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ERG11-Mediated Azole Resistance in *Candida albicans*

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ERG11-Mediated Azole Resistance in *Candida albicans*

Abstract

Although many medically important *Candida* species are commensal to the gut or colonizers of the skin, these organisms have the propensity to cause disease in the event of a waning immune system. Clinical manifestations of infections with *Candida* species can range from superficial mucosal infections to deep organ involvement usually resulting from haematogenous spread of infection. Despite significant progress made in the management of patients with fungal infections, the emergence of antifungal resistant clinical isolates creates significant problem in regards to antifungal prophylaxis and empirical treatment strategies. Antifungal resistance is associated with high mortality rates and hefty medical costs. The azole-antifungal class has been the “work horse” of antifungal pharmacotherapy for the past 20 years defined by its efficacy against *Candida* species and paucity of side effects. As the only oral option available for systemic antifungal treatment, the azoles are the most suitable option for the long treatment periods sometimes required for antifungal prophylaxis and therapy. As the azoles are fungistatic to *Candida* species, the lengthy and repeated treatment courses have resulted in azole-resistant clinical isolates resulting in treatment failure and increased patient mortality.

Candida albicans is the most prevalent etiologic cause of fungal disease. High-level azole resistance in this species is a result of the interplay of several mechanisms of resistance. Overexpression of the efflux transporter genes *CDR1*, *CDR2*, and *MDR1* is a common mechanism of drug resistance in *C. albicans* and the majority of previous investigations pertained to defining mechanisms of transcriptional regulation of efflux transporters. Alternatively to efflux transport, point mutations in the *ERG11* gene, whose gene product is the target of azoles, result in reduced target binding affinity without precluding enzymatic function. In addition to point mutations, overexpression of *ERG11* has also been shown to decrease fluconazole susceptibility. *ERG11* gene amplification by chromosome 5 duplication or the presence of a chr5L isochromosome is known to contribute to azole resistance. Additionally, the zinc-cluster transcription factor *Upc2* has been shown to regulate the expression of *ERG11* and other genes involved in ergosterol biosynthesis.

In a large group of clinical *C. albicans* isolates enriched for azole resistance, I created a transcriptional profile defining expression of genes known to cause azole resistance such as *ERG11*, *CDR1*, *CDR2* and *MDR1*. Not surprisingly, *CDR1* and *CDR2* overexpression was generally coordinately regulated and quite prevalent among these isolates. Of those isolates that did overexpress *MDR1*, even fewer isolates expressed *MDR1* to the levels previously observed in azoleresistant isolates. *ERG11* was found to be upregulated in almost three-fourths of the fluconazoleresistant isolates examined. This suggests that *ERG11* overexpression is a common contributor to fluconazole resistance in *C. albicans*. Among the *ERG11*-overexpressing isolates studied here, I repeatedly recovered eight distinct single-nucleotide substitutions in *UPC2*. Five of these substitutions in *UPC2* have not been described previously. Of the five novel mutations, four mutations resulted in increased *ERG11* expression and increased resistance to fluconazole but to various degrees. Genome-wide transcriptional analysis was performed for the four strongest *Upc2* amino acid substitutions (A643V, G648D, G648S, and Y642F). Genes commonly upregulated by all four mutations included those involved in ergosterol biosynthesis, in oxidoreductase activity, the major facilitator efflux pump encoded by the *MDR1* gene, and the uncharacterized ATP binding cassette transporter *CDR11*. These findings demonstrate that gain-of-function mutations in *UPC2* are more prevalent among clinical isolates than previously thought and make a significant contribution to azole antifungal resistance, but the findings do not account for *ERG11* overexpression in all such isolates of *C. albicans*.

Although prevalent, not all *ERG11*-overexpression in this group of isolates could be explained by GOF mutations in *Upc2*. In *C. albicans*, the Pho-G transcription factor *NDT80* has been implicated in azole

resistance not only due to its regulation of *CDR1* but also due to its regulation of genes involved in the ergosterol biosynthesis pathway. In the next set of experiments, *NDT80* alleles for genetically matched pairs of isolates 945/1619 and 1002/3795 were sequenced. In both matched sets, the fluconazole resistant isolate overexpresses *ERG11*. Sequencing of the *NDT80* allele of both matched sets revealed several mutations that resulted in amino acid substitutions when compared to SC5314. This analysis also showed that a loss of heterozygosity event occurs so that the resistant counterpart was homozygous for one allele. A strain carrying the *NDT80* allele derived from fluconazole-resistant isolate 1619 did not result in increased *ERG11* expression and increased fluconazole resistance. The mechanism by which *ERG11* is upregulated in the absence of *UPC2* gain-of-function mutations is currently under investigation.

In addition to *ERG11*-overexpression, mutations in *ERG11* that result in amino acid substitutions in lanosterol demethylase have also been associated with decreased azole susceptibility. In the third study, I examined the prevalence and variance of *ERG11* mutations in the same group clinical *C. albicans* isolates. In this collection, I identified that 55 of the 63 isolated contained missense mutations in *ERG11* that resulted in at least one amino acid substitution. From this sequencing data, I selected a group of mutant *ERG11* alleles were expressed in an azole-susceptible background so I could determine the specific contribution of the mutant *ERG11* allele on antifungal susceptibility. In this analysis, I was particularly interested in characterizing amino acid substitutions that occurred alone and also when accompanied by another mutation. In total, I characterized ten *ERG11* alleles containing one amino acid substitution and nine alleles which carried *ERG11* alleles with a combination of amino acid substitutions. Fluconazole, itraconazole and voriconazole susceptibilities for these strains were tested. Our data demonstrated many of these mutations resulted in fluconazole resistance, but most were not as significant when tested against voriconazole or itraconazole. Itraconazole, in particular seemed less effected by *ERG11* mutations which produced significant resistance to fluconazole although amino acid combination Y132F and F145L resulted in increased itraconazole resistance. Specific combinations of *ERG11* mutations resulted in increased azole resistance beyond single mutations. These data suggest that structural differences between azole effect activity against specific mutant *ERG11* alleles.

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ERG11-Mediated Azole Resistance in Candida albicans

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Stephanie Ann Flowers
December 2013

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DEDICATION

To my parents, Marcia Flowers and Allan Flowers, and to my step-parents, Roger Alaux and Jacqueline Flowers, for all their love and support. To my grandmother and “grandfather”, Virginia Clarine Walton and Burton Zoul, you are greatly missed.

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ABSTRACT

Although many medically important *Candida* species are commensal to the gut or colonizers of the skin, these organisms have the propensity to cause disease in the event of a waning immune system. Clinical manifestations of infections with *Candida* species can range from superficial mucosal infections to deep organ involvement usually resulting from haematogenous spread of infection. Despite significant progress made in the management of patients with fungal infections, the emergence of antifungal resistant clinical isolates creates significant problem in regards to antifungal prophylaxis and empirical treatment strategies. Antifungal resistance is associated with high mortality rates and hefty medical costs. The azole-antifungal class has been the “work horse” of antifungal pharmacotherapy for the past 20 years defined by its efficacy against *Candida* species and paucity of side effects. As the only oral option available for systemic antifungal treatment, the azoles are the most suitable option for the long treatment periods sometimes required for antifungal prophylaxis and therapy. As the azoles are fungistatic to *Candida* species, the lengthy and repeated treatment courses have resulted in azole-resistant clinical isolates resulting in treatment failure and increased patient mortality.

Candida albicans is the most prevalent etiologic cause of fungal disease. High-level azole resistance in this species is a result of the interplay of several mechanisms of resistance. Overexpression of the efflux transporter genes *CDR1*, *CDR2*, and *MDR1* is a common mechanism of drug resistance in *C. albicans* and the majority of previous investigations pertained to defining mechanisms of transcriptional regulation of efflux transporters. Alternatively to efflux transport, point mutations in the *ERG11* gene, whose gene product is the target of azoles, result in reduced target binding affinity without precluding enzymatic function. In addition to point mutations, overexpression of *ERG11* has also been shown to decrease fluconazole susceptibility. *ERG11* gene amplification by chromosome 5 duplication or the presence of a chr5L isochromosome is known to contribute to azole resistance. Additionally, the zinc-cluster transcription factor Upc2 has been shown to regulate the expression of *ERG11* and other genes involved in ergosterol biosynthesis.

In a large group of clinical *C. albicans* isolates enriched for azole resistance, I created a transcriptional profile defining expression of genes known to cause azole resistance such as *ERG11*, *CDR1*, *CDR2* and *MDR1*. Not surprisingly, *CDR1* and *CDR2* overexpression was generally coordinately regulated and quite prevalent among these isolates. Of those isolates that did overexpress *MDR1*, even fewer isolates expressed *MDR1* to the levels previously observed in azole-resistant isolates. *ERG11* was found to be upregulated in almost three-fourths of the fluconazole-resistant isolates examined. This suggests that *ERG11* overexpression is a common contributor to fluconazole resistance in *C. albicans*. Among the *ERG11*-overexpressing isolates studied here, I repeatedly recovered eight distinct single-nucleotide substitutions in *UPC2*. Five of these substitutions in *UPC2* have not been described previously. Of the five novel mutations, four mutations resulted in increased *ERG11* expression and increased resistance to fluconazole but to various degrees. Genome-wide transcriptional analysis was performed

for the four strongest Upc2 amino acid substitutions (A643V, G648D, G648S, and Y642F). Genes commonly upregulated by all four mutations included those involved in ergosterol biosynthesis, in oxidoreductase activity, the major facilitator efflux pump encoded by the *MDR1* gene, and the uncharacterized ATP binding cassette transporter *CDR11*. These findings demonstrate that gain-of-function mutations in *UPC2* are more prevalent among clinical isolates than previously thought and make a significant contribution to azole antifungal resistance, but the findings do not account for *ERG11* overexpression in all such isolates of *C. albicans*.

Although prevalent, not all *ERG11*-overexpression in this group of isolates could be explained by GOF mutations in Upc2. In *C. albicans*, the Pho-G transcription factor *NDT80* has been implicated in azole resistance not only due to its regulation of *CDR1* but also due to its regulation of genes involved in the ergosterol biosynthesis pathway. In the next set of experiments, *NDT80* alleles for genetically matched pairs of isolates 945/1619 and 1002/3795 were sequenced. In both matched sets, the fluconazole resistant isolate overexpresses *ERG11*. Sequencing of the *NDT80* allele of both matched sets revealed several mutations that resulted in amino acid substitutions when compared to SC5314. This analysis also showed that a loss of heterozygosity event occurs so that the resistant counterpart was homozygous for one allele. A strain carrying the *NDT80* allele derived from fluconazole-resistant isolate 1619 did not result in increased *ERG11* expression and increased fluconazole resistance. The mechanism by which *ERG11* is upregulated in the absence of *UPC2* gain-of-function mutations is currently under investigation.

In addition to *ERG11*-overexpression, mutations in *ERG11* that result in amino acid substitutions in lanosterol demethylase have also been associated with decreased azole susceptibility. In the third study, I examined the prevalence and variance of *ERG11* mutations in the same group clinical *C. albicans* isolates. In this collection, I identified that 55 of the 63 isolated contained missense mutations in *ERG11* that resulted in at least one amino acid substitution. From this sequencing data, I selected a group of mutant *ERG11* alleles were expressed in an azole-susceptible background so I could determine the specific contribution of the mutant *ERG11* allele on antifungal susceptibility. In this analysis, I was particularly interested in characterizing amino acid substitutions that occurred alone and also when accompanied by another mutation. In total, I characterized ten *ERG11* alleles containing one amino acid substitution and nine alleles which carried *ERG11* alleles with a combination of amino acid substitutions. Fluconazole, itraconazole and voriconazole susceptibilities for these strains were tested. Our data demonstrated many of these mutations resulted in fluconazole resistance, but most were not as significant when tested against voriconazole or itraconazole. Itraconazole, in particular seemed less effected by *ERG11* mutations which produced significant resistance to fluconazole although amino acid combination Y132F and F145L resulted in increased itraconazole resistance. Specific combinations of *ERG11* mutations resulted in increased azole resistance beyond single mutations. These data suggest that structural differences between azole effect activity against specific mutant *ERG11* alleles.

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CHAPTER 1. INTRODUCTION

CANDIDA ALBICANS

The historical importance of fungus and fungal pathogens

As immunocompetent mammals are naturally resistant to fungus, infections by fungal organisms seem enigmatic and dangerous. Mammals' innate immunity to fungal infections is likely due to a combination of the innate and adaptive immune system in addition to elevated body temperatures characterized by our endothermic lifestyle (19). A computational model has determined the optimal temperature that provides maximal protection against fungus to be 36.7 °C, which is close to mammalian temperatures (11). This data suggests that vertebrate endothermic physiologic temperatures could be in part selected by pathogenic fungal species (19). An example of protection conferred by both a competent immune system and an endothermic physiological temperature has been demonstrated recently in the local bat population. In August of 2013, British Broadcasting Company reported a "bat killing fungus" detected in Arkansas that is responsible for the devastation of the North American bat population. The visual symptom of infected bats is a white fungus that typically grows on the nose, ears or tails, earning the title "white nose syndrome" (31). The etiologic cause of this infection is the fungus *Pseudogymnoascus destructans*. This fungus is unable to grow at temperatures above 20°C and infects bats as their body temperature lowers due to hibernation (30). Dr. Arturo Casadevall, from Albert Einstein School of Medicine has attributed mammalian immunity to disseminated fungal infections with the dominance of mammals after the Cretaceous-Tertiary (K-T) Boundary (19). In his article entitled "Fungi and the Rise of Mammals", Dr. Casadevall describes how the "fungal bloom" resulting from catastrophic events 65 million years ago set the stage for mammals replacing reptiles as the dominant land animals (19, 192).

Historically, the progress defining the significance of medically relevant mycology was slow due to the infrequency of disease and lack of experience of medical practitioners. The first observations of *Candida* infections is thought to date back to the ancient Greek physician Hippocrates who described an oral candidiasis, or thrush, around 400BC in book three in "Of the Epidemics"(10). In this selection, what is presumed to be an observation of oral candidiasis is described as "mouths affected with aphthous ulcerations". Much later in history but prior to Louis Pasteur, the Hungarian physician David Gruby identified the human pathogen, later named *Achorion schoenleinii*, to cause the scalp condition called favus (72). He was able to demonstrate causation of human disease by culturing the microorganism on potato slices and infecting healthy tissue.

Despite the early observation and detection of diseases caused by fungal pathogens, bacterial diseases are better studied. This is most likely due to the frequency and severity of bacterial and mycobacterial epidemics that affect large populations as opposed to infrequent and isolated encounters with fungal pathogens. The current accepted genus for

C. albicans was introduced in 1923 by Christine Berkhout, who was later credited with deriving the first “rational systematics of anascosporogenous yeasts” (10). The genus *Candida* is derived from ancient Roman political candidates, or *candidatus*, who wore white togas. The species name *albicans* is derived from *Albico*, which also means “to be white” (10). Prior to the 1980s, mortality attributed to mycosis was ranked 10th in the United States when compared to mortality rates from alternate infectious etiologies. Between the time periods of 1980 to 1997, a marked increase of mortality attributed to fungal pathogens occurred, which is a time period that correlates with the onset of HIV and medical advancements that prolong the life of critically ill patients that are susceptible to these pathogens (118).

On June 5, 1981, the Centers for Disease Control and Prevention (CDC) published in their Morbidity and Mortality Weekly Report (MMWR) describing independent case studies of five homosexual, previously healthy young men presenting with what was then known as *Pneumocystis carinii*, which is an opportunistic pathogen. (1). These men presented with other opportunistic infections and three cases noted oral candidiasis on their medical problem list. This publication is considered by many the beginning of the AIDs epidemic. With the spread of AIDs, detection of mucosal and invasive fungal opportunistic pathogens exploded (118). The most common pathogens in this population were *Candida albicans* and *Cryptococcus neoformans* (6). Mucocutaneous candidiasis was the most frequently identified fungal opportunistic infection and the most common early identifier of people with Human Immunodeficiency Virus (HIV)(118). With the implementation of Highly Active Antiretroviral Therapy (HAART), the frequency of *Candida* spp. infections in HIV patients has dramatically decreased (188). However, in populations that do not have access to antiretrovirals or in cases where patients are non-compliant or are non-responders to their HAART regimen, treatment courses of azole antifungals have resulted in a subset of isolates that have become resistant to treatment (188).

Despite the successful development of treatments for HIV, the population susceptible to invasive fungal infections continues to grow and *Candida* spp are leading the charge. Advances made in invasive medical technology, immunosuppressive and chemotoxic treatments have resulted in a wide range of subjects susceptible to invasive fungal disease. In nosocomial derived bloodstream infections, *Candida* spp the rank overall as the fourth most commonly isolated pathogen in hospitals and are identified as the second most frequent cause of infection in North American intensive care units (198, 205). *C. albicans* remains the most prevalent etiological agent of *Candida* infection.

Biology and classification

C. albicans is both a commensal organism and a medically important opportunistic pathogen. This species taxonomically belongs to the Fungi kingdom, the phylum Ascomycota, which is the largest phylum within the kingdom Fungi, and the class Saccharomycotina (87). The genus *Candida* is within the class of Deuteromycetes, since many species of this genus were not observed to have a sexual cycle. *Candida* spp.

includes more than 160 species but only 20 of these are clinically relevant in human disease (10).

C. albicans is a diploid, polymorphic fungus that is member of the CTG clade defined by a unique codon exchange from a leucine to a serine (183). Its mode of reproduction is predominately clonal but still exhibits substantial heterozygosity due to its ability to undergo recombination and ploidy changes based on chromosomal loss and reformation (134). *C. albicans* possesses homologous of the mating-type genes that are characterized in *Saccharomyces cerevisiae* and can undergo a mating process which results in a tetraploid cell (76). *C. albicans* lack genes to undergo meiosis so return to the diploid state is a result of random chromosomal loss and is also a source of genomic microvariation in this species (12, 133). DNA fingerprinting techniques have been developed to identify population structure and microevolution in *C. albicans*. Results of these studies have defined five major genetically related groups, designated as clades, which are geographically linked to specific areas of the world (179).

Genetics and genetic manipulation

C. albicans generally is considered to be an obligate diploid organism but has been recently shown to have a viable haploid state that is recovered both *in vitro* and *in vivo* (70). *C. albicans* propagates predominantly by clonal methods however, a parasexual cycle has been defined for this species (12, 80). The nuclear genome is around 16 million base pairs comprised of eight chromosomes (82). The chromosomes are numbered from 1 (~4.3 Mb) to 7 (~1.03 Mb) and also chromosome R, which varies in size depending on the number of ribosomal DNA repeats. The complete sequence of *C. albicans* is available on the Candida Genome Database (CGD) website from Stanford University (www.candidagenome.org).

The success of *C. albicans* as an opportunistic pathogen is due to its ability to adapt to various niches aided by genetic changes such as point mutations, loss of heterozygosity (LOH) events, and instances of aneuploidies (134). *C. albicans* has a high level of single nucleotide polymorphisms (SNPs), which cause amino acid changes in the protein sequence. Previous work has shown that out of the approximate 6,000 total genes of the *C. albicans* genome, about 2,792 open reading frames (ORFs) carry at least one SNP resulting in heterozygous alleles in almost half the genome (18).

Over the last few decades, molecular pathogenicity and drug resistance mechanisms in *Candida* species have been intensely investigated using genetic tools and molecular techniques which were often times developed to study genetics in *S. cerevisiae*. Creating libraries of gene disruptants has been a powerful tool in the examination genetic regulatory networks of specific cellular processes (69, 132). In *C. albicans*, homozygous gene disruption requires two rounds of transformation because it is a diploid species. By this method, a linear fragment of exogenous DNA is introduced with a selectable marker flanked by specific homologous regions which facilitates the targeted incorporation into the genome (69). This disruption cassette can be constructed by using polymerase chain

reaction (PCR) or traditional cloning techniques and selectable markers can be either auxotrophic genes (*LEU2*, *ARG4* and *URA3*) or drug resistance genes such as *SAT1* (156). Using *URA3* auxotrophic strains have generally lost favor due to the observation that *URA3* expression is correlated with virulence in this species (182).

The use of DNA microarrays has been the method of choice for examining genome-wide gene transcription of *C. albicans*. Simply put, an array is a collection of single stranded DNA probes fixed on a solid surface. These probes can be deposited or synthesized *in situ* onto materials such as nitrocellulose, nylon or glass (56). Custom arrays for various species are now commercially available but variation can occur that affect gene expression levels such as labeling efficiency of specific dyes, concentration and quality of RNA starting material, and preparation of PCR products. Our lab has employed the use of microarrays to examine the differences in gene expression between fluconazole-susceptible and fluconazole-resistant strains in order to identify genes important for azole resistance (44, 50, 158). Gene expression in azole resistance will be discussed in a later section.

Unlike bacteria who can acquire antimicrobial resistance by transposable elements, antifungal resistance in pathogenic fungi is primarily due to genetic variability such as single nucleotide polymorphisms (SNPs) (125). In bacteria, whole-genome sequencing techniques have provided insight into the development and transmission of antimicrobial resistant pathogens (64). In *Candida* spp. the acquisition of drug resistance has been studied by strains that have developed resistant mutations *in vitro* or by targeted sequencing and expression analysis of genes known to cause resistance in clinical matched-paired isolates (155). Developments in sequencing technology allows us to examine sequence on a genome-wide scale and permits the detection mutations that leads to drug resistance or even accompanying mutations that may mitigate fitness costs of resistance mutations. Recently, the first study to examine the genome-wide sequence analysis of echinocandin resistance in clinical isolates of *C. glabrata* was published (178). In this study authors were able to identify mutations in previously unidentified genes conferring resistance to echinocandins, but also identified a compensatory mutation that mitigated the fitness cost of echinocandin resistance.

Pathogenicity and virulence

C. albicans has the ability to cause a range of infectious from superficial mucosal or vaginal infections to disseminated disease. The ability of *C. albicans* to cause such a diverse range of diseases is due to its arsenal of virulence and fitness attributes (117). Attributes such as phenotypic switching between a yeast and hyphal form, expression of adhesins, invasins and hydrolytic enzymes and biofilm formation are considered virulence factors that contribute to its ability to cause disease (62). Additionally, *C. albicans* displays a robust transcriptional response to changes in environment such as fluctuations in pH, nutrient acquisition and stress response which are fitness characteristics that allows this organism to colonize or invade a variety of niches within the human host.

Phenotypic changes. *C. albicans* has the ability to grow as a yeast cell or in filamentous form either as elongated cells with constrictions as septa (pseudohyphae) or as true hyphae with parallel separations in the cell wall. The ability to transition between yeast and filamentous form is important for its pathogenicity (79). The hyphal form is important for invasion of tissues while the yeast form is primarily responsible for its dissemination. Strains that have been constructed to be unable to transition to filamentous form show attenuated virulence (101). There are several environmental conditions that cue the transition of *C. albicans* to grow in filamentous form. Alkaline conditions (pH > 7), presence of serum, N-acetyl-glucosamine, physiologic temperature, low cell density ($> 10^7$ cells ml⁻¹) and CO₂ all promote hyphal growth (117). Hyphal formation is also linked to the expression of a subset of genes associated with virulence such as *HWP1*, *ALS3* and the secreted aspartyl proteases *SAP4*, *SAP5* and *SAP6*.

C. albicans isolates that have become homozygous at the mating type locus (*MTLa/a* or *MTLa/α*) can also switch between the white yeast form to an elongated opaque form (80). Opaque cells are the mating competent form of this species and can mate with other mating-competent isolates to produce progeny. When compared to white yeast cells, opaque cells exhibit a distinct genetic expression pattern and data suggest they are better colonizers of the skin (and GI tract) but also show attenuated virulence in mouse models (126). Although opaque cells have been reported to be more susceptible to killing by neutrophils than white cells, they also don't release a polymorphonuclear (PMN) chemoattractant, which suggests they avoid detection of neutrophils (93, 94). A recent study by Sasse et al. compared internalization of white yeast, filamentous and opaque cells by various phagocytic cells. Their data demonstrate that although opaque cells avoid recognition by human neutrophils on a glass slide, they cannot avoid detection and are efficiently phagocytosed by dendritic cells and by neutrophils in a collagen matrix environment (167).

Adhesins and invasins. In order for *C. albicans* to become pathogenic, three different processes occur: (i) adherence, (ii) invasion, and (iii) induction of host damage (62). *C. albicans* is able to adhere to other microbial cells, abiotic surfaces in addition to host cells. Adherence is important in both the commensal and the pathogenic state (33). In *C. albicans*, adherence is mainly regulated by adhesins such as the agglutinin-like-sequence (*ALS*) family, and also by hyphal wall protein 1 (Hwp1) (117). *ALS* genes encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins. The *ALS* family is composed of eight members (Als1-7 and Als9) of which Als3 is particularly important. In addition to Als3, Hwp1, which is a hypha-associated GPI-linked protein, is also important for adherence (117).

Invasion of *C. albicans* cells can occur by two different mechanisms: induced endocytosis by host cells and active penetration by *C. albicans* (33, 212). Als3, along with a member of the heat-shock protein 70 (Hsp70) family, Ssa1, are considered invasins and these proteins have been shown to trigger endocytosis by inducing host cytoskeletal arrangements; however, this process does not require viable fungal cells (129, 184). The more predominate form of invasion is active penetration. Hyphae penetrate the

cell wall by using physical force in combination with hydrolytic enzymes, specifically serine-aspartic-proteases (SAPS). The molecular mechanisms behind active penetration have not all been defined. Although this process results in the host secretion of pro-inflammatory cytokines and activation of host cellular immunity, hyphae invasion is assumed to play a role in gaining access to deep tissue and the blood stream (117).

Biofilms. *C. albicans* is the third leading cause of catheter related infections, which serve as reservoirs for pathogens by releasing cells into the blood stream (29). Biofilm formation in *C. albicans* occurs through a number of different phases including attachment, yeast cell proliferation, hyphal cell formation, extracellular matrix accumulation and dispersion of yeast cells from the mature biofilm (104, 117). Transcription factors such as Bcr1, Tec1 and Egf1 are important regulators of biofilm formation and strains constructed to be defective for these regulators in addition to *NDT80*, *ROB1* or *BRG1* were deficient in biofilm formation (132). Dispersion of cells from a mature biofilm contributes to its pathogenicity as dispersed cells have been shown to be more virulent in a mouse model (191). Mature biofilms also exhibit higher resistance to antifungal agents and to host immunity than planktonic cells (46, 48).

Environmental stress response. *C. albicans* possesses a number of stress responses that facilitates its survival in a number of challenging and changing niches within the host. Pathways involved in stress response are responsible not only for its survival but are also required for virulence. Phagocytic cells within the host produce highly reactive oxygen species (ROS), reactive nitrogen species (RNS) and antimicrobial peptides that cause oxidative and nitrosative stress to the pathogen (117). *C. albicans* produces catalase Cta1 and superoxide dismutases Sod1 and Sod5 to detoxify ROS. Likewise, in a response to nitrosative stress, flavohemoglobin-related protein Yhb1 mediates the detoxification of RNS (77, 116, 207). Host macrophages and neutrophils can effectively phagocytose fungal pathogens and create a nutritionally starved environment (54). Inside these cells, *C. albicans* switches from glycolysis to gluconeogenesis while lipids and amino acids can serve as nutrient sources. In addition, induction of hyphae while inside the phagocyte can pierce the immune cell and permit escape (61, 106).

