IDENTIFICATION OF MOLECULAR RESISTANCE MECHANISMS AGAINST SYSTEMICALLY USED ANTIFUNGAL DRUGS, AMPHOTERICIN B AND CASPOFUNGIN

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ABSTRACT

IDENTIFICATION OF MOLECULAR RESISTANCE MECHANISMS AGAINST SYSTEMICALLY USED ANTIFUNGAL DRUGS, AMPHOTERICIN B AND CASPOFUNGIN

The invasive fungal infections have been evolving for the last few decades and become a crucial factor that negatively affects the survival ability of the patients. Moreover, developing new resistance mechanisms of infectious organisms to the antifungal drugs make this problem more critical. In this study, we identified four different genes *PDR16* and *PMP3, RMD9* and *SWH1*which are resistant to amphotericin B and caspofungin, respectively, in *Saccharomyces cerevisiae.* We also screened whole yeast genome at transcriptional level via microarray analysis and identified metabolic pathways affected by these antifungal drugs. In addition, we applied several characterization methods including physiological and morphological analyses to understand the resistance mechanisms. We found that *Δrmd9* mutants were extremely hyperpolarized and *Δpdr16* and *Δpmp3* mutants were depolarized when compared to control group. In contrast, overexpression of *SWH1* increased the membrane potential of wild type cells. Further analyses showed that the absence of *RMD9* disrupts the cellular morphology. To show that these genes are clinically important, *Candida albicans* orthologs of *PDR16* and *PMP3* were cloned and their roles in amphotericin B drug resistance were confirmed. *RMD9* and *SWH1* have no known *C. albicans* orthologs, so the confirmation with pathogenic yeast genome could not be performed against caspofungin.

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ÖZET

SİSTEMİK MANTAR İLAÇLARINDAN AMFOTERİSİN B VE KASPOFUNGİNE KARŞI GELİŞEN İLAÇ DİRENÇLİLİK MEKANİZMALARININ BELİRLENMESİ

İnvazif mantar enfeksiyonları son yıllarda evrilerek hastanın hayatta kalma sürecini olumsuz yönde etkileyen önemli bir faktör haline gelmiştir. Bununla birlikte, enfeksiyöz organizmalar tarafından mantar ilaçlarına karşı geliştirilen yeni mekanizmalar bu problemi daha da kritik hale getirmektedir. Ekmek mayası *Saccharomyces cerevisiae* ile yapılan bu çalışmada; genomik DNA taraması sonucu *PDR16* ve *PMP3* genlerinin amfoterisin B'ye; *RMD9* ve *SWH1* genlerinin ise kaspofungine karşı dirençlilik sağladığı tespit edilmiştir. Ayrıca tüm maya genomunun transkripsiyon düzeyinde yapılan mikrodizi taraması ile bu mantar ilaçlarının hangi metabolik yolakları etkilediği belirlenmiştir. Dirençlilik mekanizmalarının anlaşılmasında karakterizasyona yönelik fizyolojik ve morfolojik testler uygulanmış; kontrol grupları ile yapılan karşılaştırmalar sonucunda *Δrmd9* mutantının belirgin bir biçimde hiperpolarize olduğu, *Δpdr16* ve *Δpmp3* mutantlarının ise depolarize olduğu gözlenmiştir. Diğer yandan, *SWH1* geninin aşırı ifadelenmesi de yabani tip hücrelerin membran potansiyelinde artışa sebep olmuştur. Yine yapılan karakterizasyon testleri sonucunda *Δrmd9* mutant hücrelerinde morfolojik açıdan anomaliteler saptanmıştır. Bu çalışmanın klinik açıdan önemini vurgulamak amacıyla *PDR16* ve *PMP3* genlerinin *Candida albicans* ortologları klonlanmış ve amfoterisin B mantar ilacına olan dirençlilikleri gösterilerek, elde edilen bulgular desteklenmiştir. *RMD9* ve *SWH1* genlerinin *C. albicans* homologları veri tabanında mevcut olmadığı için, genlerin dirençlilikleri patojenik maya genomuyla test edilememiştir.

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CHAPTER 1

INTRODUCTION

1.1. Invasive Fungal Infections (IFIs)

The Fungi Kingdom with a huge diversity is still a mystery. Although only 5% of this kingdom could be classified, it is well known that many of fungal organisms are parasites damaging the others such as plants, animals or humans as well as other fungal species (Vandeputte, Ferrari et al. 2012). Over the past few decades, invasive fungal diseases have become a critical factor affecting the quality of life, negatively. Recent studies and statistical analyzes demonstrate that majority of immunocompromised patients are open targets for IFIs (Ascioglu, Rex et al. 2002, Loeffler and Stevens 2003). Chemotherapy, organ/stem cell transplantations, complex surgeries and specific diseases which are disrupting immune systems can conclude with morbidity or mortality due to IFIs (Morace and Borghi 2010, Person, Kontoyiannis et al. 2011, Brown, Meintjes et al. 2014).

1.1.1. Chemotherapy

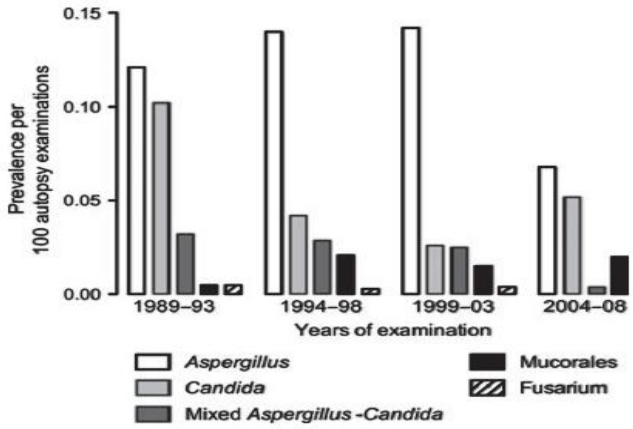


Figure 1.1. Prevalence of five common IFIs detected at autopsy of patients who had hematological malignancies (Source: Lewis, Cahyame-Zuniga et al. 2013)

Cancer is basically defined as uncontrolled cell division. Thus, control of cell cycle is mostly targeted in treatments to overcome cancer (Asghar, Witkiewicz et al. 2015). Many chemotherapeutic agents are used as an immunosupressants, since these agents eradicate rapidly dividing cells including hair follicles and immune system members (Zitvogel, Apetoh et al. 2008, Salaam, Hwang et al. 2014). In this case, many cancer patients suffer from IFIs during chemotherapy. In a 20-year study, it was recorded that a diversity of IFIs were identified at autopsy of patients who had hematological malignancies (Figure 1.1.) (Lewis, Cahyame-Zuniga et al. 2013).

1.1.2. Transplantations

Immune system has a strong fighting mechanism which may cause over-reactions of the body when it is not blocked in several cases. These over-reactions are mainly observed after solid organ or stem cell transplantations. As the immune system reacts to the new organ/tissue; mechanical complications occur, the body automatically rejects the organ and this complex process directly fails. To prevent these kinds of disasters after transplantations, the patients are supported with immunosuppressor agents (Xing, Zhong et al. 2015).

Table 1.1. Incidence, timing and outcomes of IFI after hematopoietic stem cell transplantation (HSCT) and solid organ transplantation (SOT) (Source: Vazquez, Miceli et al. 2013)

Incidence, timing and outcomes of IFI	HSCT	SOT
1-year incidence of IFI (average of studies) (%)		
Aspergillus IFI	1.6	0.7
Mucorales IFI	<0.3	0.2
Other mold IFI	<0.3	0.2
Candida IFI	1.1	1.9
Median time to IFI after transplant (days)		
Aspergillus IFI	99	184
Mucorales IFI	135	312
Fusarium IFI	123	–
Other mold IFI	–	467
Candida IFI	61	103
12-month survival after IFI (%)		
Aspergillus IFI	25.4	59
Fusarium IFI	6.3	–
Other mold IFI	28	61
Candida IFI	33.6	66

Neofytos *et al.* [2009]; Kontoyiannis *et al.* [2010]; Pappas *et al.* [2010].
IFI, invasive fungal infection; HSCT, hematopoietic stem cell transplantation; SOT, solid organ transplantation.

After transplantations IFIs are commonly observed in patients and unfortunately, most of these cases end up with mortality. Survival abilities after solid organ transplantations are nearly 60 %, whereas after hematopoietic stem cell transplantations it reduces to only 20-25% (Table 1.1.) (Vazquez, Miceli et al. 2013)

1.1.3. Complex Surgical Procedures

Intensive care units (ICUs) are mostly required for patients after complex surgical procedures in addition to chemotherapies or transplantations (Morace and Borghi 2010). In ICUs, if one or more risk factors such as long-term broad spectrum antibiotic therapies, again immunosuppressants, diabetes mellitus, long time hospitilization, mechanical ventilation, urinary tract or central venous chatheterization, age and even sex are combined, occurance of IFIs become inevitable (Passos, Sales et al. 2005, Morace and Borghi 2010, Francuzik, Sklodowska et al. 2015).

1.1.4. Immunodefeciency Diseases

Acquired Immune Deficieny Syndrome (AIDS), a result of Human Immunodeficiency Virus (HIV) infection, was first described in 1981. Following the identification of this disease, many side-effects were defined. In mid 1980s, it was reported that AIDS was not directly effective on lives. Instead, mortality was occuring due to infectious pathogens causing candidiasis, cryptococcal meningitis or sepsis etc. (Holmberg and Meyer 1986).

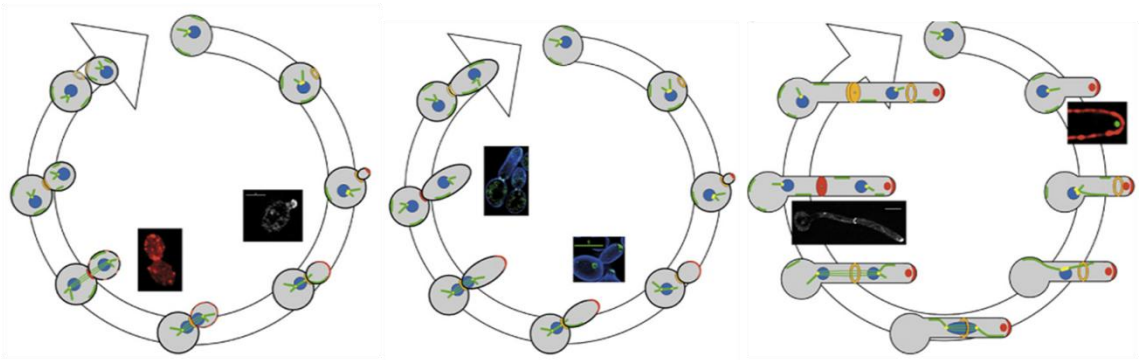
1.2. Fungal Pathogens

Although there are many different parasitic fungal species, only few of them affect humans. Yeasts, molds and dimorphic fungi are three main groups of fungal pathogens and these groups are categorized according to their morphologies, ecologies and clinical importance (Bowman, Taylor et al. 1992, Richardson 2005).

1.2.1. Pathogenic yeasts

Candida is a well known pathogenic yeast family which is also the most effective one in IFIs. Some *Candida* species are comparatively less harmless. Whereas, some of them are strongly effective in immunocompromised patients. *Candida albicans* is the main character of candidiasis that may cause mortality (Sudbery, Gow et al. 2004).

C. albicans is a polymorphic organism which means that it behaves differentially in variable conditions. Depending on the pH, temperature and nutritions this pathogenic organism can switch between yeast, hyphal and pseudohyphal morphologies (Sudbery, Gow et al. 2004, Berman 2006). In acidic environments (pH 4) and at low temperatures (30^{o}C) *Candida* cells stay in yeast form; whereas in neutral pH and at higher temperatures closer to human body, they have a transition to hyphal morphology (Figure 1.2.)



Yeast	Pseudohyphae	Hyphae	Growth conditions that yield filamentous cells that have not been well characterized
Growth below 30°C or pH 4.0 Cell density > 10^7 cells ml^{-1}	pH 6.0, 35°C Nitrogen-limited growth on solid medium (SLAD) High phosphate	Serum >34°C Lee's medium 37°C pH 7.0, 37°C *N*-acetylglucosamine	Engulfment by macrophages Spider medium Mouse kidneys Growth in agar matrix Iron deprivation Anoxia

Figure 1.2. Polymorphic *Candida*. Budding yeast form (left), pseudohyphae form (middle), hyphae form (right) and required conditions which were experimentally optimized (The figure was modified from Sudbery, Gow et al. 2004; Berman 2006).

Hyphae structures of fungal pathogens act as an anchor which provides penetration to tissues. Moreover, hyphaes directly contact to tissues and navigate the cells to suitable surfaces with weakened integrity for efficient invasion (Gow, Brown et

al. 2002). Therefore, in human body where the pH is neutral and temperature is 37^{o}C, these pathogenic *Candida* cells form hyphae structures and invade to organs easily.

In addition to *Candida* species, *Cryptococcus neoformans* is another known pathogenic yeast threatening the lives of immunocompromised patients (Boekhout, Theelen et al. 2001). *C. neoformans* is a basidiomycetous yeast that can undergo sexual reproduction and form basidiospores. In dehydrated form these spores are small enough to pass through alveolar space and colonize in lungs. After rehydration of the spores, they become strong pathogens. High amount of capsules and sheds, production of several metabolites such as melanin, mannitol; enzymes such as superoxide dismutase, protease and phospholipases are some key factors increasing the virulence of *C. neoformans* (Buchanan and Murphy 1998).

Blastomyces dermatitidis, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Sporothrix schenkii*, and *Penicillium marneffei* are other known pathogenic fungi infecting organs via dimorphic behavior (Nemecek, Wuthrich et al. 2006).

1.2.2. Pathogenic molds

Molds are filamentous fungi which can be pathogenic in several cases. *Aspergillus* is the most common one infecting plants as well as humans and causing serious defects. In contrast to patohenic yeasts, infection mechanisms of molds are typically exogenous. Inhalated spores through respiratory tract are directly transferred to lungs and there, in suitable conditions, develop pneumonia (Wald, Leisenring et al. 1997). *Aspergillus nidulans* was genetically defined in the mid of 20th century (Pontecorvo, Roper et al. 1953). This organism is used as a model, recently, which may lead to identify and/or characterize new pathogenic molds (Nayak, Szewczyk et al. 2006). Another pathogenic mold, *A. fumigatus*, has been considered as a weak pathogen since many years. However, increase in suppression of immune systems due to several reasons affected the rate of allergic bronchopulmonary aspergillosis with a direct correlation (Latge 1999, McCormick, Heesemann et al. 2010). Current studies support that *A. fumigatus* is the most common mold detected in immunocompromised patients (Husain, Alexander et al. 2003, Bidula, Sexton et al. 2015). Besides *Aspergillus*, there

are also several pathogenic *non-Aspergillus* molds causing invasive fungal infections (Husain, Alexander et al. 2003).

1.3. Antifungal Drugs

Antifungal drugs are the strongest weapons to overcome the IFIs. In clinical approaches, there are several different strategies indicating drugs with different modes of action. Four major groups of antifungal drugs are polyenes macrolides, azole groups, fluoro pyrimidines and echninocandins (Loeffler and Stevens 2003, Vandeputte, Ferrari et al. 2012).

1.3.1. Polyene Macrolides

Polyenes are 20-40 carbon macrolides and first discovered as a natural product of *Streptomyces* genus (Fjaervik and Zotchev 2005, Vandeputte, Ferrari et al. 2012). Polyenes can be in tetraene, pentaene, hexaene and even heptaene form (Thomas 1976). These macrolide structures have a common role that they have affinity to ergosterol found in plasma membrane of fungi. Thus, 8-10 molecules of a macrolide comes together and forms a pore-like structure through the plasma membrane by binding ergosterol (Figure 1.3). Essential ions and nutritions leak throughout the membrane and therefore, cell death is induced (Loeffler and Stevens 2003).

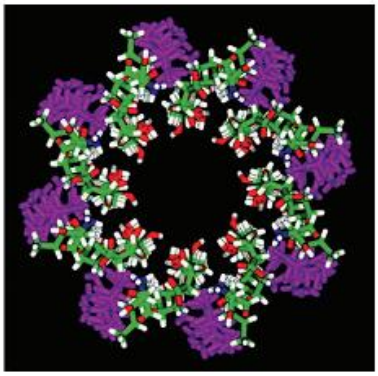


Figure 1.3. 3D image of pore structure formed by amphotericin B binding to ergosterol. Amphotericin B: white (H), green (C), red (O) and blue (N); purple (ergosterol) (Source: Vandeputte *et al.*, 2012)

In addition to pore formation of Amphotericin B, sponge model is also proposed. Thus, AmB forms an extramembraneous complex, extracts ergosterol from lipid bilayers of fungi and causes cell death (Anderson, Clay et al. 2014, Lohner 2014). The novel model of AmB sponge is shown in Figure 1.4.

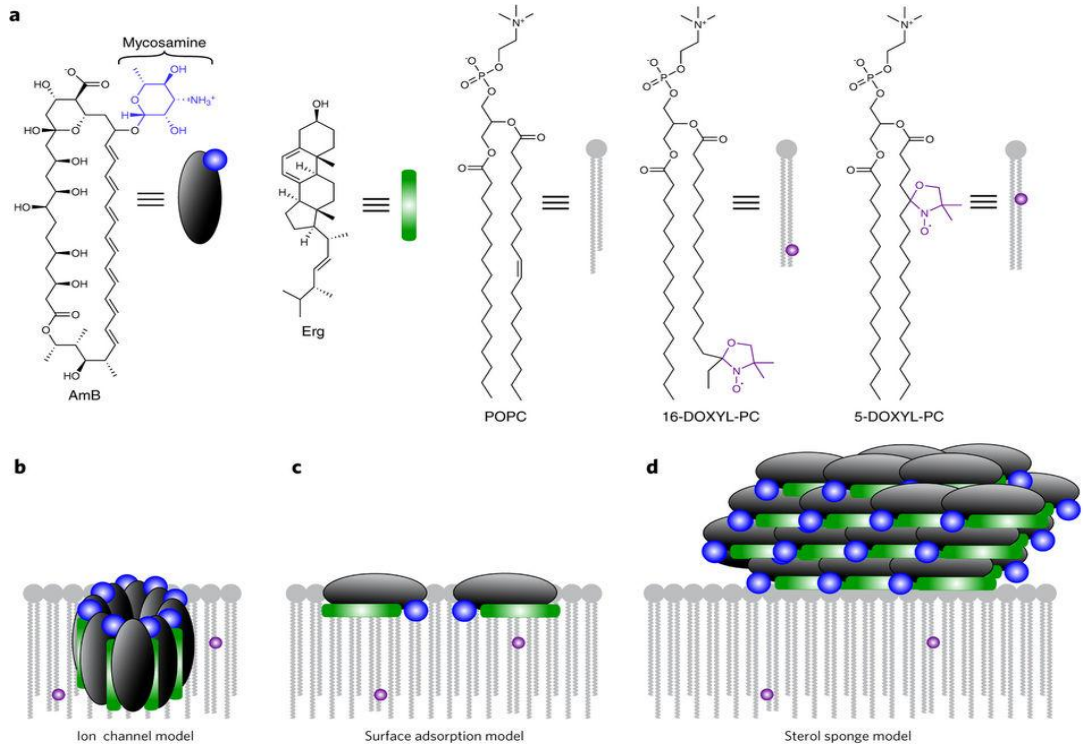


Figure 1.4. AmB cytocidal models. (a) Molecular structures of Amphotericin B (AmB), ergosterol (Erg), phosphatidylcholine (POPC), 1-palmitoyl-2-stearoyl-(16-doxyl)-*sn*-glycero-3-phosphocholine (16-DOXYL-PC) and 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine (5-DOXYL-PC); (b) Previously defended ion channel model; (c) Surface adsorption model; (d) Novel sterol sponge model (Source: Anderson *et al.*, 2014)

Two tetraenes, natamycin and nystatin (degenerated heptaene) are locally used antifungal drugs that act on oral, esophageal, gatro-intestinal or genital candidiosis as well as corneal infections. On the other hand, Amphotericin B is a systemically used antifungal drug with heptaene form (Figure 1.5.) (Fjaervik and Zotchev 2005, Vandeputte, Ferrari et al. 2012).

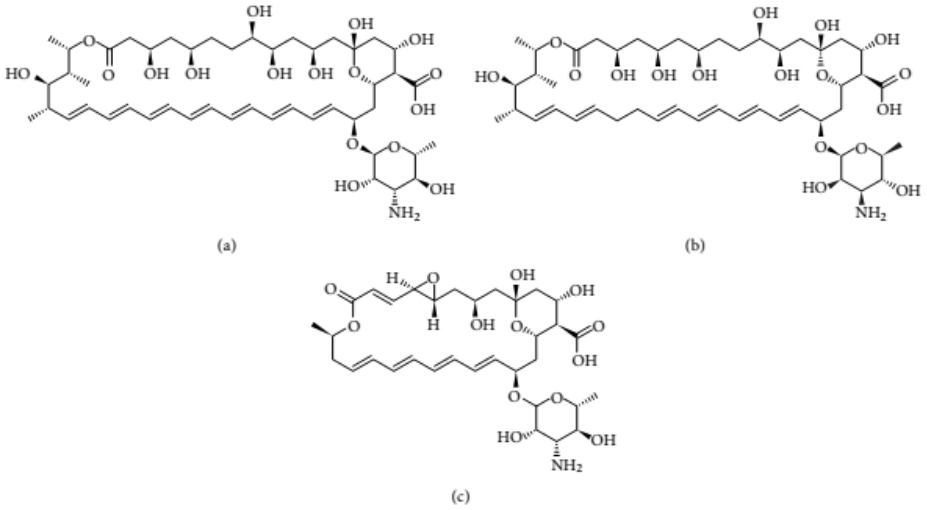


Figure 1.5. Chemical structures of polyenes, (a) amphotericin B, (b) nystatin and (c) natamycin (Source: Vandeputte *et al.*, 2012)

In the mid 20th century, nystatin was discovered by Hazen and Brown via observing an antifungal activity in fermentation broth and its name was derived from New York city (Hazen, Brown et al. 1953, Dobias and Hazen 1961). First trials of nystatin on mice demonstrated that absorption of the drug was inefficient from intestines, so other *Streptomycete* cultures were screened to find out a better one. Amphotericin A, a tetraene similar to nystatin, was identified during screening (Dutcher 1968). Whereas amphotericin B a heptaene with a greater antifungal actvity, was first discovered as a natural product of *Streptomyces nodosus* during soil screening of Orinoco river basin in Venezuela (Cohen 2010). After this time, amphotericin B is used for more than 50 years as a very common antifungal drug. Moreover, its molecular formula is enhanced within several lipid complexes in order to increase the absorption and distribution efficiency and decrease its side effects (Dismukes 2000).

Natamycin, the third polyene, was first discovered in a culture of *Streptomyces natalensis* in 1955. Further studies revealed that natamycin was a colorless product with no odor that indicates its suitable usage in food industry. Thus, natamycin is still used as a food protectant against molds (Broughton, Thomas et al. 2005).

1.3.2. Azoles

Two decades over polyene discovery, efficiency of antifungal therapies changed negatively and new drugs with broader spectrums were required. Therefore, derivatives of imidazoles with two nitrogen atoms and triazoles with three nitrogen atoms, were introduced to antifungal therapies (Smith 1990, Vandeputte, Ferrari et al. 2012). After identification of antifungal effects of azole drugs, mechanism of action was explained by several hypotheses. Bossche *et al*. explained that azoles may inhibit the ergosterol biosynthesis via interfering the p450 dependent lanosterol 14α-demethylase system (van den Bossche, Willemsens et al. 1978). Recently, it is well known that free nitrogen atom of the azole ring binds to iron atom of heme group of lanosterol 14α-demethylase enzyme which is encoded by *ERG11* gene (Vandeputte, Ferrari et al. 2012).

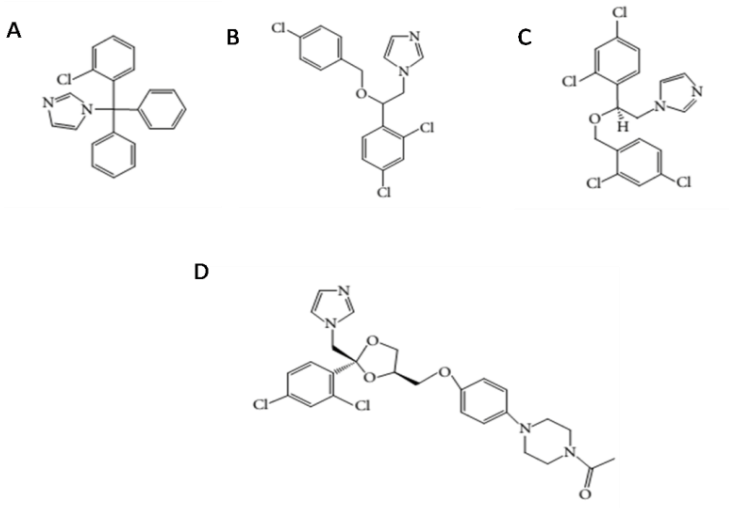


Figure 1.6. Antifungal imidazoles, (A) Clotrimazole, (B) Econazole, (C) Miconazole, (D) Ketoconazole (Source: Vandeputte *et al.*, 2012)

Historical background of azoles reaches to mid 20th century, too. Although antifungal activity of benzimidazole was found by Woolley in 1944, before the discovery of polyene activities; drug forms of azoles were produced at the end of 1950s (Fromtling 1988). Clotrimazole and econazole were the first two azole drugs, whereas the usage of both was impermanent as a result of inconsistent concentrations in blood (Figure 1.6A and 6B) (Loeffler and Stevens 2003). Then, a better one, miconazole was used in systemic infections. Moreover, miconazole was more advantageous than amphotericin B with high effectiveness and less toxicity (Figure 1.6C) (Heel, Brogden

et al. 1980). Some other imidazole derivatives, such as bifonazole, butoconazole, croconazole, fenticonazole, isoconazole, oxiconazole and tioconazole were produced and used for local therapies (Fromtling 1988). On the other hand, ketoconazole was developed for systemically usage (Figure 1.6D). However, its absorption efficiency was not enough and its intravenous form was unavailable. Moreover, it had some side effects such as decreasing the testosterone or glucocorticoid levels as well as affecting the liver and gastrointestinal activities, negatively. Therefore, triazoles took place in antifungal therapies instead of imidazoles (Vandeputte, Ferrari et al. 2012).

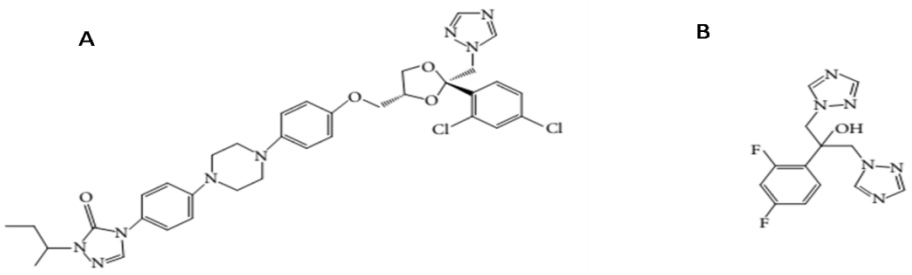


Figure 1.7. Antifungal triazoles, (A) Itraconazole, (B) Fluconazole (Source: Vandeputte *et al.*, 2012)

Fluconazole, the first water-soluble triazole, was used against candidiasis (Figure 1.7A). And few years later, itraconazole, with a broader spectrum, was developed (Figure 1.7B). Itraconazole was effective on both yeast and filamentous fungal pathogens, which were *Candida* and *Aspergillus* species, respectively (Rex, Rinaldi et al. 1995, Cornely, Maertens et al. 2007). In spite of its strength, itraconazole was disadvantageous due to its poor tolerability of cyclodextrin-containing oral solution (Cornely, Maertens et al. 2007). And common usage of fluconazole was concluded with resistance in patients.

To overcome these problems of fluconazole and itraconazole; new generation triazoles, which are posaconazole, voriconazole and ravuconazole, were developed (Figure 1.8). In few years following the millennium, voriconazole and posaconazole were approved by FDA. At the same time, ketoconazole was banned because of its toxicity. Finally, fosravuconazole (a prodrug of ravuconazole) was produced by Eisai in Japan. Recently, its third step including test on patients will be performed in Sudan by

collaboration of Eisai and Drugs for Neglected Diseases initiative (DNDi) (Zijlstra, van de Sande et al. 2016).

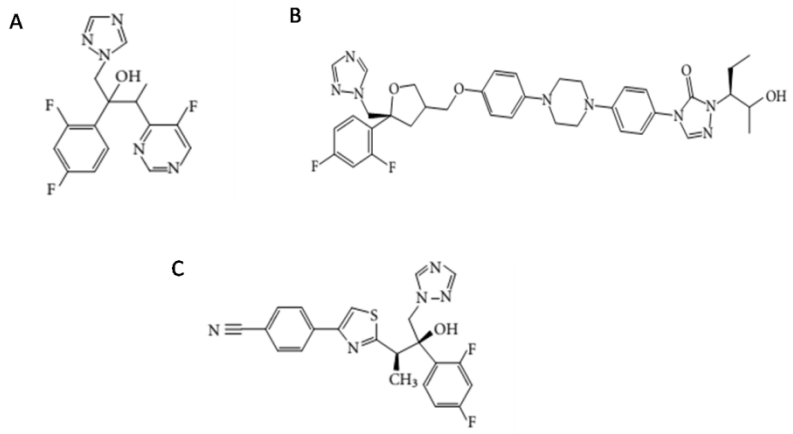


Figure 1.8. New generation triazoles, (A) Posaconazole, (B) Voriconazole, (C) Ravuconazole (Source: Vandeputte *et al.*, 2012)

1.3.3. Fluoropyrimidines

The idea of synthetic nucleoside production was first suggested by Heidelberger *et al.* in 1957. Fluoropurines and fluoropyrimidines were synthesized and further studies demonstrated that 5-fluorouracil and 5-fluoroorotic acid had tumor-inhibitory activity (Heidelberger, Chaudhuri et al. 1957). In 1968, 5-fluorocytosine (5-FC) was used as an antifungal drug against *Candida sepsis* and *Cryptococcus meningitis* (Tassel and Madoff 1968).

The uptake of 5-FC into fungal cells are provided by cytosine permease enzyme. In cells, 5-FC is immediately converted to 5-fluorouracil and fluorouridine triphosphate, respectively. This final product involves in RNA structure and therefore, disrupts the protein synthesis. In addition, 5-fluorouracil is converted to fluorodeoxyuridine monophosphate, an inhibitor of thymidilate synthase. Hence, DNA synthesis is also affected negatively (Loeffler and Stevens 2003).

5-FC is a hydrosoluble small molecule and it can be easily removed from the body (Figure 1.9). It does not have any complicated side effects that makes 5-FC favorable, whereas it is not used in monotherapy, today. High percentage of resistance development against 5-FC by fungal pathogens, forces the therapy to combinational usage (Vandeputte, Ferrari et al. 2012).

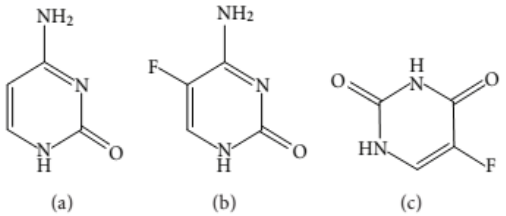


Figure 1.9. Chemical structures of fluoropyrimidines, (a) cytosine, (b) 5-fluorocytosine and (c) 5-fluorouracil (Source: Vandeputte *et al.*, 2012)

1.3.4. Echinocandins

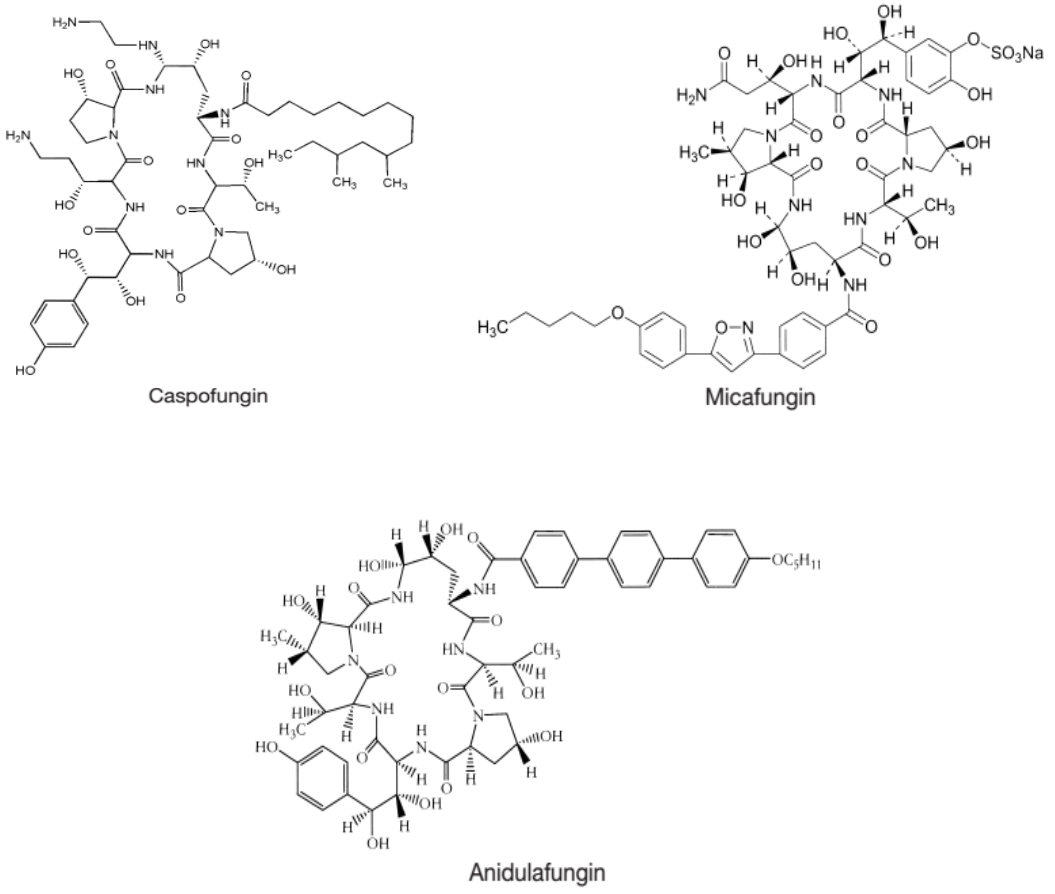


Figure 1.10. Chemical structures of echinocandins (Source: Cappelletty *et al.*, 2007)

The last group of systemically used antifungal drugs are echinocandins, which are the products of new generation technology. Echinocandins are synthetic lipoprotein molecules origining from fermentation products of several fungal organisms (Denning 2002).

