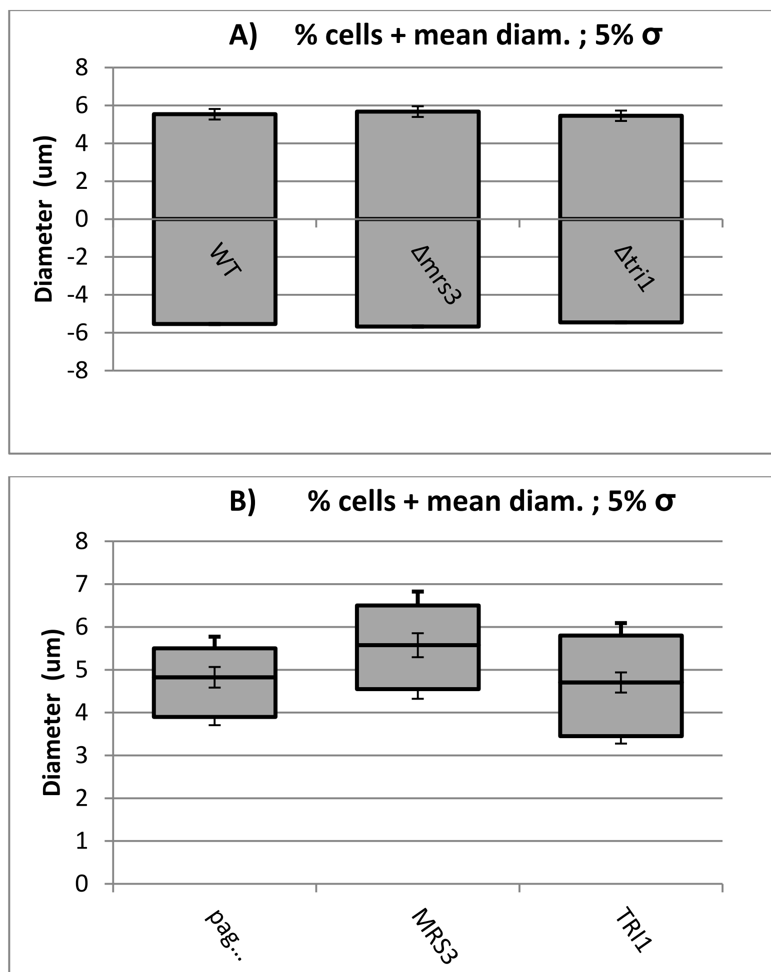

Figure 3.17. Size measurement graphics of mutant (A) and overexpression cells (B)

3.9. Cross Resistance Analysis

Ossible resistant genes were tested if they have a wide range of resistance among antifungals. 0,6µg/ml Caspofungin, 0,7µg/ml AmB, 1500µg/ml naftifin, 3µg/ml anidulafungin and azole family member 100µg/ml ketoconazole gradient plates were used for cross resistance assays. The strains that overexpress the candidate genes were spotted on drug including plates. Both MRS3 and TRI1 overexpression showed resistance only to ketoconazole (Figure 3.17.)