Stress signals are also transmitted by the mitogen-activated protein (MAP) kinase pathways, which require a series of phosphorylation events. The three MAP kinase pathways in *C. albicans* are the Mkc1 (MAP kinase from *C. albicans*), Hog1 (high osmolarity glycerol response) and the Cek1 (Candida ERK-like response) pathways (4, 32, 41). These pathways facilitate processes such as cellular integrity, biofilm formation, osmotic and thermal stress, filamentation in addition to mating. Strains deficient in these pathways are shown to have decreased virulence (41).

Infections caused by *Candida* species

Candida species are the most common cause of invasive fungal infections (IFIs), which include blood stream infections (BSIs) deep tissue infections and also organ disseminated disease (143). Numerous surveys have shown that the frequency of

candidemia and invasive candidiasis (IC) has increased world-wide in the last three decades, which merits study of infections caused by *Candida* species. According to a recent survey which describes the epidemiology and outcomes of candidemia in 3,648 patients enrolled in the Prospective Antifungal Therapy (PATH Alliance) registry, 97% of all infections are caused by five different species of *Candida*. In order of prevalence, these species are *C. albicans* (42.1%), *C. glabrata* (26.7%), *C. parapsilosis* (15.9%), *C. tropicalis* (8.7%) and *C. krusei* (3.4%) (143). This *Candida* species hierarchy varies in different areas of the world (99, 204). Among the medically relevant fungal species, *C. albicans* is the most frequently recovered from human samples in the United States. This organism is responsible for a wide range of infections, from infection of superficial mucosal surfaces to invasive life-threatening disseminated disease. Invasive fungal infections are associated with health care costs over \$32,000 per patient combined with an increase of about seven days of hospital stay (9).

Candida species are part of the normal flora that exist commensally in over 70% of the healthy population (58). Individuals of advanced age or have oral prosthesis, such as dentures, exhibit higher instances of colonization. Colonization is defined as *Candida* species generated from one or more samples from non-sterile sites and the extent of colonization has been repeatedly linked to subsequent infection (3, 97, 150, 196). Previous work has shown that isolates responsible for invasive disease are often of the same karyotype of those of which the patient is colonized (134). Patients treated extensively with broad spectrum antibiotics can lead to an over-colonization of *Candida* species and may be at higher risk for IC. Other risk factors include gastrointestinal surgery, chemotherapy, immunosuppression, or other invasive medical treatments. Mortality associated with IC has been estimated between 14.5% - 49% (139).

Mucocutaneous candidiasis can be classified into genitourinary and non-genitourinary candidiasis. The most common type of genitourinary candidiasis is vulvocandidiasis (VVC), which usually occurs in healthy women. It is estimated that 75% of women are diagnosed with VVC at least once in their childbearing years and although still less common than bacterial vaginosis, VVC is the second most common diagnosis (40%) in woman with vaginal complaints in the primary care center (2, 7). Although *Candida* species may asymptotically colonize the vaginal cavity, VVC is associated with clinical signs of inflammation. In studies where *Candida* species were identified in VVC infections, *C. albicans* was the most commonly isolated causative organism followed by *C. glabrata* (2). Recurrent vulvocandidiasis (RVVC) is defined as over four VVC diagnosis within one year and in these cases, long term antifungal suppressive therapy is indicated. Of note, incidence of the non-*albicans* species, specifically *C. glabrata*, are more prevalent in RCCV than VVC. Non-*albicans* species are also more prevalent in women with HIV, who have uncontrolled diabetes and who are advanced in age (35, 181, 195).

The diagnosis and management of candiduria remains controversial among providers. Candiduria is prevalent among critically ill patients and studies show a positive correlation between candiduria and mortality, however, the role of candiduria as an indicator of invasive disease is unclear (196). The differential diagnosis between a

urinary tract infection (UTI), colonization and sample contamination is difficult so the decision to treat is often provider specific (49). The most common risk factor for candiduria is colonization of urinary catheters, which may mask the signs and symptoms of infection. Alternatively, hematogenous spread is the most common etiology of renal candidiasis; a much more serious diagnosis is an indication to treat with aggressive antifungal therapy (71).

Oral pharyngeal candidiasis (OPC) is the most common opportunistic infection with Human Immunodeficiency Virus (HIV) (140). OPC is more common in patients with low CD4⁺ count or high HIV titers however, since the widespread implementation of highly active antiretroviral therapy (HAART), OPC rates have declined (37, 42). Even in the era of HAART therapy and fluconazole prophylaxis, *C. albicans* is still the most commonly detected species (62%), followed by *C. glabrata* (17%) and *C. dubliniensis* (12%) (140). This non-*albicans* shift in the causative etiology of OPC creates concern regarding azole resistance.

Treatment of *Candida* infections

There are currently only four structurally distinct antifungal classes available for the treatment of invasive candidiasis. Unlike bacteria, both fungal cells and mammalian cells are eukaryotic, which makes targeting fungal-specific processes more difficult. The most exploited strategy for antifungal development targets the ergosterol biosynthesis pathway or ergosterol incorporated into the fungal cell membrane. Other strategies include inhibiting RNA and DNA synthesis by compounds that are selectively transported into the fungal cell and inhibition of the fungal cell wall. Mortality rates in neutropenic patients for yeast and mold infections are still unacceptably high and time to implementation of the correct antifungal has a significant impact on patient outcomes (57).

Amphotericin B. Amphotericin B (AmB) is a polyene macrolide antifungal that was first isolated from *Streptomyces nodosus* in the 1950s. Over the past five decades, amphotericin B deoxycholate formulation (AMBd; Fungizone) has been the gold-standard for the treatment of life-threatening fungal infections and generally remains refractory to resistance. Its primary fungicidal mechanism of action is direct binding to ergosterol; the primary sterol found in the fungal cell membranes. Its dual complementary action of aggregating to form membrane-permeabilizing ion channels increases its potency but is not required for fungicidal activity (63). Pharmacodynamic studies demonstrate that fungicidal activity can be enhanced by optimizing the concentration-to-MIC ratio (89). Unfortunately, the clinical utility of AMBd is often times limited by its associated adverse effects such as nephrotoxicity and infusion-related reactions (39). Because of these treatment-limiting adverse effects, newer lipid-associated formulations of amphotericin B (Ambisome, Abelcet and Amphotec) have replaced conventional AMBd as first-line therapy for many types of infections.

Flucytosine. (5FC) is a pyrimidine analog that inhibits DNA replication and protein synthesis. Flucytosine is selectively transported into the fungal cell by cytosine permease and is then deaminated by cytosine deaminase to 5-fluorouracil. It is then converted to its active form, 5-fluorodeoxyuridylic acid to inhibit DNA synthesis (194). Due to the rapid development of resistance, 5FC is rarely used as monotherapy. In a murine model of disseminated candidiasis, time over MIC was the best pharmacodynamic parameter correlated with efficacy (8). Although the Clinical Practice Guidelines for the Management of Candidiasis recommend 5FC in combination with amphotericin B or fluconazole for a variety of infection types, concerns regarding toxicity such as bone marrow suppression and hepatic transaminase elevation often times prevent its use (139). Its current utility is primarily limited to treatment of cryptococcal meningitis (159).

Echinocandins. Echinocandins are the newest members in the spectrum of antifungals. Current approved agents by the U.S. Food and Drug Administration are caspofungin (Cancidas; Merck and Co.), micafungin (Mycamine; Astellas Pharmaceuticals) and anidulafungin (Eraxis; Pfizer Pharmaceuticals). Echinocandins are used for treatment of invasive candidiasis based on results of three defining clinical trials in which caspofungin, micafungin and anidulafungin were shown non-inferior to amphotericin B, liposomal amphotericin B and fluconazole, respectively (92, 121, 154). These agents are characterized by their broad spectrum of activity, low incidence of drug-drug interactions and a relative lack of adverse effects (211). Echinocandins are lipopeptides that bind to 1,3- β -D glucan synthase, inhibiting production of β -1,3 glucan which, along with chitin, is responsible for integrity and shape of the fungal cell wall. 1,3- β -D glucan synthase is a multi-unit enzyme that is made of a catalytic subunit Fksp, the target of echinocandins, and the regulatory unit Rho1p. All three echinocandin agents are fungicidal in a dose dependent fashion against the *Candida* species including those isolates resistant to azole antifungals (137, 146, 147, 180). Unlike the azole class, echinocandins are noted to retain activity against fungal biofilm and biofilm formation (180).

Azole antifungals. Members of the azole class are the most widely used of all antifungals. Their utility is mainly due to their activity against a broad spectrum of fungal pathogens and a mild spectrum of side effects, specifically lacking the treatment limiting nephrotoxicity experienced by patients undergoing treatment with AmB. There are four azoles currently used for the treatment of invasive fungal infections. Although these agents are all of the same class, they vary individually in regards to spectrum of activity, pharmacokinetic and toxicity profiles. Appropriate utilization of these agents is based upon these unique characteristics.

The primary target of all azole antifungals is 14- α lanosterol-demethylase, which is the product of the *ERG11* gene (209). Lanosterol demethylase is a member of the cytochrome P450 (CYP450) class of enzymes, and is a critical enzyme in the biosynthesis of ergosterol. As ergosterol is not found in the mammalian cell membrane, inhibition of this pathway is a fungal specific mechanism of action for this class. Treatment with azoles results in decreased cellular ergosterol and leads to the

accumulation of toxic methylated sterols. Azoles are considered fungistatic for *Candida* and other yeast species but some are fungicidal to *Aspergillus* species (110).

Fluconazole (FCZ; Diflucan; Pfizer). Fluconazole is available in both an oral and intravenous and has been widely studied in treating invasive fungal disease. When compared to AmB, fluconazole is defined by a limited toxicity profile, but its spectrum of activity is limited to yeast. Species such as *C. krusei* display inherent resistance to this agent and *C. glabrata* has been observed to exhibit both inherent and acquired resistance secondary to treatment (136, 144). Fluconazole has excellent bioavailability independent of pH or concomitant meals and exhibits linear kinetics (65, 214, 215). This agent also exhibits limited protein binding (~10%), a high volume of distribution and achieves appreciable concentrations in the cerebrospinal fluid (CSF)(16, 187). Fluconazole is primarily eliminated unchanged in the urine and requires dosage adjustment in renal insufficiency (15). As fluconazole is a moderate inhibitor of CYP450 enzymes 3A4, CYP2C9 and CYP2C19, it potentially interacts with the metabolism of a wide variety of drugs (131). In addition, fluconazole also inhibits uridine diphosphate (UDP)-glucuronosyltransferase (UGTs), which mediates drug metabolism by glucuronidation (190). Like all triazole antifungals, area under the concentrations curve (AUC) to MIC is the best pharmacodynamic parameter that predicts azole antifungal activity (89).

Itraconazole (ICZ; Sporanox; Janssen Pharmaceuticals). Itraconazole, voriconazole, and posaconazole are all considered extended-spectrum triazoles as they exhibit greater potency than fluconazole against most fungal species. Itraconazole is effective against most yeast however, *C. krusei* and *C. glabrata* may show cross resistance with FCZ (144). When compared to fluconazole, itraconazole gains activity against certain molds such as *Aspergillus* and dimorphic endemic fungi. Itraconazole is only available orally and is formulated as either a capsule or a solution. Oral bioavailability of itraconazole is significantly less than fluconazole (213). The oral solution exhibits better bioavailability than the capsule, and this improves under acidic fasting conditions (214). Itraconazole is highly protein bound and is metabolized in the liver by CYP3A4, which results in the formation of the pharmacologically active metabolite hydroxyitraconazole (151, 186). Itraconazole is a strong inhibitor of CYP3A4 and has the potential for numerous drug-drug interactions (78).

Voriconazole (VCZ; Vfend; Pfizer). Voriconazole is also an extended spectrum triazole and is structurally related to fluconazole. Voriconazole displays excellent activity against *Candida* spp. including *C. krusei* and is the drug of choice for treatment of invasive aspergillosis (68). Voriconazole exhibits activity against endemic fungi but notably lacks activity against Zygomycetes. Voriconazole is formulated orally and also intravenously with a cyclodextrin carrier. Like fluconazole, voriconazole is well absorbed and displays high bioavailability upon oral administration (81). Voriconazole displays non-linear kinetics and absorption is independent of pH and food administration. Voriconazole is moderately protein bound (~58%) and demonstrates CSF penetration (107). Unlike itraconazole, voriconazole is primarily metabolized by CYP2C19. The argument has been made for therapeutic drug monitoring (TDM) of voriconazole levels due to inter-patient genetic differences in CYP2C19 activity (40, 202). Renal function

should be monitored in patients receiving the intravenous formulation due to the sulphobutylether-beta-cyclodextrin carrier (138).

Posaconazole (PCZ; Noxafil; Schering Corporation). Posaconazole is the newest addition to the triazole class and is structurally related to itraconazole. Global surveillance studies have noted that posaconazole is more active than itraconazole and fluconazole, and as effective as voriconazole against a diverse population of clinically relevant *Candida* spp (148, 149). Posaconazole is available as a 40 mg/mL oral suspension and due to its lipophilicity, should be administered with a full meal or a nutritional supplement (27). Absorption of posaconazole is saturable so the administration of PCZ in divided doses has been shown to increase the relative bioavailability when compared to a single dose (45). Additionally, gastric pH also plays a role in posaconazole absorption so administration with an acidic beverage is recommended while coadministration with acid suppressing drugs may decrease absorption (25). Posaconazole is highly protein bound and distributes widely to the tissues. Metabolism is largely mediated by UGT (glucuronosyl transferase) which allelic polymorphisms have been shown to alter pharmacokinetics (60). Excretion of posaconazole has also been shown to be mediated in part by P-glycoprotein and inducers of P-glycoprotein have been shown to increase clearance (26). Posaconazole is not metabolized significantly by CYP450 enzymes but remains an inhibitor of CYP isoenzyme 3A4 which can contribute to drug interactions (91).

AZOLE RESISTANCE IN *CANDIDA ALBICANS*

Clinical impact of azole resistance

Candida spp are the principle causative organisms of oropharyngeal (OPC) candidiasis and also the fourth most common pathogen isolated from nosocomial bloodstream infections. Due to the implementation of Highly Active Antiretroviral Therapy (HAART) and fluconazole prophylaxis, the incidence of OPC among HIV⁺ patients has declined (42). There is a population that for various reasons is noncompliant with their drug regimen, do not have access to antiretrovirals or do not tolerate HAART therapy for whom OPC remains a problem. Recent studies and current IDSA treatment guidelines show that for AIDS patients, recurrent prophylactic fluconazole regimens for OPC results in decreased colonization and did not appear to cause resistance (139, 140). Data show that the likelihood of resistant yeast was increased in patients with recurrent candida esophagitis and denture use (140). In these cases, the prolonged and repeated fluconazole treatment course has created a fluconazole-resistant population of *C. albicans*. Repeated fluconazole treatment has created a non-*albicans* shift in the causative organisms of OPC, many of which have decreased susceptibilities to azoles (188).

The increased incidence of invasive candidiasis is largely a result of progress in medical technology or therapies that have created an immunosuppressed population susceptible to fungal pathogens. Invasive candidiasis has a significant impact on patient

outcomes and medical costs. The mortality attributable to invasive disease varies by source. The IDSA guidelines state that mortality attributable to disseminated candidiasis ranges between 14.5-49% in adults and 10-15% in neonates and children (139). As patients who acquire invasive candidiasis often have multiple comorbidities, the mortality attributed to candidiasis is difficult to calculate. As fluconazole has been the “work horse” of antifungal therapy, a non-*albicans* shift has also been observed in nosocomial isolates that may be less sensitive to azole treatment.

Resistance in *Candida* species is often distinguished into pathogens who exhibit intrinsic versus acquired resistance. Species such as *C. krusei* and some of the *C. glabrata* population exhibit intrinsic resistance, meaning resistance without prior introduction to the drug. *C. glabrata* and other *Candida* species can acquire resistance in response to a drug challenge. Recently, the Clinical Laboratory and Standards Institute (CLSI) have redefined their standards for in vitro antifungal susceptibility testing. Current standards now consider species-specific clinical break points (CBP) using established epidemiological cutoff values (ECV), defined as the upper cutoff value for wild-type MIC, pharmacokinetic (PK)/pharmacodynamic (PD) parameters and relationship between MIC and clinical outcome. Although previous CLSI CBPs reasonably predicted therapeutic success or failure, the new CBPs exhibit increased sensitivity in the detection of emerging resistance (145). The current interpretation of in vitro susceptibility testing of *C. albicans* after 24-hour incubation to fluconazole is “susceptible” $\leq 2 \mu\text{g/mL}$, “susceptible dose dependent” $4 \mu\text{g/mL}$ and “resistant” isolates have an MIC of $8 \mu\text{g/mL}$. Break points vary regarding species and antifungal.

Molecular mechanisms of azole resistance in *C. albicans*

The efficacy of fluconazole is decreased in clinical isolates of *C. albicans* by the interplay of several mechanisms of resistance. The azole antifungal agents inhibit the biosynthesis of ergosterol, the major membrane sterol of fungi. By binding to the heme in the active site of the cytochrome P450 enzyme, lanosterol demethylase, the azoles inhibit its activity (59). We currently do not know the mechanism of azole uptake into the fungal cell but recent kinetic data supports the hypothesis that its uptake is facilitated (113). Several mechanisms of resistance to the azole antifungal agents directly involving *ERG11* have been described in *C. albicans*. One major mechanism involves accumulation of point mutations in the gene encoding lanosterol demethylase (*ERG11*) (162). These mutations have long been accepted to confer azole resistance by producing conformational changes that prevent effective binding between the azoles and their target while not affecting the function of this enzyme in ergosterol biosynthesis (5, 15). In addition to mutations in *ERG11*, constitutive overexpression of this gene requires higher concentrations of intracellular fluconazole for sufficient inhibition. *ERG11* overexpression occurs by either gain-of-function mutations in the transcriptional regulator *UPC2* or by instances of aneuploidy, specifically, increased chromosome 5 copy number, on which *ERG11* resides (discussed below).

A less common mechanism of azole resistance is alterations in the ergosterol biosynthesis pathway which results in alternate sterols in the fungal cell membrane. This usually occurs by inactivation of sterol $\Delta^{5,6}$ -desaturase, encoded by *ERG3* (125). Erg3 converts nontoxic 14 α -methylated sterol intermediates that accumulate due to azole-mediated Erg11 inhibition, into the toxic sterol 14 α -methylelgesta-8,24(28)-dien-3 β ,6 α -diol (193). Non-functional *ERG3* alleles result in high-level azole resistance by allowing cells to avoid the synthesis of the toxic sterols. Inactivation of this enzyme has been shown to result from either a nonsense mutation in *ERG3* or a SNP, which allegedly inactivates the enzyme (119). Although strains containing these inactivating mutations are usually unable to filament and display decreased virulence, isolates containing inactive Erg3 yet are still filamentous and do not display decreased virulence have been documented (123, 193).

Overexpression of efflux pumps is a common mechanism of resistance to drugs toxic to the fungal cell. In *C. albicans*, there are two families of efflux transporters which are important in azole resistance (23, 43, 125). Two ATP Binding Cassette (ABC) transporters, *CDR1* and *CDR2*, harness the energy from ATP hydrolysis to actively transport fluconazole from the cell while the Major Facilitator Superfamily (MFS) transporter, *MDR1*, requires a proton gradient to drive efflux. The *CaCDR1* gene is a homologue to the *PDR5* ABC efflux transporter in *S. cerevisiae*. *CaCDR1* was shown to functionally complement a *S. cerevisiae pdr5 Δ* mutant by restoring fluconazole susceptibility phenotypes (152). Disruption of *CDR1* in *C. albicans* resulted in increased levels of intracellular fluconazole and increased sensitivity not only to azoles but also other drugs (164). *CDR2* is very similar to *CDR1* but disruption of this pump alone does not affect fluconazole susceptibilities (164). The disruption of *CDR2* in a mutant already deficient for *CDR1* further increases susceptibility to fluconazole (164). Similar studies were done for *CaMDR1* however, overexpression of this transporter increased resistance specifically to fluconazole and not other azoles. In a fluconazole-susceptible isolate, these efflux pumps are usually expressed in a low or non-detectable level but expression is stimulated in response to azoles and other chemicals (25). In fluconazole-resistant strains, constitutive overexpression of the *CDR1*, *CDR2* and *MDR1* genes is commonly observed. Our lab and others have shown constitutive overexpression of *CDR1*, *CDR2* and *MDR1* is due to activating mutations in zinc-cluster transcriptional regulators, Tac1 and Mrr1 respectively (3, 5). Analysis of mutants where genes encoding for these transporters are disrupted has proven that the contribution of *CDR1* is greater than *CDR2* to multidrug resistance (189). In addition, post-transcriptional studies have demonstrated that azole-resistant isolates do not only display higher transcription initiation rates, but *CDR1* mRNA is more stable when compared to the azole-susceptible counterpart (112).

The genome of *C. albicans* can tolerate changes in chromosomal rearrangement and chromosomal aneuploidies (124). Although reproduction is primarily asexual, a parasexual cycle has been identified in this species. *C. albicans* does not undergo meiosis therefore, the tetraploid result of mating undergoes recombination mediated by the endonuclease Spo11 and concerted chromosomal loss by mitosis, which results in a high degree of aneuploidy (51). *C. albicans* has also been observed to undergo substantial genetic variation in a short time in a murine model of infection (52). A study that

observed the chromosomal abnormalities that result from fluconazole exposure demonstrated the rapid acquisition of isochromosome 5L [i(5L)] in response to drug (174). Both *ERG11* and *TAC1* reside on chromosome 5 and increased chromosome 5 copy number has been shown to contribute to azole resistance in a clinical isolate (173). Genetic abnormalities have been detected on all eight chromosomes in *C. albicans* but these changes are usually transient and will normalize without drug pressure. Other conditions promote genetic abnormalities such as low nitrogen containing media in addition to growth on specific carbon sources such as l-sorbose or d-arabinose (124). These established mechanisms of resistance are all able to individually contribute to azole resistance but high-level resistance is mainly due to multiple mechanisms of resistance that accumulate over time (155).

TRANSCRIPTIONAL REGULATORS OF AZOLE RESISTANCE

Zinc-cluster transcription factors

Zinc-cluster transcription factors (TFs) are a class of exclusively fungal zinc-finger proteins comprised of six cysteine residues bound to two zinc atoms ($\text{Zn(II)}_2\text{Cys}_6$) (109). Zinc-cluster transcription factors recognize the conserved DNA motif $\text{CX}_2\text{CX}_6\text{CX}_5$ - $_{12}\text{CX}_2\text{CX}_{6-8}\text{C}$ and bind as either monomers, homodimers or heterodimers. Apart from the cysteine rich DNA binding domain (DBD), zinc-cluster TFs also contain regulatory and activation domains. Included in their target gene repertoire, many zinc-cluster TFs regulate the expression of other zinc-cluster proteins or may also auto-regulate.

The best studied zinc-cluster family member is Gal4. Gal4 regulates genes involved in the transport and metabolism of galactose via upstream activation sequences (UAS_{GAL}) in their promoters (102). Gal4 is localized in the cytoplasm and upon activation, is directed towards and imported into the nucleus facilitated by a characterized nuclear localization signal (NLS) (20). In the absence of galactose, Gal4 is inhibited by the repressor protein Gal80 (206). Relief of inhibition by Gal80 is mediated by its interaction with Gal3, a relationship which is not completely understood, but is dependent upon the presence of galactose (13, 141). Although not necessary for activation, Gal4 undergoes a series of phosphorylation events on serine residues 691, 696 and 837. These modifications are thought to help “fine tune” Gal4 activation (160). Gal4p turnover may also be post-transcriptionally regulated by the ubiquitin degradation system (128). Other zinc-cluster proteins are constitutively localized in the nucleus bound to their target promoters. Large scale and targeted protein localization studies have increased our insight of cellular location of zinc-cluster transcription factors in budding yeast (75, 115).

As DNA in chromosomes are wound tightly and organized into chromatin, zinc-cluster proteins require the participation of chromatin remodeling machinery, histone-modifying enzymes and co-activators to activate genes. Transcription of several genes in *S. cerevisiae* are known to be dependent on histone acetyltransferases such as Spt-Ada-Gcn5-acetyltransferase, known as the SAGA complex. Previous work has shown that

ScPdr1, a zinc-cluster TF and the primary regulator of the ABC transporter *PDR5*, interacts with the SAGA complex to activate transcription (55). Likewise, Ume6, a repressor zinc-cluster TF, relies on a histone deacetylase complex (HDAC) to exert its regulatory control (83).

Zinc-cluster transcription factors and azole resistance in *C. albicans*

Constitutive activation of efflux pumps *MDR1*, *CDR1* and *CDR2* are a prevalent and important mechanism of multidrug resistance in *C. albicans*. Studies performed in *S. cerevisiae* demonstrate that *CaCDR1* and *CaCDR2* can functionally complement a strain that lacks ABC transporter *PDR5* (152, 163). Regulatory elements for both genes are defined. (36) The promoter region of *CDR1* contains both a basal expression element (BEE), responsible for basal expression, and a drug responsive element (DRE), which is required for fluphenazine and estradiol induction of expression. Only a single DRE was detected in the promoter of *CDR2* but it shared a 21 base pair consensus sequence with *CDR1*. This consensus DRE (5'-CGGA(A/T)ATCGGATATTTTTTTT-3') contains two CGG triplet repeats suggesting the involvement of a zinc-cluster transcription factor in their regulation. In 2004, Coste et al, established the role of zinc-cluster transcription factor, *TAC1* (transcriptional activator of *CDR* genes) as the regulator of both *CDR1* and *CDR2* overexpression in response to fluphenazine (23). Tac1 is localized to the nucleus and GOF mutations in azole resistant isolates are prevalent, resulting in the constitutive overexpression of drug efflux transporters (22). Hyperactive *TAC1* alleles have been shown to be co-dominant with wild-type alleles and resistant phenotypes occur when strains are homozygous for a GOF *TAC1* allele (22). The mechanism by which homozygous *TAC1* alleles arise is by recombination between portions of chromosome 5 or by duplication of a portion of chromosome 5, on which *TAC1* resides. Tac1 also indirectly induces the transcription of *PDR16* encoding a phosphatidylinositol transfer protein (216). As *CDR1* and *CDR2* function as general phospholipid translocators in addition to their role in pleiotropic drug resistance, this link demonstrates the importance of these two processes for *C. albicans* survival in a wide range of environments (216). A recent study has demonstrated that artificial activation of zinc-cluster transcription factor designated Mrr2 can also increase *CDR1* transcription with similar efficacy to Tac1 (168). Unlike Tac1, Mrr2 was selective for *CDR1* expression and not *CDR2*. The clinical significance of Mrr2 in azole resistance is not yet known.