Echinocandin B was obtained from *Aspergillus* species in 1974 and then, in 1980 this cilofungin was synthesized from this precursor. With further developments LY303,366 and L743,872 were produced with antifungal activity. These two echinocandins were called pneumocandins due to their acting on both *Candida* spp. and *Pneumocystis carinii* (Denning 1997).

Recently, there are three echninocandins, which are caspofungin, micafungin and anidulafungin, were approved by Food and Drug Administration (FDA) and European Medicines Agency (EMEA) (Vandeputte, Ferrari et al. 2012) (Figure 1.10).

All these three echinocandins are pretty large molecules and their oral uptake is not possible as a result of their high molecular weights. Thus, they have only intravenous forms (Denning 2002). The echinocandins are noncompetitive inhibitors β-1,3-D-glucan synthase enzyme, which is responsible for β-1,3-D-glucan synthesis from uridine-diphosphate glucose via polymerization reaction. Without cell walls, leakage of the ions and several molecules starts through the membrane, osmolysis occurs and cells cannot survive anymore (Cappelletty and Eiselstein-McKitrick 2007, Vandeputte, Ferrari et al. 2012).

1.4. Antifungal Drug Resistance

The term of drug resistance mainly refers to be insensitivity of pathogenic organisms against the drug that they are exposed. On the other hand, there are some sub-terms that discriminates the resistance types. If the organism in host is exposed to the drug for the first time and not affected, primary resistance occurs. Whereas, after one exposure if the organism develops resistance to same drug, this situtation is called secondary or acquired resistance. In some cases, clinical applications fails due to unexpected complications and therapy does not succeed in contrast to *in vitro* tests. Therefore, clinical resistance occurs. In another resistance type, intrinsic resistance, all isolates of an organims have a natural resistance to a specific drug. Natural resistance of *Candida kruesi* against fluconazole can be defined as intrinsic resistance (Loeffler and Stevens 2003).

Pathogenic organisms develop resistance to drugs by several genetic modifications affecting their metabolic, physiological and/or morphological situations. Hence, they decrease the accumulation of the drug, reduce the affinity to the drug

and/or modify some metabolic events to overcome the toxic effects of the drug (Vandeputte, Ferrari et al. 2012).

1.4.1. Drug Efflux

ABC (ATP-binding cassette) transporters and MFS (major facilitator superfamily) transporters are the main actors of drug efflux systems. In *Candida*, CDR1 and CDR2 (*Candida* Drug Resistance 1 and 2) are two ABC transporters which are responsible for removing azole drugs from the cell (Sanglard, Ischer et al. 1997, Krishnamurthy, Gupta et al. 1998). Similarly, azole resistant clinical isolates demonstrated that CaMDR1 of *C. albicans*, CdMDR1 of *C. dubliniensis*, CtMDR2 of *C.tropicalis*, CpMDR1 of *C. parapilopsis*, ClMDR1 of *C. lusitaniae*, CgFLR1 of *C. glabrata* and AfuMDR3 of *A. fumigatus*, which were the members of MFS, played active role in azole resistance (Dias and Sa-Correia 2014). FLU1, another MFS transporter, has a similar structure to CaMDR1 and resistance to fluconazole, spesifically (Calabrese, Bille et al. 2000).

Recent studies demonstrate that all of the identified transporters have effect on azole-type antifungal drugs. In 2006, Niimi et al. explained that these transporters do not provide resistance against other antifungal drugs, mostly echinocandins (Niimi, Maki et al. 2006). Whereas, it was already demonstrated that *CDR2* conferred resistance to caspofungin three years before (Schuetzer-Muehlbauer, Willinger et al. 2003).

1.4.2. Mutations in Target Genes

The antifungal drugs which directly target some specific proteins can be disabled by down-regulation of the genes encoding these target proteins. A point mutation in *ERG11*gene encoding the cytochrome P450 14α-demethylase enzyme, significantly inhibits the interaction of azole drugs with this enzyme and therefore, pathogenic cells can resist to azoles (Marichal, Koymans et al. 1999). Another member of ergosterol biosynthesis, *ERG6,* is also effective in antifungal resistance. When *ERG6* gene is defected, it was recorded that Amphotericin B cannot disrupt the cells. Since, this drug requires ergosterol molecules during pore formation (Vandeputte, Tronchin et al. 2008).

In both *Saccharomyces cerevisiae* and *Candida albicans,* echinocandin resistance provided by a point mutation in *FKS1* gene, which encodes β-1,3 glucan synthase subunits, was reported (Douglas, Foor et al. 1994, Douglas, Marrinan et al. 1994, Kurtz, Abruzzo et al. 1996, Douglas, D'Ippolito et al. 1997).

Members of cytidine metabolism have also regulatory role in fluoropyrimidine resistance. *FUR1* (uracil phosphoribosyltransferase) gene can provide resistance to both flucytosine (5FC) and 5-fluorouracil (5FU) antifungal drugs by a single mutation (Dodgson, Dodgson et al. 2004, Papon, Noel et al. 2007). Moreover, *FCY1* (cytosine deaminase) and *FCY2* (cytosine permease) mutants are cross-resistant to fluconazole in the presence of 5FC inhibitory concentrations (Papon, Noel et al. 2007).

1.4.3. Overexpression of Defensive Genes

Despite overexpression of any defensive gene is one of the most common ways of resistance mechanisms, there are very less number of genes known as resistant.
ScPMP3 and *CaPMP3*, encoding a small plasma membrane protein in *S. cerevisiae* and *C. albicans*, respectively, provides Amphotericin B resistance in case of overexpression (Huang, Chen et al. 2013, Bari, Sharma et al. 2015). *PMP3* is an important gene which plays role in resistance to several stresses. For example in plants, overexpression of this gene protects the whole organism from salt, cold, drought and abscisic acid (Fu, Zhang et al. 2012).

Heat shock proteins, chaperones, are highly conserved stress proteins and found in all organisms (Li and Srivastava 2004). In the presence of echinocandins, calcineurin (Hsp90 client protein) is activated and therefore, mediates the activation of Hsp90. Thus, Hsp90 provides echinocandin resistance in pathogenic yeast *C. albicans* (Singh, Robbins et al. 2009).

CHAPTER 2

MATERIALS AND METHODS

2.1. Drugs, Yeast Strains and Media

Amphotericin B (Fungizone®, Bristol Myers, Squibb) and caspofungin (Sigma) was used in library screening, resistance and sensitivity tests. For cross resistance, other antifungal drugs, naftifine and ketoconazole were provided as raw material from Eczacıbaşı, voriconazole (Sigma), fluconazole (Triflucan I.V., Pfizer) and anidulafungin (Eraxis, Pfizer) were obtained commercially.

Haploid wild type BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) *S. cerevisiae* strain and its related deletion mutants were used in whole study. Diploid wild type BY4743 (MATa/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0),* FY1679-28C (MATa/α *ura3-52/ura3-52 trp1Δ63/TRP1 leu2Δ1/LEU2 his3Δ200/HIS3 GAL2/GAL2) S. cerevisiae* strains and their *Δpdr16* deletion mutants were used for confirmation in a part of cross-resistance test. BY4741 and BY4743 strains were obtained from European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF). FY1679-28C and one more BY4741 strains were kindly provided by Dr. Griac (Simova, Poloncova et al. 2013, Holic, Simova et al. 2014). SC5314 *C. albicans* pathogenic strain was obtained from Academy of Sciences of the Czech Republic (ASCR) and used to check the functions of orthologous genes.

Cells were grown in either YPD rich media (2% glucose, 2% peptone, 1% yeast extract; 2% agar in solid) or YNB selective media (2% glucose, 2% agar, 0,67% yeast nutrient base, 20 mg/L methionine, 20 mg/L uracil, 100mg/L leucine, 20 mg/L histidine; for FY1679-28C strain 20 mg/L tryptophane instead of methionine; 2% agar in solid). To obtain the transformants, related aminoacid was lacked in YNB media according to aminoacid marker of the plasmids.

2.2. Determination of Minimum Lethal Doses of the Drugs

BY4741 *S. cerevisiae* wild type cells were inoculated to YNB+all broth and grown for an overnight at 30^{o}C, 180 rpm. The next day, cells were diluted for 5 times with fresh YNB+all broth and incubated for 3 hours more. Therefore, the cells were reached to their exponential phase. Cells were harvested, washed with sterile distilled water and diluted until their OD_{600} was 0.2. Then the cells were serially diluted (10 times in each step, upto $2x10^{-4}$) and 5 µl was dropped onto YNB+all agar plates containing different concentrations of either amphotericin B or caspofungin. Plates were incubated for 3 days at 30^{o}C and images were taken.

2.3. Library Screening and Determination of Resistant Colonies

In library screening two different ways were followed. First, high copy yeast gDNA library (ATCC No.37323) was transformed to BY4741 wild type yeast cells by standard lithium acetate (LiAc) method (Gietz, Schiestl et al. 1995). After transformation procedure, the cells were either spread onto 20 x 20 cm square plates containing YNB-leu with minimum lethal doses of AmB or CSP (0.3 µg/ml and 0.4 µg/ml, respectively) or only YNB-leu. In both method plates were incubated at 30 ^{o}C for two days. Then, each grown colony on plates containing drug was selected to a fresh YNB-leu media. On the other hand, almost all colonies grown on plates which do not contain any drug were selected one by one and transferred to 96 well plates containing 200 µl of YNB-leu broth in each well. These 96 well plates were incubated at 30 ^{o}C for an overnight and therefore, a transformant collection was obtained. In this way, library screening was performed with a replicator which transfers the cells to 96 well plate containing YNB-leu with minimum lethal dose of antifungal drugs, directly. After incubation at 30 ^{o}C for 5 days, resistant colonies were selected.

2.4. Plasmid Isolation and Confirmation of Resistance

Resistant colonies were subjected to plasmid isolation procedure. First, cells were grown for overnight in suitable media and harvested. Yeast cells were exposed to

10 µl of 10U lyticase enzyme and 10 µl of 20% sodium dodecyl sulfate (SDS) for cell wall disruption. Then, plasmid isolation was done with following the protocol of Thermo Scientific GeneJET Plasmid Miniprep Kit (#K0502, #K0503).

Isolated plasmids were cleaned and amplified by transformation to JM109 *E. coli* competent cells by heat shock procedure. 400 ng of plasmid was transformed to bacteria and the cells were spread onto Luria-Bertani (LB) agar (1% tryptone, 1% NaCl, 0,5% yeast extract, 2% agar) plates including 100 µg/ml of ampicillin (amp) which is the selective marker of YEP13 plasmids. The plates were incubated at 37^{o}C for an overnight. The next day single colonies were selected, inoculated in LB + amp broth and shaken at 180 rpm 37 ^{o}C for an overnight again. After growing the cells in broth culture, the plasmids were isolated by using Thermo Scientific GeneJET Plasmid Miniprep Kit.

Plasmids were re-transformed to wild type yeast cells in order to confirm their resistance. According to spotting assay results, false positive samples were eliminated.

2.5. Identification of Resistant Genes

Plasmids whose resistance were confirmed were sequenced from both sides with suitable YEP13 primers in Middle East Technical University, Molecular Biology and Biotechnology Research and Development Center, Genome Analysis Laboratory. The sequences were searched in *Saccharomyces Genome Database (SGD)* (www.yeastgenome.org) and functional genes belong to each gene cassette were selected.

2.6. Preliminary Test of Candidate Genes

Thermo Scientific Open Biosystems Yeast ORF Collection plasmids (BG1805) carrying each gene was tested via following the galactose induction protocol published by Roth et al. (Roth). First, ORF plasmids were transformed to BY4741 wild type yeast cells by standard LiAc method and transformants were spread onto YNB-ura selective media. Yeast cells were inoculated into 4 ml of YNB-ura broth and incubated at 30 ^{o}C 180 rpm for an overnight (approximately 16 hours). The next day, cells were harvested, washed with sterile distilled water (dH_2O) for two times and resuspended in YNB-ura

with 2% D-raffinose (Sigma Aldrich) instead of glucose (YNB-ura –glu +raf). After 2 hours incubation at 30 ^{o}C 180 rpm, the same process was repeated with YNB-ura with 2% D-galactose (AppliChem) instead of glucose and raffinose (YNB-ura –glu +gal). By this way expression of genes were induced by response of GAL1 promoters to galactose. Finally, the cells were serially diluted and dropped onto gradient plates (YNB-ura –glu + gal) containing related antifungal drug. The plates were incubated at 30^{o}C for few days.

Gradient plates were prepared in two steps. First 50 ml of agar media without any drug was poured to 120 mm x 120 mm square petri dishes slanted at an angle of 45^{o}.When the agar was solidified, 50 ml of agar media including the lethal dose of antifungal drug was poured and the plate was left horizontally until the whole agar was solidified.

2.7. Gateway® Cloning of *S. cerevisiae* genes

Candidate genes were cloned into high-copy expression vectors by using Gateway Cloning Technology®. In cloning procedure plasmids from Thermo Scientific Open Biosystems Yeast ORF Collection which are suitable for Gateway cloning, were used as a gene source. In this case, ORF plasmids were isolated from bacteria and amplified. Cloning procedure of Alberti *et al.* was followed (Alberti, Gitler et al. 2007). Thus, in BP reaction 150 ng of ORF plasmid and 150 ng of pDONR (Gateway ® pDONR^{TM}221 Vector) plasmids were mixed with 1 μl of BP Clonase II enzyme (Gateway ® BP Clonase ® II Enzyme Mix) and the reaction volume was adjusted to 5 μl with TRIS-EDTA (TE) buffer. The reaction mix was incubated at room temperature for 18 hours and then the reaction was stopped with 1 μl of Proteinase K at 37^{o}C for 10 minutes. The reaction mix was transformed to OmniMAX *E.coli* competent cells. After transformation, the cells were spread onto LB agar plates, including 50 μg/ml of kanamycin (kan) which is the selective marker of pDONR plasmid. Overnight grown colonies were picked, transferred to 4 ml of LB+kan broth one by one and after incubation at 180 rpm 37^{o}C for an overnight, the plasmids were isolated by plasmid isolation kit. To confirm the gene whether it entered to pDONR, isolated plasmids were restricted with Bsp1407I (BsrGI, Fermentas) endonuclease enzyme in the presence of Tango (Yellow) Buffer at 37^{o}C for 2 or 3 hours. At the end of restriction, the samples

were run on 1% agarose gel and compared with 1 kb DNA ladder. If the empty pDONR is restricted three bands will be observed as 2899, 1442 and 652 bp whereas if the target gene was entered to pDONR the fragments will be only 2899 and the size of the cloned gene. When the BP cloning results were confirmed, the second step of the Gateway Cloning was followed. In this case, LR reaction was set up with pDONR including the related gene and the target vector pAG426GPD-ccdB with the same amounts indicated by Alberti *et al.* (Alberti, Gitler et al. 2007). For LR reaction same procedure was performed. Result of the cloning was confirmed by restriction with BsrGI endonuclease enzyme and also sequencing with Gateway primers.

One of the candidate genes (SWH1) was absent in the ORF library. Therefore, this gene was provided in pDONR plasmid by DNASU Company of the Biodesign Institute in Arizona State University. Then this gene was cloned with LR reaction by the same way.

2.8. Subcloning of AmB Resistant Genes

PDR16 and PMP3 were cloned into p425GPD (ATCC® 87359™) plasmid by subcloning method. First, genes those are already cloned into pAG426GPD-ccdB plasmid and empty p425GPD plasmid were restricted with XhoI (Fermentas) and BcuI (Spe I) (Fermentas) endonuclease enzymes from multiple cloning sites (MCS) of the plasmids. The restriction was applied to 3-5 μg of plasmids with Green Buffer (Fermentas) at 37^{o}C for an overnight. Next, the restriction products were run on 1% agarose gel, bands of the target genes and backbone of the p425GPD were excised from the gel. The products were extracted by Fermentas Silica Bead Gel Extraction Kit. Ligation reaction was set up with 150 ng of gene, 150 ng of p425 backbone and T4 DNA ligase enzyme (Fermentas) in the presence of ligation buffer (Fermentas). The reaction mix was incubated at room temperature for 5 hours and transformed to JM109 *E. coli* competent cells and selection was provided by ampicillin resistance. Selected colonies were inoculated into 5 ml of LB+amp broth and after overnight incubation their plasmids were isolated by Thermo Scientific GeneJET Plasmid Miniprep Kit. Cloning of the genes was confirmed by double digestion with XhoI and BcuI endonuclease enzymes.

2.9. Resistance Test of Candidate Genes

All cloned candidate genes were transformed to BY4741 wild type cells by LiAc method and spread onto related selective media which are YNB-ura for CSP and YNB-leu for AmB candidates. After incubation at 30 ^{o}C for 48 hours the colonies were picked and transferred onto new YNB-ura or YNB-leu plates. Resistance of the genes was tested by spotting assay. Overnight grown cells were diluted four times, incubated for 3 hours to provide them to reach the exponential phase. Cells were removed from their growing media and resuspended in sterile distilled water. Optical density (OD) of the cells was measured with spectrophotometer (Thermo Electron Corporation Multiskan Spectrum) at 600 nm wavelength. 5 µl of OD_{600} 0.02 cell suspension was spotted onto the gradient plates. Plates were photographed after 5 days of incubation at 30^{o}C. Growing rate of cells overexpressing the candidate genes were compared with wild type cells carrying empty plasmids.

2.10. Sensitivity Control of Deletion Mutants

Deletion mutant collection was provided by EUROSCARF. Deletion mutants of each selected gene were transferred to YPD plates from -80^{o}C glycerol stocks which were provided previously. As the cells were grown on YPD plates, they were inoculated into 5 ml of YNB+all restricted media and spotting assay was performed with 5 µl of OD_{600} 0.02 cell suspension onto gradient YNB+all plates including CSP or AmB.

2.11. Transcriptional Analyses

Initially, the minimum inhibitory concentration (MIC) was determined for each drug. In this experiment, the cells were inoculated into YPD rich media with or without several concentrations of the antifungal drugs. Growth curve of the cells were plotted during 15 hours by measuring the OD_{600} at each three hours. The drug dose, which reduces the growth of cells to half, was determined as MIC.

In transcriptional analyses, the cells were inoculated into YPD broth and incubated at 30 ^{o}C for an overnight (~16 hours). The next day, cells were diluted for 5 times and incubated at 30 ^{o}C, 180 rpm until they reach to their exponential phase (OD_{600}

0.5-0.8). Then, the cells were centrifuged, the media was removed and pellets were resuspended in fresh media with or without MIC of the antifungal drugs. The cells were exposed to the drugs for 3 hours. Finally, the cells were harvested and RNA isolation was performed by following the protocol of Thermo Scientific GeneJET RNA Purification Kit. 1 μg of the isolated RNA samples were treated with DNase I (Fermentas) in the presence of 1X reaction buffer with $MgCl_2$ (Fermentas). At least 500ng of the DNase I treated RNA samples were then used for transcriptional analyses. The amounts of the samples were measured by Thermo Scientific NanoDrop® spectrophotometer.

In the presence of antifungal drugs transcriptional levels of whole genome was provided by microarray analysis with agilent single-channel array which was performed by Genmar Laborotories. The raw data was provided by Agilent Feature Extraction Software and bioinformatic data analyses were evaluated by PHI Tech Bioinformatics R&D Ltd. Company. After background correction, the data was normalized via quantile procedure. The consistency of each sample was measured by Principal Component Analysis (PCA). Then, the similarities of the samples were monitored by clustering analysis. Discrimination of the varied genes was detected by fold change (log2) analysis instead of classical statistical methods. Each detected gene was finally classified due to Gene Ontology (GO) and Pathway analyses.

Real-time PCR was also performed to obtain gene specific transcriptional changes. In this method, DNase I treated RNA samples were used as a template for cDNA synthesis with Thermo Scientific First Strand cDNA Synthesis Kit. Effect of caspofungin on the expression levels of *RMD9* and *SWH1* genes and effect of Amphotericin B on the expression levels of *PDR16* and *PMP3* genes were determined by qPCR (BioRad IQ^{TM}5 Multicolor Real-Time PCR Detection System). In this method, cDNA templates were mixed with 10 μl UP water and 0.8 μl of 10 μM Forward (F) / Reverse (R) real-time primers, respectively. 12.5 μl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) was added finally and the previously optimized reaction conditions were set up. β-actin was used as an internal control. After the qPCR, threshold cycle (CT) values were recorded. The results were normalized via the formula of $2^{CTact - CTsample}$.

2.12. Cross Resistance Test

The function of AmB and CSP resistant genes on other antifungal drugs were examined. First, minimum lethal doses of nystatin (NYS), anidulafungin (AND), voriconazole (VOR), ketoconazole (KETO) and naftifin (NAF) were determined. Then, BY4741 cells over-expressing each resistant gene was tested on gradient plates containing one of the indicated antifungal drugs by spotting assay. In order to show the effect of azole drugs on *PDR16* gene, ketoconazole, voriconazole, itraconazole (ITR), thioconazole (THIO) and fluconazole (FLU) were used. In this experiment nystatin, naftifin, itraconazole, thioconazole and ketoconazole were provided as raw material in powder form from Eczacıbaşı; voriconazole was provided from Sigma Aldrich; fluconazole was provided as pharmaceutical form Triflucan IV (Pfizer) and anidulafungin was provided as pharmaceutical form Eraxis (Pfizer).

2.13. Test of the Cationic Agents

Due to mode of action of the antifungal drugs that affecting the cell wall integrity or plasma membrane structure, resistance of the genes and sensitivity of the deletion mutants were examined by spotting assay. In this technique several concentrations of sodium chloride (NaCl, AppliChem), lithium chloride (LiCl, Sigma), Hygromycin B (HygB, Sigma), tetramethyl ammonium chloride (5M TMA solution, Sigma) and spermine (Sigma) were tested. For resistance tests YNB-ura or YNB-leu plates depending on the overexpression plasmid; for sensitivity tests YNB+all plates were used.

2.14. Relative Membrane Potential Measurement

The relative membrane potential measurement was performed as described in the literature (Gaskova, Brodska et al. 1998, Kodedova and Sychrova 2015) . The wild type, Δ*pdr16* and Δ*pmp3* strains were grown in YPD medium; strains with overexpression of *PDR16* and *PMP3* were grown in YNB selective medium. Exponential phase cells were harvested by centrifugation, washed twice with sterile distilled water and resuspended in 10 mM MES buffer (pH 6.0 adjusted with

triethanolamine) to OD_{600} 0.2 for each sample. Measurements were performed in UV-grade cuvettes (Kartell). The probe diS-C3(3)(3,3´-dipropylthiacarbocyanine iodide) was added to a final concentration of 4×10^{-8} M (10^{-5} M stock solution in ethanol). The fluorescence emission spectra (λ_{ex} 531 nm, λ_{em} 560 – 590 nm) of the cell suspensions were measured by the ISS PC1 (Photon Counting) spectrofluorimeter. The staining curves recorded the dependence of the fluorescence emission maximum wavelength λmax at the time of staining. Measurements were done with three independent biological samples.

2.15. Measurement of the Cell Sizes

Diameter and volume of the wild type cells including the overexpression plasmids and deletion knockouts including the related empty plasmids were measured by CASY (Innovatis). The cells were inoculated a night before and when their OD_{600} reached to 0.4 - 0.5; 10 µl of the culture was mixed with 10 ml of CASY®ton commercial buffer. Size of the each cell was recorded by CASY and the graphs were obtained in $CASY_{EXCEL}$ program.

2.16. Fluorescence Imaging

pHluorin (in pVT100U backbone), pH sensitive green fluorescent protein (GFP) constructed by Miesenbock *et al*., was transformed into BY4741 wild type cells and their deletion mutants by electroporation technique (Miesenbock, De Angelis et al. 1998). In this transformation technique, cells were grown in YPD rich media for an overnight and the next day diluted with fresh media in order to provide them to reach exponential phase. When OD_{600} reached to 0.5 – 0.6, the cells were centrifuged, washed with sterile distilled water and the cell pellet was resuspended in 5 ml of 25 mM DTT solution. They were incubated at room temperature for 15 minutes and shaken gently. The cells were centrifuged again and the pellets were resuspended in 50 ml of chilled water. After centrifugation of the cells, the pellets were resuspended in 5 ml of TpEB (10 mM TRIS-HCl, 0.1 mM $MgCl_2$, 270 mM saccharose, pH 7.5). This step was repeated and the pellets were resuspended in 500 µl of TpEB. 100 µl of the samples were transferred to 0,1 cm electroporation cuvettes. Electroporation was applied at

625V for 24 msec. 100 µl of cold water was added and samples were incubated at room temperature for 15-20 minutes. Then, they were spreaded onto YNB-ura plates and incubated at 30 ^{o}C for 2 days. The colonies were picked, transferred to fresh YNB-ura agar plates. Finally, fluorescent and three-dimensional Nomarski images were taken under fluorescent microscope.

2.17. Intracellular pH Measurement

Wild type and deletion mutant cells expressing pHluorin (transformed with plasmid pHl-U) (Maresova, Hoskova et al. 2010) were grown in a filter sterilized selective media [0.175 % YNB (Minimal medium w/o ammonium sulphate, folic acid and riboflavin and KCl, MP Biomedicals), 0.4 % $(NH4)_2SO_4$, 2 % glucose and auxotrophic supplements including leucine, methionine and histidine] to OD_{600} ~0.5. Measurement of cytosolic pH was based on a polynomic calibration curve generated according to Orij et al. (Orij, Postmus et al. 2009) and Duskova *et al*. (Duskova, Borovikova et al. 2015). pH standards were 5.67; 5.97; 6.44; 6.59; 6.80; 7.01; 7.3 and 7.76. The intensity of the fluorescence was measured in fluorescent reader Synergy HT (Biotek) at 400 nm and 485 nm, emission was at 516 nm. To eliminate the background fluorescence, a culture of non-transformed strain was grown in parallel and the corresponding values were subtracted from the fluorescence at each excitation wavelength (softwareGen 5, BioTek Instruments). The ratio of $I_{400}/I_{485}$was used to calculate intracellular pH from the calibration curve. Each strain was measured in 8 wells (100 µl of cells per well) within one experiment (technical replicates) and the presented data are means ± SD of two independent experiments (biological replicates).

2.18. *Candida* strains and Gateway® Cloning

Candida albicans SC5314 strain was obtained from Academy of Sciences of the Czech Republic. The cells were grown in Sabouraud Dextrose Agar (SDA) media (2% dextrose, 1% peptone, 1.5% agar, pH 5.6). For each experiment, fresh cells were used to prevent the genetic variations due to their unstable character.

In resistance tests, wild type BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) *S. cerevisiae* strain was used.

Homologs of the genes were obtained from Candida Genome Database (www.candidagenome.org). The *C. albicans* homologs of amphotericin B resistant genes
PDR16 and *PMP3* were obtained as C1_03820W_A and C3_01910C_A, respectively. But these genes were renamed as *CaPDR16* and *CaPMP3* in this study. *C. albicans* homologs of caspofungin resistant genes *RMD9* and *SWH1* were not exist; therefore their resistance could not be tested.

Primers used for Gateway® Cloning method were as in the following; *CaPDR16*(F) 5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCT**CTCCACCCATACTTCTCATGTTTG-3', *CaPDR16*(R) 5'-**GGGGACCACTTTGTACAAGAAAGCTGGGT**AGGTACATGTATCATTTTAGATGG-3', *CaPMP3*(F) 5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCT**CCACATACACCATTAATTATGAA-3', *CaPMP3*(R) 5'-**GGGGACCACTTTGTACAAGAAAGCTGGGT**CATCAAGCAGTAAATCTTTGGTG-3'. Bold parts indicate Gateway® attB cloning sites. The genes were cloned into pAG426GPD-ccdB plasmid by following the manufacturer's protocol of Gateway® Cloning System.

2.19. Resistance Test of *C. albicans* Orthologs in *S. cerevisiae*

Over-expression plasmids of *C. albicans* orthologs were transformed to *S. cerevisiae* cells by lithium acetate (LiAc) method. Transformant cells were inoculated into YNB-ura selective broth and incubated by shaking at 30 ^{o}C for an overnight. To test the resistance of the orthologous genes, gradient YNB-ura agar containing 2 μg/ml of AmB was prepared. While cells were in their exponential phase were harvested and their OD_{600} was adjusted to 0.02. Resistance of the transformant cells were tested by comparing with wild-type cells carrying empty plasmid.
In addition to AmB resistance, the effect of azole drugs on *CaPDR16* gene was tested.

2.20. Transcriptional Analyses of *Candida* Genes

Minimum inhibitory concentrations (MICs) were determined in SDA broth by plotting a growth curve in the presence of several concentrations of each antifungal drug.

Candida albicans cells were exposed to 0.2 μg/ml of AmB for 3 hours while the cells were in their exponential phase. At the end of the drug exposure, the cells were harvested and RNA isolation was performed by following the protocol of Thermo Scientific GeneJET RNA Purification Kit. 1 μg of the isolated RNA samples were treated with DNase I (Fermentas) in the presence of 1X reaction buffer with $MgCl_2$(Fermentas).

Then, real-time PCR was performed to obtain gene specific transcriptional changes. In this method, 1 μg of the DNase I treated RNA samples were used as a template for cDNA synthesis with Thermo Scientific First Strand cDNA Synthesis Kit. Effect of amphotericin B on the expression levels of *CaPDR16* and *CaPMP3* were determined by qPCR. In this method, same amounts of template, primers, and SYBR Green/ROX qPCR Master Mix(2X) were mixed as given in the previous report. β-actin was used for internal control. After the qPCR, threshold cycle (CT) values were recorded.

The *Candida* primers used in qPCR were as in the following; *CaPDR16*(F) 5'-GTTGGAAAGAGTCATTGATT-3' ; *CaPDR16*(R) 5'-CTTGTCTACCAATTCCTACA-3 ;
CaPMP3(F) 5'- GCTATTTTCTTACCACCAGT-3' ; *CaPMP3*(R) 5'-CATGTAAGATAGCAGGGAA-3' and the qPCR conditions of *CaPDR16* and *CaPMP3* were 95 ^{o}C 10 min; 95 ^{o}C 15 sec, 60 ^{o}C 30 sec, 72 ^{o}C 30 sec; 4 ^{o}C 5 min, with 40 cycles.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Determination of Minimum Lethal Doses of Antifungal Drugs

To find out the exact minimum lethal doses (MLDs) of amphotericin B (AmB) and caspofungin (CSP), effective ranges of each drug was obtained according to literature (Table 1) (Letscher-Bru and Herbrecht 2003, Sabatelli, Patel et al. 2006).

Table 3.1. Selected dose ranges determined due to literature data

Drug Name	Dose Range (μg/ml)
Amphotericin B	0.2-2
Caspofungin	0.2-2

Serially diluted BY4741 wild type yeast cells were tested on YNB+all non-selective but restrictive media including different concentrations of each antifungal drug by spotting assay. Control plates were not including any drug. After 3 days of incubation, it was observed that wild type cells were not able to grow in 0.4 μg/ml of AmB or CSP (Figure 3.1).

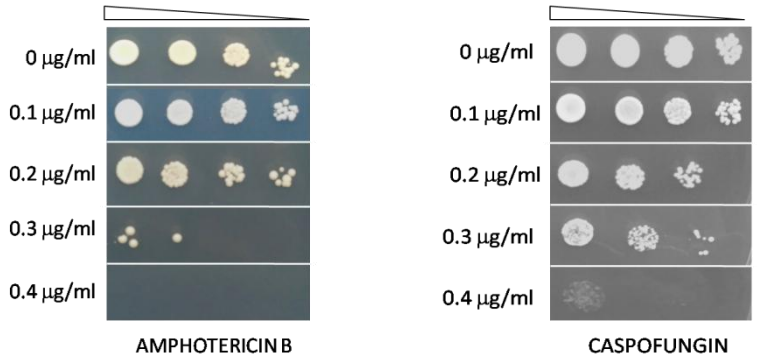


Figure 3.1. Minimum lethal doses (MLDs) of Amphotericin B (left) and Caspofungin (right) tested by spotting assay on YNB restricted media. Each line refers to different concentrations (μg/ml) of indicated drug and triangles refer to serial dilution of cells (OD_{600} from 0.2 to $2x10^{-4}$)

3.2. Library Screening and Selection of Resistant Colonies

In library screening two different protocols were followed. In the first way, gDNA library was transformed into BY4741 wild type yeast cells and these transformants were directly spread onto YNB-leu agar containing the minimum lethal dose of the each drug. After 3 days of incubation, candidate resistant colonies were selected and transferred to fresh YNB-leu agar plates (Figure 3.2.A). In the second way, library transformants were spread onto YNB-leu agar plates which do not contain any drug. After 3 days of incubation, all of the colonies were selected one by one and transferred into YNB-leu broth in 96 well plates. By this way a transformant collection was provided that can be also used in other screening studies. After selected colonies were grown for an overnight, they were transferred into new 96-well plates containing YNB-leu broth with minimum lethal dose of each drug by the help of replicator. Again the cells were incubated for 3 days and the grown cells in wells were detected by naked eye (Figure 3.2.B).