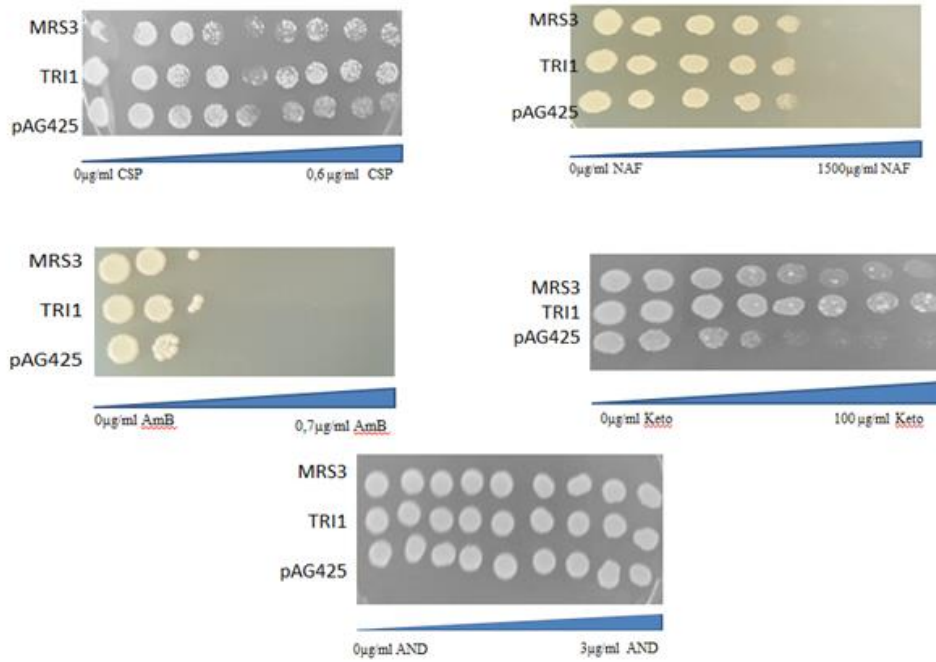

Figure 3.18. Spot assays for cross resistance to AmB, CSP, Keto, AND and NAF antifungal drugs.

3.10. *Candida albicans* Homolog Examination

3.10.1. Resistance Test by Spot Assay

C. albicans of homolog resistant genes were cloned in BY4741 yeast cells and spot assay was performed. According to results that were taken after 5 days growth, both MRS3 and TRI1 homologs showed resistant when compare with plasmid-only. (Figure 3.19)

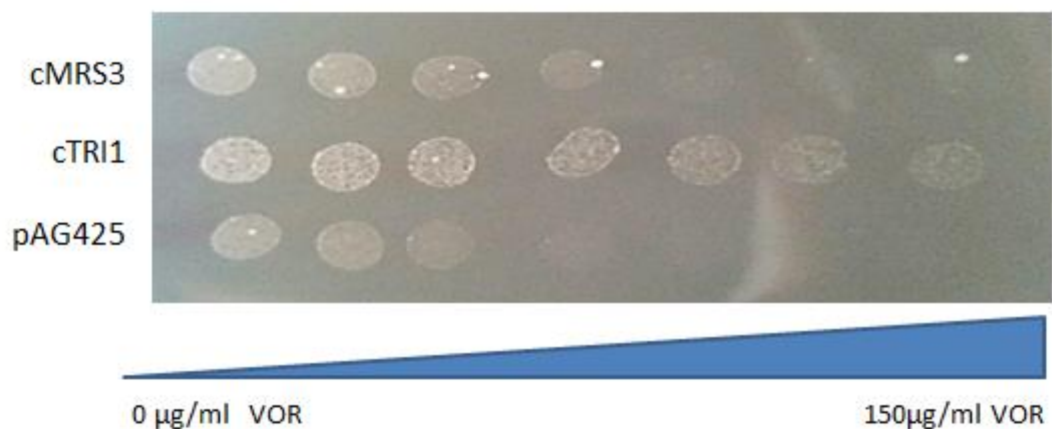

Figure 3.19. Spot assay of homolog resistant genes from C.albicans

3.10.2. Real-Time Analysis

Expression of C.albicans homolog genes were tested against Voriconazole induction by Real-time PCR. RNA isolation was done from Voriconazole treated and non-treated C.albicans cells and cDNA of these RNA’s were used for real time analysis. Graphs in Figure 3.20 indicated expression of these genes were decrease in the presence of Voriconazole.