MDR1 constitutive expression in *C. albicans* is also a frequent cause of azole resistance in clinical isolates. The mechanism of *MDR1* overexpression in azole resistance was identified using genome-wide profiling analysis of match isolate pairs of fluconazole-susceptible and fluconazole-resistant counterparts that overexpressed *MDR1* (127). Orf19.7372, designated *MRR1* was overexpressed in all three resistant isolates and disruption of *MRR1* in a fluconazole susceptible background abolished *MDR1* expression in response to fluconazole. Sequence analysis of resistance isolates revealed GOF mutations in *MRR1*. As seen with *TAC1*, the majority of *MDR1* overexpressing strains had become homozygous for the *MRR1* GOF allele either through recombination between chromosome 3 homologous, on which *MRR1* resides, or may also occur by

chromosomal duplication events, which results in two mutated *MRR1* alleles combined with a complete loss of the wild-type allele (43). An artificially activated form of the transcription factor Cap1 also results in increased *MDR1* expression and to a lesser degree, Upc2 containing a GOF mutation can increase *MDR1* expression in a *MRR1* dependent fashion (120). Adding another level of transcriptional regulation is the involvement of the MADS box transcription factor Mcm1, which is essential for *MDR1* overexpression by activated Mrr1 but unnecessary for activated Cap1 (120).

In clinical-azole resistant isolates of *C. albicans*, constitutive overexpression of *ERG11* and other genes in the ergosterol biosynthesis pathway (ERG genes) is observed. This is often a result of transcriptional activation of Upc2 (177). Previous work has identified Upc2-specific sterol response elements (SRE) core motif in the promoter region of *ERG11* and other ERG genes (135). Deletion of *UPC2* in a fluconazole-susceptible background results in hypersusceptibility to fluconazole, diminished ergosterol levels in the cell membrane and the inability to increase *ERG11* expression in response to drug treatment (135, 177). Introducing a triple hemagglutinin epitope tag at the C terminus of *UPC2*, the putative activation domain, resulted in a gain-of-function effect in the fusion protein, resulting in increased expression of Upc2 target genes (108). CHIP-Chip analysis has also identified Upc2 to be enriched in the promoters of genes involved in ergosterol biosynthesis and other pathways. In a fluconazole-resistant clinical isolate, our lab first identified a GOF mutation in *UPC2* resulting in constitutive overexpression of genes involved in the ergosterol biosynthesis pathway in addition to genes involved in the oxidative stress pathway (44). Our recent data show that a variety of *UPC2* GOF mutations are prevalent in azole-resistant clinical isolates and these mutations contribute to azole resistance (50). Notably, our lab and others have observed clinical isolates that overexpress *ERG11* yet do not contain *UPC2* GOF mutations, which suggest alternative mechanisms of *ERG11* overexpression.

FCR1 has been previously characterized as a zinc-cluster transcription factor and its contribution to pleiotropic drug resistance has been studied in *S. cerevisiae*. Talibi and Raymond expressed *C. albicans FCR1*, *FCR2* and *FCR3* genes in *S. cerevisiae* and showed that that these genes were able to increase the expression of *PDR5*, which encodes a transporter in the ATP binding cassette (ABC) superfamily and participates in the efflux of azole antifungals (185). Surprisingly, *fcrlΔ/fcrlΔ* mutant strains were more resistant to fluconazole, ketoconazole, itraconazole, and Brefeldin A. Therefore, characterization of *FCR1* as either a positive or negative regulator of antifungal resistance as yet to be clarified in *S. cerevisiae*. In addition to the *FCR1* pathway, it has been shown that azole resistant isolates ability to grow in the presence of fluconazole is abrogated in the presence of a calcineurin inhibitor (17). Targets of calcineurin include the genes *CRZ1* and *CRZ2*, which are also transcription factors and have been linked to fluconazole susceptibility. In a study by Bruno and Mitchell, mutants with the *crz1* homozygous null genotype, like when treated with cyclosporine A, would reverse the ability of azole resistant mutants to grow in the presence of drug (17).

CENTRAL HYPOTHESIS AND RESEARCH OBJECTIVES

Candida albicans is an opportunistic fungal pathogen that causes mucosal, cutaneous, and systemic infections, including oropharyngeal candidiasis (OPC), the most frequent infection in people with AIDS. Fluconazole and other azole antifungal agents have proven effective in the management of OPC; however, with increased use of these agents, treatment failures have occurred that have been associated with the emergence of azole-resistant strains of *C. albicans*. The long term goal is to improve the treatment of Candida infections by understanding the molecular basis of azole resistance. The overall objective of this dissertation was to understand mechanisms by which Erg11 mediates resistance to fluconazole. Three specific aims were developed to investigate this overall objective.

In a large group of clinical *C. albicans* isolates enriched for azole resistance, I created a transcriptional profile defining expression of genes known to cause azole resistance such as *ERG11*, *CDR1*, *CDR2* and *MDR1*. *ERG11* was found to be upregulated in almost three-fourths of the fluconazole-resistant isolates examined. These data suggest that *ERG11* overexpression is a common contributor to fluconazole resistance in *C. albicans*. Prior to this work, only 3 GOF mutations in Upc2 (G648D, A643T and A643V) have been reported in characterized genetically-matched sets of isolates. In light of this preliminary evidence, I hypothesized GOF mutations in *UPC2* were responsible for the majority of *ERG11* overexpression in this collection. My first specific aim was to investigate the prevalence and role of *UPC2* gain-of-function mutations in the overexpression of *ERG11* in fluconazole-resistant *C. albicans* isolates. Among the *ERG11*-overexpressing isolates studied, 29 isolates contained at least one amino acid substitution in Upc2. I frequently recovered and characterized eight distinct single-nucleotide substitutions in *UPC2*. Five of these substitutions in *UPC2* have not been described previously. Of the five novel mutations, four mutations resulted in increased *ERG11* expression. I determined that transcriptional activation of *ERG11* by a variety of GOF mutations in transcription factor Upc2 are prevalent in a large group of azole resistant isolates.

Although GOF mutations in Upc2 were prevalent in this collection, there were several *ERG11*-overexpressing isolates that did not contain *UPC2* mutations. Other groups have also observed *ERG11*-overexpression in clinical isolates that do not contain GOF mutations in *UPC2*. These data suggest that there are *UPC2*-independent mechanisms of *ERG11*-overexpression. In *C. albicans*, the Pho-G transcription factor *NDT80* has been implicated in azole resistance not only due to its regulation of *CDR1* but also due to its regulation of genes involved in the ergosterol biosynthesis pathway. Investigation of *CaNDT80* has identified putative binding sites in the *UPC2* promoter area and ChIP-chip data has shown enrichment of Ndt80 in the promoter region of genes involved in ergosterol biosynthesis. In light of this preliminary data, I hypothesized that GOF mutations in *NDT80* result in increased *ERG11* expression and azole resistance in clinical isolates. My second specific aim was to investigate *NDT80* mutations derived from the resistant component of two genetically matched isolate sets on *ERG11* expression and fluconazole resistance. Sequencing of the *NDT80* allele of both matched sets revealed several mutations that resulted in amino acid substitutions when compared

to SC5314. This analysis also showed that a loss of heterozygosity event occurred so that the resistant counterpart was homozygous for one allele. A strain carrying the *NDT80* allele derived from fluconazole-resistant isolate 1619 did not result in increased *ERG11* expression and increased fluconazole resistance. The mechanism by which *ERG11* is upregulated in the absence of *UPC2* gain-of-function mutations is currently under investigation.

In addition to transcriptional regulation of the *ERG11* gene, it is well known that fluconazole resistance in clinical isolates of *C.albicans* are associated with specific point mutations in *ERG11* but there are no studies that show the effects of these mutations within the pathogen itself. In my third aim, I investigated the contribution of specific amino acid substitutions to a panel of azole antifungals. To investigate this, I sequenced both *ERG11* alleles in the 63 unmatched clinical isolates with reduced susceptibility to fluconazole. Sequencing data revealed that 55 of the 63 isolates contained missense mutations in *ERG11* that resulted in at least one amino acid substitution. I selected a group of mutant *ERG11* alleles and expressed them in SC5314. In this analysis, I was particularly interested in characterizing amino acid substitutions that occurred alone and also when accompanied by another mutation. In total, I characterized ten *ERG11* alleles homozygously carrying one amino acid substitution and nine alleles which homozygously carried *ERG11* alleles with a combination of amino acid substitutions. Fluconazole, itraconazole and voriconazole susceptibilities were performed for strains carrying each mutant *ERG11* allele homozygously with an identical *ERG11* mutation. Our data demonstrate many of these mutations resulted in fluconazole resistance, but most single mutations are not as significant when tested against voriconazole or itraconazole. Itraconazole, in particular seemed less effected by *ERG11* mutations which produced significant resistance to fluconazole although amino acid combination Y132F and F145L resulted in increased itraconazole resistance. Specific combinations of *ERG11* mutations resulted in increased azole resistance beyond single mutations. These data suggest that structural differences between azole effect activity against specific mutant *ERG11* alleles.

CHAPTER 2. GAIN-OF-FUNCTION MUTATIONS IN *UPC2* ARE A FREQUENT CAUSE OF *ERG11* UPREGULATION IN AZOLE-RESISTANT CLINICAL ISOLATES OF *CANDIDA ALBICANS**

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen that causes mucosal, cutaneous, and systemic infections, including oropharyngeal candidiasis (OPC), the most frequent infection in people with AIDS (47, 88). In the United States, *Candida* is the fourth-most-common organism isolated from nosocomial bloodstream infections and is associated with a mortality rate approaching 40% (153). Fluconazole and other azole antifungal agents have proven effective in the management of OPC; however, with increased use of these agents, treatment failures have occurred that have been associated with the emergence of azole-resistant strains of *C. albicans* (157). The azole class of antifungals works by inhibiting the cytochrome P450 enzyme lanosterol demethylase, a critical enzyme in the synthesis of ergosterol which is encoded by the *ERG11* gene (90). The efficacy of fluconazole is decreased in clinical isolates of *C. albicans* by the interplay of several mechanisms of resistance (105, 125, 142, 203). Overexpression of the efflux transporter genes *CDR1*, *CDR2*, and *MDR1* is a common mechanism of drug resistance in this organism (53, 105, 165). Point mutations in the *ERG11* gene result in reduced binding affinity of azoles to their target without precluding enzymatic function (200). In addition to point mutations, overexpression of *ERG11* has also been shown to decrease fluconazole susceptibility. *ERG11* gene amplification by chromosome 5 duplication or the presence of a chr5L isochromosome is known to contribute to azole resistance (173). The zinc-cluster transcription factor Upc2 has been shown to regulate the expression of *ERG11* and other genes involved in ergosterol biosynthesis (108, 177). Previous studies show that activating mutations in Upc2 result in increased expression of *ERG11* and decreased fluconazole susceptibility; however, only three substitutions (G648D, A643T, and A643V) in Upc2 have been identified in clinical isolates that contribute to azole resistance (44, 66, 74). In all cases, these substitutions have been identified in fluconazole-resistant clinical isolates of genetically matched isolate pairs.

In this study, I examined the prevalence of overexpression of *ERG11*, as well as the overexpression of efflux pump genes *CDR1*, *CDR2*, and *MDR1*, in a group of 63 unrelated fluconazole-resistant clinical isolates. I also investigated the prevalence of *UPC2* mutations among isolates that overexpress *ERG11*. I determined which *UPC2* mutations, both previously described and novel, result in increased *ERG11* expression, altered cellular ergosterol content, and increased resistance to azoles, as well as to

*Reprinted with permission of the publisher. Flowers, S. A., K. S. Barker, E. L. Berkow, G. Toner, S. G. Chadwick, S. E. Gygax, J. Morschhauser, and P. D. Rogers. 2012. Gain-of-function mutations in *UPC2* are a frequent cause of *ERG11* upregulation in azole-resistant clinical isolates of *Candida albicans*. *Eukaryot Cell* 11:1289-99..

terbinafine. Finally, I identified genes that are coordinately differentially expressed in four strains expressing unique *UPC2* gain-of-function mutations.

MATERIALS AND METHODS

Strains and growth conditions

All *C. albicans* strains (**Table 2.1**) were stored as frozen stock in 20% glycerol at 80°C and cultured on YPD (1% yeast extract, 2% peptone, and 1% dextrose) agar plates at 30°C. YPD liquid medium was used for routine growth of strains. For selection of strains containing the *SAT1*-flipper cassette (156), nourseothricin (200 µg/ml) was added to YPD agar plates. One Shot *Escherichia coli* TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) were used as the host for plasmid construction and propagation. These strains were grown in Luria-Bertani (LB) broth or on LB agar plates supplemented with 100 µg/ml ampicillin (Sigma) or 50 µg/ml kanamycin (Fisher BioReagents, Fair Lawn, NJ) when required.

qRT-PCR

First-strand cDNAs were synthesized separately from 1 µg of total RNA in a 21-µl reaction mixture volume using the SuperScript first-strand synthesis system for reverse transcription (Invitrogen). Quantitative PCRs (qRT-PCRs) were performed in triplicate as technical replicates using the 7000 sequence detection system (Applied Biosystems). PCRs were performed, independently amplifying 18S rRNA or the *ACT1* gene (normalizing genes) and the genes of interest (GOI) from the same cDNA, using SYBR green PCR master mix (Applied Biosystems). Genespecific primers were designed using Primer Express software (Applied Biosystems) synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table 2. The PCR conditions consisted of AmpliTaq Gold activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C. Determination of the dissociation curve and detection of SYBR green fluorescence was performed by using software provided with the 7000 sequence detection system. The cycle threshold (*CT*) value of the normalizing gene was subtracted from the GOI to obtain the ΔCT value. The ΔCT value of a calibrator was subtracted from the sample ΔCT value to obtain the $\Delta\Delta CT$ value. The change in fold expression was obtained by calculating $2^{-\Delta\Delta CT}$. The standard error was calculated from ΔCT values as previously described (44).

Plasmid construction for allele sequencing

C. albicans *UPC2* coding sequences were amplified by PCR (*Pfu* DNA polymerase; Stratagene) from *C. albicans* genomic DNA using the primers UPC2-A and UPC2-E (**Table 2.2**). Products were cloned into pCR-BLUNTII-TOPO using a Zero Blunt TOPO

Table 2.1. *C. albicans* strains used in this study.

Strain	Strain background	Relevant characteristics or genotype	Source
SC5314	N/A	<i>UPC2-1/ UPC2-2</i>	ATCC
Clinical isolates (#1-#10)	N/A	azole-susceptible	University of Iowa
Clinical isolates (#11-#72)	N/A	azole-resistant	University of Iowa
Constructed laboratory strains			
UPC2M4A	SC5314	<i>upc2-1Δ::FRT/upc2-2Δ::FRT</i>	Dunkel, 2008
11A8A2A	UPC2M4A	<i>UPC2^{G648S}::FRT/UPC2^{G648S}::FRT</i>	This study
SC11A1A	SC5314	<i>UPC2/UPC2^{G648S}::FRT</i>	This study
22A1A13A	UPC2M4A	<i>UPC2^{G304R}::FRT/UPC2^{G304R}::FRT</i>	This study
SC22A3A	SC5314	<i>UPC2/UPC2^{G304R}::FRT</i>	This study
YFA3A2K1	UPC2M4A	<i>UPC2^{Y642F}::FRT/UPC2^{Y642F}::FRT</i>	This study
SCYFA2A	SC5314	<i>UPC2/UPC2^{Y642F}::FRT</i>	This study
AVA1A16A	UPC2M4A	<i>UPC2^{A646V}::FRT/UPC2^{A646V}::FRT</i>	This study
SCAVA4	SC5314	<i>UPC2/UPC2^{A646V}::SAT1-FLIP</i>	This study
25B2D1	UPC2M4A	<i>UPC2^{W478C}::FRT/UPC2^{W478C}::FRT</i>	This study
SC25A1	SC5314	<i>UPC2/UPC2^{W478C}::FRT</i>	This study
28A7A10A	UPC2M4A	<i>UPC2^{A643V}::FRT/UPC2^{A643V}::FRT</i>	This study
SC28A3A	SC5314	<i>UPC2/UPC2^{A643V}::FRT</i>	This study
SCUPC2R12A	SC5314	<i>UPC2^{G648D}::FRT/UPC2-2</i>	Heilmann, 2010
SCUPC2R14A	SC5314	<i>UPC2^{G648D}::FRT/UPC2^{G648D}::FRT</i>	Heilmann, 2010
SCUPC2R32A	SC5314	<i>UPC2^{A643T}::FRT/UPC2-2</i>	Heilmann, 2010
SCUPC2R34A	SC5314	<i>UPC2^{A643T}::FRT/UPC2^{A643T}::FRT</i>	Heilmann, 2010

Table 2.2. Primers used in this study.

Primer	Sequence
qRT-PCR	
<i>ACT1</i> -F	5'-ACGGTGAAGAAGTTGCTGCTTTAGTT-3'
<i>ACT1</i> -R	5'-CGTCGTCACCGGCAAAA-3'
<i>18S</i> -F	5'-CACGACGGAGTTTCACAAGA-3'
<i>18S</i> -R	5'-CGATGGAAGTTTGAGGCAAT-3'
<i>ERG11</i> -F	5'-CCCCTATTAATTTTGTTCCTAATTTAC-3'
<i>ERG11</i> -R	5'-CACGTTCTCTTCTCAGTTTAATTTCTTTC-3'
<i>CDR1</i> -F	5'-ATTCTAAGATGTCGTCGCAAGATG-3'
<i>CDR1</i> -R	5'-AGTTCTGGCTAAATTCGTAATGTTTTC-3'
<i>CDR2</i> -F	5'-TAGTCCATTCAACGGCAACAAT-3'
<i>CDR2</i> -R	5'-CACCCAGTATTTGGCATTGAAA-3'
<i>BMRI</i> -F	5'-ACATAAATACTTTGCCCATCCAGAA-3'
<i>BMRI</i> -R	5'-AAGAGTTGGTTTGTAATCGGCTAAA-3'
<i>UPC2</i> mutant construction	
<i>UPC2</i> -A	5'-GGGCCC <u>G</u> AGATCTTGATGTCATTAG-3'
<i>UPC2</i> -B	5'- <u>CTCGAG</u> CTATATCTTCAATGAAGT-3'
<i>UPC2</i> -E	5'- <u>CTCGAG</u> CACCACAGTAACGAATCAC-3'
<i>UPC2</i> -C	5'-CCGCGGACAGGTCAATACCGCGTAG-3'
<i>UPC2</i> -D	5'- <u>GAGCTC</u> GTTCCTCTAGTATCACTCTT-3'
<i>UPC2</i> sequencing	
<i>UPC2</i> seqA	5'-CTGCAGAGAATCACAGTGAAGTTC-3'
<i>UPC2</i> seqB	5'-CTCAGCCGGTGATTCCCTCCA-3'
<i>UPC2</i> seqC	5'-CGGTCAAACCTCAATATGCTTGAC-3'
<i>UPC2</i> seqD	5'-GTTTCCAGTGCTTTTGGACTCTCC-3'
<i>UPC2</i> seqZ	5'-CCTATCATCTACGCGGTATTGACC-3'
<i>UPC2</i> seqF	5'-TGGAGGAATCACCGGCTGAG-3'
<i>UPC2</i> seqG	5'-GTCAAGCATATTGAGATTTGACCG-3'
<i>UPC2</i> seqH	5'-GGAGAGTCCAAAAGCACTGGAAAC-3'

Underlined sequence reflects the introduction of a restriction site sequence.

PCR cloning kit (Invitrogen) and transferred into *Escherichia coli* TOP10 cells with selection on LB agar plates containing 50 µg/ml kanamycin. Plasmid DNA was purified (QIAprep; Qiagen, Germantown, MD) and sequenced on an ABI model 3130XL genetic analyzer using the *UPC2* sequencing primers (**Table 2.2**), resulting in a full-length sequence from both strands of the *C. albicans UPC2* gene. The sequencing was performed using six sets of clones derived from three independent PCRs for each strain/isolate sequenced.

Sequenced plasmids containing the *UPC2* open reading frame (ORF) whose predicted translation indicated an amino acid substitution were digested with restriction enzymes *ApaI* and *XhoI*, which excised the fulllength ORF from the plasmid, and the *UPC2* alleles were cloned upstream of the *SAT1*-flipper cassette into the *ApaI* and *XhoI* sites of plasmid pSFS2 (25). The *UPC2* downstream segments were amplified with *Ex Taq* (TaKaRa) using primers UPC2C and UPC2D and cloned downstream of the *SAT1*-flipper cassette in pSFS2 using the *SacI* and *SacII* sites. This process generated plasmids pUPC2-G648S, pUPC2-M597I, pUPC2- G304R, pUPC2-A643V, pUPC2-Y642F, and pUPC2-A646V.

Construction of *UPC2* allele strains

C. albicans strains UPC2M4A (7) and SC5314 were transformed by electroporation with gel-purified inserts from plasmids pUPC2-G648S, pUPC2-M597I, pUPC2-G304R, pUPC2-A643V, pUPC2-Y642F, pUPC2-W467C, and pUPC2-A646V derived from the plasmid pSFS2. pSFS2 contains the *SAT1*-flipper disruption cassette developed by Reuss et al. (156), consisting of the *SAT1* selectable marker which confers resistance to nourseothricin and the *FLP* flipper recombinase gene, both flanked by FRT sites (flipper recombinase target sequences). Nourseothricin-resistant transformants were selected as previously described (156). Upon induction of the *FLP* gene, the cassette is excised such that only the *UPC2* allele with a downstream FRT is left in the *UPC2* locus. Integration of constructs was confirmed by Southern hybridization.

Fluconazole susceptibility testing

MICs were obtained by using a modified CLSI protocol outlined in CLSI document M27-A3 using RPMI or YPD medium. Overnight cultures grown at 30°C were streaked onto Sabouraud's agar. Plated cultures were grown for 24 h at 30°C. Individual colonies were suspended in sterile water until an OD₆₀₀ of 0.1 was reached. The working colony concentration was made by making a 1:50 dilution and a 1:20 dilution sequentially in medium. Aliquots of 100 µl from the working stock were used to inoculate a series of fluconazole-YPD medium dilutions, the highest being 64 µg/ml. Similar procedures were used for terbinafine and amphotericin B dilutions; however, the highest concentration used for these agents was 8 µg/ml. Cultures were incubated at 35°C for 48 h, and MICs were recorded. Azole MICs for constructed strains were obtained by Etest (bioMérieux) following the manufacturer's instructions. Briefly, yeast cultures were grown in YPD

broth medium overnight at 30°C in a shaking incubator and plated to Sabouraud dextrose agar at 18 h. Isolated colonies were then selected from these plated cultures, suspended in deionized water to achieve 0.5 McFarland turbidity, and streaked evenly across YPD plates by rotating 90° in three directions. Etest strips were then applied to the inoculated agar surface, and MICs were read at both 24 and 48 h.

Ergosterol quantification analysis

Ergosterol was extracted and quantified as described previously (100). Briefly, a single colony of *C. albicans* from a fresh YPD plate was used to inoculate 50 ml of RPMI 1640 plus 2% glucose (Sigma, St. Louis, MO) and incubated for 16 h with shaking at 35°C. Stationary-phase cells were collected by centrifugation for 5 min at 2,700 rpm and washed twice with sterile distilled water. The net weight of the pellet was determined. To each pellet, 3 ml of 25% alcoholic potassium hydroxide solution (25 g of KOH, 35 ml of sterile distilled water, with final volume adjusted to 100 ml with 100% ethanol) was added, and then the pellet was vortexed for 1 min. Cell suspensions were transferred to a sterile borosilicate glass screw-cap tube and incubated at 85°C for 1 h. Sterols were then extracted from cooled tubes by the addition of a mixture of 3 ml *n*-heptane and 1 ml sterile distilled water followed by vortexing for 3 min. The heptane layer was transferred to a clean sterile borosilicate glass tube and stored at 20°C for up to 24 h. A 100-μl amount of the sterol-heptane mixture was scanned spectrophotometrically between 240 nm and 300 nm with a DU530 life science UV spectrophotometer (Beckman Coulter, La Brea, CA). The presence of ergosterol in the extracted sample resulted in a characteristic four-peaked curve. The absence of detectible ergosterol was indicated by a flat line. All samples were blanked to *n*-heptane. A decrease in the height of the absorbance peaks correlates to a decrease in ergosterol content. Statistical significance was determined by using Student's *t* test ($P < 0.05$).

Transcriptional profiling

Gene expression profiles were obtained by hybridizing labeled cRNAs generated from *C. albicans* total RNA onto Affymetrix *C. albicans* custom expression arrays (CAN07, 49-5241 array format), which have been described previously (127). Total RNA was isolated as described earlier and subsequent cRNA synthesis/labeling, as well as probe hybridization, array scanning, and data analysis, were performed as previously described (127).

Among direct comparisons between strains, genes were considered to be differentially expressed if their change in expression was at least 1.5-fold greater (for upregulated genes) or at least 1.5-fold less (for downregulated genes) in both independent experiments of each comparison. Cells that were empty were called “absent” by the Affymetrix criteria for the corresponding comparison/experiment, and thus, their expression values were not valid. Examination of gene ontology (GO) classification

enrichment of microarray data was performed using the CGD gene ontology term finder (<http://www.candidagenome.org/cgi-bin/GO/goTermFinder>).

Accession numbers

The coding sequences of the *UPC2* alleles described in this study have been deposited in GenBank with the following accession numbers: JX494823, JX494820, JX494821, JX494822, JX494818, JX494819, JX494816, JX494817, JX494824, and JX494825. Data files for each scanned Affymetrix microarray chip were submitted to the Gene Expression Omnibus database (Chalfont, United Kingdom) and given the accession number GSE39201.

RESULTS

Expression of *ERG11*, *CDR1*, *CDR2*, and *MDR1* in 62 fluconazole-resistant clinical isolates of *C. albicans*

In order to examine the prevalence of and identify possible *UPC2* GOF mutations, I obtained a collection of 72 clinical *C. albicans* isolates from the laboratory of Daniel Diekema (University of Iowa). All isolates were unrelated, and they were collected from both oral and nonoral sources, including blood, tissue, and abdominal fluid (see **Appendix A** table). Fluconazole susceptibilities were confirmed in our laboratory. I defined all isolates with a MIC of ≥ 16 $\mu\text{g/ml}$ as having resistance to fluconazole for the purpose of this study. From our collection of 72 isolates, 9 were classified as fluconazole susceptible, while the remaining isolates were resistant. We then measured the levels of expression of *ERG11*, *CDR1*, *CDR2*, and *MDR1* for all isolates in order to determine the relative prevalence of overexpression of genes indicative of specific mechanisms of azole resistance (**Figure 2.1**). I defined overexpression as an increase in expression of at least 2-fold. I observed that 77% ($n = 49$) of resistant isolates overexpressed both *CDR1* and *CDR2*. *MDR1* overexpression was increased by at least 2-fold in 76% ($n = 48$) of resistant isolates, but only 21% ($n = 13$) expressed *MDR1* at levels consistent with the levels observed in isolates with *MDR1*-mediated fluconazole resistance, in which *MDR1* expression is substantially increased. For example, in a study by Morschhäuser et al., the clinical isolates examined exhibited 400- to 1,200-fold increases in *MDR1* expression.(127) Surprisingly, *ERG11* was found to be overexpressed in 75% ($n = 47$) of fluconazole-resistant isolates in this collection.