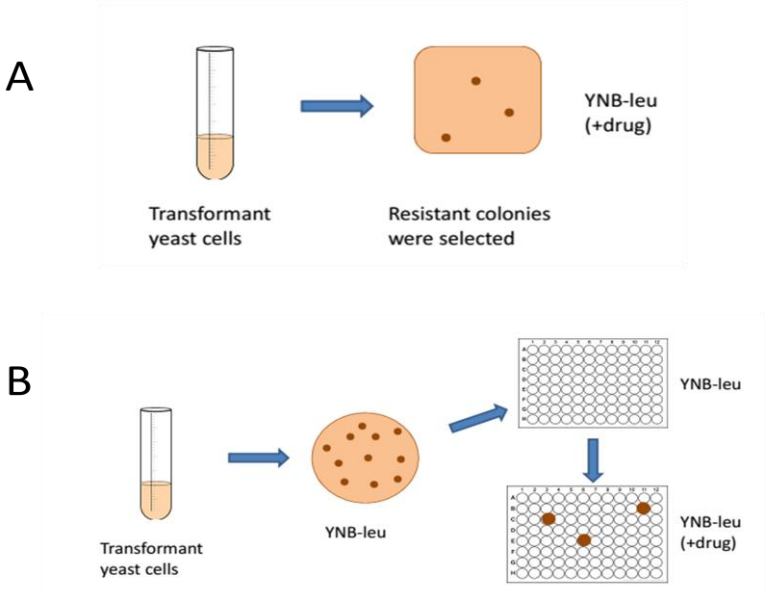


Figure 3.2. Library screening by two methods. (A) Directly selecting resistant colonies after library transformation. (B) Preparing a transformant collection and then screening each colony independently.

At the end of each two screenings, 4 AmB and 7 CSP resistant colonies were obtained in total.

3.3. Confirmation of Resistance

Plasmids were isolated from each selected resi, amplified with *E. coli* competent cells and transformed to wild type yeast cells back. Therefore, subsequently gained natural resistance of the first transformants could be eliminated.

The resistance of yeast back cells were tested by spotting assay. All 4 of the AmB resistant colonies could grow in high concentrations of AmB, when compared with control. On the other hand, 6 of the CSP resistant colonies were able to grow in high concentrations of the drug. Whereas, one of them was false positive and could not resist CSP as in yeast back form (Figure 3.3).

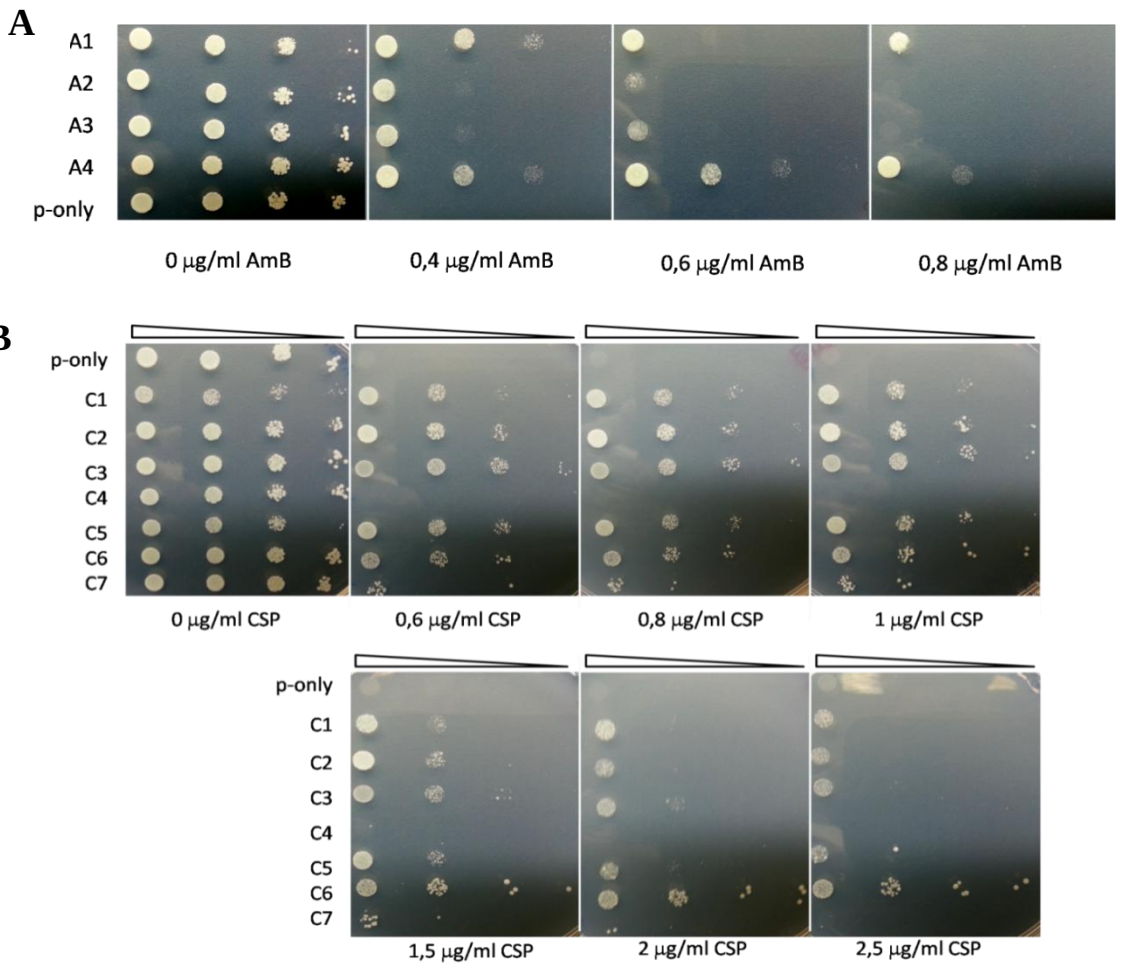


Figure 3.3. Confirmation of resistance in yeast back cells. (A) All colonies (A1-A4) were resistant to high concentrations of AmB. (B) Six of the candidates were resistant to CSP (C1-C3, C5-C7); C4 was false positive candidate that could not resist in the second time. Wild type cells containing empty plasmids were used as negative control (p-only).

3.4. Identification of Antifungal Resistant Genes

Plasmids of 4 AmB resistant and 6 CSP resistant colonies were obtained and bidirectional sequence analysis was performed (Table 3.2).

Table 3.2. Sequence analysis of resistant gene cassettes. In AmB resistant set, A1, A2 and A4 had the same sequences. In CSP set the sequence of C6 had no match in the database. F refers to forward and R refers to reverse reading.

Amphotericin B

A1, A2, A4 F

CTNTCGACAC CGTCTGGCGA CCACCCCGTC CTGTGGATCT TATTTCGTAA AGTTTTCCAT TAATATTTTC GAAGAGCTCG ATACGAGGTT CCCCCCCAAT GTTGGAAACG GTCATGCTTT TTGTTCGAGA GGCCAGAAAA GAAAGGTCAA CTATTGCGAC AGAATAAGAT TAAATTTGTA CTCGATTTGT TTCTGCTGTT GTACCAGAAT CATTAACCAG ATCTGACTTT AATTCTCATC GCGCTCGTGC TTTTGACAAA CTTGTGAGAT GGGAGTCTGT AGTGCCTTAA AAGGAAAAAA CGCGTTGTGG TTATGAATAT GAGATTTCAT AATCCAACTA TGGCATCGAC AACATTGAAA AGATAATGAG CTGGAAGTTG CTCCATTTTT TACCTTTGNA ATTTCTNCTT ATATGNATAT GCTCACTTTA CTGACTAGGA AAATGTCGTG NAAATTTTTA GAGTTCCATT TTGGCTCAAG ACAATGGCGC TACNAAAAAC GCCA

A1, A2, A4 R

GTTTTTNGCG NTATAGGCGC CNGCAACCGC ACCTGTGGCG CCGGTGATGG CCGGCCACGATGCGTCCGGC GTAGAGGATC CAAGGGTCCA AAAAGCTGCC GGAGATACGA ATAATTCATGGGACAGAGCT CAAATTATGG ATAAATTGGA GAGCTATGAAAAACCGTATG TAGGAAGCCA ACAGAGTATN AATATACTTA AAAATNATTC CTTTTAGCAT ACCCTTTTAT TTCAATGTAT AAGAGTTTTT

(Cont. on next page)

Table 3.2. (Cont.)

GGAACGCTAC TTTATAACAT TCAGGTCTGT TCACTAGAAC TCTTGTAGTT GTGCCGTCAT AAAATTAGTC TTCGCGTGGC TGAAAGAAAA GTTCCATANT ACGGGCGGCT CGAGNAGAGNAATCATTGCC GTCACAAACC NTTTTCGGGN AGAATAAAGC ANTGGATATT TGAGCTCCAN AATNAAATGG CTTTCTTTTG CGGTTACTAT CTACTNGACT TGGCTGGNTT TAACNCAGGT AGAGAGCCTC AACNTGGAAA NAAATAGAAG GGAAAGAAAT ATGCTGTAAN CTCTTN

A3F

TGGNGNCNCC CCGNNTGTGGNTNACGAGTC CGATAAAGCT TTCAAGACGG CGGATTCACT TGCAAAAGGC TGCGCAACGT GTGGCCTTTG GTGGCCAAGC GGGCGGACAA TCGTCTTCCC CCAAGAAAGG CCCATTGTCC ATCAAACCGG AGCCTGTTAA GGTAAATCGTCAAATGGATA ATGTCACAGC AGAGAAGAAA GATGTTACGC CAGCCCCATCACGCCGGTGA AGAAGAGAAG ATCCGAATCA CCGTCAATCT TTCTCAATCG TAAAAACCTG CGCTATTCAG GCCTACTCCG AAGACAAACG CTGCAGGTTCTCGACCCCAT ACAACAGCGA TCACCAACGA TCACCGTACT ACTTCTCATC CTTATCCCCA CAAAGATGTT GTAACTTCAA TATCAAGTGT CACCGCAAATCCCGTANCAA GGGCCACAGA AGAAAAATCC GGATTTTCGT ACGAATGCCG GATCTGACGG AGATAGTTTN CGCACGTGAC CGCAACGGGC CTACTACCCG TCCGTATATC TATGAGCCAC GNAATAGCAG TACCCACTCC GAA

A3R

GGGCCGATCT TCCCATCGGT GATGTCGGCG ATATAGGCGC CAGCAACCGC ACCTGTGGCG CCGGTGATGC CGGCCACGAT GCGTCCGGCG TAGAGGATCC CTTGTTAGAA GAATATTTTC CATCATTAAA ACATCAAGCA GTTTCGTTCT GTAAGGTCCT TGATTCTGCT ATTGACAACC TTGAAAACGT TCATGTCCTA GATGATTATA TTGTGAAATT AGGGAAAAAC GCCATTCCAG AATTCTCGGC ATCAAAACCG TTGGTTTCGA AGTAATGGGA AAAGCATTTA TGACTACATT

(Cont. on next page)

Table 3.2. (Cont.)

GCAGGACAGG TTTGGATCTT TTCTCACACT GGAACTCAAA AATCTATGGG GACAACTTTA CTCATATTTG GCAAATTGTA TGATTACTGC AGGGAAGGAC CCAATGGAAA AGATTCAACC AGATTTTTCG TATAATGGTG ACTCTGTGGT TTTAATTTTT CCATTCCCAA CTTGCGATGC ATGATATAAG TACAGTTAAC ANGCTACAAA NGGGNAAACT AAAAACGCTA CTATACCTCA TAATATAACA CANGTACCGA CAAATAAANT CCTACNGAAA TC

Caspofungin

C1F

GGTAGTGGAC ACTATTGAAA GTCATGAAAG CTANGTTTTA GCATCTTTAA TATATTTCGT TCAGCCCGGT TAATCTTTTC CATGAAAATA AAAACCTTTA AATCAATGAG TATGAATGCC ACGCAGCTCA TGGAATATGA TGTATTGATG CCTCAAGAAG TTTATGAATA CTTCATACTA TATAATTAAA TAGGTCCTAT CCTGTTTTCC GATGTACCGG CATTCGCAAT GGATACAAAT GAACGAGTGT TATTGTGACT ACATTGCACA GCTTAGAAAT ATCAGCACAA TCTTTAAGTC ATGATTTTTT CTTTTGTTCC NATACTCTCC ATTAATTTCC AGTATAGACT TTTGGTACGG GGTGCTCCTC CTGGACAACC ACTTAGGNCT GNATCTTCTC CTTTTGNTCC CTTTCTTTTT TGCTGCCCTT GTTTNACTTC ACACGANGTT TTCCGCGCAG CTTTTCGTAT CACATTTTCA TACCTTTGAT CGGCCTAACN CTATTGGGGG CTAAANGGACAGGGGNGGAAACATCAAGCC ATGAACAGTA ATAAGGNNGA GGCTCATTGTTNAANCAAAT AACACAGTTG NCCCCGGNAA TGCGTTTTCG NATGAGTNCN CNAGATATTT NCGANGACTC AGCCAAACTA TTNCNNTG

C1R

GGTGATGTCG GCGATATAGG CGCCAGCACC GCACCTGTGG CGCCGGTGAT GCCGGCCACG ATGCGTCCGG CGTAGAGGAT CCGCGCCCTT TGGCGTGTTC

(Cont. on next page)

Table 3.2. (Cont.)

ACCACAGAGT CGCGCCGGGT GTGGTCCAACATCAGGGACT ATCTGTTCCA TGCGGACGAC TGCACCAACT GGCGCAATCT CAAGCTGATC GACTCCGCGC TGTTCGTGGT CTGNTCTCGA CGACGTGGCG TTTGCCGCCG ATCAGCAGGA CGAGCTCACG CGTTCGATGC TGTGCGGGAC TTCTACCATC AATCTCGACC CGCACCAACA CCAGCCGCCA TTGAACGTGC AGACAGGCAC CTGTCTCAAC CGCTGGTACG ACAAGTTACAACTGATCGTG ACAAGAACGG TANGGGGGCA TCAACTTCNA AACACCGGTG TGGACGGCAC ACTGTGNTGC GGCTCGCCAC AGANATCTAC ACAGACTCGATNCTGAGCTT CGCACGCGGN GTCACNAAAA CGTCGTCGAC NTCTTTAGCG ACGACGATGG AAAAACATCG TCGTCGTCGT TGGCCTCGGC GGNTCACTCC GCCAACTTGA TCACATCCCT CGNAAACTGG AANGGCCCCT GACANTTNCT GCAATCG

C2F

GGAGCCCTTC GANCNGTCTG GCGACACACC CGTCCTGTGG TCAGTGTAGA TTCTCTAATA TTCAACGTTA GCACCTTGCT CAATACTCAA ATACGCTACA TAGGTTTCTA TTAGATGCAG CCATACTACG GGGTCCTTTT CAATTAATTG AGCTCTGAGT TTAAACGGTG GAAATTTGAC AATGGGCAGA GAAATATCTG CATTACCCCC ATTTATTTCA ATAACGCGGG NNTGTACCTT GATAACATTA TTCTTAGNGG ATTCTATCCT ATAAAAATTT CCAAGATTCA ATAGTTAACT CGAATGTCTC TCATTTATGA GAACTAGTGG TTTATATTTT CATTTTTTCT ATGGCCGCGC AAATCCATTG GCAGATATTC AGGTATATAA ACGTTACAAA GCCAAGAGGA GAATGGAAGGCCNACAAAAA ATTCTTGTAC AATTGCATAT ATTGATTCAT TACAATATTA CTGCCGTCGN CCTCTCAGTC ATAAATCATG NTTTCCCTTT CCCTCTCAGC ATGCTTTTTC TCGGCCTCTT CCATTAAGCG AGTCTTATGA GACTNGGCAC GCTCTTGAGC TAGCTTTTNG CCAACTCGCC CTTATGGACT TTTCATNCGN CTNTGGAATC TCTTCTCAGC AGCTAACCTN GGGGCCCCTT TGGGCTAACT TTATTTTNGC NNGNACNCTT GGATCAGNTA ATTNGCGGCC TTCTTTCTTT TGNANANCNG GANAGGNGAC NCNTTAGGNG GGGCCCCCCC NTTTAACCTN CCCCCCTNAN GNCAANNTCT NGNTTTGACT

(Cont. on next page)

Table 3.2. (Cont.)

TGACNGGANC NNATAAATGGGTTNGNCCTG CNTNANNGCC CCCCCCNAAT TTGGNANGAN TNGGTGGGNT TTTCTNCNCA ANCCCCAN

C2R

GCGCTCATGA GCCCGANTTN NNCCGATCT CCCCATCGGT GATGTCGGNT ATTTAGGCGC CAGCAACCGC ACCTGTGGCG CCGGTGAATG CCGGCCACGA TGCGTCCGGC GTANAGGATC TTCTTTTTTG GATTCAATTG CTTCATTGGA GCTTCGTCAC CGAAACCTTC AAAGAACACC TTGTCACCGG CCTTAGAGTC CTTTGGTGGC TCGACAAATT CAACTTTGTC GTCGTTAGAA CCACACAATA CCCATAGCAG TGGATTTAAT ACCTCTCATG TTAACTGGTT TCAGTTGCAT ACAACAACAA CATAACGTTC TTGCATAGCG TCCAAAGGAA AATGCTTGAC CAAACCAGAA NAACAGTTCT TGGGCCTTCT TCATCACCGA CATCAATTGT AGAGACATAT AGGGAGTCGG CATCTGGGTG CTTGATGGCT TTTTGAAAAA ACCAAACGGA AATCTATTGC GGATGGCTTT GGCTTTTCTG GGGCCTTATT TNGTNGCTCT TGTTGTTGTT TGGCGTTGGC GGCCTTCTTA GCTGCCTTCT GGCCTTTGCT CTCTCTCATT TCTNAAGGNT TCNTCATCNG NTTACTCTGN AAGATCTGCT TTTAGCTTCT ACAAATCTCG NCTGCNTGGT GCGGAN

C3F

CTNTCGACAC CGCTGGCGAC CACACCCGNN CCTGTGGATC CTCATTTTCC TTCGAAGCTT CCATTGGTGT GGATTGACGA GATTTGATAG GCGAGATGGA GGCCCCAGAG TTCGGTGCAC CTAGCGCCAC TTTGGATCGC CTTTTCGAAG GTCTTGCATT TAAGCAACTT TCATTTAACG GCTGCGCCAT TGATGGCATT TTTCTTTTTG TGCTGTTTTG TGAGGAATTG ACACTCGAAC GGTGTTCTCA CAGTTATTCG CTGAGATGAC GCATAGTTAG AAGGTGATCT TCCTCTAATA ATAAGAGGAG GCGTTTTCAT TTCTTGCAAT ACACAAACAT CCCTTTTGAT CCATTTTTTA AAGCCAGTTC ATCATAAGGA ATTCCGGGAT TCTCCCCATG AAAGGTATCT GGATCTACCA CTGCACCTAA AATTACATGC AAGCTAAAGT

(Cont. on next page)

Table 3.2. (Cont.)

GTTTATTTTG TTGGGATGGT TTCTTCACGC TAATTGATGA AGCAATTGAA CTCTTTCATA ACGGGCACTT TCTGATAGAA TCTTCTGGAT AGNAAACATA AGAGNCACTC CTATTCTTCA TANTA

C3R

CCNAACNGCG CTCATGAGCC CGATTTCTCC CGATCTTCCC CNTCGGTGAT GTCNNTTATT TAGGCGCCAG CAACCGCACC TGTGGCGCCG GTGATGCCGG CCACGATGCG TCCGGCGTAG AGGATCCAGT CGAATATAAA ATCTTGCTAC TGCATTATTC TACGATTTCC TGTTATCACT TATAGTACAC ATGTCTGAGT TTTTGAATGA AAATCCCGNA CATTTTAGAG GAGAACCAAC TTCCCACTAG AAAAGAAGAA TAGTACCCAGGNACCTTTTG TTAGGCGGGT TCAGCAACGA AGCTACGCTG GAAAGGAGAA GCCTTTTGCT GAAAATAGAC CATTCTTTAA AGTCTCAGGT ATTGCAAGAT ATAGAGGTCT TAGACAAGCT TCTTTCCATT CGAATTCACC CGAACTGACT TCCGATGAGG ATAGTTTGCC NGCAAAAGCG AGNATGAACCCTACCGGGTGGAGGAAGGAG NAGGAANAGC CTGATCTCCT TGATGCTCAA AAATAATGAT TNNTAGCTCA TATTCCNACC TGAGCACCGT NAGTCTGGGA CACTCTCTGT TGTAATTGGN AGANNTGGTG TNCTGATNAG GAACCNAANN AANGGCTGAG TCGGATCAAA NACCCCCCAT TNCCCTNG

C5F

GNTCNTTCGC NCTTGGAGCC CTATCGANAC CNTCTGGCGA CCACACCCGT CCTGTGGATC TTGTACGGCA CATCTATATT ATCAAACTTT TCAGCAAAGT CAGCGGCGTT TATAATATTT GAGCCATTTT TCCATTTTTC AAAAGATTTC ATGAAACTCT CCTTATGAGG GGTGATTCAT TTTATCCCTT TTCGATTCAC CCTCATTCAA AGCGATATAG TCAAGTAATT TCTTCTTTCT GAAATTATAC CGCTTATTTA TATGACTATC TTCAGGGTTT TCTTTATTCC TTTGCCGCTT TCCTTTTTGT GTGATGAGAG CGTATGCGGT AAGATATTCT CCTTCCTTAT GATTAGGACA ACGGTAGCTC TTGGATTTGC TTCGCAGAAT TTGGATAAGA

(Cont. on next page)

Table 3.2. (Cont.)

CAGAACTTCA GAACTTGTAA GATTTGAATA GTGAATGCGT TTCAAGGCAC GCATTTCACG TGGACCACTG CGGGCAACTA TCACACTTCA CCCAATTAAT GCGCCTGGNA TTATCCTTCA ACTGGGAAAG GCTGGCAAAT ATTGGGATCT TGCATTGCAC CATAGCTTAT TAGAATTTTG NTTTATGATC ATTACGNCTN CTTACTCATA AN

C5R

GGGNCCGGGN NGCCGGCCCATGCGNCGGCG NNAGGTCCTC TGCTAACTAG TTCCCAGTTC ATCAATTGCT TTTTTCAAGT TTTACCGGGT TTCTCTTTCC ACGATGTCGA AGAAGTCAAG TTGATAAGCA CAGATTATCT ATTCAAGGAT GGTNACCATA TTCCACAAGT TGGCGAAGAA GGTTACATAC CTGGTGAAGA AGAAGCGTGG GTCCGTAACA ACACATCCAC TTTGGCGCAG ATTGAATCTA ATGTCCTAGA AGATTTTGAA TTTCCAAAGG ACGAGAGAAT ATTTTATCAT TTCATGAAGT GAAGCACTTT GAAAAATGCT CANGGGCGAT GCTGGCGCAA AACTGATAAC ACACCTAAAG AATCTATGAC TAGTGTGATT TCTGATTCGG TNAGTTGTCT GAAGCCGAGT TCACTATCTA TCTCAATACA TCTCTCTGNA CATTNAGCTC GAAGGGTTCG AAAACTACAA AGCNGTTGTT GAGAACTCTC

C6F

NCNTCTGGNA CACNCCGTCT GTGGTCTGCT CGCGGATGCT GCTGGCTACC CTGTGGACAC CTACTCTGTA TTAACGAAGC GCTGGCATTG ACCCTGAGTG ATTTTTCTCT GGTCCCGCCG CATCCATACC GCCAGTTGTT TACCCTCACA ACGTTCCAGT AACCGGGCAT GTTCATCATC AGTAACCCGT ATCGTGGAGC ATCCTCTCTC GTTTCATCGG GTATCATTAC CCCCATGAAC AGAAATCCCC CTTACACGGA GGCATCAGTG ACCAAACAGG AAAAAACCGC CCTTAACATG GCCCGCTTTA TCAGAAGCCA GACATTAACG CTTCTGGAGA ACTCAACGAG CTGGACGCGG ATGAACAGGC AGACATCTGT GAATCGCTTC ACGACCACGC TGATGAGCTT TACCGCAGCT GCCTCGCGCG TTTCGGTGAT

(Cont. on next page)

Table 3.2. (Cont.)

GACGGTGAAA ACCTCTGACA CATGCAGCTC CCGGNAGACG GTCACAGCTT GTCTGTAAGC GGATGCCGGG GAGCAGACAA GCCCGTCAGG GCGCGTCANC GGTTGTTNGG CGGGTGTCNG GGGCGCAGCC ATGANCCN

C6R

GCNNTNATCGCGCTGNCNNANNAATGCANNNNGAGNANAT NNCANATTGG GTGTTANAGNGTNCACNGTN CTNTCGANCC AGTNNANTNC GTNNGNTAGA CNGGANNCAATCGNACTACG TATNGGTNGT GGNTGGTNTN NNCGNTNCCT CCNACCGTCC NTGTGCTGCA CGCCACCTAT TTCTCCNTCT NATCTATACA GCTCGTTNNN CATCNTACTC CTNTNGNCNN CTNCTGACCN TAGATTCNNC ACCTNTGAAT ACGCTNNATN NNTAATTNNN GNGTNNGANN TNNCCGCCTT AATCATANGNATCGTTNGAT ACANCNNNNC AANACCTNAC CAGANNTNTC CATGTNNTCN TGCGACGTTN CTANTATATC ACGATNGCCN AGAGGCNNCG ATCGTNNCTN ATNGNATCAG ACNTTNCCNT CGNCATANTC NACNCTANGA TTGNAAACNA CACTTTGNTG CNTCANNTNG ACAATCGNTN NNGGTTCGTA CACNNTGCAT NNNGTANTCG TNNACTNNGC NGTCTTANGG TNTNGNNAAT TNATCATGTC GAGNTNGCTA CGTCTGNNNA CCAACTNCGA CGTCGTATGC NATGGNTACG NNNCTATNAN TNCTGTGCTA TTCTCGACNC TANATTNNNC GNNNNNTACACAACANCNGA TTATNTNNGN GCGNACGCNT NTNNCTGTNT ACTCNACNNANNTGCANNTA CANACNGTNC CGNACNCCNT NNACTNCGTN ANNCNCCTNATGNNATNCNT NNACNTCANN NNTCANATAN CGTCTATNCA NGNTCGNTATCTCTAATGAT NNTNNCTANN NTAATNTNNG NNNNNANANG NCNNTNTCNT NANATCGNNT ANCNTNTATN TNNNTTGNCT NTGCNNTAGC AATCTCACGT CTTANCACAC TATCTCACCT CCCCNTATGN TGANCCTNAG NTTGTGAGTN TACGTTGANN ATGATACNNT AGTATNTACN CCTCNTNCNG NTNTGTACTAGCNACNNTATTNCGTNTGANANTNAC

(Cont. on next page)

Table 3.2. (Cont.)

C7F

AGGGCNTCGC GCTTGGNCGC GCCAGTCCTG CTCGCTTCGC TNTTNNNGCC CTATCCTTNA CGANTNCNCG CCGCNNNCCG NAGAGAGNTA NTTCNCNCCC CCTATTCCTA CTAAGGGGNT TATTAAGGAG CCCAATATTT GGGAAGGATG TTGGGCCAAGATGGCCTGCAAGCAGCTATTTCATATGCCA CAGGAAACAG GGCTGCTTTG ATTGGTTCTT TAGGTTCTAT ATTCAAGACC GTTAAGGGAG GTATGGGCAATAATGTGGAT AGAGAACGCG TGAGACAGAT CAAATTCTCA GCAGCAGATG TTGTTATGTT ATCAGGTTCG AAGGATAATC AAACTTCTGC AGATGCTGTC GAAGATGGGC AAAATACAGG TGCAATGTCC CACGCCTTCA TCAAGGTTAT GACTTTACAA CCACAGCAAT CATATTTATC TCTTTTACAG ACCATGAGGA AAGAATTGGC TGGTAAGTAT TCTCAAAACA ACAATTATCA TCGTCACACC CTATTGACGT AATCTGCAAT TTATTATGTA GAATATATGATGTGTACGTT GGTAGATGTA TTCAGACTGG TGTAGTATGT GATTCANTTT TATTTTTTAG AAGCAGAACN ATCTAGCTAT TATACTATNA CNTTAGGCAA GATTATAGGGATAGGCTTTT GGAATAAGAC NTGGGTTTTT AACGANNGAN CN

C7R

GNNCCGGNCCGGGGCCTGCC ACATACCCAC GCCGAAACAA GNGCTCATGA AGCCCGANTN NNATCCCTCT TCCCCAGNGG GGAAGNCNNT TTTNTCCCCC NCCAGCAACC GCACCTGTGG CGCCGGTGAT GCCGGCCACG AATGCGTCCG GCGTAGAGGA TCGCAACCAC GTTCAATTTC TCTTAGTTGG TTACCAATTG GAGAACCACCGTAGACGACG CAGGCCTTGA CCCAGGATCT ATAAGTAAAT TTCTTGGCTT CATCGAAAAT TTGGGGTGGG CCAACTCTCT AGTTGGAGCC ATAATGACAG CAGTTGGGTA GGCCTTTCTT TGGTAAAAGG AGCCTTGAGA CTCTGGTTGA GGAGATGGTC CAGTCTTAAA TGATTCGGAC AACACTGGGA ATAAAACCCA CCAGTCTTAC CAGAACCGGT CTGCGCACAG GCCATCAAAT CTCTGCCGTT GGCAACGATA GGGACGGAGT ATTTTTGCAC AGGTGTTGGC

(Cont. on next page)

Table 3.2. (Cont.)

TTGGTGAAAC GGGCCAATTT GATGTTTTCC AATAACAATC CGTCCAATGG AGGNGAGGAAATTCTGTGATNGGTTCAGGA ACATCCTTAC CAGAGGCGNC CACTGGAATATCATCGTAGT TATCGAAGTN ATACCANAAG ATNGNAANTT GGATCCTCGG GGACACCAAA NTGGCGATCT CGGCNTTTCG TTTNTGGGGC TGG

All these sequences were searched in *Saccharomyces* Genome Database (SGD) and the borders of the gene cassettes were obtained. According to open reading frame (ORF) maps, possible candidates were selected according to their function (Figure 3.4).