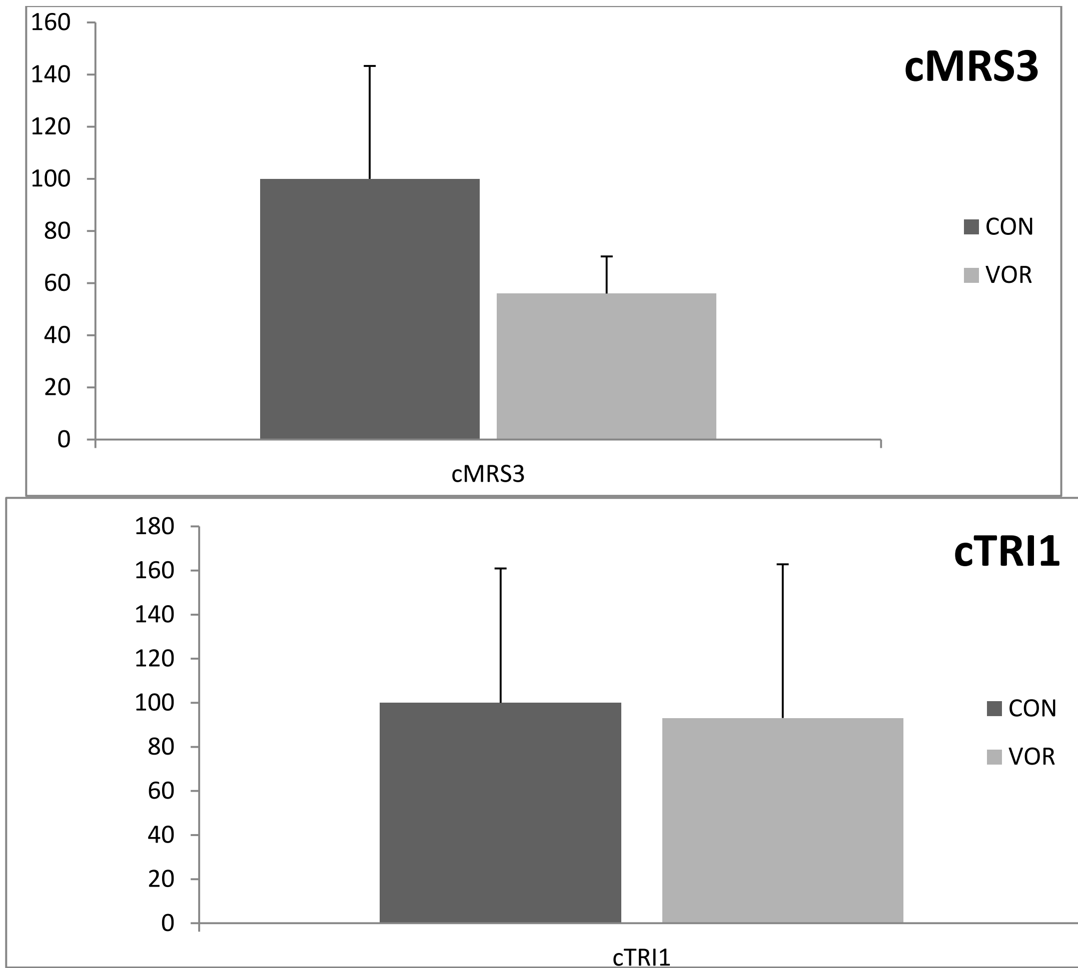

Figure 3.20. Real-time analysis of resistant homolog genes in *Candida albicans*

CHAPTER 4

CONCLUSION

In this project, identification of resistant genes against antifungal Voriconazole agent and the characterization of resistance genes was studied. A genome wide overexpression screening yielded MRS3 and TRI1 genes as resistant genes. Real-time PCR analysis indicated that Voriconazole treatment increased overexpression of MRS3 and TRI1 genes. Expression or deletion of these genes did not give any significant differences for membrane related physiological parameters.

MRS3 gene is an ion-transporter gene and also may transport other cations. Overexpression of MRS3 may cause up-regulation of other genes related to "iron regulation." Azole group of antifungals bind to iron atom of the heme domain in lanosterol demethylase. Change in iron regulation might fluctuate lanosterol demethylase level in the cell. Therefore, change in the target of Voriconazole can be related with the observed resistance.

Function of TRI1 gene is not known. It encodes for a non-essential sumoylated protein.

Data from this project will be useful for the future investigations. Because it is important that unraveling of the novel drug resistance mechanisms and the detection of these in patients will be of great importance and may be lifesaving.

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