Most but not all isolates that overexpress *ERG11* carry a GOF mutation in *UPC2*

Twenty-nine of the 47 isolates that overexpressed *ERG11* possessed one or both *UPC2* alleles with a mutation that resulted in a predicted amino acid substitution.

Figure 2.1. Expression levels of *CDR1*, *CDR2*, *MDR1*, and *ERG11* in 63 fluconazole-resistant clinical isolates.

All gene expression levels were measured by qRT-PCR in triplicate, and fold expression of genes in resistant isolates was compared to the average of the expression levels in three susceptible isolates. Results for 63 isolates with reduced susceptibility to fluconazole are represented, but only even-numbered isolates' results are labeled. Error bars show standard errors.

Although many silent mutations were observed in the *UPC2* alleles tested, eight distinct single *UPC2* mutations were also recovered, three of which had been described previously: G648D, A643T, and A643V (**Table 2.3**). Eighteen clinical isolates were found to overexpress *ERG11* yet contained no amino acid substitution in Upc2. These data suggest an alternate mechanism of activating *ERG11* expression in these isolates.

GOF mutations in *UPC2* result in increased expression of *ERG11*

In order to assess the contribution of each individual mutant *UPC2* allele to fluconazole resistance, I expressed each mutant allele alone, with the other *UPC2* allele disrupted (data not shown), as a heterozygote with a wild-type *UPC2* allele, and as a homozygote. *ERG11* mRNA abundance was measured by qRT-PCR (**Figure 2.2**). The homozygous null *upc2Δ* strain showed a significant decrease in *ERG11* expression compared to that of its parental wild-type strain. Seven of the eight mutations tested resulted in increased *ERG11* expression, with the strongest expression observed among homozygous strains. Interestingly, different mutations elicited different levels of *ERG11* expression, with the G648D substitution resulting in the highest level of expression.

GOF mutations in *UPC2* result in elevated cellular ergosterol content

In order to confirm that activation of Upc2 results in a global increase of ergosterol biosynthesis, I compared the cellular ergosterol content in the wild type to the amounts in derivatives that were heterozygous or homozygous for various *UPC2* mutations. I found that strains carrying *UPC2* GOF alleles generally contained larger amounts of ergosterol than the wild-type strain (**Figure 2.3**). For many isolates, increased ergosterol levels correlated with the increased *ERG11* transcript levels resulting from each individual *UPC2* mutation; however, this was not the case for the A643V allele, which resulted in strong *ERG11* expression but a nonsignificant increase in cellular ergosterol content. *UPC2* alleles containing the G648D, W478C, and Y642F amino acid substitutions showed statistically significant increases in ergosterol content relative to the amount in the wild-type strain, while strains carrying other *UPC2* GOF alleles trended toward larger amounts of cellular ergosterol.

GOF mutations in *UPC2* influence susceptibility to antifungals

To examine the impact of distinct *UPC2* mutations on antifungal resistance, I determined the susceptibilities of strains constructed to carry mutant *UPC2* alleles to a panel of antifungals (**Table 2.4**). Included in this panel were agents in the azole class (fluconazole, itraconazole, and voriconazole). Also included were terbinafine, which inhibits another ergosterol biosynthesis enzyme (squalene epoxidase), and amphotericin B, which targets ergosterol in the fungal cell membrane. All strains constructed were compared to the background strain, strain SC5314, and the *upc2Δ/upc2Δ* strain

Table 2.3. Occurrence of Upc2 amino acid substitutions in predicted translated sequence in fluconazole resistant clinical *ERG11*-overexpressing isolates.

Upc2 substitution	Isolate	Genotype	Fluconazole MIC (µg/mL)	Expression of efflux transporter genes ^a			
				<i>ERG11</i> (±SE)	<i>CDR1</i> (±SE)	<i>CDR2</i> (±SE)	<i>MDR1</i> (±SE)
G648D	21	Heterozygous	32	6.2 (±0.2)	14.7 (±5.5)	883.3 (±301.9)	10.4 (±3.1)
	38	Heterozygous	64	3.8 (±0.3)	3.9 (±0.7)	111.0 (±57.1)	1.4 (±0.2)
	47	Heterozygous	128	7.9 (±1.2)	11.8 (±5.1)	327.0 (±166.3)	8.2 (±3.1)
	52	Heterozygous	256	3.7 (±1.4)	1.2 (±0.3)	17.1 (±3.2)	0.3 (±0.1)
	59	Heterozygous	>256	7.2 (±1.0)	6.5 (±0.3)	48.5 (±0.3)	8.0 (±0.2)
	71	Heterozygous	>256	9.4 (±1.1)	5.6 (±1.9)	149.8 (±56.5)	4.6 (±1.8)
G648S	25	Heterozygous	64	3.6 (±0.6)	1.1 (±0.5)	2.8 (±1.1)	160.5 (±78.9)
	35	Heterozygous	>256	4.4 (±1.3)	1.2 (±0.4)	0.7 (±0.3)	79.9 (±22.8)
	55	Homozygous	>256	2.6 (±0.6)	1.7 (±1.5)	26.2 (±17.4)	0.9 (±3.2)
	56	Homozygous	>256	2.1 (±1.0)	8.0 (±3.1)	167.8 (±94.0)	2.5 (±0.9)
	57	Homozygous	>256	19.4 (±12.4)	10.2 (±3.5)	263.3 (±106.0)	12.9 (±5.2)
	58	Homozygous	>256	6.6 (±1.5)	9.7 (±4.2)	226.6 (±131.7)	3.8 (±1.2)
	65	Heterozygous	>256	4.2 (±0.3)	9.8 (±1.5)	294.9 (±128.4)	2.4 (±0.1)
	67	Heterozygous	>256	4.7 (±0.7)	7.4 (±2.8)	105.1 (±43.3)	1.2 (±0.5)
	69	Heterozygous	>256	5.6 (±0.9)	7.2 (±0.5)	136.8 (±69.3)	1.4 (±0.5)
A643T	13	Homozygous	16	2.4 (±1.1)	6.4 (±2.9)	185.9 (±80.9)	2.4 (1.6)
A643V	30	Heterozygous	64	4.6 (±1.6)	1.4 (±0.3)	18.0 (±15.7)	18.9 (±16.7)
	68	Heterozygous	>256	4.9 (±0.4)	6.3 (±2.2)	64.5 (±31.8)	216.3 (±98.9)

Table 2.3. Continued.

Upc2 substitution	Isolate	Genotype	Fluconazole MIC ($\mu\text{g/mL}$)	Expression of efflux transporter genes ^a			
				<i>ERG11</i> ($\pm\text{SE}$)	<i>CDR1</i> ($\pm\text{SE}$)	<i>CDR2</i> ($\pm\text{SE}$)	<i>MDR1</i> ($\pm\text{SE}$)
A646V	45	Heterozygous	128	3.5 (± 0.3)	4.8 (± 0.3)	147.8 (± 71.7)	15.5 (± 7.7)
	67	Heterozygous	>256	4.7 (± 0.7)	7.4 (± 2.8)	105.1 (± 43.3)	1.2 (± 0.5)
Y642F	33	Homozygous	36	12.6 (± 1.8)	6.8 (± 2.0)	201.5 (± 129.0)	1.5 (± 0.5)
	34	Homozygous	37	7.5 (± 0.6)	5.4 (2.5)	126.8 (± 62.6)	1.2 (± 0.5)
W478C	15	Heterozygous	16	8.1 (± 2.7)	15.9 (± 3.8)	1415.1 (± 400.1)	4.0 (± 3.0)
	27	Heterozygous	64	6.2 (± 2.8)	14.2 (± 4.7)	629.7 (± 255.3)	1.1 (± 0.1)
	28	Heterozygous	64	18.1 (± 7.3)	18.1 (± 6.3)	1439.7 (± 570.8)	23.2 (± 20.6)
G304R	18	Heterozygous	32	5.6 (± 1.2)	10.4 (± 2.4)	575.0 (± 117.2)	1.0 (± 0.3)
	19	Heterozygous	32	5.2 (± 1.4)	10.0 (± 4.8)	401.3 (± 204.5)	4.6 (± 3.0)

^aq-RT-PCR was performed in triplicate. (SE) is calculated standard error.

Figure 2.2. *ERG11* transcription levels in strains carrying mutant *UPC2* alleles are represented as averages from three independent qRT-PCR assays. Error bars show standard errors. *ERG11* expression was quantified for each mutant strain listed and is compared to that of wild-type strain SC5314.

Figure 2.3. Ergosterol quantification in *C. albicans* laboratory strains expressing mutant *UPC2* alleles.

Increased ergosterol content was shown for strains expressing 2 *UPC2* mutant alleles compared to the expression levels in heterozygote and wild-type strains. The heptane extraction layer from 16-h cultures was scanned spectrophotometrically between 240 and 300 nm. The presence of ergosterol in an extracted sample resulted in a 4-peak curve. A decrease in absorbance peaks correlates to a decrease in ergosterol content. Each panel represents the results for a different Upc2 GOF mutation, as follows: G648S (A), A643V (B), Y642F (C), G648D (D), A646V (E), A643T (F), W478C (G), and G304R (H). *UPC2* mutant alleles were expressed as homozygotes with the same GOF mutation (○) or as heterozygotes with the wild-type allele (▲). Their absorbencies were compared to those of the wild-type strain SC5314 (●). The results for the homozygous revertants shown in panels C, D, and G showed statistically significant greater ergosterol contents than were found in the wild type ($P < 0.05$).

Table 2.4. MICs of strains expressing *UPC2* mutant alleles to a panel of antifungals in YPD.

Strain	Relevant genotype	FCZ (µg/mL)		ICZ (µg/mL)		VCZ (µg/mL)		TBF (µg/mL)		AMB (µg/mL)	
		24hr	48hr	24hr	48hr	24hr	48hr	24hr	48hr	24hr	48hr
SC5314	<i>UPC2-1/UPC2-2</i>	0.5	4	0.016	0.023	0.008	0.032	0.5	2	0.0313	0.125
UPC2M4A	<i>upc2-1Δ::FRT/upc2-2Δ::FRT</i>	0.023	0.032	0.002	0.004	0.002	0.002	0.016	0.016	0.0313	0.5
UPC2M2K21A	<i>UPC2-IS1-1-caSAT1/upc2-2Δ::FRT</i>	0.25	1.5	0.012	0.023	0.004	0.008	0.25	1	0.0313	0.25
11A8A2A	<i>UPC2^{G648S}::FRT/UPC2^{G648S}::FRT</i>	4	>256'	0.5	0.75	0.25	>32'	2	4	0.016	0.125
SC11A1A	<i>UPC2/UPC2^{G648S}::FRT</i>	1	>256'	0.032	0.19	0.047	>32'	2	4	0.0625	0.25
28A7A10A	<i>UPC2^{A643V}::FRT/UPC2^{A643V}::FRT</i>	2	>256'	0.125	>32	0.25	>32'	1	4	0.016	0.125
SC28A3A	<i>UPC2/UPC2^{A643V}::FRT</i>	1	2	0.023	0.047	0.047	>32'	1	2	0.0313	0.25
YFA3A2K1	<i>UPC2^{Y642F}::FRT/UPC2^{Y642F}::FRT</i>	2	>256	0.047	0.5	0.064	>32'	1	4	0.0625	0.5
SCYFA2A	<i>UPC2/UPC2^{Y642F}::FRT</i>	0.75	1	0.023	0.032	0.023	0.19	1	2	0.0313	0.25
AVA1A16A	<i>UPC2^{A646V}::FRT/UPC2^{A646V}::FRT</i>	1.5	>256	0.064	0.25	0.064	>32	1	4	0.0313	0.25
SCAVA4	<i>UPC2/UPC2^{A646V}::SAT1-FLIP</i>	0.5	1.5	0.016	0.032	0.016	0.064	1	2	0.0313	0.5
22A1A13A	<i>UPC2^{G304R}::FRT/UPC2^{G304R}::FRT</i>	0.19	0.75	0.012	0.023	0.008	0.032	1	2	0.0313	0.5
SC22A3A	<i>UPC2/UPC2^{G304R}::FRT</i>	0.75	>256	0.012	0.094	0.023	>32	1	4	0.0313	0.25
25B2D1	<i>UPC2^{W478C}::FRT/UPC2^{W478C}::FRT</i>	1	2	0.047	0.047	0.016	0.5	0.25	1	0.0313	0.25
SC25A1	<i>UPC2/UPC2^{W478C}::FRT</i>	0.5	1	0.016	0.016	0.008	0.032	0.5	1	0.016	0.125
SCUPC2R14A	<i>UPC2^{G648D}::FRT/UPC2^{G648D}::FRT</i>	8	>256'	>32	>32	0.064	>32'	2	4	0.016	0.125
SCUPC2R12A	<i>UPC2^{G648D}::FRT/UPC2-2</i>	1.5	>256'	0.25	>32	0.047	>32'	1	2	0.0313	0.25
SCUPC2R34A	<i>UPC2^{A643T}::FRT/UPC2^{A643T}::FRT</i>	1.5	>256'	2	>32	0.064	>32'	2	2	0.016	0.125
SCUPC2R32A	<i>UPC2^{A643T}::FRT/UPC2-2</i>	2	>256'	0.016	0.032	0.012	>32'	1	2	0.016	0.125

FCZ-fluconazole; ICZ- itraconazole; MCZ- miconazole; VCZ- voriconazole; TBF- terbinafine ; AMB- amphotericin B.

Susceptibility was tested at 24 and at 48 hours. Azole MICs were determined by Etest method while terbinafine and amphotericin B determined by broth microdilution methods.

UPC2M4A. Strain UPC2M4A was highly susceptible to terbinafine, as well as all the azoles tested. Interestingly, this strain exhibited a 4-fold-higher MIC of amphotericin B than SC5314 at 48 h. Strains expressing *UPC2* alleles carrying the G648D, G648S, A643T, A643V, Y642F, W478C, and A646V substitutions in the predicted protein sequence all exhibited increased resistance to fluconazole compared to that of strain SC5314, irrespective of the additional presence of a wild-type allele. Strains expressing the G304R mutant allele did not show a decrease in susceptibility beyond that of the wild-type, indicating that this mutation does not result in gain of function. Correlating with increased *ERG11* expression, fluconazole susceptibility also decreased, with the most prominent change occurring in the homozygous strains. A similar trend was exhibited for the other azole agents and also for terbinafine susceptibilities. For amphotericin B, strains homozygous for an activated *UPC2* mutant allele generally demonstrated a reduction in MIC at 48 h compared to that of the parental wild-type strain. However, all strains tested were susceptible to this agent. These results demonstrate that seven of the eight mutations in *UPC2* confer increased resistance to ergosterol biosynthesis inhibitors.

Genome-wide transcriptional-profile analysis of strains carrying activated *UPC2* alleles

I identified genes whose expression is influenced by Upc2 by comparing the transcriptional profiles of strains that were engineered to carry one of the four GOF *UPC2* alleles with the strongest effects on *ERG11* expression. The 11A8A2A (*UPC2^{G648S}/UPC2^{G648S}*) constructed strain expressed 170 upregulated genes and 9 downregulated genes compared to the expression profile of SC5314. Strain SCUPC2R14A (*UPC2^{G648D}/UPC2^{G648D}*) expressed 520 upregulated and 292 downregulated genes. There were 119 genes upregulated and 81 genes downregulated for strain YFA3A2K1 (*UPC2^{Y642F}/UPC2^{Y642F}*), while strain 28A7A10A (*UPC2^{A643V}/UPC2^{A643V}*) expressed 352 upregulated and 169 downregulated genes. There were 61 commonly upregulated genes and 5 commonly downregulated genes among the four strains carrying unique *UPC2* gain-of-function alleles (**Tables 2.5 and 2.6**). Genes commonly upregulated in all four mutant strains included those involved in ergosterol biosynthesis and in oxidoreductase activity, the major facilitator efflux pump encoded by the *MDR1* gene, and the uncharacterized ATP-binding cassette transporter *CDR11*.

DISCUSSION

In *Candida albicans*, it is well established that activating mutations in transcription factors regulating genes encoding efflux pumps mediate resistance to azole antifungals. It has been shown that specific mutations in the transcription factor gene *TAC1* mediate the expression of the genes encoding ABC transporters *CDR1* and *CDR2*, resulting in increased azole resistance in *C. albicans* (22, 23). Likewise, Mrr1 has been identified as the regulator of the major facilitator superfamily (MFS) transporter Mdr1 in azole-resistant isolates, and specific mutations in this transcriptional regulator result in its

Table 2.5. Genes upregulated by at least 1.5- fold in strains carrying UPC2 GOF alleles

Function ^a	CGD name	Orf19 designation	Change in fold expression ^b							
			G648S		G648D		Y642F		A643V	
			Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
Lipid metabolic process	<i>ERG1</i>	orf19.406	1.8	1.8	2.7	2.3	2.2	2.0	2.3	2.3
	<i>ERG2</i>	orf19.6026	1.7	1.5	2.3	1.9	1.9	1.9	2.1	2.0
	<i>ERG24</i>	orf19.1598	1.7	1.7	2.3	2.1	1.7	1.8	2.1	2.3
	<i>ERG27</i>	orf19.3240	1.8	2.1	3.3	3.4	2.2	2.2	3.1	3.2
	<i>ERG7</i>	orf19.1570	1.5	1.6	2.8	2.1	1.8	1.6	2.7	2.4
	<i>NCP1</i>	orf19.2672	1.6	1.7	2.6	2.0	1.7	1.9	1.9	1.9
		orf19.3483	2.0	2.4	5.3	4.7	2.9	2.2	3.5	3.3
		orf19.1881	1.5	1.6	2.4	2.2	1.5	1.5	2.4	2.1
		orf19.6025	2.2	2.1	3.9	5.2	3.8	3.1	5.2	5.2
Transcription factor activity		orf19.5210	1.5	2.1	2.7	2.7	1.8	1.5	2.9	2.3
Transport	<i>MDR1</i>	orf19.5604	5.9	5.5	15.1	8.0	6.6	6.1	5.9	5.7
		orf19.5535	1.5	1.6	2.2	2.7	1.5	1.9	2.7	3.2
	<i>FRP1</i>	orf19.5634	5.4	5.7	17.3	17.5	8.1	5.9	16.3	14.7
	<i>FTH1</i>	orf19.4802	2.6	2.6	5.3	5.2	2.3	1.9	6.6	5.2
	<i>GYP1</i>	orf19.3811	1.6	1.6	3.7	3.5	1.8	1.5	3.7	3.4
		orf19.2350	1.7	1.7	3.4	4.4	2.0	1.9	3.2	3.0
	<i>HXT5</i>	orf19.4384	1.8	2.1	2.6	2.0	1.8	1.5	2.3	1.7
Protein modification process		orf19.7329	1.6	2.1	3.1	3.0	1.7	1.8	2.5	2.3
		orf19.6025	2.2	2.1	3.9	5.2	3.8	3.1	5.2	5.2
		orf19.7547	1.8	1.5	2.8	2.7	2.0	1.5	3.1	2.3
		orf19.2285	1.7	2.5	1.8	1.5	1.8	2.4	2.1	2.1
Biosynthetic process	<i>COQ4</i>	orf19.3008	1.5	1.5	2.3	2.8	1.9	1.6	2.6	2.5

Table 2.5. Continued.

Function ^a	CGD name	Orf19 designation	Change in fold expression ^b							
			G648S		G648D		Y642F		A643V	
			Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
Biosynthetic process	<i>HEM14</i>	orf19.4747	2.1	1.6	3.6	3.0	2.2	1.8	2.9	2.3
Pathogenesis	<i>SLD1</i>	orf19.260	1.8	1.5	2.2	1.8	1.7	1.6	2.0	1.9
	<i>PHO100</i>	orf19.4424	1.5	3.4	4.8	2.7	1.6	1.5	2.0	2.3
	<i>SET3</i>	orf19.7221	2.8	2.7	6.3	5.7	4.4	3.6	4.8	4.8
Metabolic process		orf19.3617	1.7	1.9	1.7	2.2	1.7	1.5	1.8	1.5
		orf19.329	2.1	2.3	2.4	2.6	2.0	1.6	2.5	2.5
		orf19.4031	1.8	1.8	2.5	2.5	1.6	1.5	2.9	2.5
		orf19.6025	2.2	2.1	3.9	5.2	3.8	3.1	5.2	5.2
		orf19.1865	2.4	2.2	3.7	2.9	2.4	2.2	3.4	3.1
		orf19.496	2.4	2.3	4.4	3.8	2.5	2.0	3.6	3.1
	<i>ARO9</i>	orf19.1237	1.5	3.4	5.8	4.2	1.5	1.9	2.1	2.8
	<i>IDP2</i>	orf19.3733	2.2	1.6	3.3	2.8	2.6	2.0	3.2	2.5
Response to stress	<i>DDR48</i>	orf19.4082	2.2	4.3	5.8	4.7	2.7	2.9	3.4	4.3
	<i>FMA1</i>	orf19.6837	1.8	3.2	2.7	5.3	1.5	3.3	1.7	3.0
	<i>YMX6</i>	orf19.5713	1.6	2.6	2.9	2.0	1.6	1.6	1.7	1.8
		orf19.288	1.8	1.5	3.0	2.5	2.1	1.5	2.5	2.0
Biological activity unknown	<i>TEF4</i>	orf19.2652	16.4	16.6	55.8	90.2	29.9	32.7	65.5	67.8
		orf19.3627	4.0	1.9	7.0	4.0	4.4	1.9	5.1	1.9
	<i>PGA7</i>	orf19.5635	2.3	3.7	5.7	7.4	2.7	3.8	4.8	6.1
	<i>BUL1</i>	orf19.5094	1.9	1.9	4.1	3.1	2.5	1.8	2.1	1.9
	<i>PGA45</i>	orf19.2451	1.7	1.5	2.1	2.2	1.7	1.9	2.0	2.3
		orf19.344	5.1	6.2	20.2	16.2	7.2	6.6	9.6	9.0
		orf19.5777	1.7	2.4	4.8	3.8	1.9	1.5	3.1	2.5

Table 2.5. Continued.

Function ^a	CGD name	Orf19 designation	Change in fold expression ^b							
			G648S		G648D		Y642F		A643V	
			Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
Biological activity unknown		orf19.5799	1.7	2.0	3.4	2.4	1.5	1.5	2.4	2.6
		orf19.7456	1.7	2.6	4.4	4.1	2.2	2.1	3.9	3.5
		orf19.7043	2.5	1.9	5.2	3.6	2.8	2.3	4.9	3.0
		orf19.4013	2.6	2.2	4.2	3.5	3.1	2.8	4.8	3.8
		orf19.4014	1.5	2.1	2.3	2.4	1.7	1.6	2.6	2.6
		orf19.6840	2.0	2.1	4.1	4.4	2.6	2.4	3.1	2.6
		orf19.286	2.1	2.5	2.3	2.2	1.9	1.9	3.5	1.9
		orf19.7504	2.1	1.8	3.0	3.3	1.9	2.0	2.2	2.1
		orf19.3737	1.6	1.9	2.9	3.4	2.0	1.8	3.1	2.6
		orf19.1964	2.0	1.8	2.5	2.2	1.7	1.6	2.2	1.6
		orf19.1800	2.3	2.2	4.6	3.9	2.5	2.2	4.5	3.4
		orf19.7263	1.8	1.9	2.9	2.8	1.6	1.7	2.1	1.9
		orf19.2496	1.7	1.8	2.6	2.5	1.8	1.7	2.0	1.5
	<i>ATO9</i>	orf19.3261	1.8	2.2	2.7	3.4	1.6	2.3	4.5	4.5

^aDescriptions of gene from the Candida Genome Database (<http://www.candidagenome.org>).

^bFold change defined as the average ratio of gene expression levels in the isolates compared in two independent microarray experiments.

Table 2.6. Genes downregulated by at least 1.5-fold in strains carrying *UPC2* GOF alleles.

Function ^a	CGD name	Orf19 designation	Change in fold expression ^b							
			G648S		G648D		Y642F		A643V	
			Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
Transport	<i>HGT10</i>	orf19.5753	0.6	0.5	0.2	0.2	0.5	0.6	0.2	0.3
Pathogenesis	<i>SAP7</i>	orf19.756	0.4	0.4	0.3	0.2	0.4	0.4	0.3	0.4
Biological activity unknown		orf19.716	0.4	0.5	0.4	0.4	0.6	0.6	0.4	0.4
		orf19.3621	0.3	0.5	0.3	0.3	0.6	0.4	0.5	0.5
		orf19.4873	0.4	0.5	0.6	0.5	0.6	0.6	0.6	0.6

^aDescriptions of gene from the Candida Genome Database (<http://www.candidagenome.org>).

^bFold change defined as the average ratio of gene expression levels in the isolates compared in two independent microarray experiments.

constitutive activation, leading to the overexpression of Mdr1 (43, 127). Fluconazole-resistant isolates that overexpress *MDR1* have become homozygous for the mutated *MRR1* allele and work in a semidominant fashion if expressed with a wild-type allele (43). Overexpression of these transporters is known to occur among azole-resistant clinical isolates and contributes significantly to this process.

Considerably less is known about the prevalence of constitutive *ERG11* overexpression and its clinical impact on azole resistance. *ERG11* encodes the azole target, lanosterol demethylase, a key enzyme in the ergosterol biosynthesis pathway. *ERG11* is transcriptionally regulated by the zinc cluster transcription factor Upc2. In *C. albicans*, Upc2 is orthologous to two transcription factors in *Saccharomyces cerevisiae*, Upc2 and Ecm22, which regulate ergosterol biosynthesis and uptake of exogenous sterols (28, 34). The expression of these genes is affected by sterol depletion, but the exact mechanism of activation is currently unknown. Previous work in *S. cerevisiae* shows that ScUpc2 and ScEcm22 are localized to intracellular membranes outside the nucleus, and under sterol-depleting conditions, perinuclear localization is increased (115). Gain-of-function mutations in the activation domain located near the C terminus have been recovered from both ScUpc2 and ScEcm22; however, mediation of *ERG11* expression during hypoxic conditions or when chemically treated with azole antifungals is mediated by ScUpc2 (34).

In *C. albicans*, only three mutations that increase the expression of *ERG11* and result in decreased susceptibility to fluconazole have been described in Upc2. In the present study, I examined a large collection of unrelated azole-resistant *C. albicans* clinical isolates for possible *UPC2* GOF mutations. As fluconazole resistance in *C. albicans* has most often been associated with OPC, the opportunity to examine resistant isolates cultured from both oral and nonoral sources was somewhat unique. Among the isolates studied here were 16 documented nonoral isolates, 12 of which were observed to exhibit reduced susceptibilities to fluconazole. Although our analysis cannot define the clinical implications for this observation, these data underscore that fluconazole resistance extends beyond oral manifestations of candidiasis.

I further delineated the mechanisms of resistance in these isolates by determining the relative levels of expression of *CDR1*, *CDR2*, *MDR1*, and *ERG11*. Not surprisingly, *CDR1* and *CDR2* overexpression was generally coordinately regulated and quite prevalent among these isolates. Of those isolates that did overexpress *MDR1*, even fewer isolates expressed *MDR1* to the levels previously observed in azole-resistant isolates (127). *ERG11* was found to be upregulated in almost three-fourths of the fluconazole-resistant isolates examined. This suggests that *ERG11* overexpression is a common contributor to fluconazole resistance in *C. albicans*.