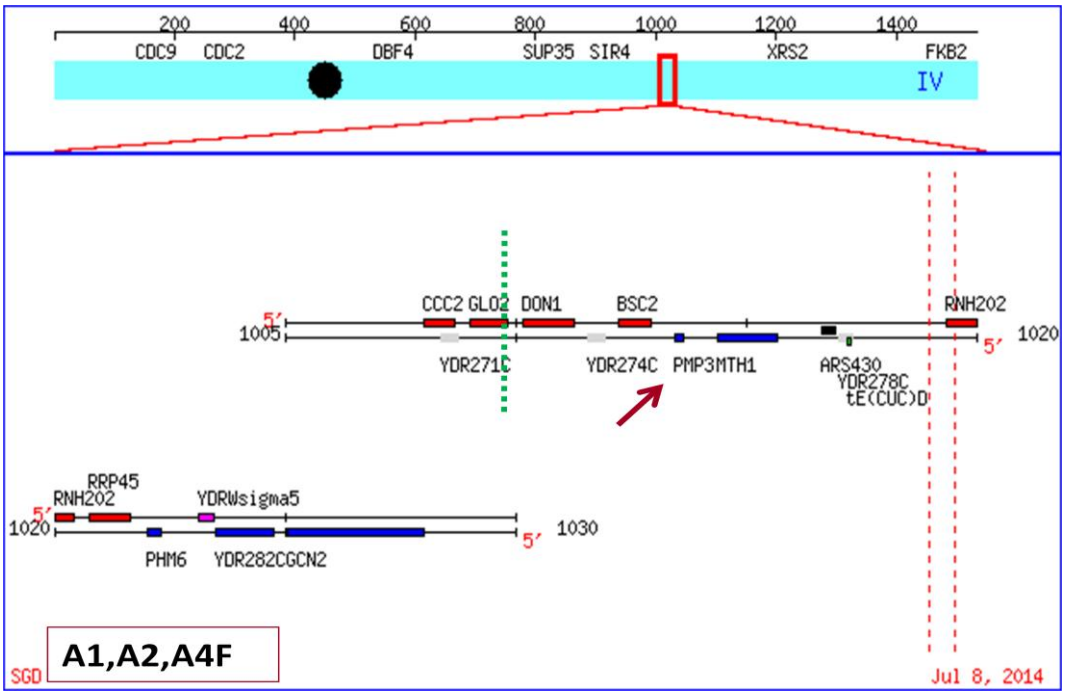
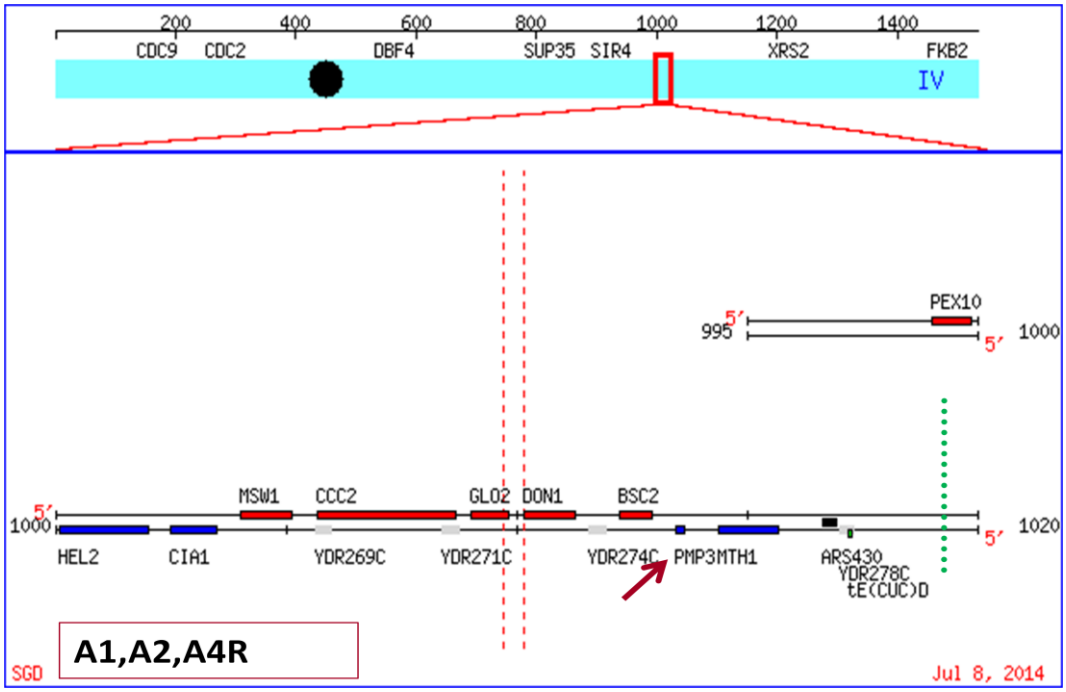


Figure 3.4. ORF maps of gene cassettes. Each sample and its sequence direction (F: forward or R: reverse) was indicated in the red box on left bottom of the maps. The regions between the two dashed red lines are referring to sequenced gene parts and single green dashed line indicates the other border of the gene cassette. Red arrows indicate the selected candidate genes. The selection was done according to genomic function and/or literature information.

(Cont. on next page)



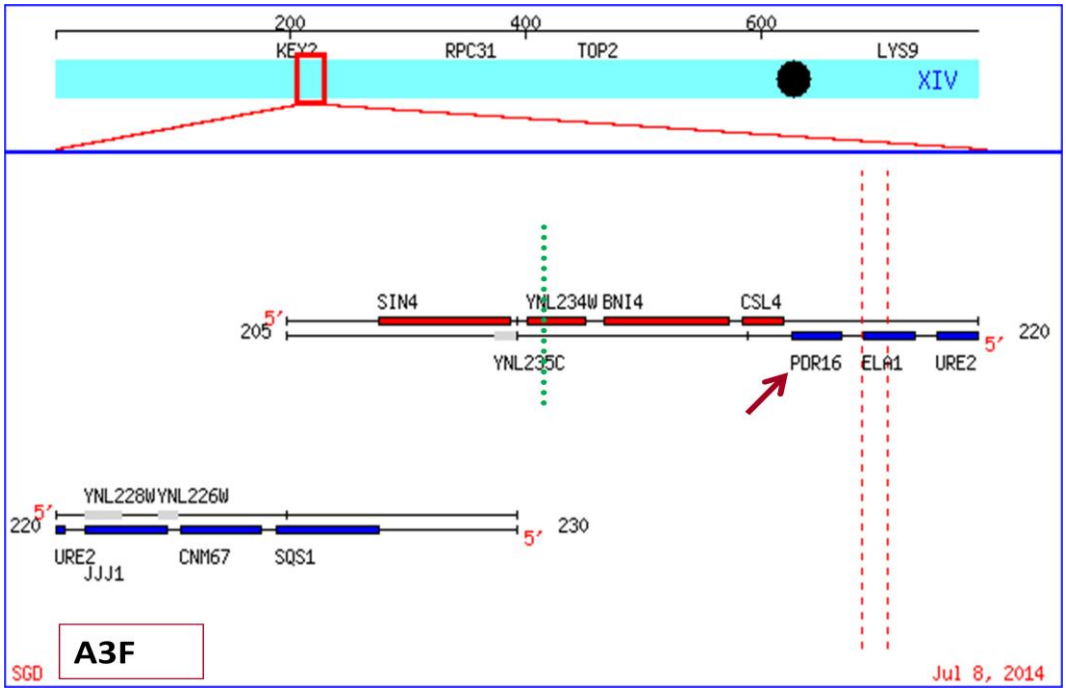


Figure 3.4. (Cont.)

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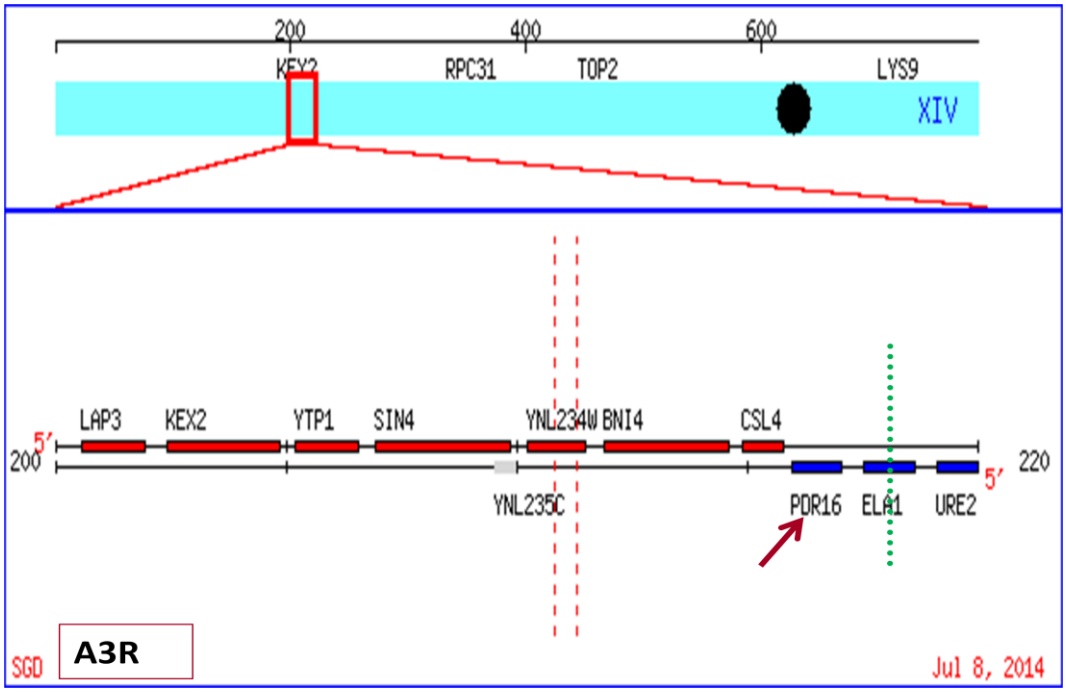

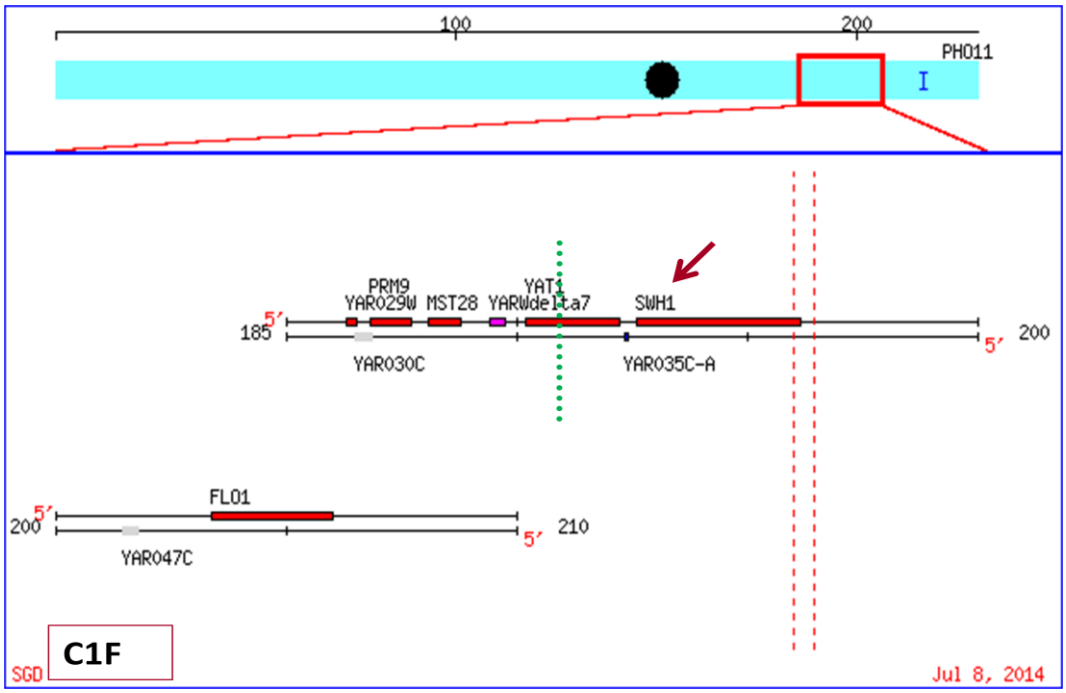

Figure 3.4. (Cont.)

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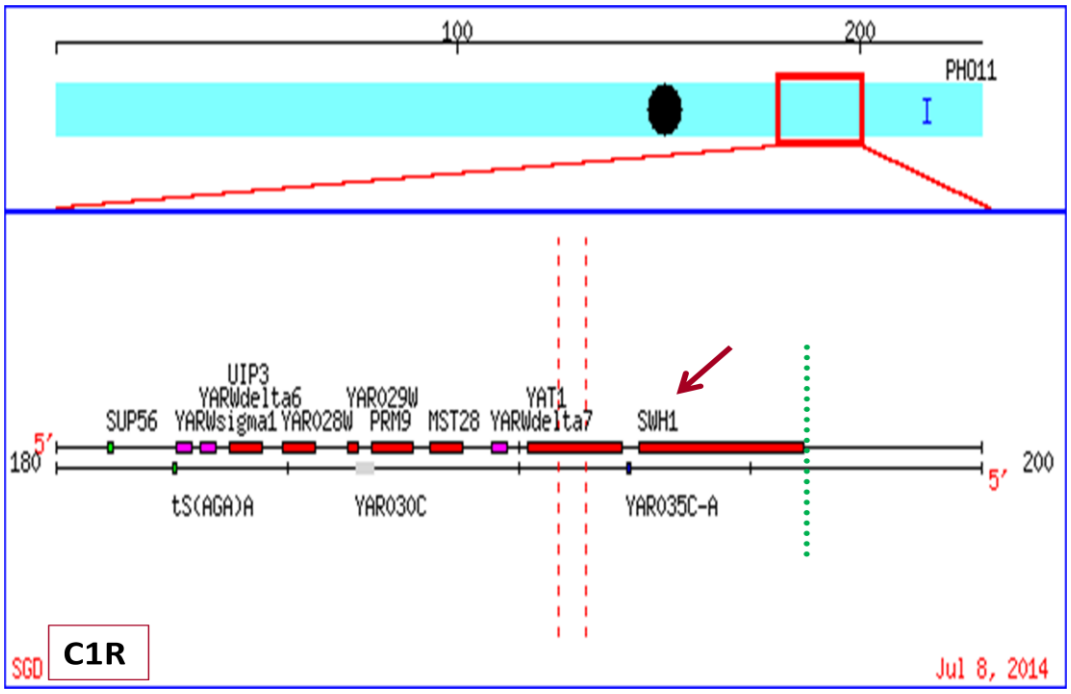

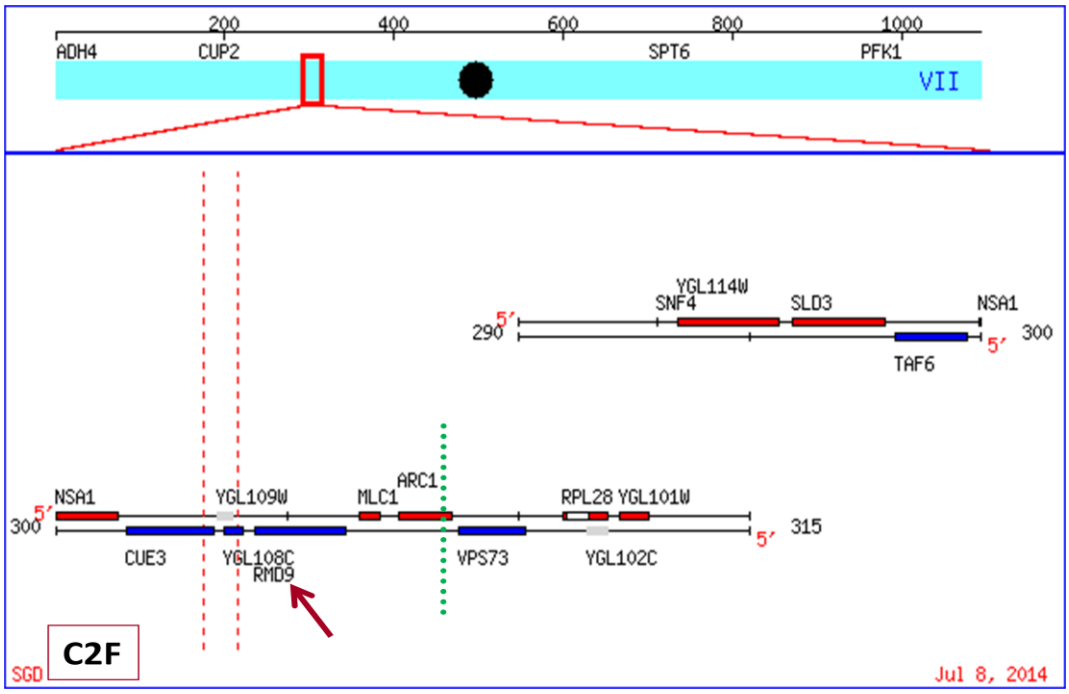

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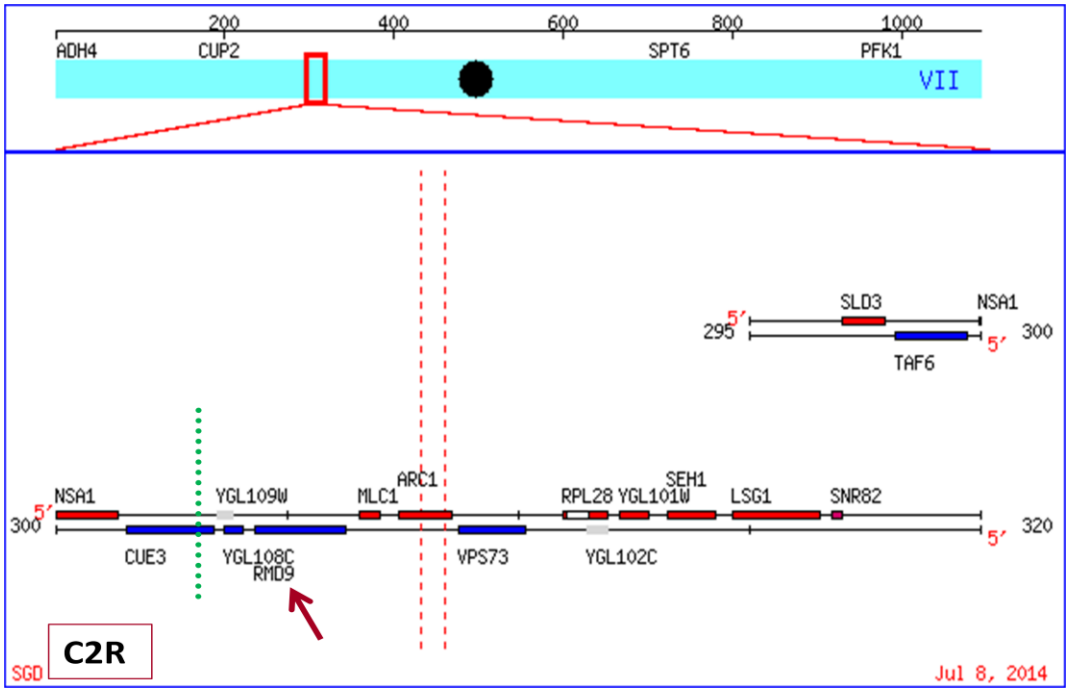

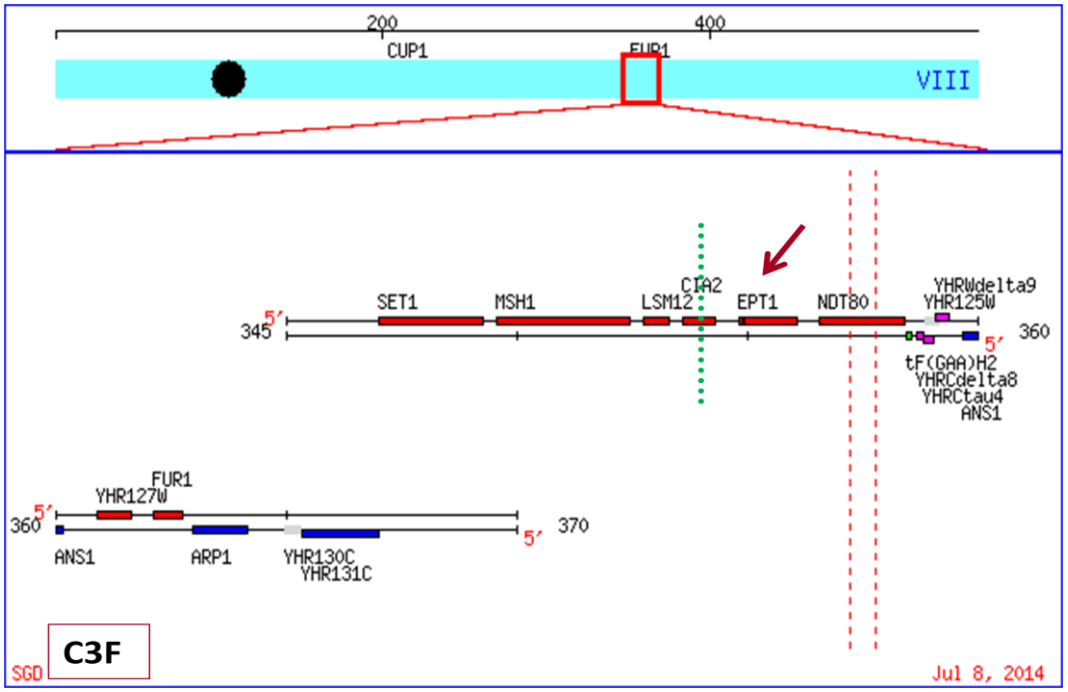

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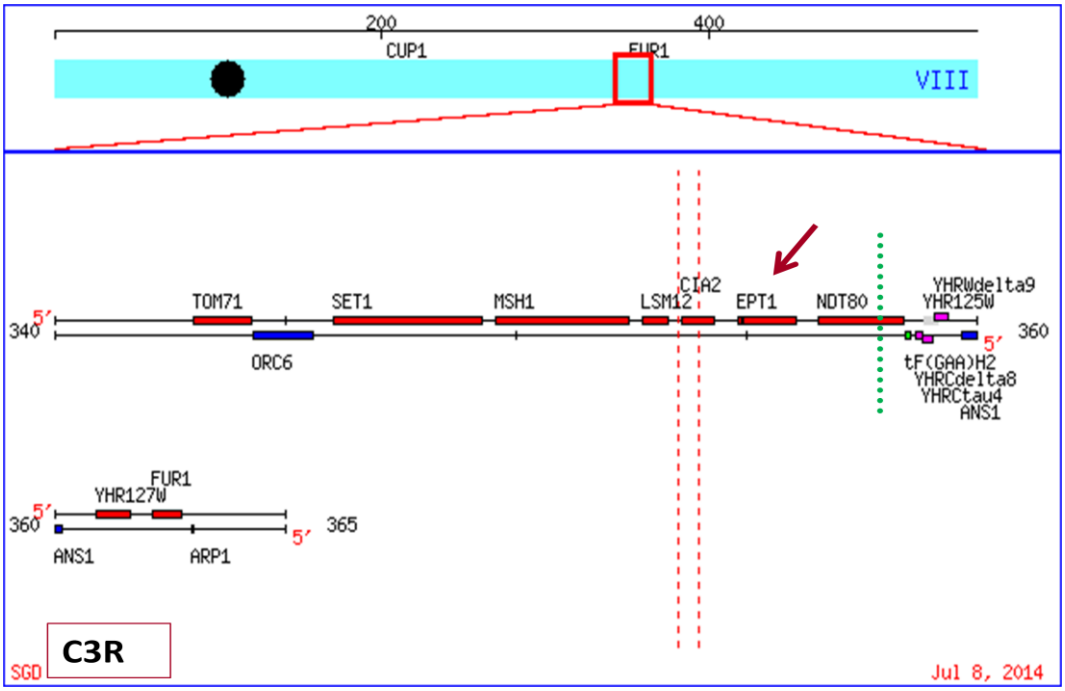

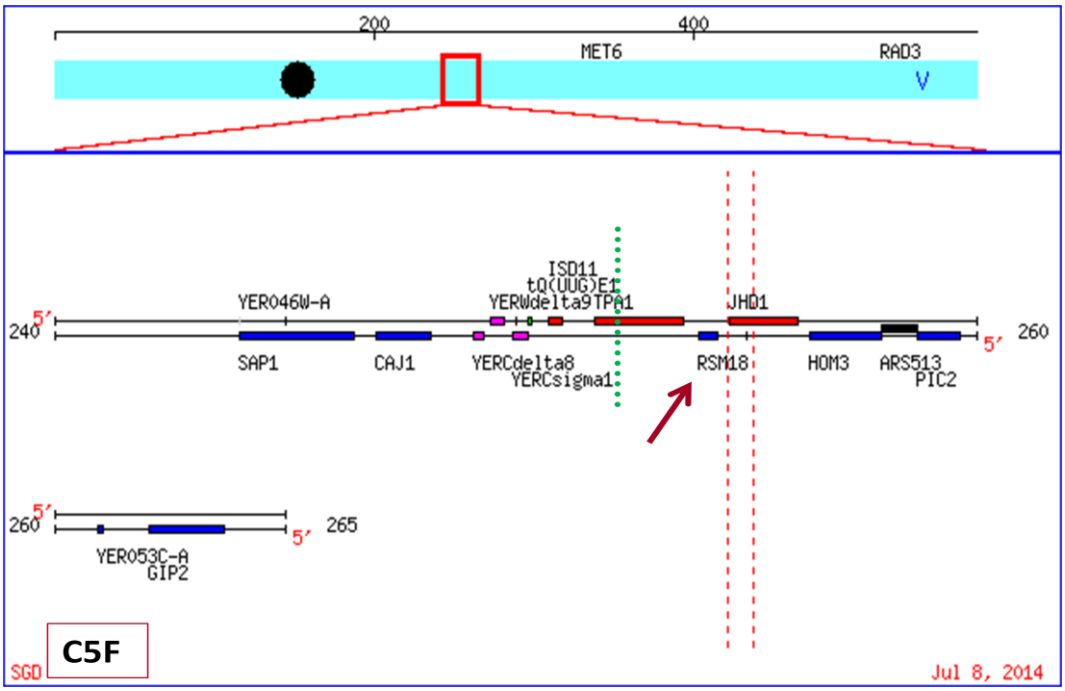

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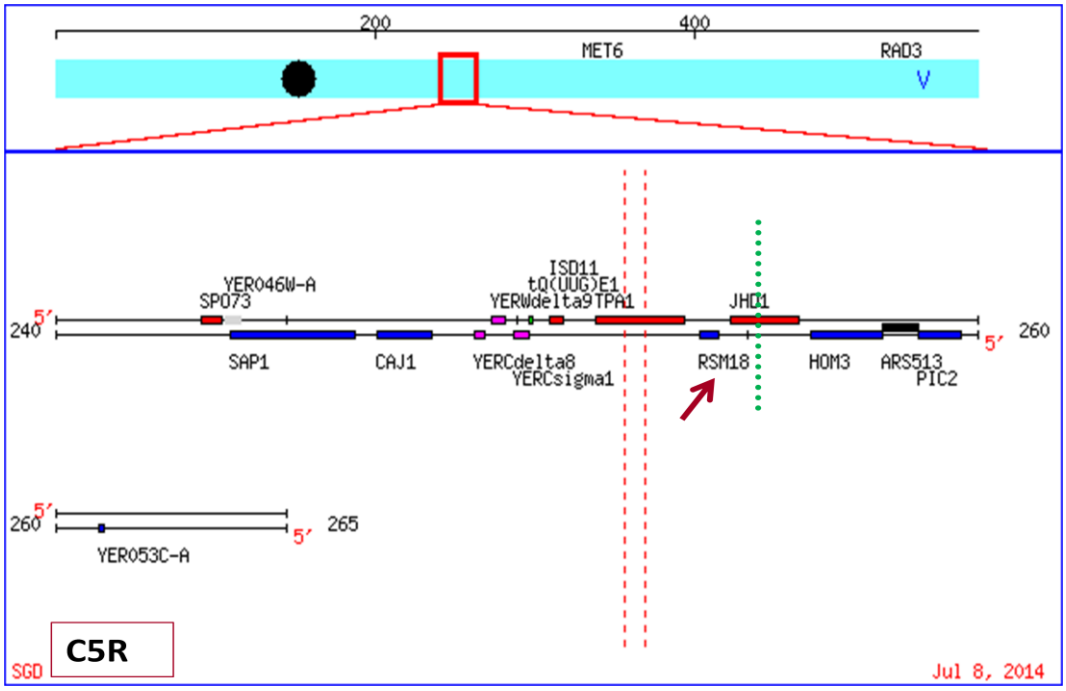

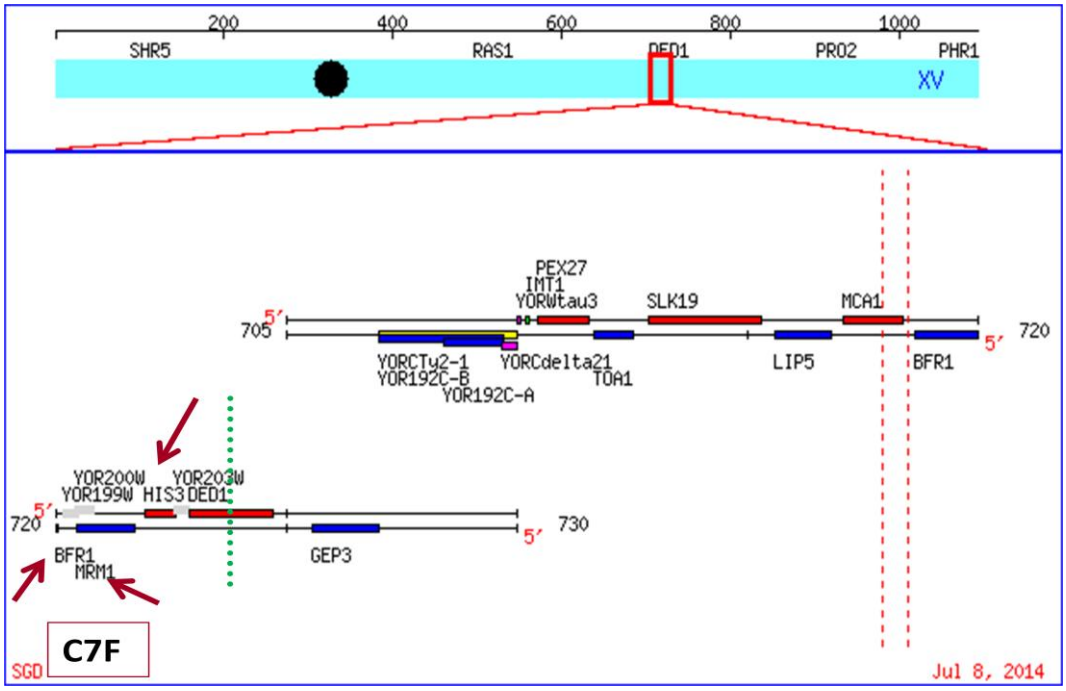

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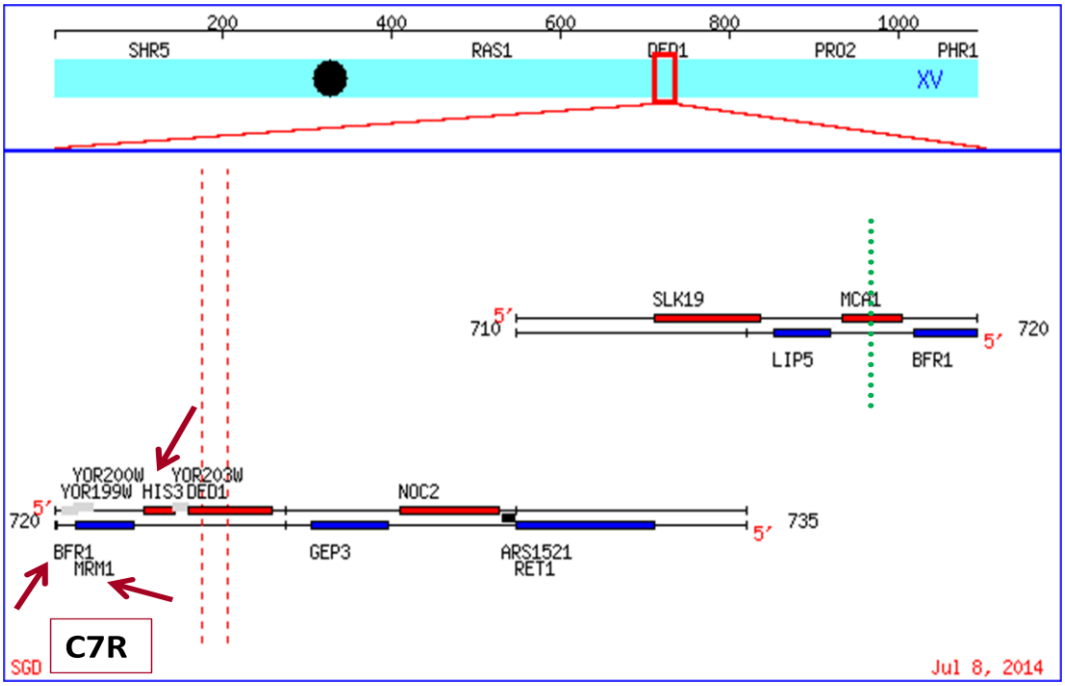

Figure 3.4. (Cont.)

To describe each candidate gene in more details; their systematic names, common names, paralogs, descriptions and functions were indicated according to SGD in Table 3.3.

Table 3.3. Selected candidate genes from obtained resistant gene cassettes

Drug Name	Sample No	Common Name	Systematic Name	Paralogs	Description	Function
Amphotericin B	A1, A2, A4	PMP3	YDR276C	-	Plasma Membrane Proteolipid	Small plasma membrane protein; confers resistance to amphotericin B and is a potential target of this common antifungal drug; related to a family of plant polypeptides that are overexpressed

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Table 3.3. (Cont.)

Amphotericin B						under high salt concentration or low temperature; not essential for viability; deletion causes hyperpolarization of the plasma membrane potential
	A3	PDR16	YNL231C	-	Pleiotropic Drug Resistance	Phosphatidylinositol transfer protein (PITP); controlled by the multiple drug resistance regulator Pdr1p; localizes to lipid particles and microsomes; controls levels of various lipids, may regulate lipid synthesis; homologous to Pdr17p; protein abundance increases in response to DNA replication stress
Caspofungin	C1	SWH1	YAR042W	OSH2	-	Protein similar to mammalian oxysterolbinding protein; contains ankyrin repeats and FFAT motif; interacts with ER anchor Scs2p at the nucleusvacuole junction; regulated by sterol binding

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Table 3.3. (Cont.)

Caspofungin	C2	RMD9	YGL107C	YBR238C	Required for Meiotic nuclear Division	Mitochondrial protein required for respiratory growth; mutant phenotype and genetic interactions suggest a role in delivering mt mRNAs to ribosomes; located on matrix face of the inner membrane and loosely associated with mitoribosomes
	C3	EPT1	YHR123W	CPT1	Ethanolamine Phospho Transferase	sn-1,2-diacylglycerol ethanolamine- and cholinephospho tranferase; not essential for viability
	C5	RSM18	YER050C	-	Ribosomal Small subunit of Mitochondria	Mitochondrial ribosomal protein of the small subunit; has similarity to *E. coli* S18 ribosomal protein
	C7	BFR1	YOR198C	-	BreFeldin Resistance	Component of mRNP complexes associated with polyribosomes; involved in localization of mRNAs to P bodies; implicated in secretion and nuclear segregation;

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Table 3.3. (Cont.)