Among the independent azole-resistant, *ERG11*-overexpressing isolates studied here, I repeatedly recovered eight distinct single-nucleotide substitutions in *UPC2*, of which the G648S substitution occurred in nine isolates and the G648D substitution occurred in six isolates. Five of these substitutions in *UPC2* have not been described previously. Of the five novel mutations, four mutations resulted in increased *ERG11* expression and

increased resistance to fluconazole but to various degrees. I observed that the homozygous strain carrying the G304R *UPC2* allele was more susceptible to azoles than the strain carrying the G304R substitution heterozygously; however, neither strain resulted in any decrease in antifungal susceptibility or increase in *ERG11* expression. As has been previously observed in *UPC2* GOF mutations, amino acid substitutions resulting from activating mutations were localized near the C terminus, where the activation domain of zinc-cluster transcription factors is found (109). Mutations in this region of the protein are theorized to work by one of two mechanisms: (i) mutations in this domain could relieve the transcription factor from a repressor that would otherwise keep Upc2 inactive in the absence of an activating signal or (ii) a mutation in this area could interfere with the transmembrane region of the protein, causing localization to the nucleus and constitutive activation of its target promoters.

In total, seven of the eight Upc2 mutations observed resulted in increased expression of *ERG11*, but each mutation increased transcription to a different degree. For many strains, increased expression of *ERG11* was directly correlated with the amount of ergosterol within the fungal cell, and this trend was also observed in the decreased azole susceptibility seen in strains that overexpressed *ERG11*. Unlike *TAC1* GOF mutations, *UPC2* GOF mutations were found to occur with a wild-type allele in many isolates. I observed that strains constructed to express two mutant alleles had increased levels of *ERG11* expression and decreased levels of azole susceptibility compared to the levels in strains containing both an activated Upc2 and a wild-type allele. As seen with *TAC1* or *MRR1* mutations, a loss-of-heterozygosity event that results in two GOF alleles, either by a mitotic recombination event between homologous chromosomes or by a loss of a chromosome combined with a duplication of the homologous chromosome, would provide an advantage when challenged with antifungals (22, 127). Interestingly, I saw an opposite trend in the amphotericin B susceptibility of strains expressing activated *UPC2* alleles, in which *UPC2* GOF homozygous strains with higher *ERG11* expression had increased susceptibility to this agent compared to that of the heterozygous strains or the homozygous null *upc2Δ* strain. This is probably due to higher levels of cellular ergosterol, which is the target of amphotericin B.

An important observation to note is the discrepancy between the levels of *ERG11* expression of the clinical isolate containing a *UPC2* mutation and the laboratory strain constructed to carry the same mutant *UPC2* allele. This difference may be due to additional levels of *ERG11* regulation that are developed in a clinical isolate, such as alternate transcriptional regulations or alternate single-nucleotide polymorphisms that influence ergosterol biosynthesis. Furthermore, not all *ERG11* overexpression in clinical isolates was a result of a GOF mutation in *UPC2*; therefore, it is likely that other regulators of ergosterol biosynthesis are contributing factors. Alternatively, instances of aneuploidy, specifically, chromosome 5 duplication or chr5L isochromosome formation, are known mechanisms of *ERG11* overexpression in *C. albicans* (173). However, the *ERG11* expression levels I observed in many clinical isolates in this study exceed what would be expected from an extra copy of *ERG11* due to the presence of a chr5L isochromosome. It is possible that some isolates that do not carry mutations in *UPC2* actually overexpress *UPC2* instead, due to a mutation in the *UPC2* promoter region, gene

duplication, or a *trans*-acting mutation. Whether this has occurred is being explored in ongoing investigations.

As azole resistance is mechanistically multifactorial in clinical isolates, I expected the fluconazole susceptibility of strains constructed to express specific *UPC2* mutations to reflect the contribution of increased expression of genes of the ergosterol biosynthesis pathway, including *ERG11*, as a result of an activated *UPC2* allele. In these clinical isolates, I have observed a high prevalence of mutations in the *ERG11* gene itself (data not shown). It is likely that in clinical isolates, activated forms of Upc2 occur in conjunction with mutant *ERG11* alleles and that its subsequent overexpression results in a combinatorial effect on azole susceptibility. The combinatorial effects of activated *UPC2* alleles and mutant *ERG11* alleles on fluconazole susceptibility have not been explored.

Although microarray analysis of the G648D *UPC2* GOF mutation has been performed previously, I expanded this analysis with four of the seven strongest *UPC2* GOF mutations (G648D, G648S, Y642F, and A643V) for a more comprehensive examination (44). All four mutations caused the coordinate upregulation of 61 genes. As expected, many of the ergosterol biosynthesis genes (*ERG1*, *ERG2*, *ERG7*, *ERG24*, *ERG26*, and *ERG27*) were upregulated by at least 1.5-fold over their expression in SC5314 in two separate experiments. *ERG11* was upregulated in three of the four laboratory strains carrying mutations G648D, Y642F, and A643V, but elevated transcription of *ERG11* could only be detected in one of the two experiments with the strain carrying the G648S allele (**Appendix B**). Interestingly, *UPC2* was not found to be upregulated in any of the four strains analyzed by microarray analysis, although Upc2 is thought to autoregulate and has been shown to bind its own promoter in experiments using chromatin immunoprecipitation with microarray technology (ChIP-chip) (217).

Aside from genes involved in the ergosterol biosynthesis pathway, other core sets of target genes upregulated by Upc2 included those in the gene ontology (GO) functional grouping of oxidoreductase activity. Additionally, iron ion binding was a commonly found GO category identified by the GO term finder. Not surprisingly, *MDR1* was overexpressed in all four strains with independent Upc2 GOF mutations; however, gene expression levels were not increased to those observed in fluconazole-resistant isolates containing an *MRR1* GOF mutation. Upc2 has previously been shown to bind to the promoter of the drug efflux pump *MDR1* but was not a cause of Mdr1-mediated drug resistance (169, 217). In the present study, *CDR11* expression was increased in all independent *UPC2* GOF mutations, and it increased 4.8-fold when two alleles of *UPC2* carrying the predicted G648D substitution in its translated sequence were expressed. Interestingly, the gene encoding an uncharacterized ABC transporter, *CDR11*, was previously shown to be upregulated by 1.5- to 3.5-fold in a laboratory strain carrying a single *UPC2* allele containing a G648D amino acid substitution; this gene has been hypothesized to play a role in sterol transport, because of its similarity to sterol transporters in *S. cerevisiae*) (44).

In conclusion, I found overexpression of *ERG11* to be prevalent among a collection of clinical isolates with reduced susceptibility to fluconazole and have identified and

characterized novel *UPC2* gain-of-function mutations that contribute to azole resistance in clinical isolates of *C. albicans*. Overexpression of *ERG11* could be explained by a gain-of-function mutation in *UPC2* in many but not all cases. Gain-of-function mutations led to increased resistance to azole antifungals and terbinafine, to increased cellular ergosterol levels, and to increased expression of genes involved in ergosterol biosynthesis and oxidoreductase activity, as well as the transporter genes *MDR1* and *CDR11*. The mechanism by which *ERG11* is upregulated in the absence of *UPC2* gain-of-function mutations and the potential contribution of sterol uptake to Upc2-mediated azole resistance are under investigation.

CHAPTER 3. *NDT80* MUTATIONS FOUND IN CLINICAL ISOLATES OF *CANDIDA ALBICANS* DO NOT CONTRIBUTE TO AZOLE RESISTANCE BY *ERG11* OVEREXPRESSION

INTRODUCTION

Not all *ERG11* overexpression in azole-resistant isolates can be explained by GOF mutations in *UPC2*. Our lab and others have documented isolates with increased *ERG11* expression that do not carry a mutated *UPC2* allele. These data suggest that there are alternate mechanisms of *ERG11* overexpression in these isolates (50, 66). *NDT80* is a Pho-G transcription factor that is an important regulator of meiosis in *S. cerevisiae* (67). In *C. albicans*, *NDT80* has been implicated in azole resistance not only due to its regulation of *CDR1* but also due to its regulation of genes involved in the ergosterol biosynthesis pathway (21, 170, 171, 199). Recent ChIP-chip analysis has shown that *NDT80* binds to the promoter region of *ERG* genes and also to genes involved in hyphal growth, the mitotic cycle and carbohydrate metabolism (171). A putative Ndt80 binding site has also been found in the regulatory region upstream of the *UPC2* allele (73). Sellam and colleagues compared *ERG* gene expression of a strain constructed to be deficient for *NDT80* to that of wild-type and an *NDT80* revertant strain (171). The mutant strain deficient for *NDT80* was unable to stimulate expression of *ERG11* and other *ERG* genes to the extent of either the wild-type strain or the *NDT80* revertant strain in response to fluconazole. This data indicated the potential role of *NDT80* in *ERG* gene regulation as it relates to fluconazole treatment.

In a previous search for *UPC2* GOF mutations, genetically matched isolate pairs 945 and 1619, and 1002 and 3795 were shown to constitutively overexpress *ERG11* in the fluconazole-resistant respective counterpart (66). Sequence analysis performed on *UPC2* did not detect any mutations leading to amino acid substitutions in these isolates. Due to the possible role of *NDT80* in *ERG* gene regulation, I hypothesized that the *NDT80* allele expressed in the fluconazole-resistant isolates may contain activating mutations resulting in *ERG11* overexpression and azole resistance.

MATERIALS AND METHODS

Strains and growth conditions

C. albicans strains were cultured on YPD (1% yeast extract, 2% peptone, and 1% dextrose) agar plates at 30°C and stored as frozen stock in 20% glycerol at -80°C (Table 3.1). YPD liquid medium was used for routine growth of strains. Nourseothricin (200 µg/ml or 25 µg/ml) was added to YPD agar plates for selection of strains containing the *SAT1*-flipper cassette. For plasmid construction and propagation, One Shot *Escherichia coli* TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) were used as a host. These strains were grown in Luria-Bertani (LB) broth or on LB agar plates supplemented

Table 3.1. *NDT80* strains and primers used in this study.

Strains and primers	Relevant genotype/sequence
SC5314	Wild-type
ScNDT80 ⁹⁴⁵ A	<i>NDT80</i> allele derived from azole-susceptible isolate
ScNDT80 ⁹⁴⁵ B	<i>NDT80</i> allele derived from azole-susceptible isolate
ScNDT80 ¹⁶¹⁹ A	<i>NDT80</i> allele derived from azole-resistant isolate
ScNDT80 ¹⁶¹⁹ B	<i>NDT80</i> allele derived from azole-resistant isolate
qRT-PCR	
<i>NDT80</i> -F	5'-CCT TTG GCT CAG CAA CA-3'
<i>NDT80</i> -R	5'-GCT TGG TCT TGG TTT TGT GC-3'
<i>ACT1</i> -F	5'-ACGGTGAAGAAGTTGCTGCTTTAGTT-3'
<i>ACT1</i> -R	5'-CGTCGTCACCGGCAAAA-3'
<i>ERG11</i> -F	5'-CCCCTATTAATTTTGT TTTCCCTAATTTAC-3'
<i>ERG11</i> -R	5'-CACGTTCTCTTCTCAGTTTAATTTCTTTC-3'
<i>NDT80</i> mutant construction	
<i>NDT80</i> -A	5'- <u>GGG CCC</u> GCA CCA TCA ATT TGG AGA CC-3'
<i>NDT80</i> -B	5'-ACG GCA GCT TCT TCA <u>CTC GAG</u> ACG TT-3'
<i>NDT80</i> -C	5'-CAC TTC ATT <u>GGG CCC</u> GTT CAC TAG-3'
<i>NDT80</i> -D	5'-GCC ACT AAG <u>GTG TGA</u> GCT CAA GAT CC-3'
<i>NDT80</i> -E	5'- <u>CTC GAG</u> GAT TGG TAC ACG ACA CCT GG-3'
<i>NDT80</i> sequencing	
<i>NDT80seqA</i>	5'-CAACAGAAACACCACCACCA-3'
<i>NDT80seqB</i>	5'-GCTGACGCTCAATCACAAGC-3'
<i>NDT80seqC</i>	5'-GAGTTGCCTGACCACGAGAC-3'
<i>NDT80seqD</i>	5'-ATCAACCTTTCTTCGTCATCATC-3'
<i>NDT80seqE</i>	5'-GTCCTCGTGGTCAGGCAACTC-3'
<i>NDT80seqF</i>	5'-GCTTGTGATTGAGGGTCAGC-3'

Underlined sequence reflects the introduction of a restriction site sequence.

with 50 µg/ml kanamycin (Fisher BioReagents, Fair Lawn, NJ) or 100 µg/ml ampicillin (Sigma) when required.

RNA isolation

RNA was isolated using a small-scale version of the hot phenol method of RNA isolation described by Schmitt et al. Briefly, overnight cultures were diluted to an OD₆₀₀ of 0.2 and then incubated at 30°C with shaking for an additional three or six hours to mid-log phase. Cells were collected by centrifugation, resuspended in AE buffer and then transferred to a 2-mL microcentrifuge tube containing acid phenol (pH 4.3) with 1% SDS. Cells were incubated at 65°C for 10 minutes, then lysates were clarified by centrifugation. The supernatant was then transferred into a new tube containing 900 µl of chloroform and mixed. The sample was then subjected to centrifugation again, and the top aqueous layer was transferred to a new tube containing one volume of isopropanol and 0.1 volume 2M sodium acetate. The RNA pellet was subsequently washed with 500 µl of 70% ethanol and collected by centrifugation. The RNA pellet was resuspended in DNase/RNase-free H₂O. Quantity and purity were determined spectrophotometrically at absorbances of A₂₆₀ and A₂₈₀.

Quantitative RT-PCR

First-strand cDNAs were synthesized separately from 1 µg of total RNA in a 21-µl reaction volume using SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Quantitative PCRs were performed in triplicate as technical replicates using the 7000 sequence detection system (Applied Biosystems). PCRs were performed, independently amplifying 18S rRNA or *ACT1* gene (normalizing genes) and the genes of interest (GOI) from the same cDNA, using the SYBR Green PCR master mix (Applied Biosystems). Gene-specific primers were designed using Primer Express software (Applied Biosystems) synthesized by Integrated DNA Technologies (Coralville, IA) and listed in **Table 3.2**. PCR conditions consisted of AmpiTaq Gold activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C. The dissociation curve and detection of fluorescence of SYBR Green was performed by software provided with the 7000 sequence detection system. The cycle threshold value (C_T) of the normalizing gene was subtracted from the GOI to obtain the ΔC_T value. The ΔC_T value of a calibrator was subtracted of the sample ΔC_T value to obtain the ΔΔC_T value. Change in fold expression was obtained by calculating 2^{-ΔΔC_T}. Standard error was calculated from ΔC_T values as previously described.

Plasmid construction for allele sequencing

CaNDT80 coding sequences were amplified by PCR (*Pfu* DNA polymerase; Stratagene) from *C. albicans* genomic DNA using the primers NDT80-A and NDT80-E (**Table 3.2**). Products were cloned into pCR-BLUNTII-TOPO using a Zero Blunt TOPO

Table 3.2. *NDT80* sequencing data from matched isolate sets 580, 2440 and 945, 1619 were compared to the SC5314.

Isolate	Allele	Mutations in sequence as compared to SC5314
580 FCZ ^S	1	C72T, G240A, G247A, A249G, Deletion of CAA between bp 259-261, C783T, C804T, C735G, G741A, C783T, C804T, C825T, T1269C, A1398G, C1491A
	2	G243A, Deletion of CAA between bp 259-261, C783T, C804T, C735G, G741A, C783T, C804T, C825T, T1269C, C1491A
2440 FCZ ^R	1	G243A, Deletion of CAA between bp 259-261, C783T, C804T, C735G, G741A, C783T, C804T, C825T, T1269C, A1328G , C1491A
	2	G243A, Deletion of CAA between bp 259-261, C783T, C804T, C735G, G741A, C783T, C804T, C825T, T1269C, C1491A
945 FCZ ^S	1	A240G, A246G, A249G, Addition of 2 X CAA, A393T, G430A , A486C , C741G, G747A, C810T, C831T, T1276C, A1404G, C1497A
	2	A240G, A246G, Addition of 1X CAA, C741G, G747A, T1276C
1619 FCZ ^R	1	A240G, A246G, A249G, Addition of 2 X CAA, A393T, G430A , A486C , C741G, G747A, C810T, C831T, T1276C, A1404G, C1497A
	2	A240G, A246G, A249G, Addition of 2 X CAA, A393T, G430A , A486C , C741G, G747A, C810T, C831T, T1276C, A1404G, C1497A

Bolded mutations lead to amino acid substitutions. The G340A leads to A145T a.a. substitution. The A486C mutation leads to a Q162H aa substitution. The A1328G mutations lead to a Y444C a.a. substitution. Shaded boxes defines *NDT80* sequence of the second allele for each clinical isolate.

PCR Cloning Cloning Kit (Invitrogen) and transferred into *Escherichia coli* TOP10 cells with selection on LB agar plates containing 50 µg/ml kanamycin. Plasmid DNA was purified (QIAprep; Qiagen; Germantown, MD) and sequenced on an ABI Model 3130XL Genetic Analyzer using the *NDT80* sequencing primers (**Table 3.2**), resulting in full-length sequence from both strands of the *CaNDT80* gene. The sequencing was performed using six sets of clones derived from three independent PCRs for each strain/isolate sequenced.

Sequenced plasmids of the *ERG11* orf whose predicted translation indicated an amino acid substitution were digested with restriction enzymes *ApaI* and *XhoI*, which excised the full-length orf from the plasmid, and the *ERG11* alleles were cloned upstream of the *SAT1*-flipper cassette into the *ApaI* and *XhoI* sites of plasmid pSFS2. The *NDT80* downstream segments were amplified with ExTaq (Takara) using primers NDT80C and NDT80D and cloned downstream of the *SAT1*-flipper cassette in pSFS2 using the *SacI* and *SacII* sites. This process generated plasmids pNDT80⁹⁴⁵ and pNDT80¹⁶¹⁹.

Construction of *NDT80* allele strains

C. albicans strain SC5314 were transformed by electroporation with gel purified inserts from plasmids derived from the plasmid pSFS2. Plasmid (p)SFS2 contains the *SAT1*-flipper disruption cassette developed by Reuss et al (156)., consisting of the *SAT1* selectable marker which confers resistance to nourseothricin and the FLP flipper recombinase gene both flanked by FRT sites (flipper recombinase target sequences). Nourseothricin-resistant transformants were selected as previously described (156). Upon induction of the FLP gene, the cassette is excised such that only the *NDT80* allele with a downstream FRT is left in the *NDT80* locus. Integration of constructs was confirmed by Southern hybridization.

Fluconazole susceptibility testing

Minimum inhibitory concentrations (MICs) were obtained by using a modified CLSI protocol outlined in M27-A2 using RPMI. Overnight cultures grown at 30°C were streaked onto Sabouraud's agar. Plated cultures were grown for 24 hours at 30°C. Individual colonies were suspended in sterile water until the optical density at 600 nm of 0.1 was reached. The working colony concentration was made by making a 1:50 dilution and a 1:20 dilution sequentially in media. 100 µl from the working stock was used to inoculate a series of fluconazole/YPD media dilutions, the highest being 64 µg/ml. Cultures were incubated at 35°C for 48 hours and MICs were recorded.

RESULTS

Sequencing *NDT80* of matched isolate sets 945/1619 and 1002/3795 revealed multiple single nucleotide polymorphisms and a loss of heterozygosity event in the *NDT80* alleles of the resistant isolate

NDT80 in both matched isolate sets, the *NDT80* allele was sequenced for both the susceptible and resistant isolates (**Table 3.2**). In both matched pairs, the allele expressed in the resistant isolate contained mutations that result in amino acid substitutions within *Ndt80* when compared to the strain published in the Candida Genome Database (<http://www.candidagenome.org/>) and also when compared with the respective susceptible isolate. Additionally, sequencing of both matched sets show that in the resistant isolate, a loss of heterozygosity event had occurred so that the resistant counterpart was homozygous for one allele.

Mutations observed in *NDT80* of resistant isolates do not result in fluconazole resistance or increased *ERG11* expression

In the fluconazole-susceptible isolate SC5314, a series of strains were constructed to test the role of mutant *NDT80* alleles derived from clinical isolates on fluconazole resistance and *ERG11* expression. For these experiments I used the matched isolate set 945 and 1619 as the resistant isolate 1619 had the greatest expression of *ERG11* (66). Fluconazole-susceptible isolate 945 was heterozygous for *NDT80*, containing two different alleles of *NDT80*, one of which was expressed homozygously in the resistant isolate 1619. In constructed strains, each allele was expressed homozygously in isolate SC5314 (**Figure 3.1**). Additionally, a strain disrupted for *NDT80* was also constructed for MIC comparison. When compared to SC5314, neither strain constructed to carry the *NDT80* allele observed in the susceptible or the resistant isolate resulted in increased resistance to fluconazole or increased *ERG11* expression. I observed a two-fold decrease in fluconazole MIC in the strain in which *NDT80* was disrupted (data not shown).

DISCUSSION

In *C. albicans*, GOF mutations in zinc-cluster transcription factors resulting in increased expression of genes involved in azole resistance are well documented. As these mutations in *Tac1*, *Mrr1* and *Upc2* all contribute to azole resistance by distinct mechanisms, *Ndt80* has been implicated in the regulation of ABC efflux-transporters *CDR1* and *CDR2* in addition to ergosterol biosynthesis genes. Due to this global association of *NDT80* with azole resistance, I hypothesized that fluconazole-resistant isolates that overexpress *ERG11* independent of *UPC2* GOF mutations may contain mutations in *NDT80* that resulted in an azole-resistant phenotype.

Figure 3.1. *ERG11* expression of strains constructed to carry *NDT80* alleles derived from clinically matched isolates 945 and 1619. *ERG11* expression was measured in triplicate by qRT-PCR and fluconazole MICs were tested for strains expressing *NDT80* alleles derived from fluconazole-susceptible isolate 945 or fluconazole-resistant isolate 1619.

Previous work shows that specific mutations in the transcription factor gene *TAC1* regulate expression of the genes encoding ABC transporters *CDR1* and *CDR2*. Mrr1 mediates expression of the major facilitator superfamily (MFS) transporter Mdr1 in azole-resistant isolates, and specific mutations in this transcriptional regulator result in its constitutive activation, leading to the overexpression of Mdr1. Fluconazole-resistant isolates that overexpress *MDR1* have become homozygous for the mutated *MRR1* allele and work in a semidominant fashion if expressed with a wild-type allele. Loss of heterozygosity in *TAC1* GOF mutations has also been observed in fluconazole-resistant isolates which overexpress *CDR1* and *CDR2*. In this study, matched isolate sets exhibited a similar loss of heterozygosity of *NDT80* alleles in the resistant counterpart, however, neither allele resulted in overexpression of *ERG11* when expressed in SC5314.

Since the completion of this study, the role of *C. albicans* *NDT80* in drug resistance has been further explored. In this work, *NDT80* has been shown to be dispensable to the upregulation of both *MDR1*, *CDR1* and *CDR2* in response to chemical inducers benomyl and fluphenazine respectively. Additionally, this work demonstrated that disruption of *NDT80* did not affect the regulation of efflux transporters or ergosterol biosynthesis genes in strains that carry GOF mutations in zinc-cluster transcription factors that mediate these processes. In other work, strains deficient for *NDT80* have been shown to be deficient in biofilm formation. As the purpose of this study was to explore the role of *NDT80* in *ERG11* expression, strains constructed in this study have not been tested for biofilm formation or other processes.

CHAPTER 4. CLINICALLY DERIVED MUTATIONS IN *ERG11* CONTRIBUTE TO AZOLE RESISTANCE IN *CANDIDA ALBICANS*

INTRODUCTION

In the opportunistic fungal pathogen *Candida albicans*, *ERG11*, an important gene in the ergosterol biosynthesis pathway and target of the azole antifungals, has been shown to contribute to azole resistance in a number of ways. Overexpression of *ERG11*, either by a gain of function mutation (GOF) in the transcriptional regulator, Upc2, or increased copy number of chromosome 5, on which *ERG11* resides, results in reduced azole susceptibility (50, 173). In addition, mutations in *ERG11* that result in an amino acid substitution in lanosterol demethylase has been shown to alter the ability of the azole antifungals to bind to and inhibit this enzyme, especially in the presence of lanosterol, resulting in resistance to this class of antifungal (85, 86, 200, 201). There have been many reports of mutations in *ERG11* associated with reduced azole susceptibility suggesting that lanosterol demethylase is tolerant to structural changes. Previous reports have defined three specific ‘hot spot’ regions that correspond to amino acids 105 to 165, 266 to 287, and 405 to 488 in the translated sequence that are particularly permissive to substitutions (114). In order to show that *ERG11* mutations can contribute to azole resistance, investigators have used several approaches including heterologous expression of mutated *ERG11* alleles in other species, enzyme inhibition with fluconazole in cell free extracts and by biochemical analysis that demonstrate these mutations contribute to azole antifungal resistance (85, 86, 122, 162). A number of different mutations have been associated with the azole-resistant phenotype but the majority of these mutations have not been studied in *C. albicans* background. Advances in molecular biology and our knowledge of the *C. albicans* genome now allow us to study these mutations in this pathogen itself.

In this study, I examined the prevalence and variance of *ERG11* mutations in a group of 63 characterized clinical *C. albicans* isolates with reduced fluconazole susceptibility. A select group of mutant *ERG11* alleles were then expressed in an azole susceptible background in order to determine the contribution of distinct mutations in *ERG11* on the susceptibility of different azole antifungals.

MATERIALS AND METHODS

Strains and growth conditions

C. albicans strains were cultured on YPD (1% yeast extract, 2% peptone, and 1% dextrose) agar plates at 30°C and stored as frozen stock in 20% glycerol at -80°C. YPD liquid medium was used for routine growth of strains. Nourseothricin (200 µg/ml or 25 µg/ml) was added to YPD agar plates for selection of strains containing the *SAT1*-flipper cassette. For plasmid construction and propagation, One Shot *Escherichia coli* TOP10

chemically competent cells (Invitrogen, Carlsbad, CA) were used as a host. These strains were grown in Luria-Bertani (LB) broth or on LB agar plates supplemented with 50 µg/ml kanamycin (Fisher BioReagents, Fair Lawn, NJ) or 100 µg/ml ampicillin (Sigma) when required.

Plasmid construction for allele sequencing

CaERG11 coding sequences were amplified by PCR (*Pfu* DNA polymerase; Stratagene) from *C. albicans* genomic DNA using the primers ERG11-A and ERG11-E (**Table 4.1**). Products were cloned into pCR-BLUNTII-TOPO using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and transferred into *Escherichia coli* TOP10 cells with selection on LB agar plates containing 50 µg/ml kanamycin. Plasmid DNA was purified (QIAprep; Qiagen; Germantown, MD) and sequenced on an ABI Model 3130XL Genetic Analyzer using the *ERG11* sequencing primers (**Table 4.2**), resulting in full-length sequence from both strands of the *CaERG11* gene. The sequencing was performed using six sets of clones derived from three independent PCRs for each strain/isolate sequenced.

Sequenced plasmids of the *ERG11* orf whose predicted translation indicated an amino acid substitution were digested with restriction enzymes *ApaI* and *XhoI*, which excised the full-length orf from the plasmid, and the *UPC2* alleles were cloned upstream of the *SAT1*-flipper cassette into the *ApaI* and *XhoI* sites of plasmid pSFS2 (156). The *UPC2* downstream segments were amplified with ExTaq (Takara) using primers UPC2C and UPC2D and cloned downstream of the *SAT1*-flipper cassette in pSFS2 using the *NotI* and *SacII* sites. This process generated plasmids pERG11-2, pERG11-5, pERG11-6, pERG11-7, pERG11-7N, pERG11-8, pERG11-8O, pERG11-9, pERG11-10, pERG11-13, pERG11-15/16, pERG11-19, pERG11-20, pERG11-21, pERG11-22, pERG11-27, pERG11-29 and pERG11-30.

Construction of strains carrying specific *ERG11* alleles

C. albicans strain SC5314 were transformed by electroporation with gel purified inserts from plasmids (plasmids go here) derived from the plasmid pSFS2. pSFS2 contains the *SAT1*-flipper disruption cassette developed by Reuss et al. consisting of the *SAT1* selectable marker which confers resistance to nourseothricin and the *FLP* flipper recombinase gene both flanked by FRT sites (flipper recombinase target sequences). Nourseothricin-resistant transformants were selected as previously described.(156) Upon induction of the *FLP* gene, the cassette is excised such that only the *ERG11* allele with a downstream FRT is left in the *ERG11* locus. Integration of constructs was confirmed by Southern hybridization.

Table 4.1. *C. albicans* strains carrying mutant *ERG11* alleles used in this study.

Strain	Strain background	Relevant characteristics or genotype	Source or reference
SC5314	N/A	<i>ERG11-1/ERG11-2</i>	ATCC
Clinical Isolates 10-72	N/A	Azole resistant	University of Iowa
Constructed laboratory strains			
5A2A43A	SC5314	<i>ERG11^{Q21L}::FRT/ERG11^{Q21L}::FRT</i>	This study
5B6A19A	SC5314	<i>ERG11^{Q21L}::FRT/ERG11^{Q21L}::FRT</i>	This study
20E1III1G1	SC5314	<i>ERG11^{Y132F}::FRT/ERG11^{Y132F}::FRT</i>	This study
20B5A14A	SC5314	<i>ERG11^{Y132F}::FRT/ERG11^{Y132F}::FRT</i>	This study
10C1B1M1	SC5314	<i>ERG11^{K143R}::FRT/ERG11^{K143R}::FRT</i>	This study
10B1A32A	SC5314	<i>ERG11^{K143R}::FRT/ERG11^{K143R}::FRT</i>	This study
2A1A18A	SC5314	<i>ERG11^{F145L}::FRT/ERG11^{F145L}::FRT</i>	This study
2B1A51A	SC5314	<i>ERG11^{F145L}::FRT/ERG11^{F145L}::FRT</i>	This study
21C1M1A1	SC5314	<i>ERG11^{S405F}::FRT/ERG11^{S405F}::FRT</i>	This study
21B12A61B	SC5314	<i>ERG11^{S405F}::FRT/ERG11^{S405F}::FRT</i>	This study
20NA50A8A	SC5314	<i>ERG11^{G448E}::FRT/ERG11^{G448E}::FRT</i>	This study
7A5A5A	SC5314	<i>ERG11^{F499V}::FRT/ERG11^{F499V}::FRT</i>	This study
7B4A29A	SC5314	<i>ERG11^{F449V}::FRT/ERG11^{F449V}::FRT</i>	This study
15A3A108A	SC5314	<i>ERG11^{G450E}::FRT/ERG11^{G450E}::FRT</i>	This study
16A14A47A	SC5314	<i>ERG11^{G450E}::FRT/ERG11^{G450E}::FRT</i>	This study
22AABA56A	SC5314	<i>ERG11^{D446E}::FRT/ERG11^{D446E}::FRT</i>	This study
22B12A58A	SC5314	<i>ERG11^{D446E}::FRT/ERG11^{D446E}::FRT</i>	This study

Table 4.1. Continued.

Strain	Strain background	Relevant characteristics or genotype	Source or reference
19A1A1C1	SC5314	<i>ERG11</i> ^{G464S} ::FRT/ <i>ERG11</i> ^{G464S} ::FRT	This study
19B1A71A	SC5314	<i>ERG11</i> ^{G464S} ::FRT/ <i>ERG11</i> ^{G464S} ::FRT	This study
6A1A47A	SC5314	<i>ERG11</i> ^{K143R,E266D} ::FRT/ <i>ERG11</i> ^{K143R,E266D} ::FRT	This study
27A5A33A	SC5314	<i>ERG11</i> ^{Y132F, F145L} ::FRT/ <i>ERG11</i> ^{Y132F, F145L} ::FRT	This study
27B7A63A	SC5314	<i>ERG11</i> ^{Y132F, F145L} ::FRT/ <i>ERG11</i> ^{Y132F, F145L} ::FRT	This study
7NA35A40A	SC5314	<i>ERG11</i> ^{F145L, E266D} ::FRT/ <i>ERG11</i> ^{F145L, E266D} ::FRT	This study
13A38A25A	SC5314	<i>ERG11</i> ^{D278N, G464S} ::FRT/ <i>ERG11</i> ^{D278N, G464S} ::FRT	This study
9A14A21	SC5314	<i>ERG11</i> ^{Y132F, K143R} ::FRT/ <i>ERG11</i> ^{Y132F, K143R} ::FRT	This study
9B4B34A	SC5314	<i>ERG11</i> ^{Y132F, K143R} ::FRT/ <i>ERG11</i> ^{Y132F, K143R} ::FRT	This study
8OB29A48A	SC5314	<i>ERG11</i> ^{G307S, G450E} ::FRT/ <i>ERG11</i> ^{G307S, G450E} ::FRT	This study
30A5A53A	SC5314	<i>ERG11</i> ^{M258L, G464S} ::FRT/ <i>ERG11</i> ^{M258L, G464S} ::FRT	This study
30B5A57A	SC5314	<i>ERG11</i> ^{M258L, G464S} ::FRT/ <i>ERG11</i> ^{M258L, G464S} ::FRT	This study
8A4A1A	SC5314	<i>ERG11</i> ^{I483V, G450E} ::FRT/ <i>ERG11</i> ^{I483V, G450E} ::FRT	This study
8B4A47A	SC5314	<i>ERG11</i> ^{I483V, G450E} ::FRT/ <i>ERG11</i> ^{I483V, G450E} ::FRT	This study

Table 4.2. *ERG11* primers used in this study.

Purpose, primer	Sequence
<i>ERG11</i> mutant construction	
<i>ERG11</i> -A	5'- <u>GGGCCC</u> GGGTTATTTGAGAACAGCC-3'
<i>ERG11</i> -B	5'-ATCCGTTCTCGAGCACTAAGGGACAA-3'
<i>ERG11</i> -C	5'-GTAATCAATTGAGCTCTTTTAACTTT-3'
<i>ERG11</i> -D	5'-GATTATAGTTCCGCGGTGGTTTTACC-3'
<i>ERG11</i> -E	5'-TGATGGTTTTTGTCCACTGGCTCGAG-3'
<i>ERG11</i> sequencing	
T7	5'- TAATACGACTCACTATAGGG-3'
<i>ERG11</i> seqB	5'-TATTTTCACTGCTTCAAGATCT-3'
<i>ERG11</i> seqC	5'-CCAAAAGGTCATTATGTTTTAG-3'
<i>MI3R</i>	5'-CAGGAAACAGCTATGACC-3'
<i>ERG11</i> seqE	5'CATTTAGGTGAAAAACCTCATT-3'
<i>ERG11</i> seqF	5'-TACTCCAGTTTTTCGGTAAAGGG-3'

^aUnderlined sequence reflects the introduction of a restriction site sequence

Azole susceptibility testing

Minimum inhibitory concentrations (MICs) were obtained by using a modified CLSI protocol outlined in M27-A2 using RPMI. Overnight cultures grown at 30°C were streaked onto Sabouraud's agar. Plated cultures were grown for 24 hours at 30°C. Individual colonies were suspended in sterile water until the optical density at 600 nm of 0.1 was reached. The working colony concentration was made by making a 1:50 dilution and a 1:20 dilution sequentially in media. 100 µl from the working stock was used to inoculate a series of azole/RPMI media dilutions, the highest being 64 µg/ml for fluconazole. Similar procedures were used for voriconazole and itraconazole dilutions, however the highest concentrations used for these agents was 8 µg/ml. Cultures were incubated at 35°C for 48 hours and MICs were recorded.

RESULTS

Many clinical isolates with reduced fluconazole susceptibility carry mutations in *ERG11*

Of the 63 isolates that were determined to have a reduced susceptibility to fluconazole (MIC $\geq$ 8 µg/mL), 55 carried a mutation in *ERG11* that lead to an amino acid substitution. Although silent mutations were observed in the *ERG11* alleles tested (data not shown), we recovered 26 distinct positions where mutations occurred that resulted in an amino acid substitution either alone or in combination with other mutations. Each isolate was homozygous for mutations in the *ERG11* allele. Nine of these substitutions, either due to their position (Q21R, M258L, L403F and I483V) or the specific amino acid substitution (A114V, D446E, Y447S, F449I and I471M) have not been described in previous reports. Among the isolates that carry amino acid substitutions in Erg11, the number of substitutions varied between the isolates and ranged between 1 (n = 28) and 4 (n = 2). Most isolates in this collection carried a single amino acid substitution in Erg11. In total, 30 unique *ERG11* alleles were recovered from sequence analysis (**Table 4.3**). The most common polymorphisms detected were at positions E266 (n=10), Y132 (n=9), G464 (n=8) and K143 (n=7) (**Table 4.2**).

Single mutations in *ERG11* contribute to azole resistance in *C. albicans*

In order to assess the contribution of each individual mutant *ERG11* allele to azole antifungal resistance, we expressed specific mutant alleles homozygously with an identical mutant *ERG11* allele (**Table 4.4**). Each strain was constructed in duplicate and susceptibilities were tested against fluconazole, itraconazole, and voriconazole. Azole susceptibilities of constructed strains were compared to the wild-type susceptible parent strain, SC5314 at 48 hours.

Table 4.3. Occurrence of Erg11 amino acid substitutions in predicted translated sequence in fluconazole resistant clinical *ERG11*-overexpressing isolates.

A.A. substitution (mutation)	ID	Zygotity	FCZ MIC (µg/mL)	UPC2 mutation*	Other resistance mechanism*
None	36		64	G648S	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	37		64		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	53		>256		↑ <i>ERG11</i>
	55		>256		↑ <i>ERG11</i>
	56		>256		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	57		>256		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	58		>256		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	60		>256		↑ <i>ERG11</i>
Q21R (A62G)	16	Hererozygous	16		↑ <i>MDR1</i>
Y132F (A395T)	48	Homozygous	256		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
K143R (A428G)	13	Homozygous	16	A643T	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	12	Homozygous	16		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	14	Homozygous	16		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
F145L (T435G)	22	Homozygous	32		↑ <i>ERG11</i>
F449V (T1345G)	40	Homozygous	128		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>

Table 4.3. Continued.

A.A. substitution (mutation)	ID	Zygotity	FCZ MIC (µg/mL)	UPC2 mutation*	Other resistance mechanism*
S405F (C1214T)	18	Homozygous	32		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	19	Homozygous	32		
	20	Homozygous	32		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	41	Homozygous	128		↑ <i>ERG11</i> , <i>MDR1</i>
D446E (T1338A)	25	Homozygous	32	G648S ^h	↑ <i>ERG11</i> , <i>MDR1</i>
	35	Homozygous	64	G648S ^h	↑ <i>ERG11</i>
	44	Homozygous	128		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i>
G448E (G1341A)	31	Homozygous	64		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	65	Homozygous	>256	G648S ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	66	Homozygous	>256		↑ <i>CDR1</i> , <i>CDR2</i>
	67	Homozygous	>256	G648S ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	69	Homozygous	>256	G648S ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
G450E (G1349A)	24	Homozygous	32		↑ <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i>
	34	Homozygous	64	Y642F	↑ <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i>
	63	Homozygous	>256		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i>
	64	Homozygous	>256		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i>
G464S (G1390A)	32	Homozygous	64		↑ <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i>
	38	Homozygous	64	G648D ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>

Table 4.3. Continued.

A.A. substitution (mutation)	ID	Zygotity	FCZ MIC (µg/mL)	UPC2 mutation*	Other resistance mechanism*
G464S (G1390A)	39	Homozygous	64		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
continued	42	Homozygous	128		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
A114S (G340T), Y257H (T769C)	17	Homozygous	32		↑ <i>CDR1</i> , <i>CDR2</i>
F126L (C378A), Y132F (A395T)	30	Homozygous	64	A643V ^h	↑ <i>ERG11</i>
Y132F (A395T), K143R (A428G)	50	Homozygous	256		↑ <i>CDR1</i> , <i>CDR2</i>
	61	Homozygous	>256		↑ <i>CDR1</i> , <i>CDR2</i>
Y132F (A395T), F145L (T435G)	29	Homozygous	64		↑ <i>CDR1</i> , <i>CDR2</i>
K143R (A428G), E266D (A798C)	10	Homozygous	16		↑ <i>ERG11</i> , <i>CDR1</i>
F145L (T435G), E266D (A798C)	33	Homozygous	64	Y642F	↑ <i>CDR1</i> , <i>CDR2</i>
M258L (A772T), G464S (G1390A)	70	Homozygous	>256	A646V ^h	↑ <i>ERG11</i>
E266D (A798C), G464S (G1390A)	45	Homozygous	128	A646V ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	15	Homozygous	16	W478C ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	27	Homozygous	64	W478C ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>

Table 4.3. Continued.

A.A. substitution (mutation)	ID	Zygotity	FCZ MIC (µg/mL)	UPC2 mutation*	Other resistance mechanism*
D278N (G832A), G464S (G1390A)	43	Homozygous	128		↑ <i>CDR1</i> , <i>CDR2</i>
	51	Homozygous	256		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
	62	Homozygous	>256		↑ <i>CDR1</i> , <i>CDR2</i>
G307S (G919A), G448R	26	Homozygous	32		
	54	Homozygous	>256		↑ <i>ERG11</i> , <i>MDR1</i>
G450E (G1349A), I483V (A1309G)	23	Homozygous	32		↑ <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i>
A114V (C341T), E226D (A798C), H283R (A848G)	21	Homozygous	32	G648D ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
Y132F (A395T), T229A (A685G), F449L (T1345A)	59	Homozygous	>256	G648D ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
Y132F (A395T), V437I (G1309A) , F449L (T1345C)	71	Homozygous	>256	G648D ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
G307S (G919A), L403F (A1209T), G448R(G1342C)	46	Homozygous	128		
G307S (G919A), V437I (G1309A), Y447S (A1340C)	49	Homozygous	256		↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>

Table 4.3. Continued.

A.A. substitution (mutation)	ID	Zygoty	FCZ MIC (µg/mL)	UPC2 mutation*	Other resistance mechanism*
A114V (C341T), D153E (T459G), E266D (A798C), G450E (G1349A)	72	Homozygous	>256		↑ <i>CDR1</i> , <i>CDR2</i>
A114V (C341T), Y132F (A395T), E266D (A798C), V437I (G1309A)	47	Homozygous	128	G648D ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>
A114V (C341T), Y132F (A395T), E266D (A798C), V437I (G1309A)	47	Homozygous	128	G648D ^h	↑ <i>ERG11</i> , <i>CDR1</i> , <i>CDR2</i>

*Data previously published in **Flowers, S. A., K. S. Barker, E. L. Berkow, G. Toner, S. G. Chadwick, S. E. Gyga, J. Morschhauser, and P. D. Rogers.** 2012. Gain-of-function mutations in *UPC2* are a frequent cause of *ERG11* upregulation in azole-resistant clinical isolates of *Candida albicans*. *Eukaryot Cell* **11**: 1289-99 (50). ^hAmino acid substitution recovered in one of two *UPC2* alleles.

I initially examined the effects of mutant *ERG11* alleles containing a single amino acid substitution in the predicted protein sequence (**Table 4.4**). I identified 10 distinct *ERG11* alleles containing a single amino acid substitution (Q21L, Y132F, K134R, F145L, F449V, S405F, D446E, G448E, G450E and G464S). With the exception of amino acid substitutions Q21L and G448E, most *ERG11* mutations resulted in decreased susceptibility to fluconazole when compared to SC5314. Strains homozygously expressing K143R resulted in the strongest decrease in fluconazole susceptibility. Strains that carried two alleles containing amino acid substitutions Y132F, F145L, S405F, D446E, F449V G464S, and G450E had at least a four-fold increase in fluconazole MICs. No single mutation significantly affected itraconazole or voriconazole susceptibilities. These data suggest that structural differences between specific azoles affect activity against specific mutant *ERG11* alleles.

Multiple mutations in *ERG11* can result in decreased azole susceptibility

Previous observations show that combinations of *ERG11* mutations can lead to considerable increases in MICs to fluconazole. To examine the effects of multiple mutations on azole susceptibility, I selected a group of clinically occurring *ERG11* alleles that carried two amino acid substitutions. I was specifically interested in evaluating azole-susceptibilities of strains containing two amino acid substitutions, in which at least one substitution has been characterized alone (see above).

The K143R + Y132F was the strongest characterized combination resulting in increased FCZ MICs and VCZ MICs of ten-fold and four-fold respectively over what was observed for SC5314. Interestingly, this combination did not affect ICZ susceptibilities. Other notable increases in FCZ MICs were detected when characterizing the Y132F + F145L and the G307S + G450E combinations, both resulting in an increase in FCZ MIC of eight-fold over that of SC5314. Notably, the Y132F + F145L combination also demonstrated significant effects on both ICZ and VCZ with an increase of MICs by four-fold and eight-fold respectively. Notably, this was the only mutant *ERG11* allele characterized in this study that produced a significant decrease in ICZ susceptibility.

I characterized other combinations of polymorphisms that arose clinically in our collection. I483V + G450E in combination increased FCZ MICs six-fold over SC5314 and two-fold over what was observed for G450E as a single polymorphism. This combination did not result in significant changes to either ICZ or VCZ MICs. I observed a similar trend in investigating the effects of the addition of M258L when combined with G464S, in which the combination increased FCZ MICs by two-fold over G464S alone.

Table 4.4. Forty-eight hour MICs to FCZ, ICZ and VCZ measured by using a modified CLSI broth microdilution methods of *C. albicans* constructed to carry mutant *ERG11* alleles homozygously.

Erg11 SNP	48 hour MIC (µg/mL)		
	FCZ*	ICZ*	VCZ*
SC5314	0.25	0.016	0.016
Q21L	0.5	0.016	0.016
Y132F	2	0.016	0.03
K143R	4	0.06	0.03
F145L	1	0.03	0.016
S405F	2	0.03	0.016
D446E	1	0.016	0.016
F449V	1-2	0.03	0.016
G448E	0.5	0.03	0.016
G450E	1	0.03	0.016
G464S	1	0.03	0.016
K143R, E226D	4	0.016	0.016
Y132F, F145L	4-8	0.06	0.25
I483V, G450E	2	0.03	0.016-0.03
Y132F, K143R	8	0.03	0.06
F145L, E266D	1	0.03	0.016
D278N, G464S	4	0.016	0.016
E266D, G464S	1	0.016	0.016
M258L, G464S	2	0.016	0.03
G307S, G450E	4	0.016	0.016

*FCZ, fluconazole; ICZ, itraconazole; VCZ, voriconazole

Amino acid substitution E266D does not contribute to azole resistance

E266D was the most prevalent polymorphism detected by sequence analysis and occurred only in combination with other amino acid substitutions. To investigate the contribution of E266D to azole resistance, I compared the susceptibilities of *ERG11* alleles carrying one amino acid substitution alone to an allele containing an identical amino acid substitution combined with E266D. In our collection, K143R, F145L and G464S all clinically occurred as a single mutation and occurred combined with E266D in different isolates. In all three instances, I did not observe E266D to confer any additional effect on azole susceptibility beyond what was observed with the original amino acid substitution.

DISCUSSION

Resistance to azole antifungals in *C. albicans* is attributable to a number of distinct mechanisms. It is well established that activating mutations in transcription factors regulating genes encoding efflux pumps mediate resistance to azole antifungals. Previous work has shown specific mutations in the transcription factor gene *TAC1* mediate expression of ABC transporters *CDR1* and *CDR2*, resulting in increased azole resistance in *C. albicans* (22). Similarly, Mrr1 has been identified as the regulator of the major facilitator superfamily (MFS) transporter Mdr1 in azole-resistant isolates, and GOF mutations in this transcriptional regulator produce constitutive activation (43). I have previously characterized the expression of genes involved in azole resistance in this large group of clinical isolates with decreased fluconazole susceptibility (50). I identified several mutations in the transcription factor *UPC2*, which resulted in the constitutive activation genes involved in the ergosterol biosynthesis pathway, including *ERG11*. In this study, I determined the frequency, variability and the specific contributions of *ERG11* mutations from this characterized group of clinical isolates to azole resistance.

The cytochrome P450 (CYP) superfamily of enzymes contains more than 2500 members that can be roughly placed into two groups stratified by their function (<http://drnelson.uthsc.edu>) (98). In the first group, members metabolize a wide variety of xenobiotics while those in the second group generally participate in key biosynthetic processes such as sterol biosynthesis. Substrate specificity for this second group of P450s is narrow. Sterol 14 α -demethylase (Cyp51 or Erg11) is considered to be the most ancient CYP family because it is the only P450 class that is found in different kingdoms such as animals, plants and bacteria (130, 210). In 2009, the first observed virally encoded *CYP450* gene was observed and exhibited low-level sequence homology to *CYP51* genes although its function remains unknown (95). Despite only the 22-33% of sequence homology demonstrated between kingdoms, there are only four known substrates for this class of enzymes. This substrate preservation between kingdoms suggests that the Cyp51 structure is highly conserved regardless of low sequence homology (**Figure 4.1**). There are 41 amino acids conserved between the animal, bacterial and fungal Cyp51 enzymes (98). Of those 41 amino acids, there are three positions that are conserved in all CYP450

Figure 4.1. Cyp51 secondary structure is conserved despite low sequence homology between kingdoms.

Alignment of *C. albicans*, *T. cruzi*, *M. tuberculosis*, and Wheat Cyp51 proteins performed by UniProt www.uniprot.org. Pink highlights alpha helical structure and purple highlights beta sheets as predicted by crystal structure when available. Light blue highlighted heme coordinating cysteine is conserved in all CYP450 enzymes. *indicates conserved amino acid residues in all represented organisms. The following accession numbers were used: *C. albicans*-P10613; *T. cruzi*-Q7Z1V1; *T. aestivum*-P93596; *M. tuberculosis*-P0A512.

1	MAIVETVIDGINYFLSLSVTQQISILLGVPPFVYNLWV-QYLYSLRKDRAPLVFYWIPWFG	59	<i>C. albicans</i>
1	-----MFI-----EAIVLALTALILYSVSVKSFNTTRPTDPPVYPVTVPFLG	43	<i>T. cruzi</i>
1	-----MSAVALPRVSGGDEHG	17	<i>M. tuberculosis</i>
1	-----RPPPTIPGAPVVG	13	Wheat
			* *
60	SAASYGQQPYEFFESCRQKY-GDVFSFMLLGKIMTVYLGPKGHEFVFNAKLSDVSAEDAY	118	<i>C. albicans</i>
44	HIVQFGKNPLEFMQRCKRD LKS GVFTISIGGQRTIVGD PHEHSRFFSPRNEILSPREVV	103	<i>T. cruzi</i>
18	HLEEFRTDPIGLMQVRDEC-GDVGTFLAGKQVLLSGSHANEFFFRAGDDLDQAKAY	76	<i>M. tuberculosis</i>
14	GLLRFLRGPIPLIRAERYARL-GPVFTVPILTRITFLIGPDVSAHFFKSNESDMSQQEVY	72	Wheat
	: * :: . * : : : : . * . : . *		
119	KHLTPVFGKGVYDCPNLSRLMEQKKFAKFALTTDSFKRYVPKIREILNYFVTDESFKL	178	<i>C. albicans</i>
104	T-IMTPVFGEGVAYAAPYP RMREQLNFLAEELTIAKFQNFVPAIQHEVRKFMAEN-----	157	<i>T. cruzi</i>
77	F-FMTPIFGEGVVDASPERRKEML--HNAALRGEQMKGHAATIEDQVRRMIAD-----	127	<i>M. tuberculosis</i>
73	R-FNVPTFGPGVVDVDYQVRQEQRFFTEALRANKLRSYVDQMVAAEEYFSK-----	125	Wheat
	: . * * * * : * * : : : : : : :		
179	KEK-THGVANVMKTQPEITIFTASRSLFGDEMRRIFD-RSFAQLYSDLDKGFTPINFVFP	236	<i>C. albicans</i>
158	-WKDEGVINLLEDGCGAMINTACQCLFGEDLRKRLNARHFAQLLSKMESSLI PAAVFMF	216	<i>T. cruzi</i>
128	-WGEAGEIDLDDFAELTIYTSSACLIGKKFRDQLD-GRFAKLYHELERGTDPLAYVDP	184	<i>M. tuberculosis</i>
126	-WGESGTVDLKYELEHLIILTASRCLLGREVREKLF-DDVSALFHDLDNGMLPISVIFP	182	Wheat
	* : : * * : . * : * : : : * : : * . *		
237	N---LPLPHYWRDAAQKKISATYMKIKSRR-ERGDIDPNRDLIDSLIHSTYKDGWK-	291	<i>C. albicans</i>
217	WLLRLPLPQSARC REARAE LQKILGETIVAREKEEASKDNMTSDLLGGLLHAYVRDGTIR	275	<i>T. cruzi</i>
185	Y---LPIESFRRRDEARNGLVALVADIMNGRI-ANPPTDKSDRDLMDVLIAVKAETGTPR	240	<i>M. tuberculosis</i>
183	Y---LPIPAHRRRDQARTRLAEIFATIIKSRK-AS---GQSEEDMLQCFIDSKYKNGRQ-	234	Wheat
	** : * * : : : : * : : : : *		
292	MTDQEIANLLIGILMGQHTSASTSAWFLHLGKPH--LQDVIYQEVVELLKEKGGLN	349	<i>C. albicans</i>
276	MSLHEVCGMIVAAMFAGQHTSTITTSUSMLHLMHPKNKKWLDKLHKEIDEP-----A	328	<i>T. cruzi</i>
241	FSADEITGMFISMMFAGHTSSGTASWTLIELMRHRD--AYAAVIDELDELY----GDGR	294	<i>M. tuberculosis</i>
235	TTESEVTGLLIAALFAGQHTSSITSTWTGAYLLKFQ--YFAEAVEEQKEVMK---RHGD	289	Wheat
	* * * * * * * * * *		
350	DLTY-EDLQKLPSVNMNTIKETLRMHMPLHSIFRKVTNPLRIPE---TNYIYVPGHYVLVS	405	<i>C. albicans</i>
329	QLNVDNVMDEMPPFAERCVRRESIRDPDLLMVMRMVKAEEVKVGS-----YVVPKGDIIACS	383	<i>T. cruzi</i>
295	SVSF-HALRQIPQLENVLKETLRLHPPLIILMRVAKGEFEVQG-----HRIHEGDLVAAS	348	<i>M. tuberculosis</i>
290	KIDH-DILAEMDVLRYCIKEALRLHPPLIMLLRQSHSDFSVTTREGKEFDIPKGHIVATS	348	Wheat
	. : . : : : . : : : * . * * : * : : : * . : : * *		
406	PGYAHTSERYFDNPEDFDPTRWDTAAAKANSVSFNSDDEVYDGFVKVSKGVSSPYLPFGG	465	<i>C. albicans</i>
384	PLLSHHDEEAFFNPRLWDPERDEK-----VDGAFIGFGA	417	<i>T. cruzi</i>
349	PAISNRIPEDFPD PHDFVPARYEQPROED-L-----LNRWTWIPFGA	389	<i>M. tuberculosis</i>
349	PAFANRLPHIFKNPD SYDPDRFAAGREEDKV-----AGAFSYISFGG	390	Wheat
	* : . . * * : * : * * : : *		
466	GRHRCIGEQFAYVQLGTILTTFFVYNLRWTIDG-YKVPDPDYSSMVVLPTEPAEIIWEKRE	524	<i>C. albicans</i>
418	GVHKCIGQKQFALLQYKTI LATAFREYDFQLLR-DEVPDPDYHTMVVGPTLNQCLVKYTRK	476	<i>T. cruzi</i>
390	GRHRCVGAFAAIMQIKAI FSVLLREYEFEMAQPPESYRNDH SKMVVQLAQPA CVRYRRRT	449	<i>M. tuberculosis</i>
391	GRHGLGEPFAYLQIKAIWTHLLRNFEFELVSPFP--ENDWNAMVVGIGKEVMVNYKRRK	448	Wheat
	* * * : * * : * : : : : : * * * : : *		
525	TCMF-	528	<i>C. albicans</i>
477	KKLPS	481	<i>T. cruzi</i>
450	GV---	451	<i>M. tuberculosis</i>
449	LIVDN	453	Wheat

enzymes. One of these positions is a cysteine thiolate that coordinates the heme group while the other two are the ExxR salt bridge in the K-helix (98).