Caspofungin	C7					multicopy suppressor of BFA (Brefeldin A) sensitivity
		MRM1	YOR201C	-	Mitochondrial rRNA Methyl-transferase	Ribose methyl transferase; modifies a functionally critical, conserved nucleotide in mitochondrial 21S rRNA
		HIS3	YOR202W	-	HIStidine	Imidazoleglycerol-phosphate dehydratase; catalyzes the sixth step in histidine biosynthesis; mutations cause histidine auxotrophy and sensitivity to Cu, Co, and Ni salts; transcription is regulated by general amino acid control via Gcn4p

3.5. Preliminary Test of the Candidate Genes

Expression vectors of all genes, except *SWH1*, was provided from Thermo Yeast ORF Collection in a bacterial glycerol stock. The plasmids were isolated and transformed into wild type yeast cells. The presence of GAL1 promoter in BG1805 vector required galactose induction before the experiments (Figure 3.5.A). Resistance of each candidate gene was tested by spotting assay. When the cells reached to their exponential phase, after overnight incubation in YNB-ura including glucose and dilution with fresh media, cells were centrifuged and supernatant was removed. Then the cells were incubated in selective media including D-raffinose and D-galactose, respectively, in every two hours. Then, the cells were dropped onto YNB-ura without glucose, with galactose gradient plates containing lethal doses of related antifungal drugs (Figure 3.5.B).

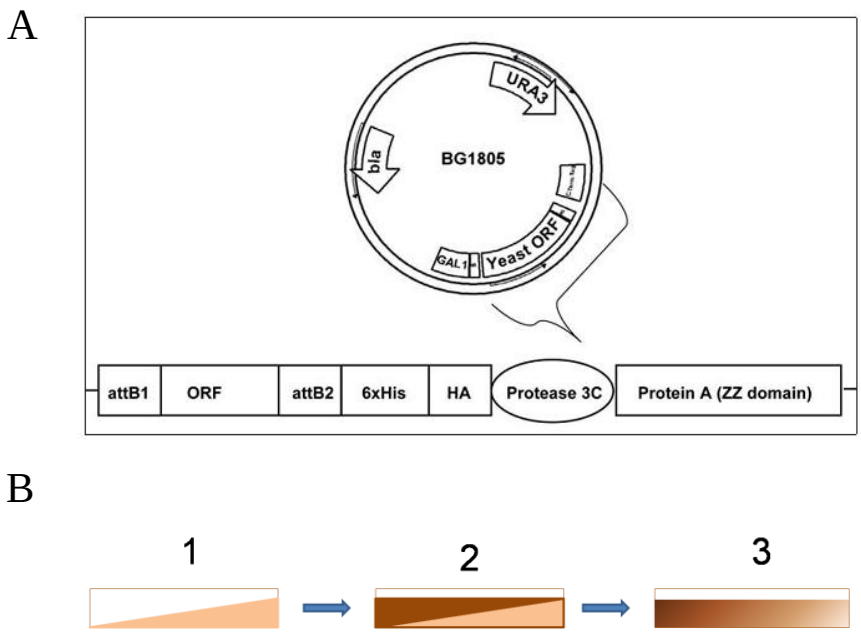

Figure 3.5. (A) Schematic map of ORF Collection plasmid. attB1 and attB2 sites demonstrate the availability for Gateway cloning. Expression of the gene is promoted by GAL1 (Source: Thermo Scientific Open Biosystems Yeast ORF Collection Technical Manual). (B) Steps of gradient plate preparation, bright color refers to only media, dark color refers to media including the lethal dose of the drug. After first media was solidified with 45^{o} slope, the second media was poured and during its solidification a transient between two media occurred.

After few days of incubation, the images were taken. None of the caspofungin candidates were resistant than control cells carrying empty plasmid (Figure 3.6.A). Also *PMP3* could not grow as well as control, whereas *PDR16* expressing cells were significantly resistant to higher doses of amphotericin B (Figure 3.6.B). According to these results, it was thought that the genes might have cytotoxic effect due to extremely expression caused by GAL1 induction. Since GAL1 is an inducible promoter and in some cases it may cause cytotoxic protein aggregation in yeast cells (Mumberg, Muller et al. 1994, Kissova, Polcic et al. 2000, Li, Wang et al. 2000). Another reason might be the effect of glucose. If the glucose could not be removed efficiently from the cells, the expression was directly repressed (Mumberg, Muller et al. 1994). Therefore, it was decided to clone these candidate genes into pAG426GPD-ccdB vectors by Gateway® Cloning Technology.

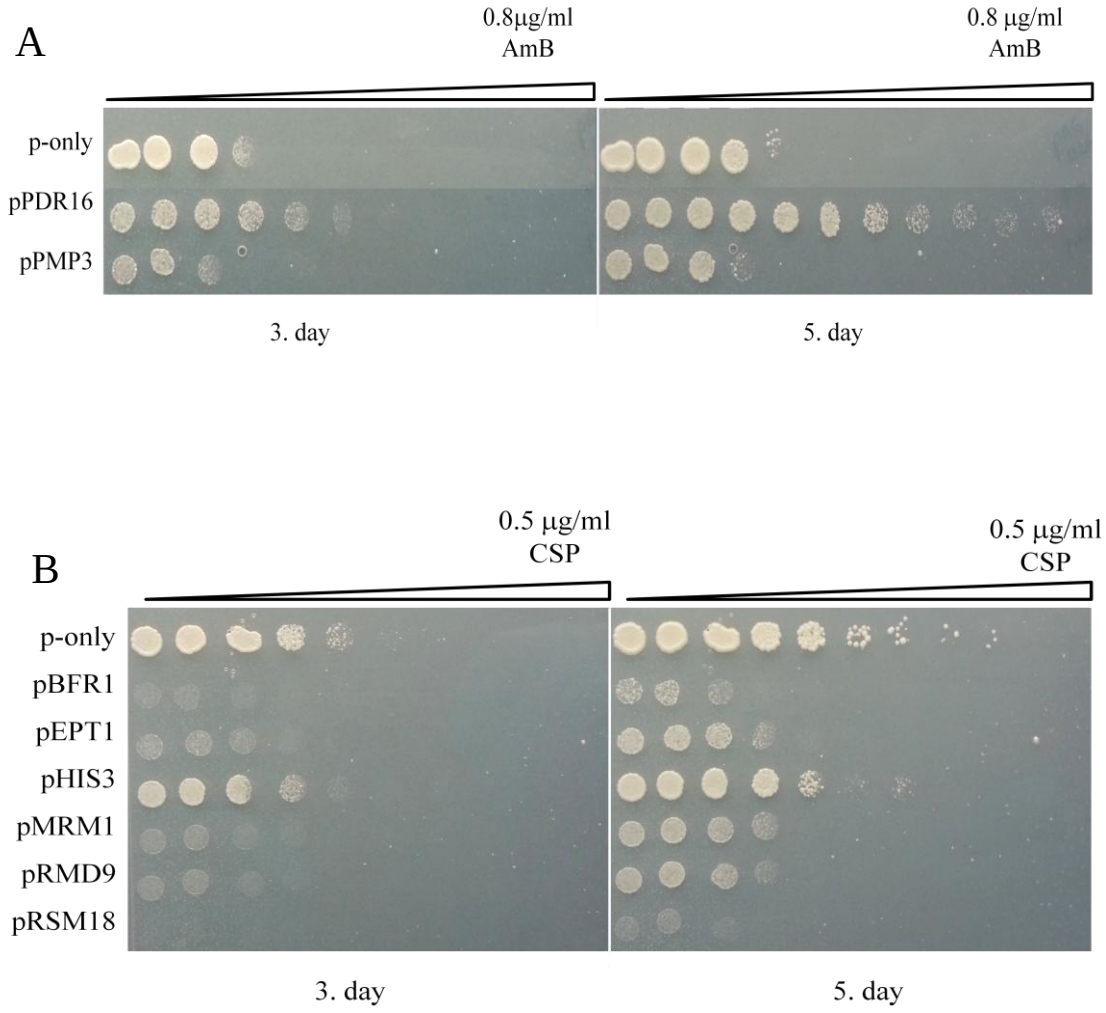

Figure 3.6. Gradient spotting assay of ORF transformant cells. (A) AmB resistant candidates, (B) CSP resistant candidates were tested on gradient plates. Wild-type cells containing empty plasmids were used as negative control (p-only). Images were taken on both 3rd and 5th days.

3.6. Gateway® Cloning of Candidate Genes to Expression Vectors

Each candidate gene was cloned from ORF plasmids into high-copy overexpression plasmids via Gateway® technology. attB1 and attB2 sites of the genes were exchanged with the attP1 and attP2 sites of the pDONR plasmid by a homolog recombination called BP reaction. The elimination of failed reactions were provided by a potent toxin ccdB (Bernard, Gabant et al. 1994, Bernard 1996). When the target gene was entered into the pDONR plasmid, the attB sites were renamed as attL and the second exchange was occurred between attL-containing entry clone and

attR sites of the destination vector, pAG426GPD-ccdB. The cloning procedure which was published by Alberti et al. was followed (Alberti, Gitler et al. 2007). The schematic demonstration of Gateway® cloning was given in Figure 3.7.

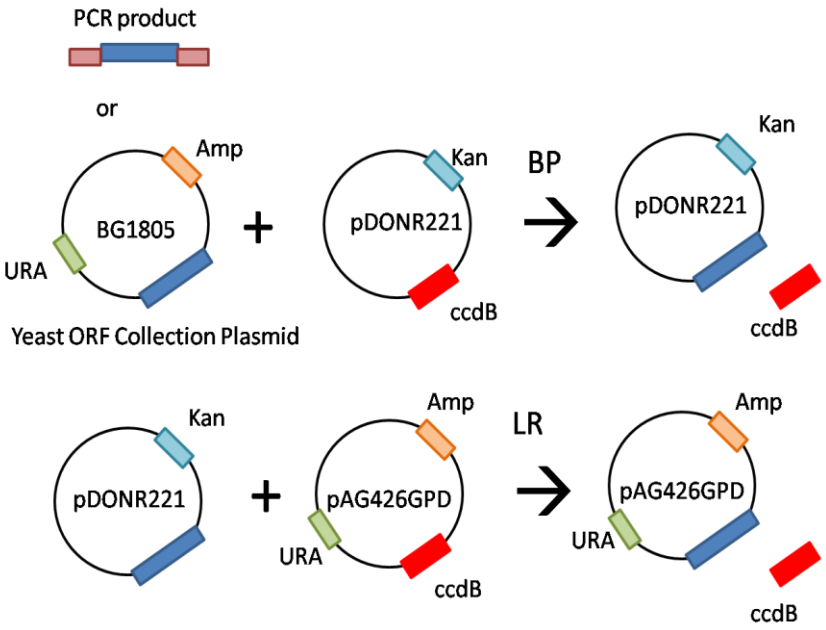

Figure 3.7. Schematic demonstration of Gateway® Cloning Technology.

In both BP and LR steps, the results were confirmed by restriction reaction with Bsp1407I (BsrGI). And moreover final clones had sequence analysis to be sure that correct genes were cloned. After that, overexpression plasmids carrying each candidate gene were transformed into wild-type yeast cells. An empty plasmid was also transformed to provide a negative control. The resistance of these genes was tested by spotting assay.

RMD9 and *SWH1* provided resistance to wild type cells against CSP when compared with control. In addition, *PDR16* was resistant to AmB in contrast to *PMP3* (Figure 3.8.). Interestingly, during this study it was demonstrated that *PMP3* is significantly resistant to AmB by Huang *et al.* (Huang, Chen et al. 2013). Therefore, these two AmB candidate genes were cloned into another expression vector p425GPD (ATCC®87359™) by subcloning technique to prove their resistance.

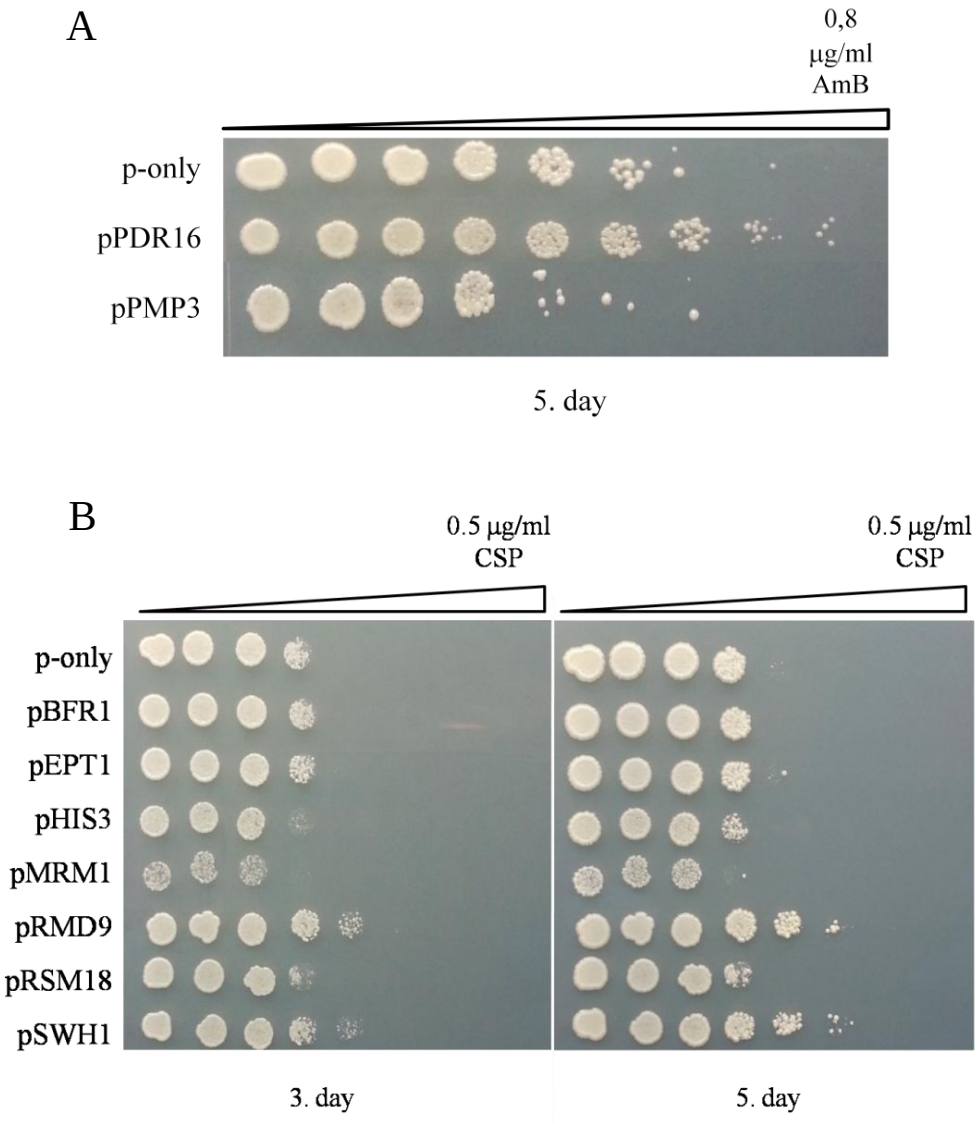

Figure 3.8. Resistance test of cloned genes. (A) AmB resistance, (B) CSP resistance test of candidate clones were tested on gradient plates. Wild-type cells containing empty plasmid were used as negative control (p-only).

3.7. Subcloning of AmB Candidate Genes

Both *PDR16* and *PMP3* genes were double digested with XhoI and BcuI (SpeI) restriction enzymes and excised from the pAG426GPD backbone. In a similar way, another expression vector p425GPD was restricted with the same enzymes to provide specific sticky ends. Then, the gene and the backbone of p425GPD were ligated to each other and the results were checked in agarose gel (Figure 3.9).

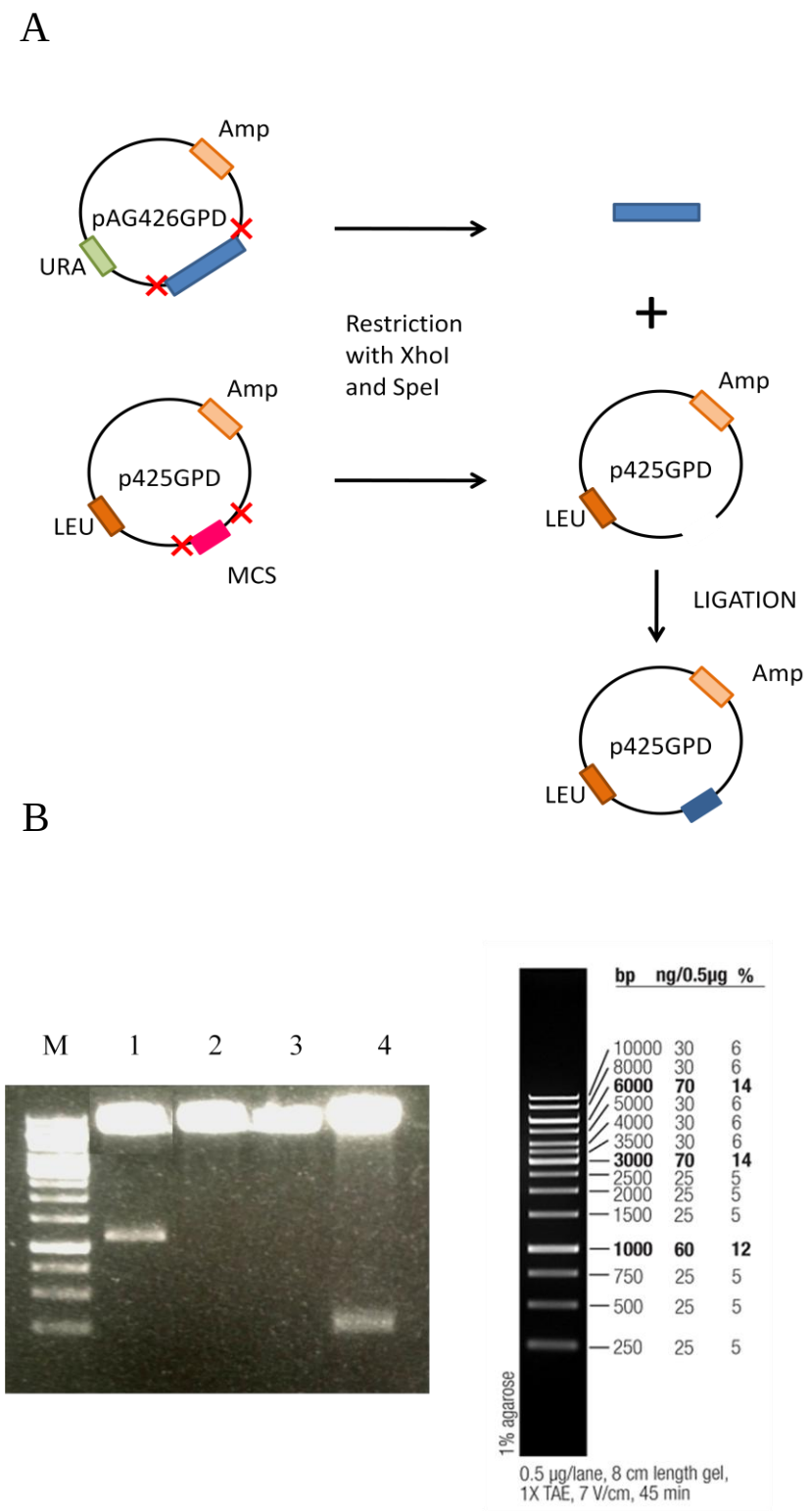

Figure 3.9. (A) Schematic demonstration of subcloning procedure. (B) Gel image of subcloned *PDR16* and *PMP3* genes. [M: 1 kb DNA marker, the exact sizes are given on right; 1: p425GPD + *PDR16* (7.76kb + 1055 bp); 2-3: false positive results; 4: p425GPD + *PMP3* (7.76kb + 168 bp)].

After subcloning was performed succesfully, the plasmids carrying AmB candidates and an empty one were transformed to wild type yeast cells and again their resistance was tested by spotting assay. In this instance, both of the genes could resist the AmB more than control group (Figure 3.10).

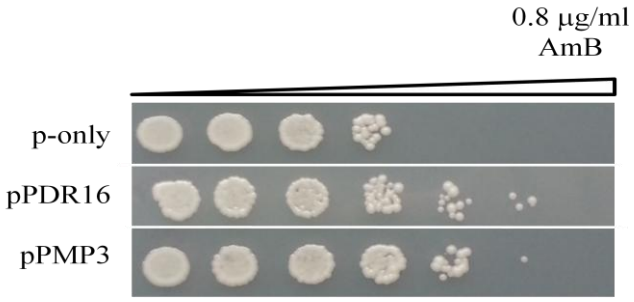

Figure 3.10. *PDR16* and *PMP3* were resistant to AmB on their 5th day when compared to control group (p-only).

3.8. Sensitivity Test of Deletion Mutants

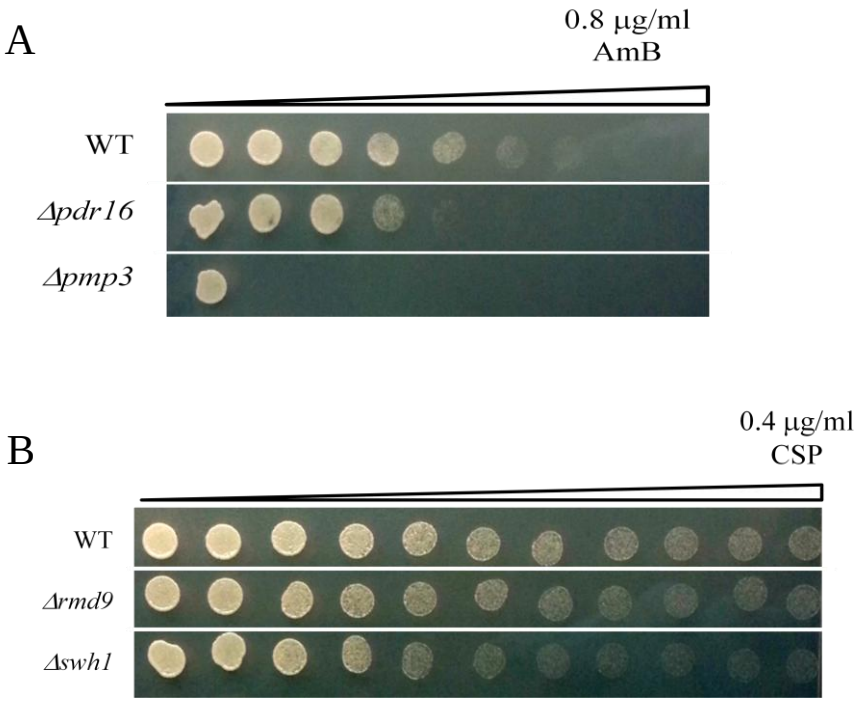

Figure 3.11. Sensitivity test of deletion mutants. (A) *Δpdr16* and *Δpmp3* could not grow as well as control in the presence of AmB; (B) *Δrmd9* and *Δswh1* had no significant change in the presence of CSP when compared to wild type control cells (WT).

In the previous parts, it was demonstrated that overexpression of *RMD9* or *SWH1* and *PDR16* or *PMP3* was siginificantly resistant to CSP and AmB, respectively. With a plain logic, the deletion mutants of each gene was tested by spotting assay to check whether their absence make the cells sensitive to the drugs. Haploid deletion mutants and wild type cells as a control were spotted onto gradient YNB+all agar including CSP or AmB. After 3 days of incubation, it was observed that *Δpmp3* was significantly and *Δpdr16* was partially sensitive to AmB than wild type cells. On the other hand, neither *Δswh1* nor *Δrmd9* had no difference with control in the presence of CSP (Figure 3.11).

3.9. Global and Gene-Based Transcriptional Analyses

Minimum inhibitory concentration (MIC) was determined to understand the acute response of resistant genes within the drugs. Therefore, BY4741 wild type yeast cells were inoculated into YPD rich media including several concentrations of drugs with a standard OD_{600} value. In every 3 hours, their growth rate (OD_{600}) was measured by spectrophotometry (Figure 3.12).

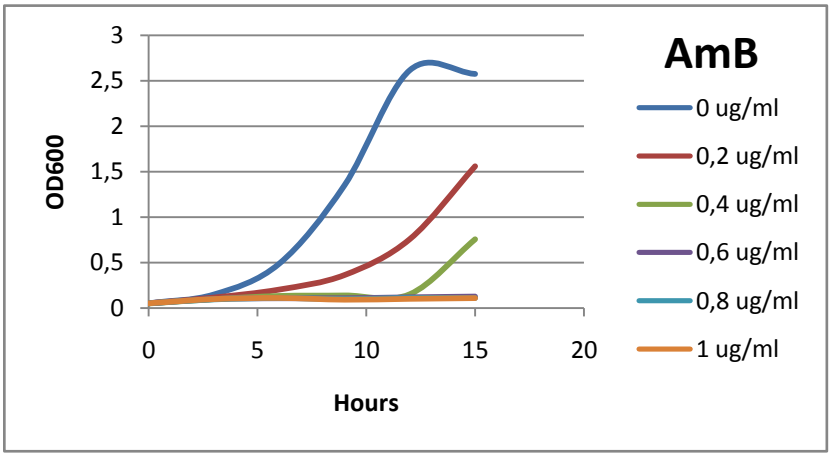

Figure 3.12. Minimum inhibitory concentrations of AmB and CSP. OD_{600} values were measured in every 3 hours during 15 hours of time period.

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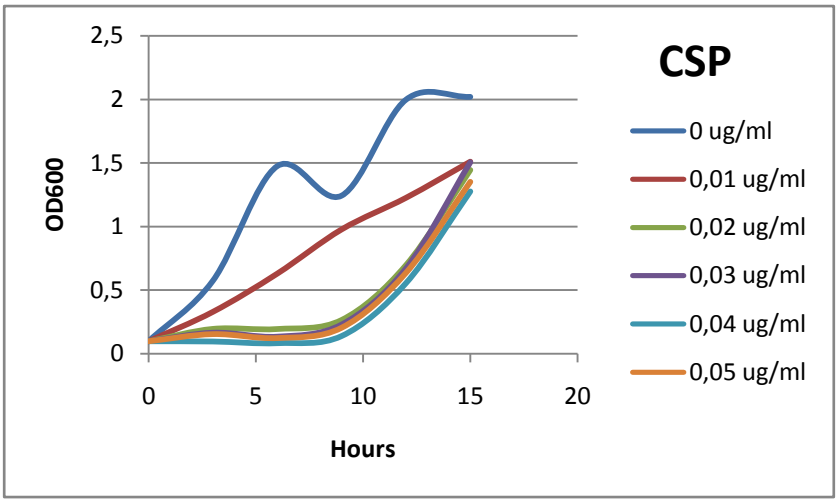

Figure 3.12.(Cont.)

In order to obtain the effects of the drugs on genes at transcriptional levels, first wild type yeast cells were exposed to 0.2 µg/ml of AmB or 0.01 µg/ml of CSP or nothing (no drug) for 3 hours and then their total RNAs were isolated. cDNAs were synthesized and used for further analyses.

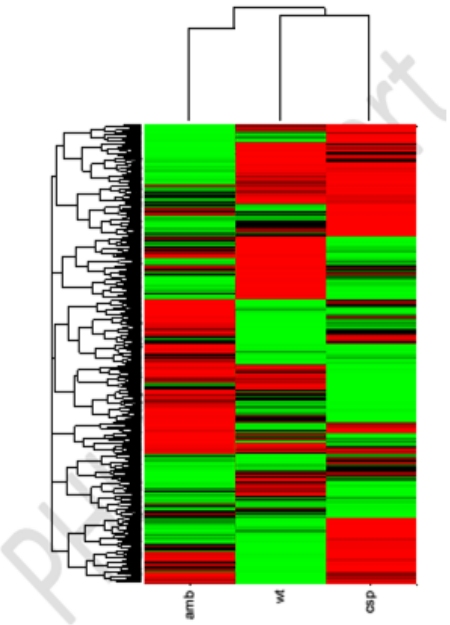

Figure 3.13. Clustering analysis of microarray data. Left column refers to gene levels of yeast cells which were exposed to amphotericin B, right column refers to the yeast cells which were exposed to caspofungin and the column in the middle refers to control cells which were exposed to nothing. Red color indicates the gene levels above the average and green color indicates the gene levels below the average.

Amphotericin B disrupts the homeostasis of the cell by forming pores in the membrane and changing the ionic balance. Besides, caspofungin inhibits the cell wall production and by this way, it weakens the cells. In addition to general aspects, whole genomes of the *S. cerevisiae* yeast cells were monitored by microarray to attain the cellular processes which are significantly affected from the drugs. According to clustering analysis, profile of the caspofungin and control were quite similar rather than amphotericin B (Figure 3.13).

Up-regulated and down-regulated genes more than two fold were evaluated in GO and Pathway analyses. GO Biological Process Term for up and down genes was summarized in Table 3.4.

Table 3.4. GO Biological Process Term for up and down genes for amphotericin B or caspofungin vs. control

Amphotericin B vs. Control	
Up-regulated Genes	
Energy reserve metabolic process	Glucan metabolic process
Generation of precursor metabolites and energy	Glucan biosynthetic process
Cellular carbohydrate metabolic process	Cellular polysaccharide metabolic process
Glycogen metabolic process	Disaccharide metabolic process
Carbohydrate transport	Cellular carbohydrate biosynthetic process
Energy derivation by oxidation of organic compounds	Maltose metabolic process
Single-organism carbohydrate metabolic process	Cellular polysaccharide biosynthetic process
Carbohydrate metabolic process	Polysaccharide biosynthetic process
Hexose transport	Oligosaccharide metabolic process
Monosaccharide transport	Single-organism carbohydrate catabolic process
Cellular glucan metabolic process	
Down-regulated Genes	
Alpha-amino acid biosynthetic process	Ion transport
Alpha-amino acid metabolic process	Sulfur compound biosynthetic process
Cellular amino acid biosynthetic process	Sulfate assimilation
Cellular amino acid metabolic process	Metal ion homeostasis
Organic acid biosynthetic process	Sulfur compound metabolic process

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Table 3.4. (Cont.)