Sterols are an essential component of the eukaryotic membrane that functions to maintain fluidity as well as other regulatory processes. The azole class of antifungals function not only to inhibit ergosterol biosynthesis but also result in the accumulation of toxic methylated sterol precursors (24). The primary sterol in the fungal cell membrane is ergosterol and CYP51 in *C. albicans* is the rate limiting step in this biosynthetic process.

CaCyp51 catalyzes a three-step reaction that ultimately results in the demethylation of lanosterol. Each step requires one molecule of oxygen and NADPH. The azoles inhibit lanosterol demethylase by binding of the nucleophilic N₄ atom of the azole ring to the heme iron at its sixth coordinate position (162). The normal substrate for this enzyme is lanosterol and azole derivatives sit in the same binding pocket. Biochemical analysis show all azoles bind selectively to CaCyp51; however, K_d values show a two-to four-fold lower affinity to fluconazole when compared to itraconazole or voriconazole (200).

In agreement with previous studies, the majority of our clinical isolates carried point mutations in *ERG11* that lead to amino acid substitutions (122). In our collection, 87% of all isolates carried at least one nonsynonymous mutation that resulted in an amino acid substitution. Previous work has also defined mutations that occur in *ERG11* susceptible isolates which means the presence of an *ERG11* mutation may not be adequate to predict azole susceptibility. Substitutions in our collection were recovered in 26 distinct positions in which all but five were located in previously defined “hot spot” regions where *CaERG11* mutations frequently occur. In our constructed strains, most *ERG11* alleles containing single nucleotide changes resulted in meaningful changes in fluconazole MICs but no single mutation conferred significant changes in either itraconazole or voriconazole susceptibilities. K143R produced the strongest increase in fluconazole MICs in this study as a single amino acid substitution. Y132F also conferred increased resistance to fluconazole. Previous analysis performed with heterologous expression of CaCyp51(Y132H) in *S. cerevisiae* revealed a spectral change indicative of the inability of the azole nitrogen to interact in a typical fashion with the Cyp51 heme (85). Both the K143 and Y123 positions are conserved among fungal species and have been associated with resistance in previous work (5, 162).

Sanglard et al. have formally determined that Cyp51 amino acid substitutions S405F, Y132H, and G464S independently resulted in increased azole resistance when heterologously expressed in *S. cerevisiae* (162). The S405 position is a highly conserved residue of the Cyp51 in different organisms and this mutation is exclusively recovered from azole-resistant strains (122). In our collection, S405F was recovered in four distinct clinical isolates and was only recovered as a single polymorphism. In another conserved position, the G464S amino acid substitution also resulted in increased fluconazole resistance in strains carrying two mutant *ERG11* alleles. Previous spectral analysis of the G464S mutation reveals that this modification exerts its effects by a change in the heme environment(86).In this work, Kelly et al demonstrated the G464S substitution reduced affinity for fluconazole but the enzyme retained its catalytic activity.

In a fungal specific major insertion loop, I recovered multiple amino acid substitutions that occurred as single mutations and also combined with other amino acid substitutions. Modeling of amino acid residues at these positions is difficult due to the lack of fungal crystal structures. Homology modeling of this insertion loop was investigated previously in *P. pastoris* (5). Among fungal species, this unique sequence length varies and in *C. albicans*, this loop is comprised of 31 amino acid residues (5). Sequence analysis done for this loop in different fungal species show that the N-terminal portion of is variable in sequence and length while the C-terminal portion contains acidic residues followed by a more invariable portion with the motif DYG[FY]Gx[VI][ST]KG (**Figure 4.2**) (5). In this collection, amino acid substitutions were recovered at positions D446, G448, F449, and G450. These positions all occur within the C-terminal invariable motif within the fungal specific major insertion loop (**Figure 4.3**). Interestingly, mutations that occur independently at these position all increase fluconazole MICs by about fourfold in strains that express homozygous mutant *ERG11* alleles. No amino acid substitution at these positions either occurring as a single mutation, or when combined with another amino acid substitution affects susceptibilities to either itraconazole or voriconazole.

I also examined the collective effects of multiple mutations in *ERG11* that were derived from clinical isolates. Specifically, I was interested in characterizing the effects of amino acid substitutions that occurred with a mutation that had been characterized alone. The K143R + Y132F combination resulted in the strongest increase in FCZ MIC by increasing fluconazole and voriconazole MICs by ten-fold and four-fold respectively over what was observed for SC5314. This combination occurred independently in two isolates that were highly resistant to fluconazole (MIC ≥ 256 $\mu\text{g/mL}$) accompanied with increased expression of ABC transporters *CDR1* and *CDR2* in both isolates. F145L + Y132F were the only amino acid substitutions that significantly affected MICs to all azoles. Previous modeling in *Aspergillus fumigatis* defined F145 as a position that interacts with posaconazole (208). Structurally, itraconazole is similar to posaconazole and notably, this combination was the only combination of amino acid substitutions that significantly affected susceptibility to itraconazole. This combination of substitutions was also shown to catalytically impair Cyp51 when derived from a clinical *C. albicans* isolate. Investigation of Cyp51 function in *Aspergillus fumigatis* showed that positions G464S and G307 disturb the heme environment (208). Our observed susceptibility phenotypes show increased fluconazole MICs to G464S and the combination of G307S + G450E. The combination of G307S + G450E increased fluconazole MICs beyond what was observed with G450E as a single mutation.

Amino acid substitutions I483V, M258L, D278N, were demonstrated to have additive effects on fluconazole resistance when combined with an amino acid substitution that has been characterized alone. Substitution I483V has not been described previously in the literature and does not seem to be located near the active site when considering CaCyp51 secondary structure. When combined with substitution G450E, fluconazole MICs increase by two-fold over that which was observed for G450E alone. A similar trend was

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404 VSPGYAHTSERYFDNPEDFDPTRWDTAAAKANSVSFN-----SSDEVVDYGFVKVSKGVSS 458 C. albicans
408 VSPGYTHLRDEYFPNAHQFNIHRWNKDSASSYS-----VGEEVDYGFKAISKGVSS 458 S. cerevisiae
398 SSPGVTARDERHFDPRLRWDPHRWESRVEV-----EDSSDTVDYGYGAVSKGTRS 447 P. italicum
404 VSAGYAHTSDRWFEHPEHFNPRRWE SDDTKASAVSFN-----SEDTVDYGFVKISKGVSS 458 C. tropicalis
381 AAPGLTATEEEYFTHATDFDPKRWNDRVNE-----DENAEQIDYGYGLVTKGAAS 430 S. pombe
342 ISPGYTMLSERYFPNASEFQPHRWDEIKSIDGGISFGAEGENAKETVDYGFVKISKGVAS 401 C. krusei
347 ASPAISNRIPEDFPDPHDFVPPARYEQPRQEDLL-----NRW 382 M. tuberculosis
      : . : . * . : *::

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Figure 4.2. Sequence alignment highlighting the fungal specific major insertion loop in various fungal species and contrasted with the non-fungal species, *M. tuberculosis*.

Alignment was generated by UniProt www.uniprot.org. The red bracketed sequence defines the invariable sequence where we have observed amino acid modifications which affect fluconazole susceptibility. The following accession numbers were used: *C. albicans*-P10613; *C. tropicalis*-P14263; *S. cerevisiae*-P10614; *P. italicum*-Q12664; *S. pombe*-Q09736; *C. glabrata*-P50859; *C. krusei*-Q02315; *M. tuberculosis*-P0A512.

Figure 4.3. Observed amino acid substitutions in Erg11 in *C. albicans* compared to other medically important *Candida* species and organisms in other kingdoms.

The alignment was generated by using the UniProt alignment function www.uniprot.org. Amino acid numbering is based off the *C. albicans* sequence. Conserved amino acids are highlighted in blue. Amino acid substitutions highlighted in red are those observed in this study. Substitutions observed in black were noted in the literature. The following accession numbers were used: *C. albicans*-P1-613; *C. tropicalis*-P14263; *S. cerevisiae*-P1-614; *C. glabrata*-P50859; *C. krusei*-Q02315; Human-Q16850; *M. tuberculosis*-POA512; *T. Aestivum*-P93596.

observed for M258L and D278N, which both independently occurred with a G464S mutation resulting in a twofold and a fourfold increase in fluconazole MICs, respectively, over that which was observed for G464S alone. Neither the M258L nor the D278N modifications have been described previously in the literature. D278E was previously shown to occur in azole resistant isolates however, the exchange of an aspartic acid (D) for a glutamic acid (E) may be a more conserved substitution when compared to the asparagine (N) modification observed in our collection (**Table 4.3**) (111).

Not all mutations investigated contributed to azole resistance. *ERG11* is observed in our studies and others to be highly permissive to genetic polymorphisms. The most common mutation observed in this collection was E266D (n=10) and was always recovered with an additional substitution. In this study, I investigated the E266D substitution when combined with K143R, F145L and G464S independently. In all three cases, the addition of the E266D polymorphism did not confer additional effects on azole resistance beyond that which was observed for K143R, F145L and G464S alone. In the current study, I sequenced only *ERG11* alleles from isolates exhibiting decreased susceptibilities to fluconazole. It is most likely that mutations in *ERG11* occur in both azole-susceptible and resistant isolates that do not affect azole resistance. Another possibility is that mutations may arise in conjunction with mutations creating azole resistance that also affect fitness. Previous work has shown that specific *ERG11* mutations affect fitness and catalytic activity of the enzyme (166, 201). Further investigation of how *ERG11* mutations affect fitness is warranted.

One isolate contained a Q21L amino acid substitution in one *ERG11* allele with a wild-type *ERG11* allele. In membrane bound lanosterol demethylases, the far N-terminal region acts as a transmembrane portion of the enzyme that anchors this enzyme to the endoplasmic reticulum (38). This is the only mutation that I recovered in the far N-terminal region, and it was expressed with a wild-type allele. When expressed in SC5314, I did not observe a change in any azole susceptibilities at 48 hours. This data suggests that mutations occurring near the putative transmembrane region of the enzyme may not interfere with enzyme-substrate interaction.

Clearly, the susceptibility of clinical isolates is a product of the interplay of multiple mechanisms of resistance. Mutations in the *ERG11* genes have been shown to be a significant and prevalent mechanism of resistance in *C. albicans*. Notably, 19 clinical isolates carried *ERG11* mutations in addition to Upc2 GOF mutations. It is probable that the combination of these two mechanisms have a combinatorial effect on azole susceptibility. Our data clearly demonstrate that many *ERG11* mutations result in fluconazole resistance, but most are not as significant when tested against voriconazole or itraconazole. Itraconazole, in particular seems less effected by *ERG11* mutations which produce significant resistance to fluconazole. Despite this general observation, I have identified a specific combination of amino acid substitutions that significantly reduce itraconazole and voriconazole susceptibility. The association of clinically derived mutations in *ERG11* resulting in decreased azole susceptibility are of clinical importance. In this study, I limited our characterization to specific mutant *ERG11* alleles. Further

characterization of clinically derived *ERG11* mutant alleles to azole resistance is needed help guide antifungal pharmacotherapy and assist with future antifungal development.

CHAPTER 5. DISCUSSION AND CONCLUSIONS

GENERAL DISSERTATION OVERVIEW

In Chapter 2, I describe the characterization of known resistance mechanisms in a large group of clinical *C. albicans* isolates that were enriched for decreased fluconazole susceptibility. A transcriptional profile for each isolate was created for genes known to cause azole resistance such as *ERG11*, *CDR1*, *CDR2* and *MDR1*. Stratified into groups based on fluconazole susceptible vs. resistant phenotypes ($\text{MIC} \geq 8$), transcription profiles of genes known in azole resistance were then compared.

As *CDR1* and *CDR2* are transcriptionally co-regulated by Tac1, it was not surprising to observe the coordinate expression of these transporters. Increased expression of *CDR1* was observed in 77% of isolates with increased resistance to fluconazole as opposed to the overexpression of *MDR1*, which encompassed only 21%. There was no correlation between transcriptional expression of the ABC transporters and the MFS transporter *MDR1* in this group. This observation may be due to specific fitness costs associated with the overexpression of both these transporters in one isolate. In 2012, Sasse et al systematically investigated the effects of GOF mutations in the transcriptional regulators *MRR1*, *TAC1*, *UPC2*, and also mutations in *ERG11*, on associated fitness costs in *C. albicans* (166). In this work, the authors demonstrated that although combinations of mechanisms of drug resistance resulted in a step-wise acquisition of high level resistance, there was also an associated decrease in fitness when tested in non-selective conditions *in vitro* and *in vivo*. Mutations associated with the greatest decrease in fitness were the combination of a GOF mutation in *TAC1* and *MRR1* in isolates constructed to carry two mechanisms of resistance. The addition of a GOF *UPC2* allele resulted in a further decrease in fitness. In the absence of any compensatory mutations that might arise in a clinical isolate, this work demonstrated that loss of the wild-type regulation of gene expression is associated with a decrease in fitness. I believe these fitness costs may explain why simultaneous overexpression of *MDR1* and *CDR1/CDR2* are not observed in this group of clinical isolates.

In addition to efflux transporter expression, I also measured the transcriptional profile of *ERG11*, whose gene product is the target of azole antifungals. These data showed increased expression of *ERG11* occurred in 75% of our clinical isolate collection. For those that overexpressed *ERG11* when compared to fluconazole-susceptible isolates, I sequenced a known regulator of ergosterol biosynthesis *UPC2*. Previous to this study, only three distinct *UPC2* GOF mutations had been identified to occur clinically. Our sequenced data introduced five previously undescribed GOF mutations in *UPC2* and also established that these mutations occurred frequently in azole-resistant isolates.

As has been described for activating mutations in zinc-cluster transcription factors in *S. cerevisiae*, all of the mutations observed in this study occurred in the C-terminal putative activation domain of *CaUPC2*. In *C. albicans*, *UPC2* is orthologous to *UPC2* and *ECM22* in *S. cerevisiae*, which regulate ergosterol biosynthesis and uptake of

exogenous sterols. Both transcription factors are localized to the intracellular membrane of the endoplasmic reticulum but under sterol-depleted activating conditions, they localize around the nucleus (115). Gain-of-function mutations have been identified in both *ScUPC2* and *ScECM22* and in each instance, mutations identified occur in the C-terminal activation domain (197). There are two theories that describe how GOF mutations result in constitutive activation of *UPC2*. First, mutations in the C-terminus may interfere with the transmembrane region of the protein resulting in constitutive localization to the nucleus and constitutive activation of target genes. The second theory is that mutations in the C-terminal area could relieve Upc2 of a repressor that would otherwise keep Upc2 in an inactive state in non-activating conditions (177).

The most widely characterized zinc-cluster transcription factor in yeast is Gal4. Gal4 regulates genes involved in the transport and metabolism of galactose (20). As has been shown in *UPC2* localization studies in *S. cerevisiae*, Gal4 is localized to the cytoplasm and upon activation, is directed towards and imported into the nucleus. Gal4 is inhibited by repressor protein Gal80 in the absence of galactose. Previous work has shown that the carboxy-terminal 28 amino acids are responsible for the sequence specific interaction between these two proteins (161). A single amino acid substitution in this region is sufficient to constitutively activate target genes by relieving interaction of Gal4 with Gal80 (161). Notably, a mutation in Gal80 has been shown to compensate for a GOF mutation in Gal4, resulting in a wild-type phenotype. Adding another level of regulation in Gal4 activation is the detection of mutant Gal3 proteins that show altered Gal80-binding characteristics and result in constitutive activation of Gal4 (14).

A repressor protein for *UPC2* or *ECM22* has not been identified in either *S. cerevisiae* or in *C. albicans* but it is plausible that these mutations interfere with this interaction. In addition to relief of a repressor, post-transcriptional modifications occur with Gal4 by the phosphorylation of a series of C-terminal serine residues which are thought to “fine tune” activation and may regulate ubiquitin degradation (160). Activating mutations observed in CaUpc2 have not been shown to occur on any serine residues but using an online phosphorylation site identifier (<http://www.cbs.dtu.dk/services/NetPhos/>), highlights potential phosphorylation sites identified at positions S469, S533 and S575.

In strains constructed to carry a GOF allele in *UPC2*, I observed that homozygous replacement of the GOF *UPC2* allele showed a greater effect on azole susceptibility than mutations expressed with a wild-type allele. This is consistent to what is observed in GOF mutations in *TAC1* and *MRR1*. When comparing genome-wide transcriptional analysis of four distinct *UPC2* GOF mutations I observed a core set of genes to be upregulated. Expectedly, among these genes were those involved in oxidoreductase activity and those involved in ergosterol biosynthesis.

Summary and discussion of Chapter 3

In Chapter 3 I explored the hypothesis that mutations in *NDT80* might result in overexpression of *ERG11* in azole resistant isolates that do not carry mutations in *UPC2*. Ndt80 is a transcriptional regulator that in *C. albicans* has been shown to regulate *ERG11* expression. *ERG11* is a direct target of Ndt80 and its disruption results in reduced *ERG11* expression and increased azole susceptibility. Clinical isolates 945 and 1619 are genetically matched isolates comprised of a susceptible and resistant constituent, in which the resistant isolate overexpresses *ERG11* and does not carry mutations in *UPC2*. I therefore sequenced *NDT80* in both isolates and found several mutations to be present. Suspecting these mutations as being the cause of *ERG11* overexpression in these isolates, I expressed mutant *NDT80* alleles derived from the fluconazole-susceptible and –resistant isolates in SC5314. I did not observe a change in fluconazole resistance or *ERG11* expression in strains carrying mutant *NDT80* alleles. While this does not preclude the involvement of mutations in *NDT80* in azole resistance, such mutations do not explain the overexpression of *ERG11* in these clinical isolates.

Summary and discussion of Chapter 4

In Chapter 4, I characterized clinically derived mutations in the *ERG11* gene and their effects on the activity of a panel of structurally different azole antifungals. I was able to demonstrate that distinct amino acid substitutions in Erg11 produced a variable increase in resistance to fluconazole, itraconazole and voriconazole. I also observed trends to placement of the resulting amino acid substitutions in the enzyme and the level of resulting resistance to fluconazole.

Both voriconazole and itraconazole susceptibilities were not as affected by mutations in *ERG11* when compared to fluconazole. It is likely that structural differences resulting in stronger binding of newer generation azoles make them less vulnerable to the effects of *ERG11* mutations. Itraconazole seemed to be the least susceptible to *ERG11* mutations, with only the F145L + Y132F combination resulting in a significant increase in MIC. Although itraconazole retains its activity against *ERG11* mutations and previous work has shown that itraconazole is not a substrate for Mdr1 efflux transport, the utility of this agent in the treatment of invasive candidiasis is wanting due to its erratic bioavailability and the lack of an intravenous formulation.

As discussed earlier, despite the low sequence homology of Cyp51 enzymes between species, the secondary structure is highly conserved and specific amino acid positions are conserved within fungal organisms and even between kingdoms. Its logical that as these positions are conserved between species, their role in the structure and function of Cyp51 likely important. In this collection, we observed and characterized mutations occurring both in conserved and non-conserved positions in the Cyp51 sequence. A general observation was that amino acid substitutions occurring in conserved positions resulted in fluconazole resistance. The exception to this observation was the Q21L substitution, which is conserved in fungal species yet did not result in increased MICs to azoles.

Likewise, amino acid substitutions occurring in non-conserved positions, such as E266D did not affect fluconazole susceptibility and are not associated with azole-resistant isolates in this study or in previous publications (122). Over 150 missense mutations in *ERG11* have been documented in the literature and characterizing mutations as I have by constructing strains that carry these mutations is laborious and slow. Generally, mutations characterized in this study that result in a change in azole resistance are those that arise in clinically resistant isolates and are in conserved positions in the Cyp51 enzyme. As sequencing can now be efficiently and cost-effectively performed on a large scale, correlating mutations to azole resistance can be done statistically in the future once sufficient data is available.

Although several homology models for fungal Cyp51 structures have been published, a crystal structure for this enzyme is not public. Many publications on fungal Cyp51 structure cite the difficulty of obtaining a crystal for membrane bound enzymes. Despite this difficulty, X-ray structures for 25 membrane bound Cyp51 enzymes are available, including that for humans(38). As Cyp51 for *C. albicans*, as well as other fungal species, is the most important and exploited target for antifungals, the addition of a CaCyp51 crystal structure would aid in the development of better azole antifungals.

DISCUSSION OF METHODS

The studies discussed in this dissertation describe the use of a variety of molecular techniques to quantify gene transcription involved in mechanisms of resistance and the construction of strains that carry mutant genes derived from resistant isolates in a wild-type background strain. With these constructed strains, I was able to specifically identify the specific contribution to azole resistance and to identify key determinants of azole resistance in this species.

Gene-quantification

To quantify genes involved in azole resistance, I used quantitative real-time PCR (RT-PCR), which is the standard method of gene quantification. This method has several advantages over other methods of gene quantification due to its increased sensitivity and quantitative capabilities. To quantify gene expression, I used the Comparative C_t ($2^{-\Delta\Delta C_t}$) method. Using this model, I can calculate changes in gene expression as a relative fold difference between an experimental and a calibrator sample. This method requires similar amplification kinetics of both the target and reference gene because different efficiencies will generate errors with this method. Therefore, for each gene measured, a validation assay using serial dilutions of cDNA target are assayed for each gene of interest and the results are plotted with the log-input concentration for each dilution on the x-axis, and the difference for C_t dilution on the y-axis. This particular collection was comprised of a large number of unrelated clinical isolates. Most expression experiments of this nature are performed with genetically match-paired isolates that differ only in azole-susceptibility phenotypes. As none of our isolates are related, I averaged the gene

expression data from azole susceptible isolates to compared expression of genes of interest to our resistant isolates.

Studies have shown that genes involved in azole resistance are regulated at the transcriptional level in response to azole drugs, and not to gene duplication events (105). Although transcription of specific genes is correlated with azole resistance, using methods that only quantify RNA could be a limitation to this work. Manoharlal et al. demonstrated that in addition to increased transcription initiation of *CDRI*, *CDRI* mRNA stability half-life was threefold higher in an azole-resistant strain when compared to an azole-susceptible counterpart (112). Although correlating gene expression with resistance in *C. albicans* is standard in the literature, this technique neglects regulation that could occur post-transcriptionally or post-translationally that could be contributing to an azole-resistant phenotype.

Genetic manipulation in *C. albicans*

The *SAT1*-flipper technique was utilized to perform targeted gene inactivation and replacement. The *SAT1* flipping strategy allows for genetic manipulation to occur in prototrophic *C. albicans* strains using a dominant drug selection marker that is recyclable, which is important in diploid species(156). This method uses a *CaSAT1*-selectable marker which enables resistance to the aminoglycoside nourseothricin and also a *CaFLP* gene, which encodes a site-specific recombinase for cassette excision and selection marker recycling. Previous to the technique, targeted gene disruption occurred in specific auxotrophic background strains using nutritional markers for the selection of transformants. However, use of auxotrophic markers made it difficult to interpret mutant phenotypes as these genes were shown to modulate processes such as virulence.

Although the *SAT1* flipper method has resolved difficulties associated with the use of auxotrophic strains, there are other complications associated with genetic manipulation of *C. albicans*. As stated in the introduction, genetic plasticity in the *C. albicans* genome is well described and development of aneuploidy and chromosomal rearrangements occur readily under a wide variety of events including antifungal drug exposure. Aneuploidies of selected chromosomes are not uncommon in laboratory strains (96, 172). Chromosomal aneuploidies and truncations have been described in laboratory strains as a result of genetic modification like what is described in this dissertation. It is clear that detection of chromosomal ploidy is important in this species. As we do not have a cot-effective mechanism to routinely detect this in our lab, I have constructed duplicates of each strain to ensure reproducibility of the phenotypes I have observed in these mutant strains.

Measuring antifungal susceptibility

To measure antifungal susceptibility, we used a modified version of the Clinical Laboratory and Standards Institute microbroth dilution method. The modification of this

protocol is the addition of 2% dextrose to RPMI media, which has been observed to decrease the amount of trailing associated with azole fungistatic activity in this species. Reducing the amount of trailing is important for interpretation of data. In addition to standard broth dilution methods, we occasionally used azole treated E-test strips (Biomérieux) as these interpretations of MICs are less subjective. At times, instead of RPMI, we test azole susceptibilities in rich YPD media in order reveal smaller changes resulting from certain genes. Potential drawbacks of using YPD media for susceptibility testing are that media varies based on preparation and one cannot make associations of susceptibility to clinical breakpoints, as those are performed in RPMI.