Carboxylic acid biosynthetic process	Serine family amino acid biosynthetic process
Small molecule biosynthetic process	Cation homeostasis
Carboxylic acid metabolic process	Ornithine metabolic process
Organic acid metabolic process	Cysteine metabolic process
Oxoacid metabolic process	Ion homeostasis
Arginine biosynthetic process	Inorganic anion transport
Organonitrogen compound biosynthetic process	Pyridoxal phosphate metabolic process
Glutamine family amino acid biosynthetic process	Pyridoxal phosphate biosynthetic process
Aspartate family amino acid biosynthetic process	Aldehyde biosynthetic process
Glutamine family amino acid metabolic process	Sulfate assimilation, phosphoadenylyl sulfate reduction by phosphoadenylyl-sulfate reductase (thioredoxin)
Arginine metabolic process	Sulfate reduction
Aspartate family amino acid metabolic process	Single-organism biosynthetic process
Organonitrogen compound metabolic process	Inorganic ion homeostasis
Small molecule metabolic process	Transition metal ion transport
Iron chelate transport	Cysteine biosynthetic process
Methionine biosynthetic process	External encapsulating structure organization
Sulfur amino acid metabolic process	Cell wall organization
Iron ion homeostasis	Single-organism process
Transition metal ion homeostasis	Pyridoxine metabolic process
Sulfur amino acid biosynthetic process	Pyridoxine biosynthetic process
Siderophore transport	Copper ion import
Iron coordination entity transport	Vitamin B6 biosynthetic process
Serine family amino acid metabolic process	Chemical homeostasis
Hydrogen sulfide metabolic process	Ornithine biosynthetic process
Hydrogen sulfide biosynthetic process	Iron ion transport
	Zinc ion transport
Caspofungin vs. Control	
Up-regulated Genes	
Cellular carbohydrate metabolic process	Disaccharide metabolic process
Down-regulated Genes	
Cell cycle process	Cell cycle

According to microarray data, both of the antifungal drugs induced the carbohydrate metabolism. Besides, the AmB significantly reduced the aminoacid metabolism, its biosynthetic pathways playing a key role and some ionic transports. However, CSP directly reduced cell cycle process.In addition to global microarray

analysis, the effect of drugs on resistant genes were also analyzed by real-time PCR. CT values were recorded and relative changes were calculated comparing with internal control β-actin (Figure 3.14)

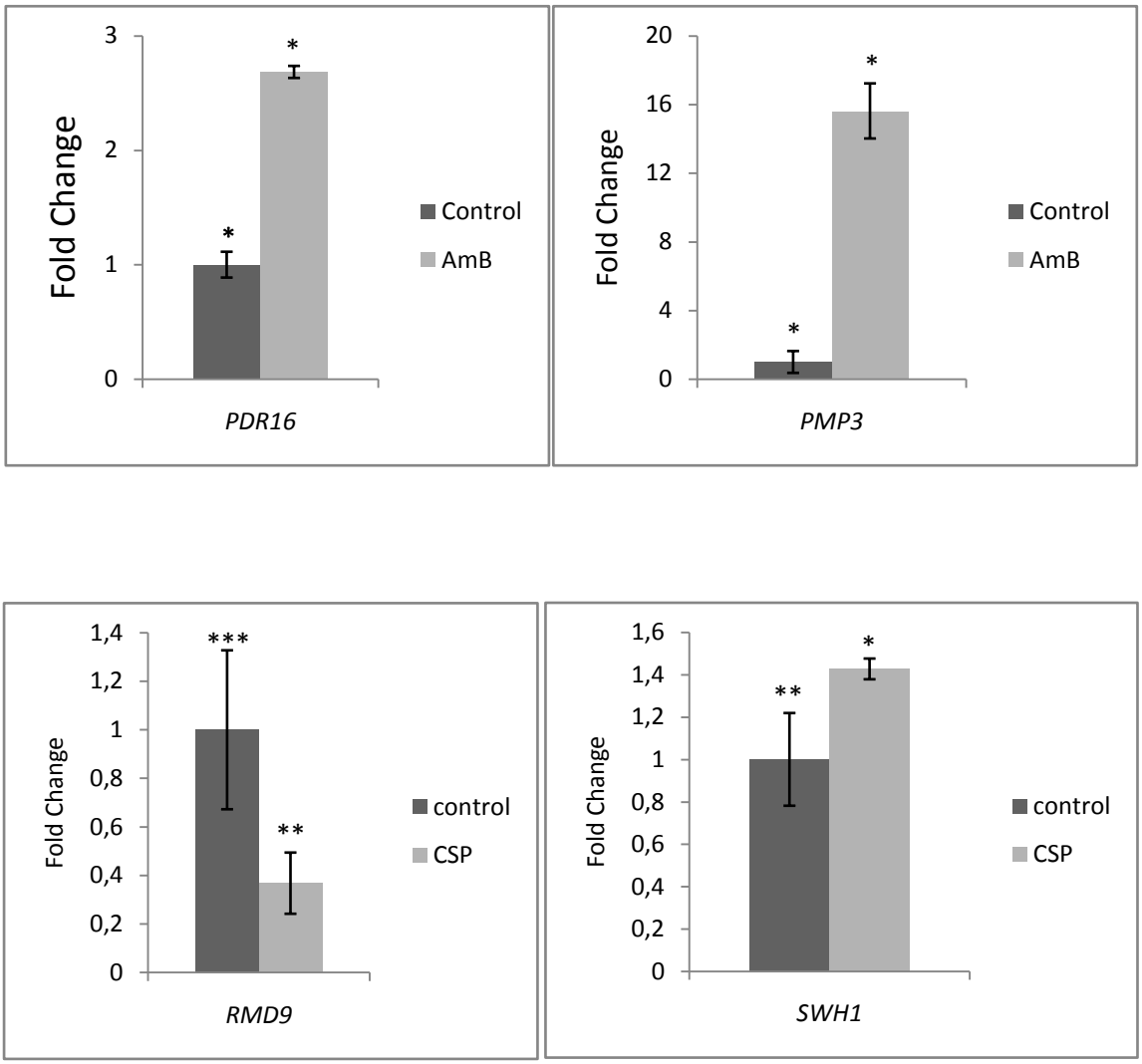

Figure 3.14. Relative transcriptional levels of *PDR16*, *PMP3*, *RMD9* and *SWH1* genes. *PDR16* and *PMP3* were exposed to AmB and *RMD9* and *SWH1* were exposed to CSP (*p<0.05; **p<0.1; ***p>0.1)

PDR16 was almost 2.5 fold and *PMP3* was 15 fold up-regulated in the presence of AmB. *SWH1* was also 1.5 fold up-regulated in response to CSP, whereas *RMD9* was 3 fold down-regulated in CSP. The significance of the results was calculated by Student's t-test.

3.10. Cross-Resistance Test

In some cases, one gene can have multiple function. Thus, AmB and CSP resistant genes identified in this study were examined whether their resistance is specific to only one drug or they can resist different antifungal drug groups. Each gene was tested on both analogs or the drugs belonging to other classes of antifungals (Figure 3.15).

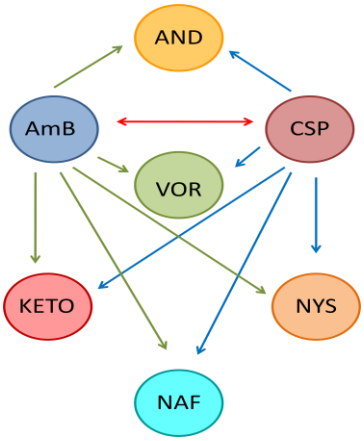

Figure 3.15. Schematic demonstration of cross resistant test. AmB and CSP resistant genes were tested on each other's drug in addition to other antifungal types (AND: Anidulafungin, VOR: Voriconazole, KETO: Ketoconazole, NYS: Nystatine, NAF: Naftifine).

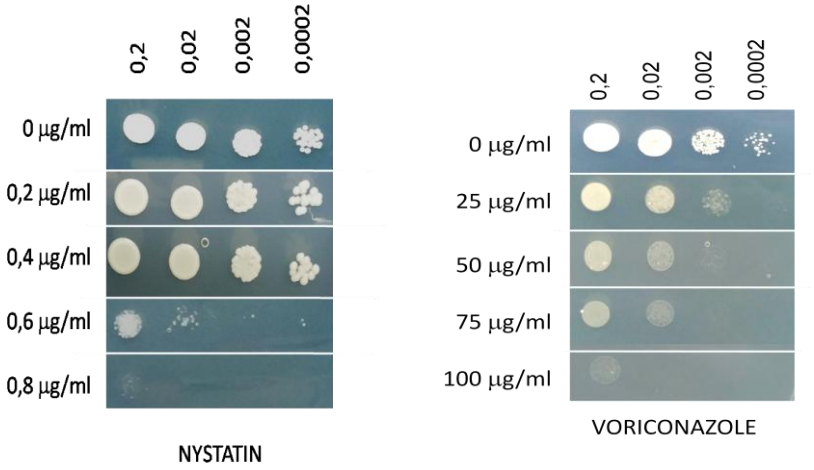

Figure 3.16. Minimum lethal doses (MLDs) of other antifungal drugs. These values were required for cross-resistance test. In all images changing concentrations of each drug was indicated in the left part of the lines. Serially diluted OD_{600} values (from 0.2 to $2x10^{-4}$) were indicated above the spots, respectively

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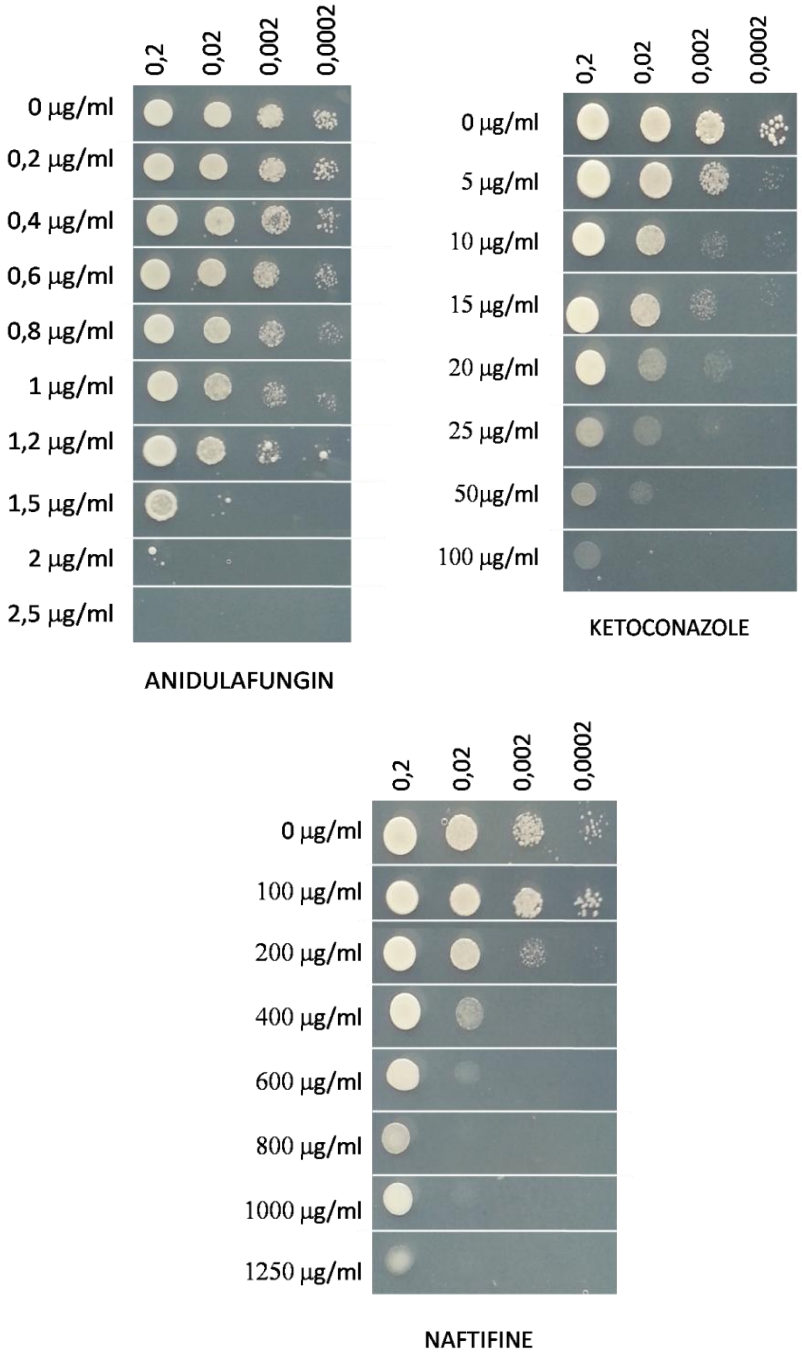

Figure 3.16. (Cont.)

In order to test the resistant genes on each drug, minimum lethal doses were determined by spotting assay. Wild type yeast cells were exposed to several concentrations of each drug and after 3 days of incubation the images were taken (Figure 3.16). At the end of 3 days of incubation, minimum lethal dose of AND, KETO, NAF, NYS and VOR were obtained as 2.5 µg/ml, 100 µg/ml, 1250 µg/ml, 0.8 µg/ml and 100 µg/ml, respectively. In cross-resistance test, gradient plates were prepared with

related selective media and maximum drug concentration was adjusted to higher doses than MLD.

At the end of this assay, it was observed that none of the genes could resist the other antifungal drugs except *RMD9* in AmB. In addition, interestingly, overexpression of *PDR16* and *SWH1* caused a significant sensitivity to yeast cells in the presence of azole drugs, both VOR and KETO (Figure 3.17).

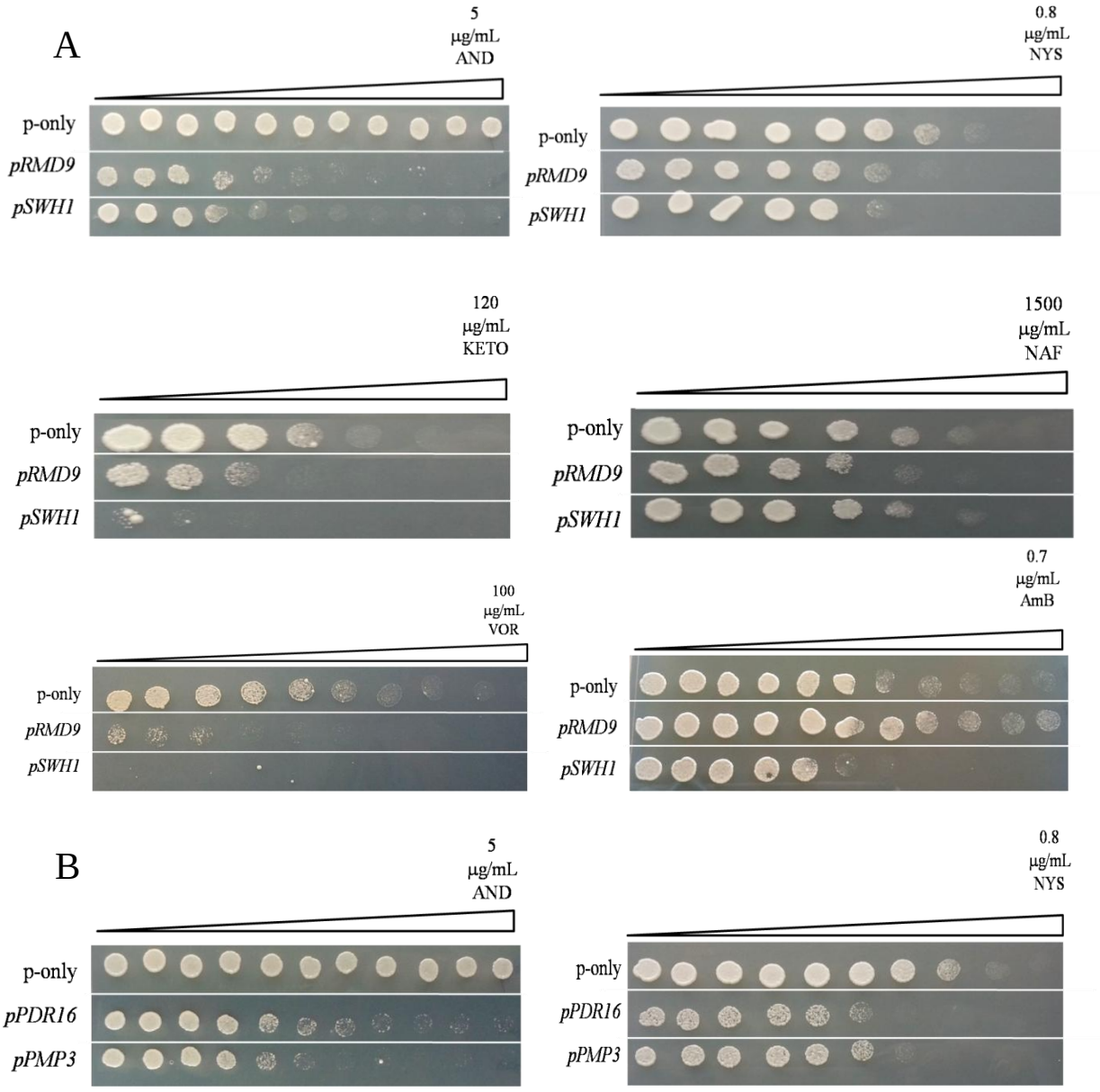

Figure 3.17. Effect of other antifungal drugs on resistant genes. (A) Cross resistance test of CSP resistant genes, (B) Cross resistance test of AmB resistant genes. No significant change was observed except *RMD9* resistance to AmB, *PDR16* and *SWH1* over-sensitivity to azole drugs, KETO and VOR. In all tests, wild type cells containing empty plasmids were used as negative control (p-only).

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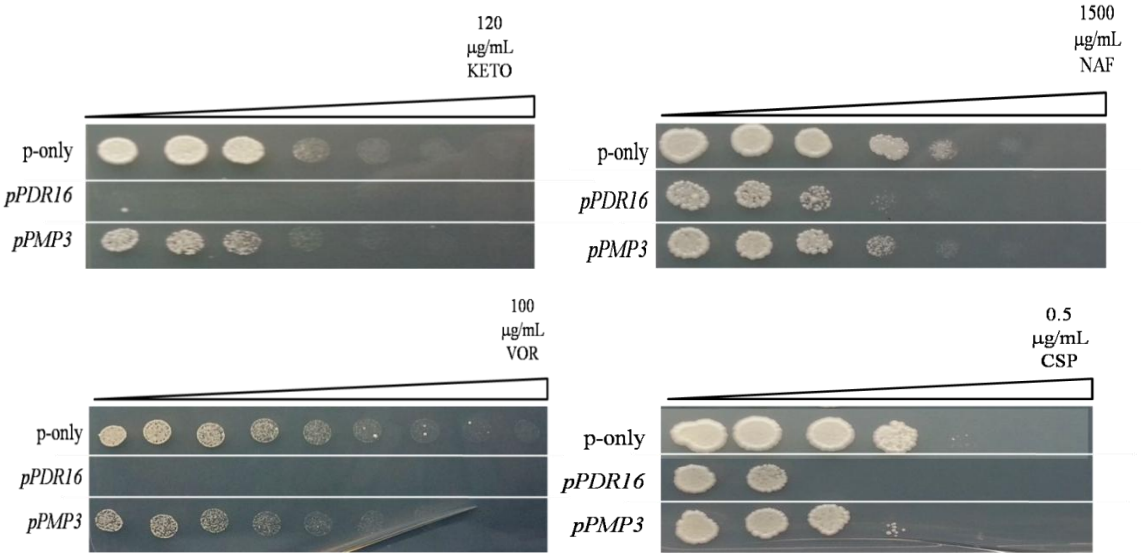

Figure 3.17. (Cont.)

The results of this experiment conduced to further analyses which aimed to prove the resistance of *RMD9* on AmB and sensitivity of *PDR16* and *SWH1* on azole drugs. First of all, *pRMD9* was transformed to both haploid and diploid wild type yeast cells and *Δrmd9* deletion mutants. For control, empty plasmid pAG426GPD-ccdB was also transformed to same type of the cells. When the wild type cells overexpressing *RMD9* were exposed to 0.4 and 0.5 µg/ml of AmB, they were partially resistant. Interestingly, *Δrmd* deletion mutants were also resistant to the same doses of AmB (Figure 3.18).

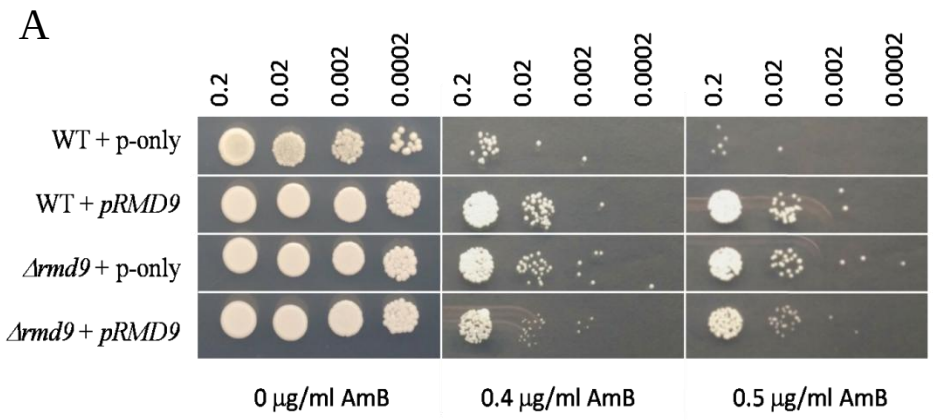

Figure 3.18. Effect of amphotericin B on *RMD9* gene. Both overexpression and deletion of *RMD9* were resistant to AmB in (A) haploid BY4741 and (B) diploid BY4743 cells. Serially diluted OD_{600} values (from 0.2 to $2x10^{-4}$) were indicated above the spots, respectively

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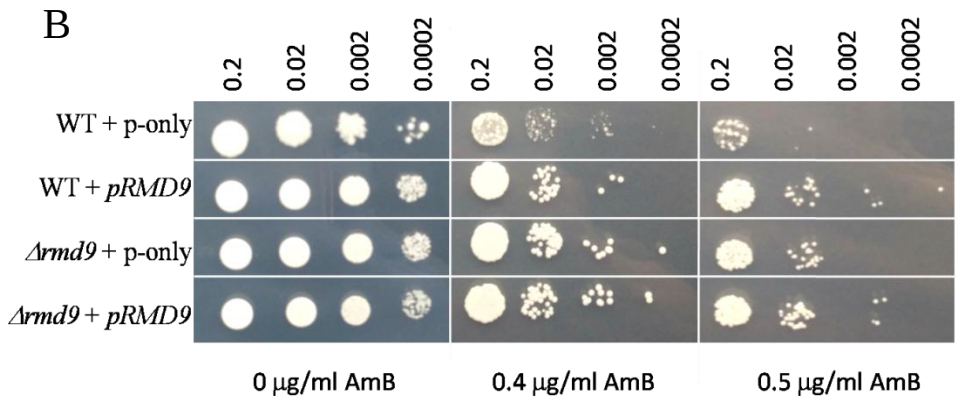

Figure 3.18. (Cont.)

Secondly, similar transformant set was designed with *SWH1* and tested on related selective media containing three different azole drugs, voriconazole, ketoconazole or itraconazole. In haploid but not in diploid cells, the expectations were confirmed and *Δswh1* deletion mutants were significantly resistant to the azoles (Figure 3.19).

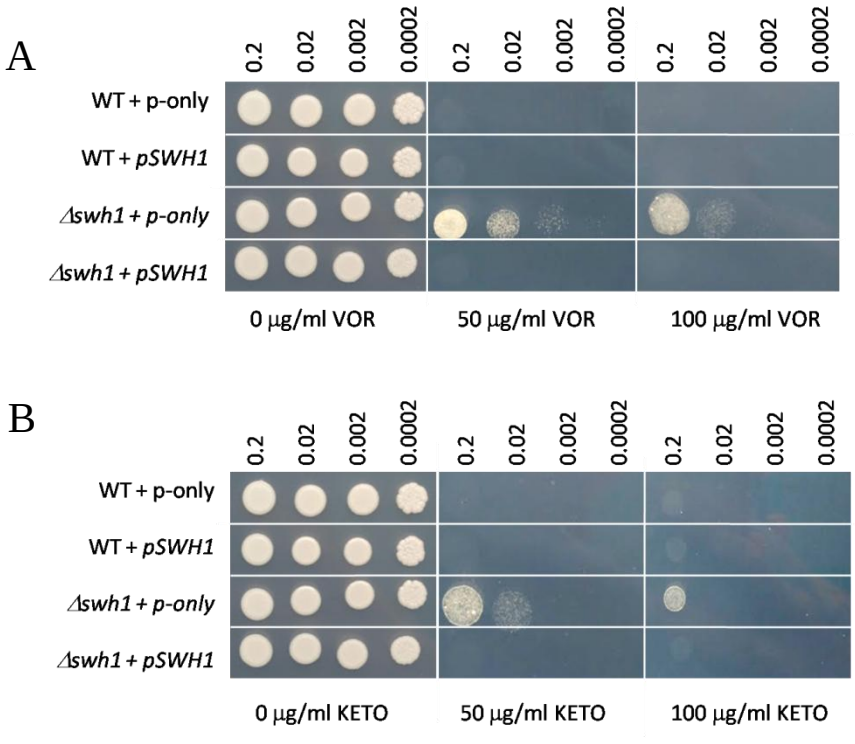

Figure 3.19. Effect of azole drugs on *SWH1* gene, (A) Voriconazole (VOR), (B) Ketoconazole (KETO), (C) Itraconazole (ITR). Serially diluted OD_{600} values (from 0.2 to $2x10^{-4}$) were indicated above the spots, respectively

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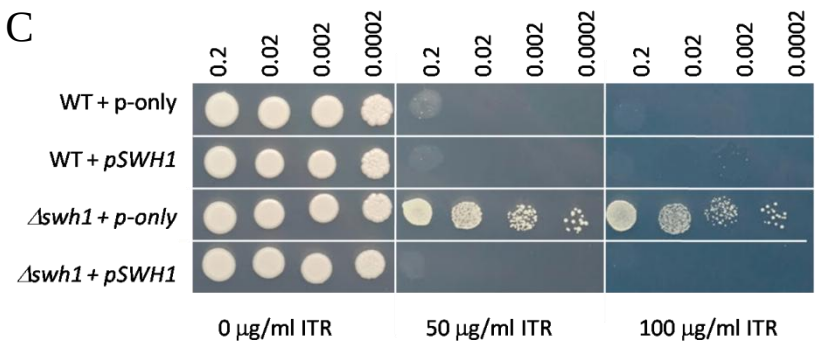

Figure 3.19. (Cont.)

Finally, same transformation set with *PDR16* was also tested with these three different azole drugs as well as *SWH1*. For this time, the deletion mutants of *PDR16* both haploid and diploid cells, were extremely resistant (Data not shown). Whereas in 2013 and 2014, Griac *et al*. published that *Δpdr16* cells were sensitive to several azole drugs including, miconazole, fluconazole, ketoconazole, voriconazole, azaconazole and paclobutrazole. They also explained that when *PDR16* overexpressing plasmid was transformed to *Δpdr16* cells, the sensitivity was lost (Simova, Poloncova et al. 2013, Holic, Simova et al. 2014). Contradiction of the data obtained in this study and Griac *et al*.'s publications might be originated from strain differences. To overcome this inconsistency, BY4741, FY1679-28C, US50-18C wild type strains and their *Δpdr16* deletion mutants were provided from Griac *et al*. directly. Only plasmid (p425GPD) and its *PDR16* overexpressing version were transformed into four different strains, BY4741 (own strain, coded as AK), BY4743 (own strain), BY4741 (obtained from Griac's lab, coded as PG) and FY1679-28C and their *PDR16* deletion mutants. In this study, unfortunately, US50-18C could not be used due to its unsuitable selection markers.

Total set including wild type cells with empty plasmid referring to control, wild type cells with *PDR16* overexpression plasmid, *Δpdr16* deletion mutants with empty plasmid and *Δpdr16* deletion mutants with *PDR16* overexpression plasmid referring to complementation, of all these four different strains were tested on three different azole drugs; voriconazole (VOR), ketoconazole (KETO) and itraconazole (ITRA). This experiment was repeated several times with fresh transformants in each time.

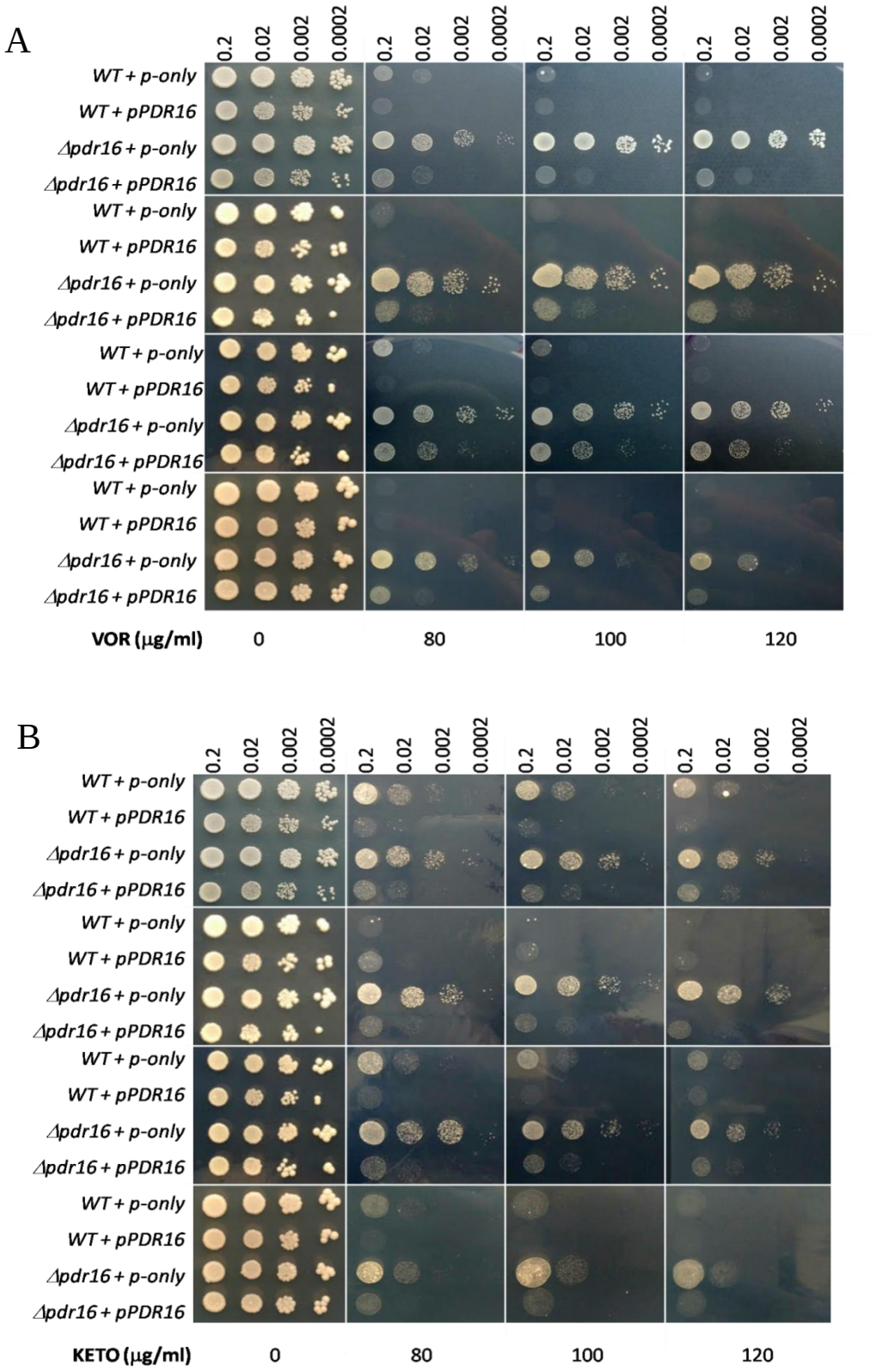

Figure 3.20. Effect of azole drugs on *PDR16* gene, (A) Voriconazole (VOR), (B) Ketoconazole (KETO) and (C) Itraconazole (ITR). The order of the strains were BY4741 (AK), BY4741 (PG), BY4743 and FY1679-28C from top to down.

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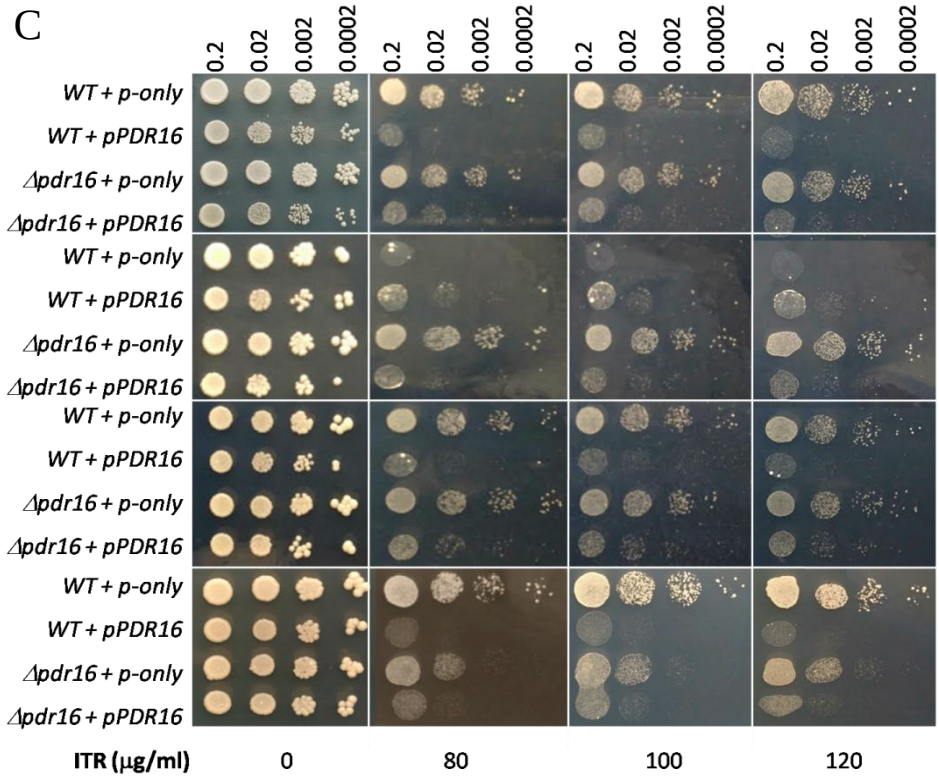

Figure 3.20. (Cont.)

In all sets, it was observed that overexpression of *PDR16* sensitized the cells in a significant range in the presence of all three azole drugs. Moreoever, deletion of this gene provided a hyperresistance against KETO and VOR, but not ITRA (Figure 3.20).

3.11. Effects of Cytotoxic Cations on Identified Genes

AmB disrupts plasma membrane and CSP inhibits cell wall biosynthesis. Thus, all of four identified genes resistant to either AmB or CSP, might have a direct or indirect relation with ion transport systems. According to microarray data, it was also supported that AmB affects ion transportation in cell (Table 3.4).

In this part, the effect of cytotoxic cations on the cells overexpressing each gene or deletion mutants based on these informations. Toxic concentrations of Na^+, Li^+ , Hygromycin B (HygB), tetramethyl ammonium (TMA) and spermine were mixed with related selective media and after 3 days images were taken.

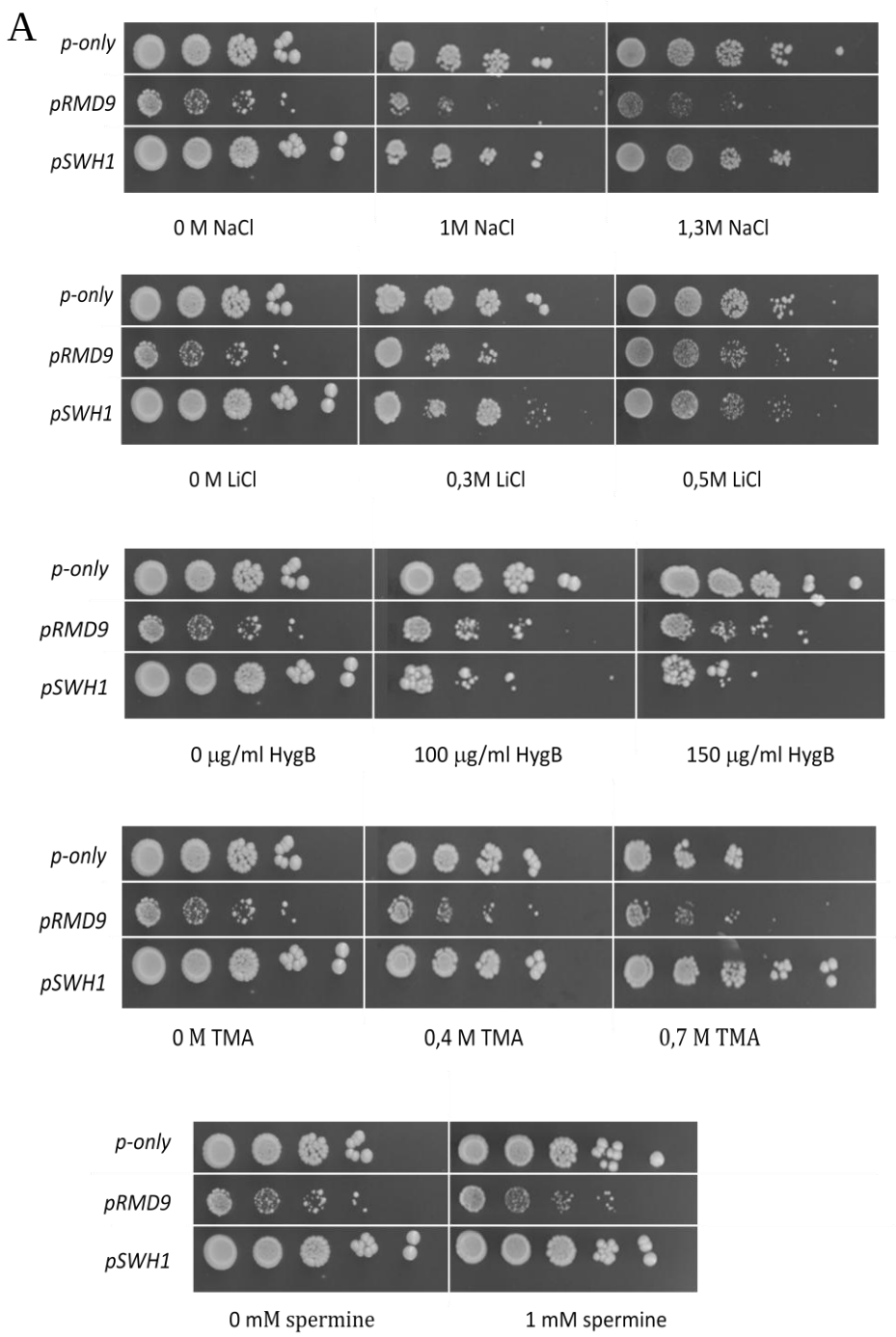

Figure 3.21. Effect of cytotoxic cations on antifungal resistant genes; *RMD9, SWH1, PDR16* and *PMP3.* (A) Wild type yeast cells overexpressing *RMD9* and *SWH1* were tested on YNB-ura, p-only refers to wild type cells containing empty plasmid pAG426GPD-ccdB, (B) Wild type yeast cells overexpressing *PDR16* and *PMP3* were tested on YNB-leu, p-only refers to wild type cells containing empty plasmid p425GPD, (C) Haploid deletion mutants of all genes and wild type (WT) yeast cells were tested on YNB+all media.

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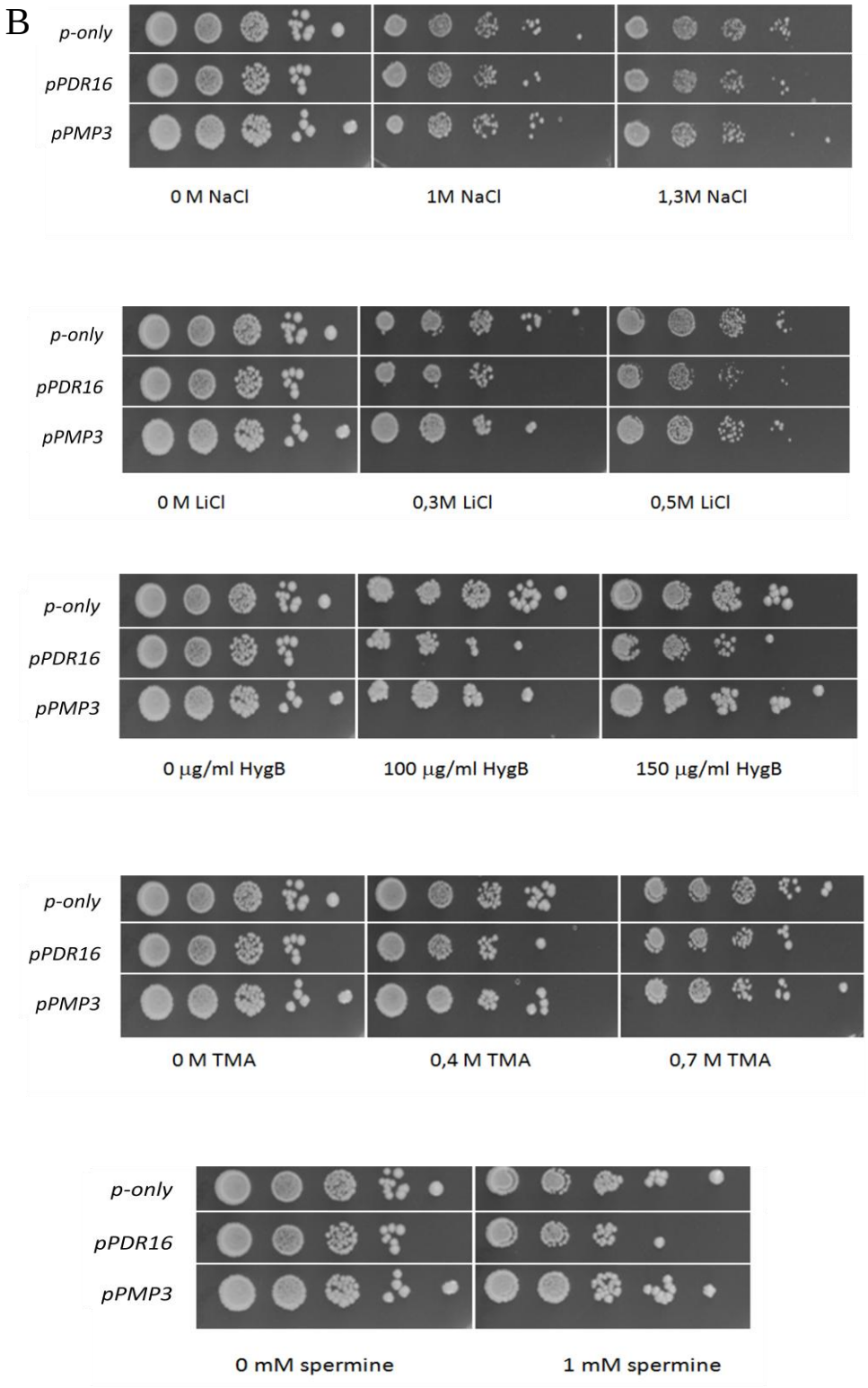

Figure 3.21. (Cont.)

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C

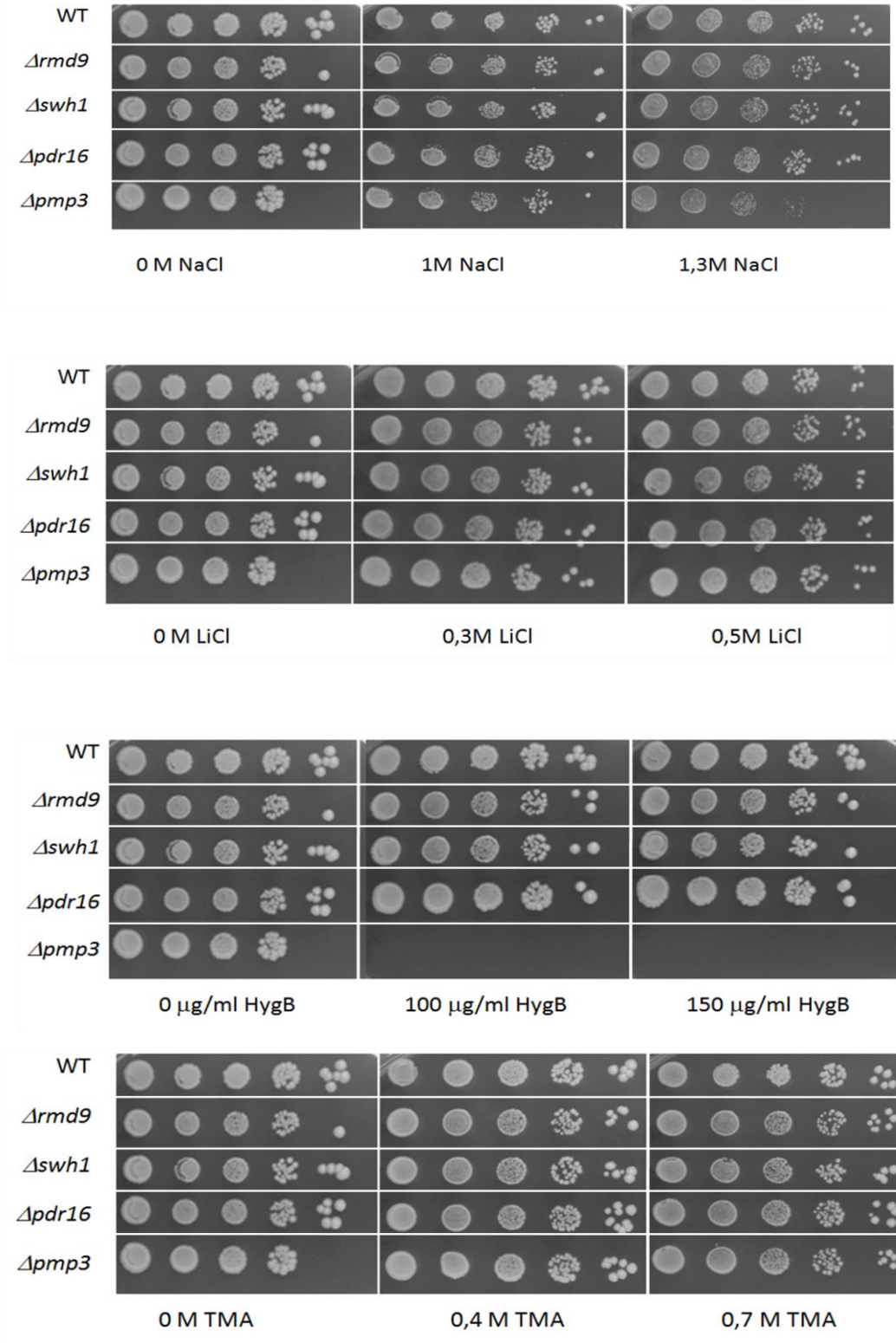

Figure 3.21. (Cont.)

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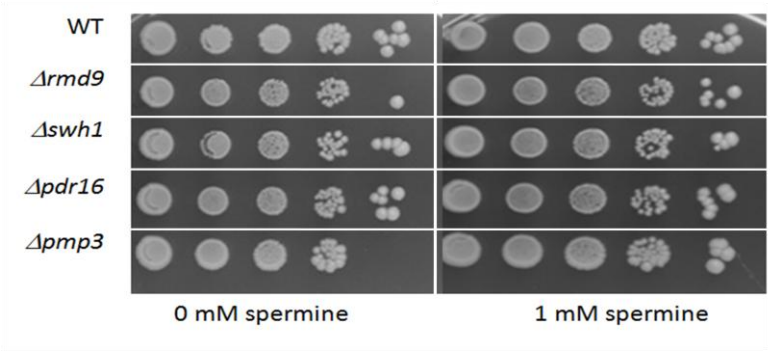


Figure 3.21. (Cont.)

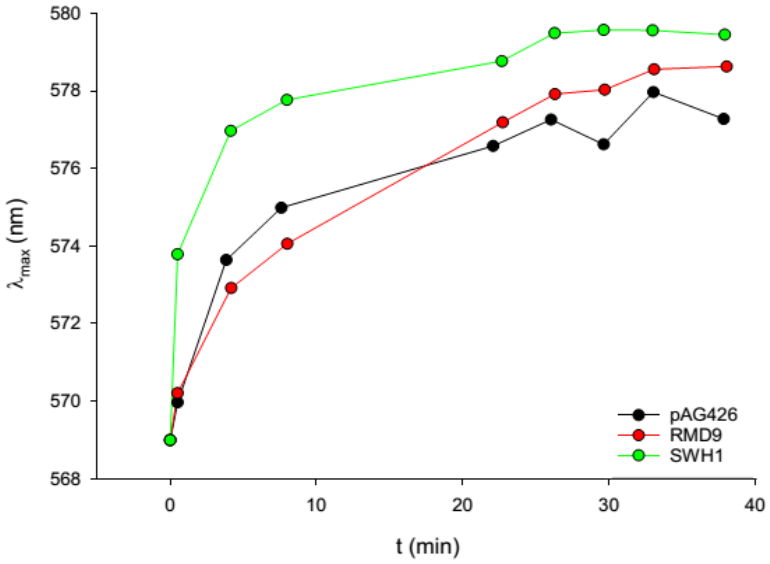
As shown in Figure 3.21.A and C, both in the presence and absence of *RMD9* had no significant change against cytotoxic cations. However, cells overexpressing *SWH1* were sensitive to HygB, but partially resistant to TMA (Figure 3.21.A). Besides, *Δswh1* cells were not affected from the cations (Figure 3.21.C). Overexpression of *PDR16* made the cells slightly sensitive to all cytotoxic cations except Na^+, but *PMP3* did not change anything in cells (Figure 3.21.B). The strongest effect of several cations were observed in *Δpmp3* cells. Especially, HygB was overtoxic for these cells and Na^+ ions have a significant inhibitory effect (Figure 3.21.C).

3.12. Relative Membrane Potential Measurement

Membrane potential of a yeast cell is usually kept in homeostasis to control the efflux and influx of the ions. When the membrane is hyperpolarized somehow, the cell activates the influx of Ca^{2+} and H^+ cations to balance the potential (Maresova, Muend et al. 2009). In drug resistance mechanisms, the hyperpolarization effect of the drug can be tolerated by this way.

To obtain a relation between the genes and membrane potential both overexpressions and deletion mutants of each gene were analyzed. Comparisons of overexpressions were done according to the cells containing the related empty plasmid. On the other hand, comparisons of deletion mutants were done with wild type yeast cells. The membrane potential of each cell was measured by fluorescence probing method which detects the influx rate of the fluorescent probe 3,3′-dipropylthiacarbocyanine iodide (diS-C_3(3)). Membrane potential of each cell is demonstrated in Figure 3.22.

A



B

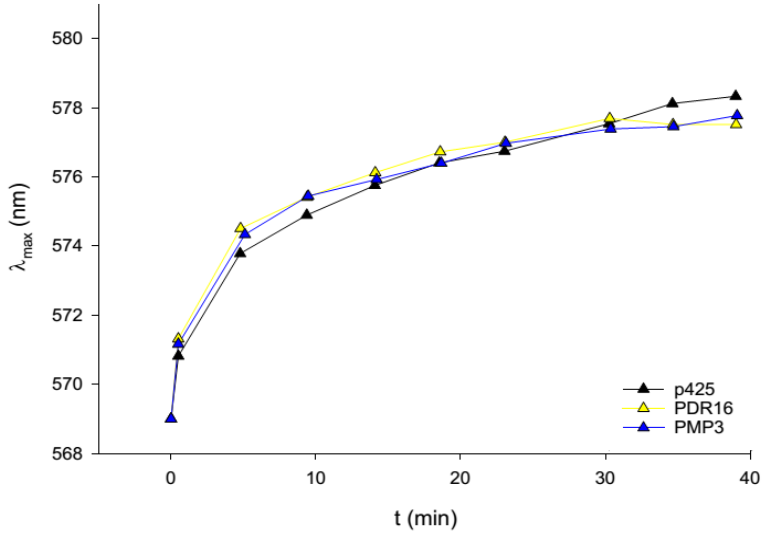


Figure 3.22. Membrane potential measurement of yeast cells. Membrane potentials of (A) Wild type cells with empty plasmid pAG426GPD-ccdB, *pRMD9* or *pSWH1*, (B) Wild type cells with empty plasmid p425GPD, *pPDR16* or *pPMP3*, (C) Wild type yeast cells and their *RMD9* and *SWH1* deletion mutants, (D) Wild type yeast cells and their *PDR16* and *PMP3* deletion mutants, were measured by fluorescence probing method.

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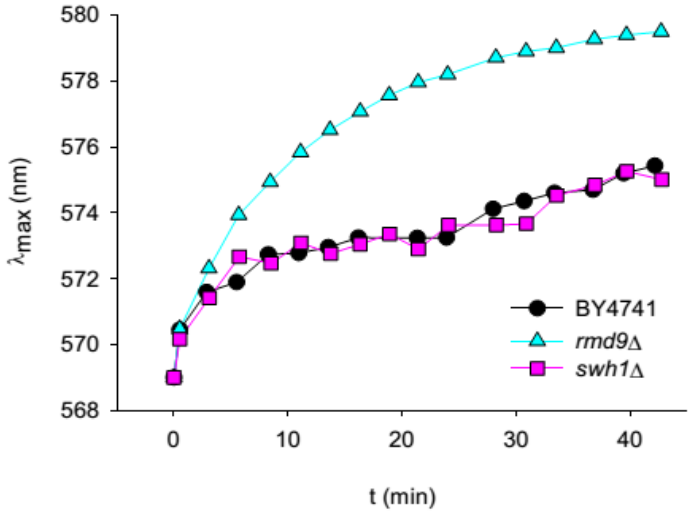
C



D

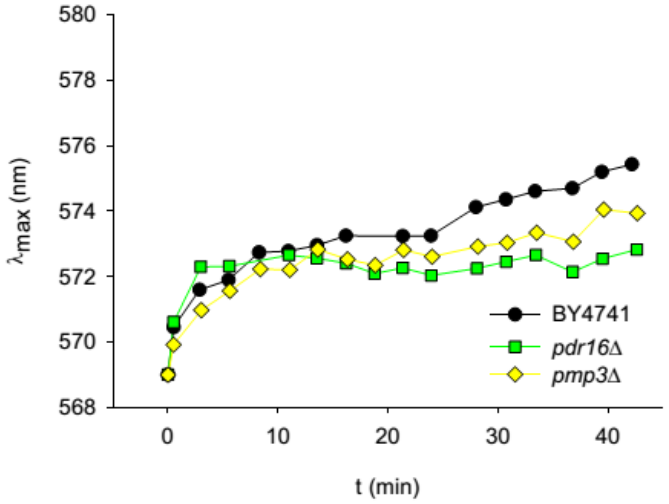


Figure 3.22. (Cont.)

Fluorescence probing method of membrane potential measurement is based on the increase of fluorescence during the enterance of cyanine probe into the cytoplasm through the plasma membrane (Pena, Uribe et al. 1984). According to the graphs in Figure 3.22, when the cyanide moves into the cell very quickly, it indicates that the membrane is hyperpolarized. On the other hand, if the cyanide moves slower, it means that the membrane potential of the cell is depolarized. On this basis, when *SWH1* gene was overexpressed, the cells became hyperpolarized and no change was observed in

cells overexpressing *RMD9* (Figure 3.22.A). In Figure 3.22.B, it was demonstrated that the cells overexpressing either *PMP3* or *PDR16* did not have any change in membrane potential when compared to control cells. In deletion mutants, the most affected one was *Δrmd9* cells which were extremely hyperpolarized. Deletion of *SWH1* did not affect the membrane potential, in contrast to its overexpression (Figure 3.22.C). Finally, both of the *Δpdr16* and *Δpmp3* plasma membranes were depolarized as time goes on (Figure 3.22.D).

3.13. Cell Size Measurements

In this section, changes in cell size due to overexpression or deletion of resistant genes were obtained. In both measurement, wild type cells with empty plasmid were the control group to eliminate the possible differences that will occur due to the plasmid. The diameters of the cells with the number of 200-1000 were measured by CASY system (Figure 3.23).

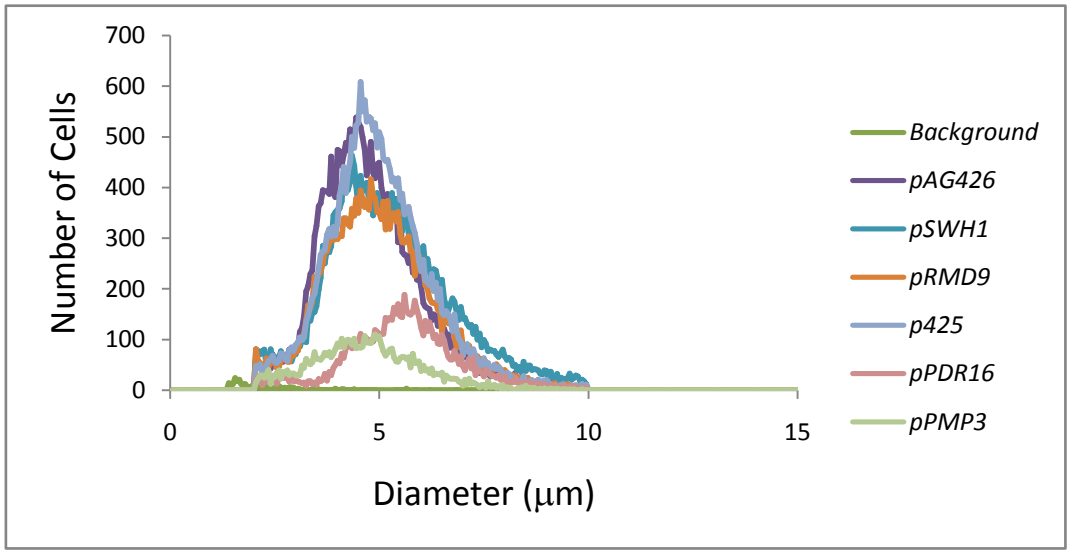


Figure 3.23. The average diameter of the cells measured by CASY, overexpression of the genes were compared with control (up) and deletion mutants with empty plasmid were compared with wild type cells (down).

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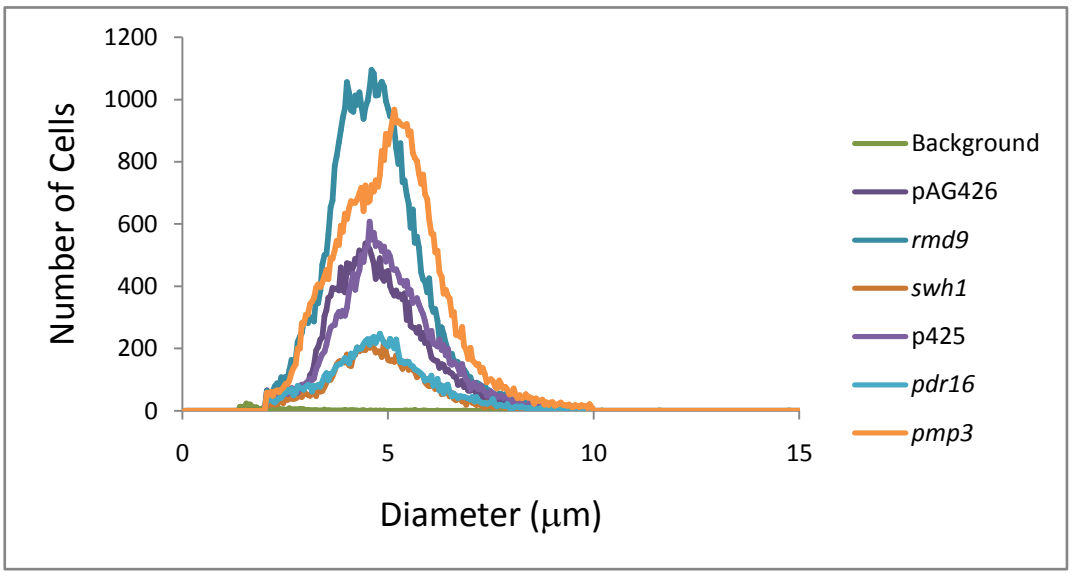

Figure 3.23. (Cont.)

The average diameter of each cell group was calculated by CASY$_{EXCEL}$ program (Table 3.5).

Table 3.5. The average diameters of the cells. In CSP resistant group p-only refers to wild type cells with pAG426GPD-ccdB, in AmB group p-only refers to wild type cells with p425GPD empty plasmids.

Cell type	**Average Diameter (µm)**	**Cell type**	**Average Diameter (µm)**
WT +p-only	4.80	WT + p-only	4.80
WT + pRDM9	5.01	*Δrmd9* + p-only	4.72
WT+ pSWH1	5.23	*Δswh1* + p-only	4.81
WT + p-only	4.99	WT + p-only	4.99
WT + pPDR16	5.57	*Δpdr16* + p-only	4.82
WT + pPMP3	4.70	*Δpmp3* + p-only	5.03

As shown in the Table 3.5, the cells with only pAG426GPD-ccdB overexpression plasmid had approximately 4.8 µm of diameter. Overexpression of *RMD9* increased the diameter ~0.2 µm and reached to 5.01 µm, but *SWH1* was more effective and its overexpression caused ~0.45 µm increase in diameter. In other words, the average diameter of the wild type cells overexpressing *SWH1* was 5.23 µm. The

wild type yeast cells with another empty plasmid p425GPD had a different size, 4.99 μm, from the other control group. Although, overexpression of the *PMP3* seems a reductive effect on the cell diameter (4.7 μm), the number of measured cells was less than 200 (Figure 3.23). Hence, the less number of cells decrease the significancy of the result and it cannot be exactly defined that cells get smaller when overexpress *PMP3*. On the other hand, *PDR16* overexpression had a great impact and the average diameter of the cells reached to 5.57 μm.

In deletion mutants, cell sizes did not change as much as in overexpressions. Only *Δrmd9* and *Δpdr16* had 0.1 μm decrease (Figure 3.24).

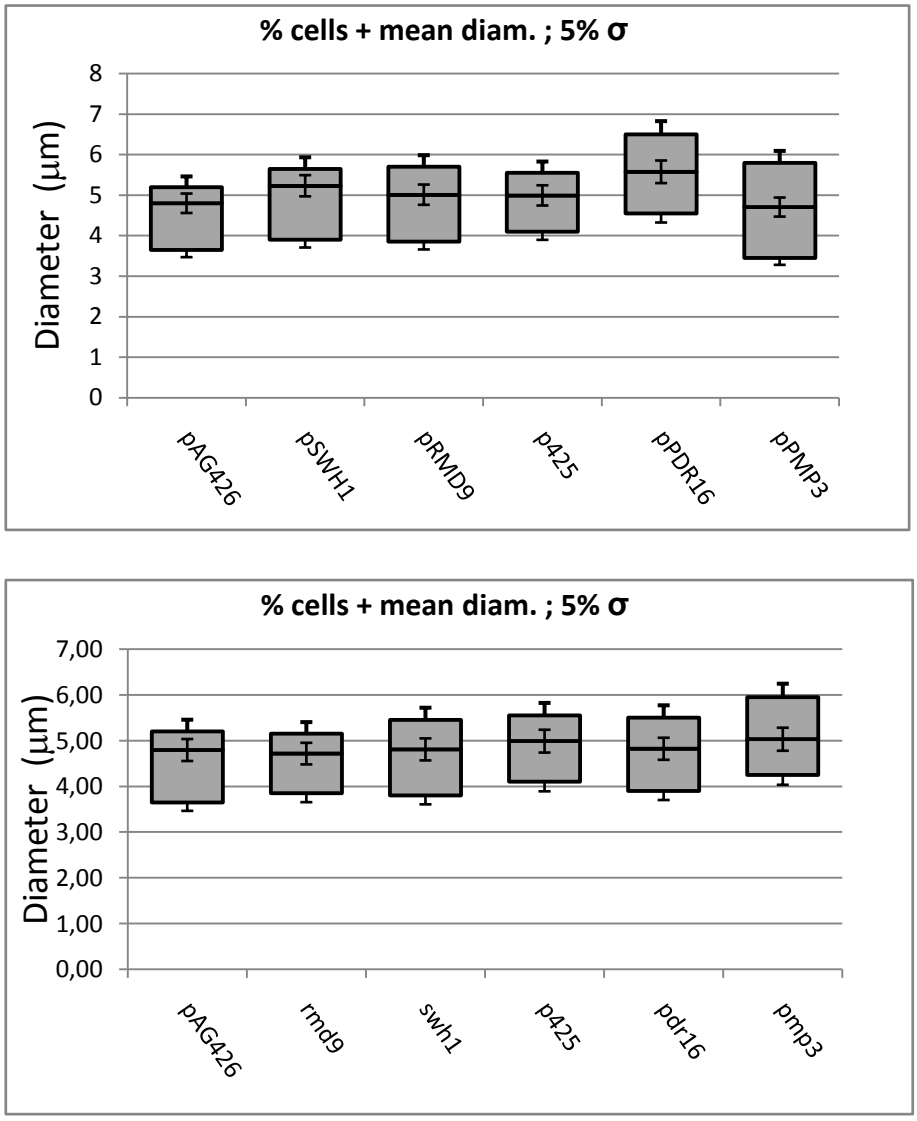

Figure 3.24. Graphical demonstration of average cell sizes. Diameters (μm) of cells overexpressing each gene (up) or deletion mutants of each gene (down) were compared with control cells.

3.14. Fluorescent Imaging of Deletion Mutants

Green fluorescent protein (GFP) was first isolated from jellyfish *Aequorea victoria* in early 60s by Shimomura *et al.* (Shimomura, Johnson et al. 1962). In 1994, Chalfie *et al*. discovered its mapping property in transparent model organism *Caenorhabditis elegans (Chalfie, Tu et al. 1994)* and then two variants of GFP, highly sensitive to pH, was produced by site directed mutagenesis in *E. coli* (Miesenbock, De Angelis et al. 1998). This pH sensitive GFP was called pHluorin.

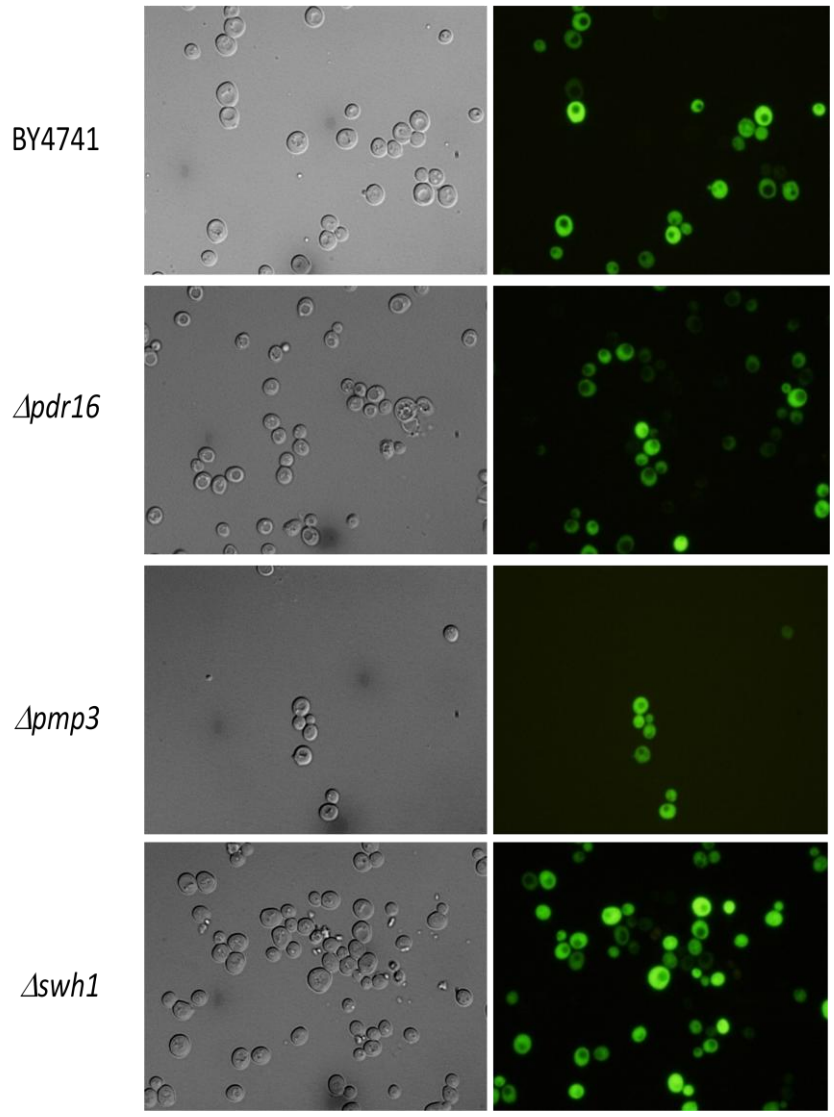

Figure 3.25. Fluorescent (right column) and Nomarski (left column) images of deletion mutants and wild type yeast cells.

In this experiment, pHluorin was transformed to BY4741 wild type yeast cells and their four deletion mutants; *Δrmd9, Δswh1, Δpdr16* and *Δpmp3*. Under fluorescent microscope the cells were observed as green circular bodies with black vacuoles. In addition to fluorescent ones, three-dimensional (3D) images were taken under Nomarski lens and the morphological variations if any, were also detected. The absence of *PDR16, PMP3* and *SWH1* did not affect the cellular morphology (Figure 3.25).

In contrast to others, *Δrmd9* cells had disrupted vacuoles. Both on fluorescent and Nomarski yeast images non-circular crumbled black dots indicated abnormal vacuoles were observed (Figure 3.26).

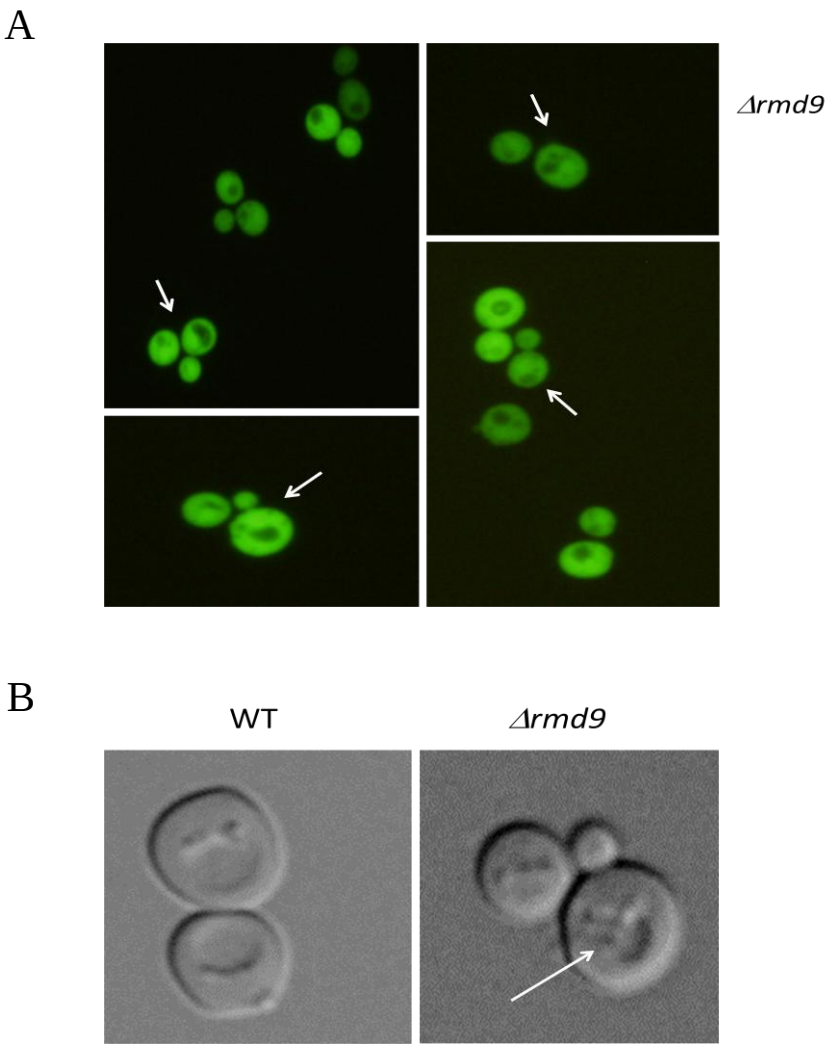

Figure 3.26. Images of *Δrmd9* deletion mutants taken under fluorescent microscope and Nomarski lens. (A) Fluorescent images of *Δrmd9* taken from different positions of the cell preparate. White arrows indicate the vacuoles with abnormal shape. (B) 3D Nomarski image of *Δrmd9*. Wild type (WT) on the left has circular vacuoles in contrast to *Δrmd9* on the right. White arrow indicates the disrupted vacuole.

3.15. Intracellular pH Measurement

Intracellular pH was measured by using pH sensitive GFP, pHluorin. In this method, a calibration curve was plotted with buffer solutions with different pH values. The stabilization of intracellular pH with these buffers were provided by digitonin detergent which increases the plasma membrane permeability and facilitates the transferring of buffer into the cytosol (Fiskum, Craig et al. 1980). Therefore, in different pH values pHluorin had different fluorescence intenstiy and the calibration curve was plotted due to fluorescence values obtained at 400 and 485 nm wavelength (Figure 3.27).

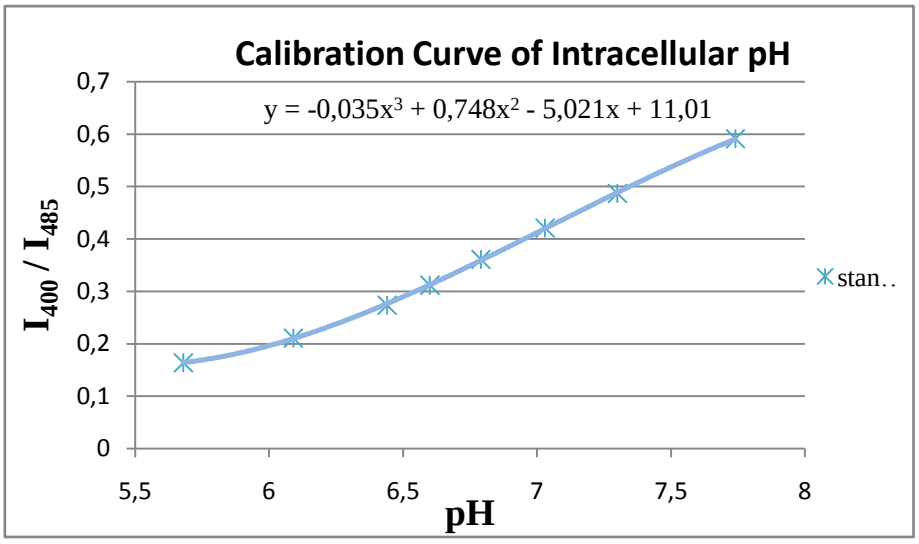

Figure 3.27. Calibration curve required for intracellular pH measurement. Internal pH of BY4741 wild-type yeast cells were stabilized by digitonin and specific buffers.

The fluorescent intensities of wild type yeast cells and all deletion mutants including pHluorin plasmids were measured at 400 nm and 485 nm wavelengths and I_{400}/I_{485} ratios were obtained. The intracellular pH of these cells were calculated from the equation. Significance of the results were evalutated by Student's t-test.

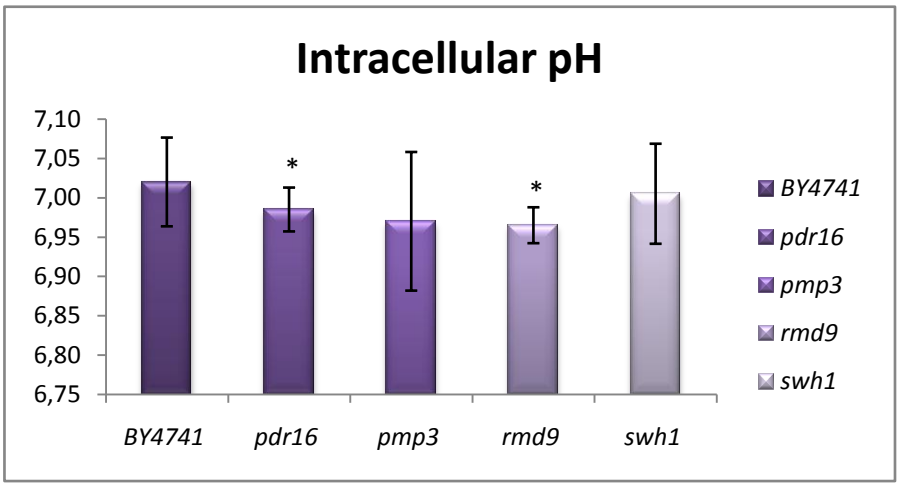

Figure 3.28. Intracellular pH of wild type yeast cells vs. deletion mutants (*p<0.05)

According to Figure 3.28, intracellular pH of the wild type yeast cells was almost neutral (7.02). However, the absence of either *RMD9* or *PMP3* made the cytosol slightly acidic. The pH of these cells were approximately 0.05 lower that may affect the metabolic processes.

3.16. Testing the Resistance of *Candida albicans* Orthologous Genes: A Clinical Proof

Candida albicans is the most common pathogenic species isolated from the patients suffering from candidiasis (Papon, Courdavault et al. 2013). Thus, in this study the resistance of *C. albicans* orthologs was also tested in order to proove their clinical importance.

The orthologous genes were obtained from www.candidagenome.org database. C1_03820W_A and C3_01910C_A were the systematic names of *C. albicans PDR16* and *PMP3* orthologs, respectively. In this study, these two orthologous genes were renamed as *CaPDR16* and *CaPMP3,* to make the results more understandable. On the other side, *C. albicans* orthologs of CSP resistant genes *RMD9* and *SWH1* have not been known yet. Unfortunately, the pathogenic resistance of these two genes could not be confirmed.

CaPDR16 and *CaPMP3* genes were first amplified by PCR using the related primers with Gateway® adaptors. PCR products were extracted from agarose gel and

the genes were cloned into pAG426GPD-ccdB Gateway® expression vectors by following the BP and LR reactions, respectively. The results were confirmed by both restiction with Bsp1407I (BsrGI) enzyme and DNA sequencing (Figure 3.29).

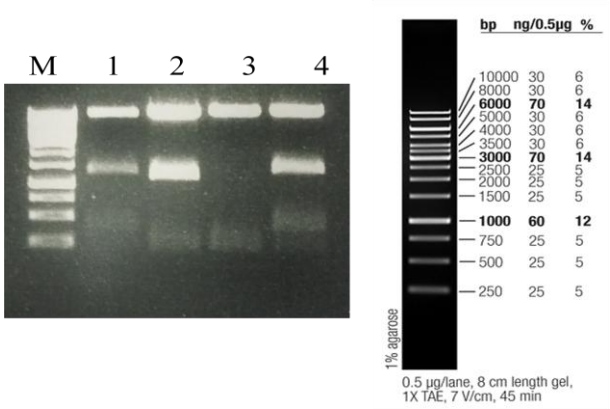

Figure 3.29. Agarose gel image of cloned *Candida* genes (M: 1kb Marker, the exact sizes are given on right; 1-4: Empty vector pAG426GPD-ccdB (Expected fragments: 6408bp, 1286bp, 402bp, 229bp); 2: pAG426GPD + *CaPDR16* (Expected fragments: 6408 bp, 1110 bp, 229 bp); 3: pAG426GPD + *CaPMP3* (Expected fragments: 6408 bp, 168 bp).

Two cloned genes and an empty vector as a control, were transfomed into *S. cerevisiae* wild type cells. The resistance of the genes were tested by gradient spotting assay and observed that both of the genes were extremely resistant to AmB (Figure 3.30).

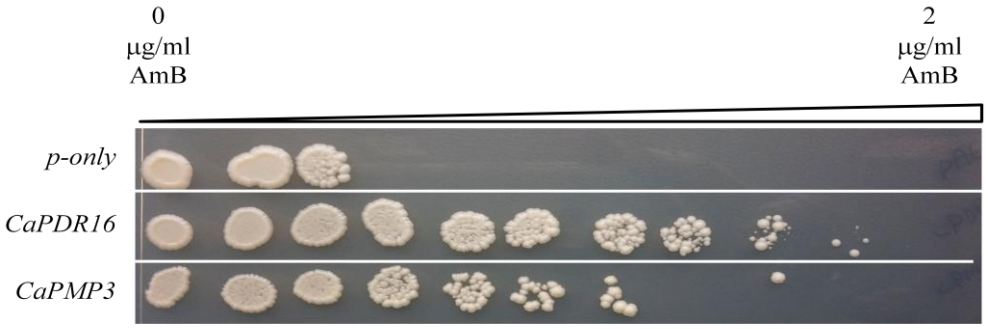

Figure 3.30. Wild type *S. cerevisiae* cells with *Candida* orthologous genes were significantly resistant to AmB when compared to wild type cells containing empty plasmids.

In the previous experiments, the relation between *PDR16* gene of *S. cerevisiae* and azole antifungal drugs were tested in details and determined that overexpression of

PDR16 sensitized the cells against the azoles. In this case, the effect of *CaPDR16* which is the *C. albicans* ortholog was also tested to support the hypothesis.

Overexpression vector pAG426GPD-ccdB and its *CaPDR16* overexpression version were transformed into BY4741 *S. cerevisiae* cells and their *Δpdr16* haploid deletion mutants. Then, spotting assay was performed and the cells were grown on related selective media with several concentrations of either VOR or KETO for three days. Similar to *PDR16* gene, the overexpression of *CaPDR16* also caused hypersensitivity to cells (Figure 3.31).

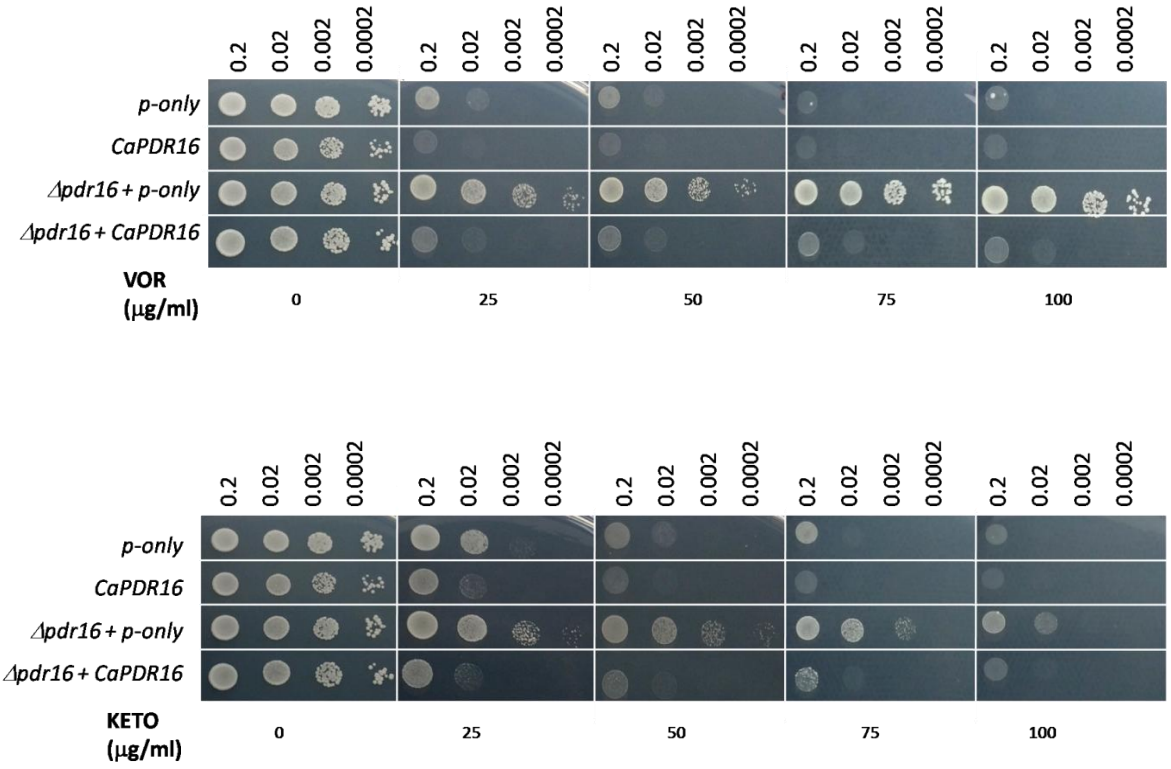

Figure 3.31. Test of *CaPDR16* orthologous gene on azole antifungal drugs, voriconazole (up) and ketoconazole (down).

3.17. Transcriptional Analyses of *Candida* Orthologs

The acute response of *Candida* genes was obtained by real-time PCR at transcriptional level. First, wild type SC1543 *Candida* cells were inoculated into specific SDA media and grown at 37^{o}C for an overnight. The next day OD_{600} of the cells were adjusted to 0.1 and cells were exposed to several concentrations of AmB.

Changes in OD_{600} were measured in every 3 hours of a whole day and minimum inhibitory concentration of AmB on *Candida* cells were determined as 0.2 µg/ml (Figure 3.32).

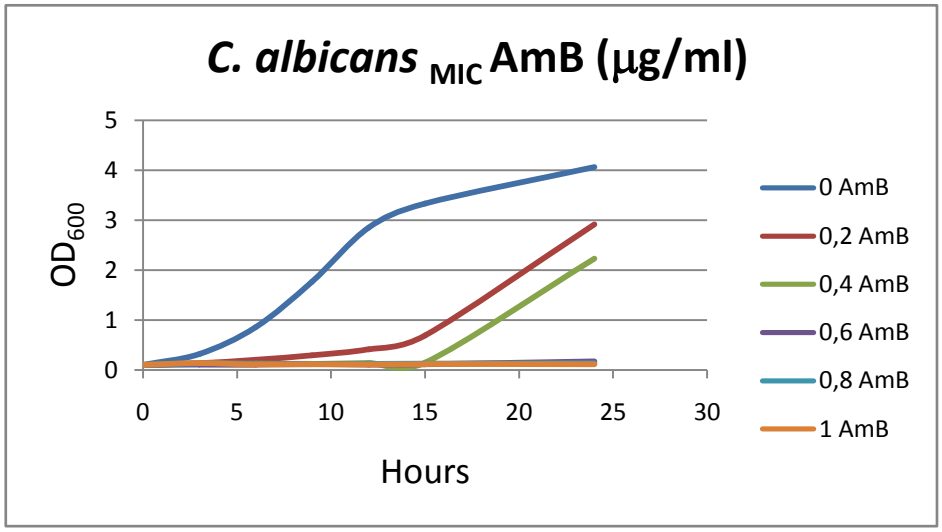

Figure 3.32. Minimum inhibitory concentration of AmB on *Candida* cells

Candida cells in their logarithmic phase were exposed to 0.2 µg/ml of AmB for 3 hours and then their total RNAs were isolated. After conversion of mRNAs to cDNAs by reverse transcription, the levels of *CaPDR16* and *CaPMP3* were recorded in either cells exposed or non-exposed to AmB.

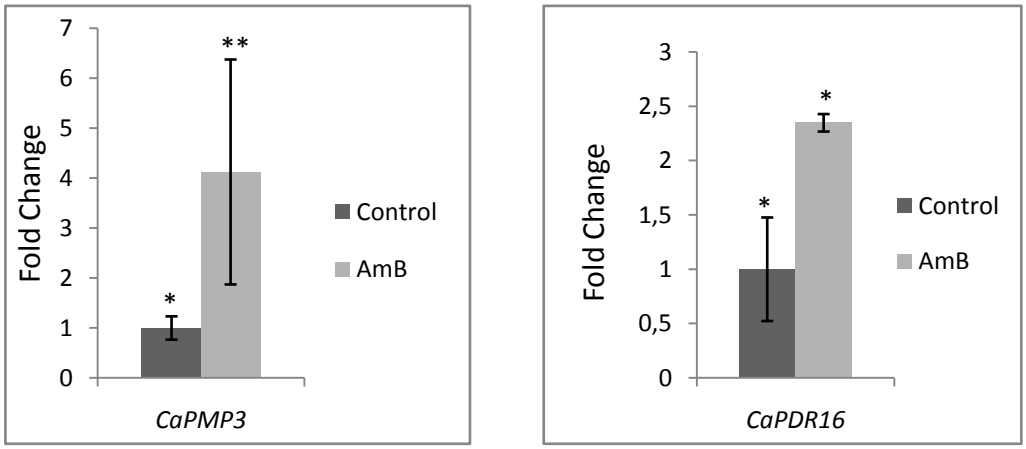

Figure 3.33. Transcriptional analyses of *Candida* orthologs in response to AmB exposure. Both AmB resistant genes were up-regulated and fold changes were shown in tha graphs (*p<0.05, **p<0.1).

Similar to *S. cerevisiae* genes, *Candida* orthologs were significantly up-regulated in response to AmB. As shown in Figure 3.33, *CaPMP3* and *CaPDR16* were almost 4 and 2.3 fold up-regulated, respectively. Two independent biological samples were analyzed and the experiments were repeated at least three times in each sample with triplicate readings. Although, non-stabile genomic property of *Candida* decreased the precision of the experiment, the significancy of the results were reliable ($p<0.05$), except *CaPMP3* in AmB ($p<0.1$).

Test of *Candida* genes proved that these two genes, *PDR16* and *PMP3* play crucial role in defence mechanisms of pathogenic yeasts against AmB. Same tests should be repeated for the *Candida* orthologs of *RMD9* and *SWH1* when they were identified and indicated in *Candide* Genome Database. Therefore, their clinical importance can be revealed.

CHAPTER 4

CONCLUSION

In recent years, the number of patients in intense units are dramatically increasing. And this increment accelerates the number of death due to invasive fungal infections (IFIs). To cope with the high number of IFIs and their mortality and morbidity risks is getting harder as a result of resistance mechanisms developed by fungal pathogens. Unfortunately, most of these resistant mechanisms still not known.

In this study, identification and characterization of resistance mechanisms against two systemically used antifungal drugs; amphotericin B and caspofungin, was aimed. Genomic DNA library of non-pathogenic bakery's yeast *S. cerevisiae* was screened and genes providing resistance were determined.

Caspofungin (CSP), is the first member of new generation antifungal drugs, echinocandins. In general, CSP inhibits the β-1,3 glucan synthase enzyme and therefore disrupts the cell wall integrity. By this way, cells lose their growing ability and become sensitive to osmotic changes (Letscher-Bru and Herbrecht 2003).

One of the CSP resistant genes identified in this study was *RMD9.* Rmd9p is a mitochondrial protein and related with mitochondrial inner membrane. Nouet et al. explained that Rmd9p had a role in the resistance and/or processing of mRNAs encoding the subunits of electron transport chain (ETC) (Nouet, Bourens et al. 2007). Thus, most probably the cells can deal with the oxidative stress caused by CSP via overexpressing this *RMD9* gene (Kelly, Rowan et al. 2009). In transcriptional analyses, *RMD9* was down-regulated as an acute response to CSP which prevented the regulation of electron transport chain members. Similarly, in *Δrmd9* deletion mutants the ETC could not work efficiently and this abnormality might cause a decrease in cytosolic pH. The relation between oxidative phosphorylation and cytosolic pH was also explained by Pál L.Vághy (Vaghy 1979).

Another possible reason of decrease in cytosolic pH might be the energy production of cells via fermentation, since they could not use their ETC system efficiently due to the absence of their regulatory protein Rmd9. Therefore, the cells produced several organic acids during fermentation and caused acidity in the cytoplasm

(Kawahata, Masaki et al. 2006). Moreover, as seen in fluorescent images, the cells might disrupt their vacuolar structures via autophagy to produce much more energy and nutrient in *Δrmd9* deletion mutants (Teter and Klionsky 2000).

Besides, the increment in proton concentration of the cytoplasm might affect the osmotic balance of the cells. Thus, they trigger the efflux of protons and this situtation might cause a hyperpolarization in plasma membrane. In literature, it was shown that there is a correlation between the cytoplasm acidification and membrane hyperpolarization, which also supports the situation of *Δrmd9* deletion mutants (Brummer, Felle et al. 1984). The abnormal efflux of protons might provide a protection to cells against amphotericin B (AmB). Because, AmB makes holes through the plasma membrane and causes leakage of essential ions to the outside (Ghannoum and Rice 1999). Hence, the excess protons leave the cells more easily by the help of AmB holes and the cells could survive.

In contrast, when yeast cells overexpress *RMD9,* they could produce enough energy for metabolic processes and therefore they might survive in the presence of antifungal drugs. It is also known that β-1,3-glucan synthase enzyme, direct target of CSP, is activated by ATP (Shematek and Cabib 1980). And this information supports the idea of how *RMD9* could resist to antifungal drugs, AmB and CSP.

Other CSP resistant gene was identified as *SWH1*. The protein encoded by this gene has a homology with mamalian oxysterol binding protein (Schmalix and Bandlow 1994). The exact function of *SWH1* is not known yet, but its structural similarity with Osh1p make the researchers to think it may play role in nucleus-vacuole (NV) junction and endoplasmic reticulum (ER) traffic (Levine and Munro 2001). CSP disrupts the cell wall integrity, hence *SWH1* could provide resistance by membrane fusion pathways and ER stress response (Boyce and Yuan 2006).

According to the data obtained in this study, the absence of this gene did not have any significant effect on cell physiology such as membrane potential or intracellular pH. In otherwise, the overexpression of *SWH1,* the stress molecules such as CSP or TMA can be exported to the outside of the cells via several pathways related with exocytosis. In addition, cells overexpressing *SWH1* might have a regulatory effect on Na^+/Ca^{2+} exchangers. It is known that ER is a calcium storage organelle and the membrane potential of the plasma membrane varies due to cytosolic Ca^{2+} and Na^+ concentrations (Koch 1990, Blaustein and Lederer 1999). In this case, the

hyperpolarization of plasma membrane when *SWH1* was overexpressed might be a result of variations in cytosolic ion concentrations. In size measurements, it was observed that cells overexpressing this gene were quite larger than wild type control cells. This enlargement might be a result of lipid droplets and membranous structures produced by ER(Beller, Thiel et al. 2010). Since, it was shown that accumulation of membranous structures in cells by a kind of stimulation, causes enlargement in yeast cells (Biemans, Thines et al. 1991).

In cross resistance test, cells overexpressing *SWH1* were sensitive to azole drugs, voriconazole and ketoconazole. After this determination, deletion mutants and their complementaries were also tested in different azole drugs with several concentrations. Not in diploid, but in haploid cells; *Δswh1* cells were extremely resistant to azole drugs. Azole antifungals inhibits cytochrome p450-dependent 14-α-lanosterol demethylase enzyme (CYP51) and stop ergosterol biosynthesis which has a crucial role in cell viability (Johnson and Kauffman 2003). If cells can resist these azole drugs in the absence of *SWH1* gene, it can be concluded that *SWH1* has a regulatory role in lipid metabolism.

As well as gene based screening, general response of cells to CSP were obtained by microarray analysis. Both of *RMD9* and *SWH1* could not be specificly detected in microarray screening, however the functions of genes were not totally irrelated with the general response. Two hours of CSP exposure activated the carbohydrate metabolism, inhibited the cell cycle process and demonstrated that cells require energy to deal with the drug stress. Thus, cells stop their too much energy consuming activities initially. In contrast, they used their whole energy to remove the CSP. Activation of *SWH1* might be a parallel solution of cells to send the drug back to the outside by exocytosis which is a metabolic energy requiring process (Almers 1990, Ohyama, Hosaka et al. 2002). Moreover, this exocytosis event might be triggered by increased cytosolic Ca^{2+} (Almers 1990, Ohyama, Hosaka et al. 2002). It is interesting that *RMD9* was down regulated after two hours of CSP exposure. This can be explained as an acute response of the cells. Most probably, the cells first degraded their energy reservoirs, carbohydrates and in this period they only activated their sugar metabolism. It was not analyzed but predicted that cells would up-regulate their *RMD9* gene in longer exposures to produce energy from sugar monomers.

The second drug of this study was amphotericin B (AmB), a member of polyene antifungals. In larger polyenes such as AmB, there are two mode of action models. In

the first one, eight or ten polyene molecules come together, hydroxyl residues stay inside of the plasma membrane and pore like structures are formed through the plasma membrane. By this way, the cells lose their essential molecules and cytoplasmic compounds through these pores and cannot survive anymore (Ghannoum and Rice 1999). In the second model, the polyene molecules aggregate on the plasma membrane and absorbs the sterol like a sponge. Without sterols, the cells cannot continue their viability (Anderson, Clay et al. 2014, Lohner 2014). In this study, *PDR16* and *PMP3* genes were identified as AmB resistant according to gDNA library screening.

According to Van den Hazel *et al.,* Pdr16p is a phosphotidylinositol transfer protein controlled by regulator of pleiotropic drug resistant protein, Pdr1p. This regulator protein Pdr1p localizes in lipid particles and microsomes and therefore regulates the lipid levels. It was demonstrated that plasma membrane of *Δpdr16* deletion mutants have different lipid compositions. On this basis, it was concluded that *PDR16* controls the sterol concentrations in cells directly or has a role in sterol biosynthesis indirectly. It may activate the enzymes or regulate the concentrations of sterol precursors required for sterol biosynthesis (van den Hazel, Pichler et al. 1999).

In 2009, Anderson *et al*. screened the yeast transcriptome and obtained that six genes including *PDR16* were up-regulated in response to AmB (Anderson, Sirjusingh et al. 2009). However, functional and physiological tests of *PDR16* against AmB was performed in this study, for the first time. Moreover, its *Candida* ortholog in response to AmB has not been studied until this time.

When the role of *PDR16* on sterol biosynthesis is considered, it can be concluded that the damage caused by AmB are repaired by the ability of this gene. In both *S. cerevisiae* and *C. albicans*, cells overexpressing *PDR16* were significantly resistant to AmB. Furthermore, the deletion mutant of *PDR16* was quite sensitive to this antifungal drug that confirmed the expectations.

In order to monitor the behaviors of cells overexpressing *PDR16* in the presence of other antifungal drugs, the cross-resistance test was performed. Overexpression of this gene could not provide any resistance to the other antifungals. However, during this test something very interesting was observed. *PDR16* caused an extreme sensitivity to cells in azole drugs, both KETO and VOR, which contradicted with literature (Simova, Poloncova et al. 2013, Holic, Simova et al. 2014). The strain difference was considered first and two more strains were provided from Griac *et al.* who defended the opposite aspect. Hence, not only the local strains, but also foreigner strains were tested with three

different azole drugs, ketoconazole, voriconazole and itraconazole (Simova, Poloncova et al. 2013, Holic, Simova et al. 2014). In all of the strains, overexpression of *PDR16* made the cells hypersensitive to these three azoles. Furthermore, *Δpdr16* deletion mutants were significantly resistant to KETO and VOR, but not to ITRA. According to this information, the idea of strain difference causing the contradiction was rebutted. Besides, *CaPDR16* ortholog had the same phenotype in response to azoles, which totally supported the previous data.

In transcriptional analyses of both organisms, *PDR16* and its ortholog were significantly up-regulated in response to AmB and these results were also supported by the literature data (Anderson, Sirjusingh et al. 2009). Further physiological tests including membrane potential and cytosolic pH measurements demonstrated that cells were affected from the absence of *PDR16.* In other words, the plasma membrane of *Δpdr16* deletion mutants were depolarized and a slight decrease was observed in cytosolic pH. These physiological changes might be a conclusion of varied lipid compositions of the plasma membrane.

The second AmB resistant gene *PMP3* was first identified by Navarre *et al.* that encodes a small hydrophobic polypeptide consists of 55 aminoacids (Navarre and Goffeau 2000). While this study was proceeding, the AmB resistance of this gene was published by Huang *et al*. (Huang, Chen et al. 2013). Therefore, it was confirmed that *PMP3* was the right one selected from the gene cassette. *PMP3* encodes a membrane related small protein and its plant homolog *RCI2* is a responsive gene to low-temperature and salt-stress (Nylander, Heino et al. 2001). Thus, somehow *PMP3* might have a regulatory role on osmolytic balance of the cells. Both in previous studies and also in this study, it was demonstrated that *Δpmp3* was significantly sensitive to several cations including sodium, Hygromycin B etc. (Navarre and Goffeau 2000, Nylander, Heino et al. 2001) as well as AmB. In other words, *PMP3* might prevent the lose of essential ions caused by AmB.

According to transcriptional analyses, *PMP3* was almost 15 times upregulated which means that it was an emergent gene providing an acute protection against external stress. Moreover, the activation of carbohydrate metabolism obtained from microarray analysis, might be related with both energy requirement and accumulation of organic osmolytes which are sugar based polyols to provide a self-protection against ionic imbalance (Burg and Ferraris 2008).

The relation of this gene with osmolytic balance was also confirmed by characterization tests. When *PMP3* was overexpressed no significant change was observed. Whereas in its deletion mutant, plasma membrane of the cells was slightly depolarized. In addition, the intracellular pH was lower than the wild type control cells. In the absence of *PMP3* the cells might not keep their osmotic balance and have abnormal changes. The membrane potential of *Δpmp3* cells were previously measured by Navarre *et al.* in 2000 and they recorded hyperpolarization instead of depolarization (Navarre and Goffeau 2000). Here in this study, this experiment was repeated three times with different biological samples and similar results were obtained in each measurement. Thus, the incompatibility of the results and literature might be explained by different probes used in this study and by Navarre *et al.*

Finally, *C. albicans* ortholog of *PMP3 (CaPMP3)* was cloned into expression vector and its AmB resistance was confirmed in *S. cerevisiae* cells. Similar to bakery's yeast, *CaPMP3* was significantly upregulated in *C. albicans* when exposed to AmB. By this way, its clinical importance was approved one more time.

In summary, two resistant gene couples were identified against two different systemically used antifungal drugs, AmB and CSP, in this study. Even though some of the data was published before the whole study was completed, most of the results were obtained for the first time and they will have an important contribution to literature.

As a future perspective, the cross resistance of these genes with other antifungal drugs as well as specific chemicals causing stress may be tested. The relation between *PDR16* and azole drugs may be studied in details and the behavior of the cells may be tested in different environmental conditions. In addition, the *Candida* orthologs of *RMD9* and *SWH1* may be obtained by scanning of whole *Candida* genome and their CSP resistance may be tested. Finally, some key points of microarray may be examined in details. Extremely up-regulated and down-regulated genes may be cloned into expression vectors and tested with related antifungal drug.

In conclusion, all of these data will be useful in the future investigations and novel drug discoveries. These identified genes can be potential targets during drug therapies. And the antifungal drug resistance can be partially extinguished.

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VITA

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