The ability to incorporate exogenous sterols into the fungal cell membrane has been shown for *S. cerevisiae* and *C. glabrata* but has not yet been shown for *C. albicans*. If this process occurs, it may be that testing MICs in media without exogenous sterols may underrepresent resistance phenotypes. If a particular isolate has an increased ability to incorporate sterols from the environment, inhibition of ergosterol synthesis could be bypassed. As yeast extract is a component of YPD, this may be partly contributing the increased growth and higher MICs resulting from susceptibility testing in this media.

OVERALL THEME AND CONCLUSIONS

Throughout the research presented in this dissertation, there is one recurring theme which deserves attention. The work presented here is the largest comparison of resistance mechanisms that have been defined in a group of azole-resistant clinical isolates of *C. albicans*. Prior to this work, the primary focus of azole resistance was on efflux transporters *CDR1*, *CDR2* and *MDR1*. It was not surprising that overexpression of efflux transporters was well represented in this collection. Notably, expression data in this work revealed the considerable involvement of *ERG11* overexpression which had previously taken a back seat to the involvement of efflux transporters. Overexpression of *ERG11* seems to be something specific for *C. albicans*, as smaller population studies have not shown increased *ERG11* expression to be prevalent in *C. glabrata* or *C. dubliniensis*. Also in this group, we defined the significance of individual mutant *ERG11* alleles on azole resistance. Numerous sequencing data has demonstrated *ERG11* is highly tolerant of mutations in both azole-susceptible and resistant isolates. These data corroborated with sequencing data for this collection. Large scale phenotypic characterization of these mutations done in this work demonstrated *ERG11* mutations were a prevalent contributor to azole resistance in *C. albicans*.

FUTURE DIRECTIONS

The long term goal of our research is to improve the treatment of *Candida* infections by understanding the molecular basis of azole resistance. Ultimately, part of goal will be to develop improved options for the treatment of candidiasis. Currently, there are only 4 systemic therapies available for the treatment of fungal disease. Despite the increasing need for new antifungal classes to treat candidiasis, antifungal drug discovery is limited

by the detection of novel and specific antifungal targets. The azole class of antifungals is presently our only oral option for the treatment of systemic disease but as shown in this work, there are clinical isolates that have become highly resistant to these agents. In Chapter 4, I investigated several mutations in *ERG11*, some of which caused an increase in voriconazole and itraconazole susceptibility to the susceptible dose-dependent range. In this study, I was only able to characterize a subset *ERG11* alleles observed, but there are probably specific mutant alleles, like those which contain 3 or 4 amino acid substitutions, which may confer a greater effect on azole resistance. The more information obtained on *ERG11* mutations and their specific effect azole resistance may shed light on how to better target Erg11 either by construction of improved members of the azole class or by development of a new compound.

Although my contribution to characterizing *ERG11*-mediated azole resistance in *C. albicans* is nearly complete, there are areas in this project that warrant further exploration. I present here some future directions made possible from the groundwork prepared by these data.

Whole genome sequencing to investigate *UPC*-independent mechanisms of *ERG11*-overexpression

Our lab and others have observed *ERG11* overexpression in isolates that do not contain GOF mutations in *UPC2*. In Chapter 3, I investigated the role of mutant *NDT80* alleles derived from resistant isolates in *ERG11* overexpression but was unable to demonstrate a role for this transcription factor in regulating azole resistance. The mechanism of *ERG11* overexpression in these isolates remains unknown. The strategy of targeted gene sequencing based on genome-wide expression data or educated guesses are time consuming and largely yield negative results. As there are potentially several genes involved in azole resistance that have not been identified, investigations of this nature can now be undertaken on a genome-wide scale.

As transcription factors have played a primary role in many key drug resistance and pathogenic processes, exploring mutations that occur in these genes containing missense polymorphisms in resistant isolates will be an obvious and fruitful choice to investigate further. As someone who has spent considerable time exploring sequencing data of this type, I have further suggestions of possible genes and pathways to explore in future studies.

First, I recommend that in addition to mutations in *ERG11*, polymorphisms occurring in other components of the ergosterol biosynthesis pathway be explored. Studies have already shown that azole- and amphotericin B- resistant species of *Candida* display alternate sterol profiles. Additionally, as microarray data show an increased expression of genes important to ergosterol biosynthesis in response to chemical inhibition, I suspect that decreased ergosterol production resulting from mutations in the *ERG* pathway may also result in increased *ERG11* expression.

A next generation sequencing study performed in a series of clinical *C. glabrata* isolates has already identified a gene mutation that arises after *FKS* mutations that result in ameliorating fitness costs of echinocandin resistance (178). As similar fitness defects occur in azole-resistant *C. albicans* strains, detection of genes that restore fitness in clinical isolates would represent an additional mechanism of resistance in this species.

In the future, proteins required for pathogenicity in fungus may be considered a potential drug targeting strategy. One unit of chromatin is defined as 146bp of DNA wrapped around a histone complex comprised of two histone H2A-H2B dimers and one histone (H3-H4)₂ tetramer(103). Histone proteins play a role in tightly packaging DNA into nucleosomes and association to a tightly wound chromatin is associated with gene silencing. Data from genome-wide transcription profiles suggest that wide-spread transcriptional networks are activated in response to antifungals and pathogenesis, which suggests some level of chromatin regulating events (175). Specific histone modifications result in either the relaxing of the histone tail permitting access of transcriptional machinery to coding genes or tightening of the histone complex resulting in gene silencing. The role of various histone modification enzymes have already been investigated regarding pathogenesis and even antifungal resistance in *Candida* species as nicely reviewed by Lopes de Rosa and Kaufman (84). Identification of mutations that modulate chromatin structure in resistant isolates would further delineate drug resistance mechanisms and perhaps identify future drug targets.

Employing genotypic testing as a tool to guide antifungal therapy

As mentioned earlier, the CLSI guidelines have been recently modified for both azoles and echinocandins. Newer clinical breakpoints take into account species specific epidemiological cutoff values (ECV), resulting in species specific breakpoints to help guide therapy. Revising these breakpoints may allow us to detect *Candida* species and isolates that are a risk for the development of resistance. Although recent data show that *C. albicans* as a species remains largely susceptible to the azole class, this work demonstrates that azole resistance does arise in clinical isolates. As a means to quickly and efficiently guide treatment as well as to track resistance patterns in *Candida* species, genotypic data could potentially play a large role in guiding antifungal treatment in the future.

As an example of this type of analysis, Shields et. al described the relationship between echinocandin MICs and patient responses to therapy in *C. glabrata* (176). Authors extracted information from medical records (prior echinocandin exposure, duration of treatment, patient outcomes) and tied this with microbiologic data such as caspofungin susceptibilities and occurrence of *FKS* mutations. This study produced a paradigm defining a method-specific caspofungin susceptibility threshold that was sensitive and specific enough to detect the presence of *FKS* mutations in *C. glabrata*. Of direct clinical importance, authors also identified prior echinocandin use and caspofungin MIC over the defined threshold (MIC > 0.25 µg/mL for E test) as the most sensitive predictors of treatment outcomes.

In *C. albicans*, azole resistance in clinical isolates is multifactorial but there is extensive data defining key mutations in genes resulting in a resistant phenotype. In a similar manner, it would be significant to use these data to further define treatment paradigms in an effort to detect resistant organisms and to improve patient outcomes.

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APPENDIX A. *C. ALBICANS* STRAINS TABLE

Strain #	Modified strain identification	Source if known	Relevant characteristics or genotype ^a	Source	UPC2 polymorphisms observed (amino acid substitutions)
Clinical isolates					
SC5314	SC5314		UPC2-1/ UPC2-2	ATCC	
20644.082	1		FLU ^S isolate	University of Iowa	
20205.043	2	Blood	FLU ^S isolate	University of Iowa	
20339.016	3	Esophageal	FLU ^S isolate	University of Iowa	G1791A (M597I)
20389.007	4		FLU ^S isolate	University of Iowa	
20646.057	5	Blood	FLU ^S isolate	University of Iowa	
20313.046	6	Throat	FLU ^S isolate	University of Iowa	
20510.016	7	Abdom.Fluid	FLU ^S isolate	University of Iowa	
20464.007	8	Sput	FLU ^S isolate	University of Iowa	
20646.057	9	Blood	FLU ^S isolate	University of Iowa	
20324.046	10	Blood	FLU ^S isolate	University of Iowa	C449T (P150L); A817G (T273A)
20512.088	11		FLU ^R isolate	University of Iowa	
20512.089	12		FLU ^R isolate	University of Iowa	
20533.031	13		FLU ^R isolate	University of Iowa	G1927A (A643T)
20637.057	14	Blood	FLU ^R isolate	University of Iowa	
20513.024	15		FLU ^R isolate	University of Iowa	G1434C (W478C)
20288.098	16		FLU ^R isolate	University of Iowa	
20642.062	17		FLU ^R isolate	University of Iowa	
20513.009	18		FLU ^R isolate	University of Iowa	
20513.01	19		FLU ^R isolate	University of Iowa	G910A (G304R)
20513.011	20		FLU ^R isolate	University of Iowa	G910A (G304R)

Appendix A. Continued.

Strain #	Modified strain identification	Source if known	Relevant characteristics or genotype ^a	Source	UPC2 polymorphisms observed (amino acid substitutions)
20513.016	21		FLU ^R isolate	University of Iowa	G1943A (G648D)
20245.061	22	Abdom.Fluid	FLU ^R isolate	University of Iowa	
20323.066	23	Throat	FLU ^R isolate	University of Iowa	
20339.013	24	Esophageal	FLU ^R isolate	University of Iowa	
20339.027	25	Oral	FLU ^R isolate	University of Iowa	G1942A (G648S)
20339.063	26	Esophageal	FLU ^R isolate	University of Iowa	
20513.025	27		FLU ^R isolate	University of Iowa	G1434C(W478C)
20513.026	28		FLU ^R isolate	University of Iowa	
20408.049	29	Tissue	FLU ^R isolate	University of Iowa	
20531.041	30	Blood	FLU ^R isolate	University of Iowa	C1928T (A643V)
20628.078	31		FLU ^R isolate	University of Iowa	
20288.080	32	Tissue	FLU ^R isolate	University of Iowa	
20323.052	33	Throat	FLU ^R isolate	University of Iowa	
20339.014	34	Esophageal	FLU ^R isolate	University of Iowa	
20339.028	35	Esophageal	FLU ^R isolate	University of Iowa	G1942A (G648S)
20339.044	36	Oropharynx	FLU ^R isolate	University of Iowa	A1925T (Y642F)
20339.045	37	Buccal	FLU ^R isolate	University of Iowa	A1925T (Y642F)
20339.027	38	Oral	FLU ^R isolate	University of Iowa	G1943A (G648D)
20339.062	39	Esophageal	FLU ^R isolate	University of Iowa	
20408.059	40	Blood	FLU ^R isolate	University of Iowa	
20348.024	41	Sputum	FLU ^R isolate	University of Iowa	
20512.090	42		FLU ^R isolate	University of Iowa	
20339.003	43	Buccal	FLU ^R isolate	University of Iowa	
20339.025	44	Oral	FLU ^R isolate	University of Iowa	

Appendix A. Continued.

Strain #	Modified strain identification	Source if known	Relevant characteristics or genotype ^a	Source	UPC2 polymorphisms observed (amino acid substitutions)
20339.048	45	Esophageal	FLU ^R isolate	University of Iowa	C1937T (A646V)
20339.064	46	Esophageal	FLU ^R isolate	University of Iowa	
20339.072	47	Esophageal	FLU ^R isolate	University of Iowa	G1943A (G648D)
20512.099	48		FLU ^R isolate	University of Iowa	
20377.041	49	Pleural Fl	FLU ^R isolate	University of Iowa	
20323.080	50	Throat	FLU ^R isolate	University of Iowa	
20339.008	51	Oral	FLU ^R isolate	University of Iowa	
20339.058	52	Esophageal	FLU ^R isolate	University of Iowa	G1943A (G648D)
20672.028	53	Blood	FLU ^R isolate	University of Iowa	
20495.091	54	Abscess	FLU ^R isolate	University of Iowa	C406T (P136S), A671G (N224S), A749G (N250S), T1952C (M651T)
20513.014	55		FLU ^R isolate	University of Iowa	G1942A (G648S)
20513.021	56		FLU ^R isolate	University of Iowa	G1942A (G648S)
20513.022	57		FLU ^R isolate	University of Iowa	G1942A (G648S)
20513.023	58		FLU ^R isolate	University of Iowa	G1942A (G648S)
20430.009	59	Blood	FLU ^R isolate	University of Iowa	G1943A (G648D)
20288.030	60	Tissue	FLU ^R isolate	University of Iowa	
20323.083	61	Throat	FLU ^R isolate	University of Iowa	
20339.006	62	Oral	FLU ^R isolate	University of Iowa	
20339.011	63	Esophageal	FLU ^R isolate	University of Iowa	
20339.015	64	Oral	FLU ^R isolate	University of Iowa	
20339.020	65	Oropharynx	FLU ^R isolate	University of Iowa	G1942A (G648S)
20339.021	66	Oropharynx	FLU ^R isolate	University of Iowa	
20339.022	67	Oropharynx	FLU ^R isolate	University of Iowa	G1942A (G648S)

Appendix A. Continued.

Strain #	Modified strain identification	Source if known	Relevant characteristics or genotype ^a	Source	<i>UPC2</i> polymorphisms observed (amino acid substitutions)
20339.030	68	Oropharynx	FLU ^R isolate	University of Iowa	A817G (T273A), C1928T (A643V)
20339.032	69	Oropharynx	FLU ^R isolate	University of Iowa	G1942A (G648S)
20339.049	70	Esophageal	FLU ^R isolate	University of Iowa	C1937T (A646V)
20339.070	71	Oral	FLU ^R isolate	University of Iowa	G1943A (G648D)
20513.02	72		FLU ^R isolate	University of Iowa	

FLU^S; fluconazole susceptible. FLU^R; fluconazole resistant

APPENDIX B. GENES UPREGULATED IN STRAINS CARRYING *UPC2* GOF ALLELES BY AT LEAST 1.5 FOLD

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
<i>ANP1</i>	orf19.3622	1.9	1.5	1.5	1.2	1.3	1.4	1.1	1.0	contributes_to alpha-1,6-mannosyltransferase activity (IEA)
<i>FRE9</i>	orf19.3538	2.1	1.5	1.2	1.4	1.0	1.3	1.2	1.7	ferric-chelate reductase activity (IEA)
<i>GST1</i>	orf19.3121	7.8	2.0	38.0	1.2	16.4	0.9	13.5	0.9	transcription corepressor activity (IEA)
<i>IFC1</i>	orf19.3746	1.9	1.8	0.9	0.9	1.3	0.7	1.2	0.8	oligopeptide transporter activity (IGI, ISS)
<i>LTE1</i>	orf19.2238	18.2	2.0	9.8	0.5	19.1	1.3	10.1	0.8	guanyl-nucleotide exchange factor activity (IEA)
<i>NBP35</i>	orf19.747	1.8	1.5	2.1	1.4	1.6	1.0	2.1	1.2	nucleotide binding (NAS) 4 iron, 4 sulfur cluster binding (IEA)
<i>PHO114</i>	orf19.4584	1.7	1.6	2.2	1.4	1.8	0.9	2.0	1.3	acid phosphatase activity (IEA)
<i>PLB1</i>	orf19.689	1.6	2.0	1.9	1.2	1.3	1.1	1.4	1.1	lysophospholipase activity (IDA, IMP, ISS)
<i>PLB4</i>	orf19.1443	1.6	1.5	2.0	1.3	1.4	1.3	1.5	1.3	lysophospholipase activity (ISS)
<i>PRR2</i>	orf19.1341	1.5	2.5	2.9	1.1	0.9	0.9	1.1	1.6	receptor signaling protein serine/threonine kinase activity (IEA)
<i>RBT1</i>	orf19.1327	1.9	1.6	1.2	1.1	1.6	1.9	1.1	1.2	
<i>SMC3</i>	orf19.262	1.5	1.7	1.3	0.9	1.8	0.8	1.4	0.7	ATPase activity (IEA)
<i>SUA72</i>	orf19.3519	1.5	1.7	2.3	0.8	1.3	1.0	1.7	1.2	general RNA polymerase II transcription factor activity (IEA)
<i>ZRT1</i>	orf19.3112	1.5	1.6	1.6	1.4	1.1	0.8	1.2	1.0	low-affinity zinc ion transmembrane transporter activity (IEA)
	orf19.11618	1.5	1.6	1.6	0.7	0.9	1.0	1.8	0.8	
	orf19.1768	1.6	1.7	1.3	1.0	1.6	1.1	1.6	0.9	
	orf19.302	1.7	1.5	1.3	1.1	1.0	0.7	1.4	1.2	contributes_to Rab guanyl-nucleotide exchange factor activity (IEA)
	orf19.3156	2.1	1.5	1.9	1.0	1.7	1.0	1.5	1.0	
	orf19.3813	1.5	1.8	2.7	1.4	1.4	1.0	2.4	1.1	
	orf19.3926	1.6	15.1	1.2	5.6	0.7	8.9	0.3	1.0	endoribonuclease activity (IEA)
	orf19.3976	1.5	2.0	0.8	0.9	1.6	1.0	1.5	1.0	structural constituent of cytoskeleton (IEA)
	orf19.4276	1.8	2.5	2.2	0.6	1.8	1.7	1.2	1.0	
	orf19.4711	3.8	1.7	4.9	1.4	1.7	1.0	3.7	0.9	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.4735	1.5	1.6	1.5	1.1	1.2	0.8	1.4	0.8	
	orf19.4923	1.5	2.4	1.4	1.9	1.1	1.1	1.3	1.9	sugar transmembrane transporter activity (ISS)
	orf19.5693	1.5	1.5	1.6	1.4	1.1	1.4	1.3	1.4	GPI-anchor transamidase activity (IEA)
	orf19.6351	1.7	1.7	1.3	1.4	2.4	0.9	1.5	1.0	
	orf19.6899	1.6	2.5	0.6	0.4	0.7	0.9	0.3	0.4	
	orf19.6950	1.5	2.2	0.9	1.0	1.5	1.2	1.1	1.0	
	orf19.6979	1.8	1.5	2.2	0.9	1.6	1.1	1.3	1.0	
	orf19.6982	1.7	1.5	1.4	1.3	1.7	1.1	1.4	1.1	
	orf19.7193	1.7	1.5	1.0	1.1	1.3	0.8	1.0	0.8	
	orf19.7303	3.5	2.8	1.8	0.7	1.4	1.2	3.8	1.3	
	orf19.911	2.3	1.6	1.8	1.2	1.4	1.2	1.9	0.9	ubiquitin-protein ligase activity (IEA)
	orf19.980	1.7	1.5	1.9	1.3	0.8	1.3	0.8	1.1	
ADO1	orf19.5591	1.4	1.3	1.6	1.6	1.4	1.5	1.5	1.4	adenosine kinase activity (IEA)
AMS1	orf19.2768	1.1	2.8	4.2	2.0	1.3	0.8	1.3	1.3	alpha-mannosidase activity (IEA)
ASR1	orf19.2344	0.6	3.2	2.1	1.8	0.6	1.0	0.9	1.2	
BUD16	orf19.1828	2.5	0.9	2.7	1.5	2.1	0.8	2.1	0.9	
CAS1	orf19.1135	1.1	1.2	1.5	1.5	1.2	1.0	1.4	1.5	
CAT1	orf19.6229	1.2	1.7	1.7	1.8	1.3	1.5	1.4	1.8	catalase activity (IDA, IMP, ISS) protein binding (IDA)
CAT2	orf19.4591	1.1	1.4	1.5	1.6	1.1	1.1	1.5	1.4	
CAT8	orf19.5097	1.6	0.7	2.0	2.1	1.0	1.1	2.4	1.1	
CDC23	orf19.6437	1.1	1.1	1.6	1.5	1.3	1.1	1.5	1.4	protein binding (IEA) ubiquitin-protein ligase activity (IEA)
CTA4	orf19.7374	1.4	1.3	2.0	1.6	1.7	1.4	1.9	1.3	transcription activator activity (IDA, IGI)
CYB2	orf19.5000	1.1	1.3	1.8	1.5	1.2	0.9	1.5	1.1	L-lactate dehydrogenase (cytochrome) activity (NAS)
DAL9	orf19.6956	0.9	1.6	2.0	1.6	0.9	1.2	1.3	1.7	anion:cation symporter activity (ISS)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
<i>ECM29</i>	orf19.6773	1.1	1.2	1.5	1.5	1.3	1.0	1.2	1.2	
<i>ERG10</i>	orf19.1591	1.2	1.5	2.0	1.7	1.1	1.0	1.7	1.4	acetyl-CoA C-acyltransferase activity (IEA)
<i>ERG10</i>	orf19.1591	1.3	1.5	1.6	1.6	1.4	1.7	1.4	1.8	acetyl-CoA C-acyltransferase activity (IEA)
<i>FGR16</i>	orf19.2303	2.4	1.1	1.8	1.5	1.7	1.0	2.1	1.2	
<i>FGR34</i>	orf19.7579	1.0	1.4	1.5	1.5	1.0	0.9	1.2	1.2	
<i>FMO2</i>	orf19.859	1.3	1.8	2.5	1.9	0.6	0.7	1.5	1.2	
<i>FRP2</i>	orf19.7112	0.8	1.4	1.6	1.5	1.1	1.1	1.1	1.3	ferric-chelate reductase activity (IEA)
<i>GAP2</i>	orf19.6993	0.6	1.5	2.2	1.7	0.6	0.7	1.1	1.6	L-proline transmembrane transporter activity (IEA)
<i>GAT1</i>	orf19.1275	0.7	1.8	1.6	2.0	0.5	1.0	0.9	2.7	specific RNA polymerase II transcription factor activity (IMP, ISS)
<i>GDH3</i>	orf19.4716	1.0	1.6	1.9	1.6	1.0	1.3	1.2	1.6	glutamate dehydrogenase (NADP+) activity (IEA)
<i>GPD1</i>	orf19.1756	1.1	1.3	1.7	1.5	1.2	1.0	1.5	1.4	glycerol-3-phosphate dehydrogenase (NAD+) activity (IEA)
<i>GRE2</i>	orf19.3150	0.9	1.5	1.7	1.5	1.0	1.2	1.2	1.4	oxidoreductase activity (NAS)
<i>GST1</i>	orf19.3121	0.8	1.1	2.4	1.6	1.1	1.6	1.0	1.4	transcription corepressor activity (IEA)
<i>HIS5</i>	orf19.4177	1.3	1.5	1.5	1.5	1.0	0.8	1.7	1.2	histidinol-phosphate transaminase activity (IEA)
<i>HOS3</i>	orf19.2772	1.1	1.1	1.6	1.5	1.3	0.9	1.4	1.2	histone deacetylase activity (ISS)
<i>HSX11</i>	orf19.4592	2.0	1.4	2.4	1.6	1.9	1.1	2.1	1.2	ceramide glucosyltransferase activity (IDA, IGI, IMP)
<i>HXK1</i>	orf19.2154	1.3	1.4	1.6	1.6	0.9	1.0	1.3	1.5	N-acetylglucosamine kinase activity (IDA, IMP) hexokinase activity (ISS)
<i>IFR1</i>	orf19.1763	1.1	3.3	4.0	1.9	1.6	1.1	2.1	1.4	
<i>IFU5</i>	orf19.2568	1.1	1.7	2.2	1.7	1.1	1.2	1.3	1.6	
<i>INO4</i>	orf19.837.1	1.1	1.0	1.6	1.7	1.4	1.1	1.7	1.2	
<i>INP51</i>	orf19.1373	1.9	1.0	2.5	1.5	1.7	0.8	2.6	1.3	inositol-polyphosphate 5-phosphatase activity (IEA)
<i>KNS1</i>	orf19.4979	0.7	1.8	2.0	2.2	0.9	0.8	1.4	2.1	protein serine/threonine kinase activity (IEA)
<i>LEU2</i>	orf19.7080	1.0	1.5	1.5	1.5	0.9	1.2	1.1	1.3	3-isopropylmalate dehydrogenase activity (IDA, IGI, NAS)
<i>LIP8</i>	orf19.1345	1.4	1.9	1.6	1.6	0.8	1.5	1.3	2.0	lipase activity (IDA)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
MIT1	orf19.4077	1.2	1.1	2.2	1.9	1.4	1.0	1.8	1.3	mannosyltransferase activity (IMP)
MLS1	orf19.4833	0.8	2.1	1.9	1.5	0.8	1.0	1.0	1.3	malate synthase activity (ISS)
MUQ1	orf19.2107	0.9	1.5	1.6	1.5	1.1	1.1	1.3	1.4	choline-phosphate cytidyltransferase activity (IEA)
NAG6	orf19.2151	1.2	1.3	1.7	1.5	1.1	1.1	1.4	1.3	GTP binding (ISS)
NCE4	orf19.5320	1.2	0.8	1.8	1.6	1.6	0.8	2.0	1.1	
NUF2	orf19.1941	1.1	1.1	1.6	1.5	1.4	0.8	1.6	1.1	structural constituent of cytoskeleton (IEA)
OYE32	orf19.3131	1.0	2.5	2.3	1.7	1.0	1.2	1.3	1.8	NADPH dehydrogenase activity (IEA)
PAN1	orf19.886	1.1	1.8	1.8	1.9	1.0	1.3	1.3	1.7	cytoskeletal adaptor activity (IEA) protein binding, bridging (IEA)
PDC12	orf19.4608	2.3	1.3	3.8	1.5	3.0	1.0	3.3	1.0	
PDK2	orf19.7281	0.9	1.3	1.5	1.6	1.0	0.8	1.2	1.3	
PEP7	orf19.5662	1.5	1.1	1.9	1.7	1.7	0.8	1.6	1.4	phosphatidylinositol 3-phosphate binding (IEA)
PGA60	orf19.5588	0.8	4.6	1.7	2.4	1.0	0.2	1.0	5.0	
PLB5	orf19.5102	0.9	1.6	1.9	1.5	0.8	0.9	1.2	1.5	phospholipase A2 activity (IMP) lysophospholipase activity (ISS)
PLD1	orf19.1161	1.3	1.2	1.8	1.5	1.3	1.1	1.7	1.4	phospholipase D activity (IMP, ISS)
POT1-2	orf19.2046	1.1	1.5	1.5	1.7	1.2	1.0	1.5	1.3	acetyl-CoA C-acyltransferase activity (IEA)
PPH3	orf19.4378	1.2	1.2	1.5	1.5	1.2	1.0	1.8	1.4	
PRR2	orf19.1341	1.7	1.4	3.3	1.5	0.6	1.9	2.7	0.8	receptor signaling protein serine/threonine kinase activity (IEA)
RAD10	orf19.2469	1.7	1.2	1.5	1.5	1.7	1.1	1.6	1.1	DNA strand annealing activity (ISA)
RAD14	orf19.6517	1.0	1.5	1.6	1.6	0.9	1.0	1.2	1.7	damaged DNA binding (IEA) zinc ion binding (IEA)
RAM1	orf19.5046	1.0	0.9	1.5	1.5	1.0	1.0	0.9	1.0	protein farnesyltransferase activity (IEA)
RDH54	orf19.5367	1.5	1.2	1.7	1.6	1.2	0.9	1.6	1.1	DNA topoisomerase activity (IEA) DNA translocase activity (IEA)
RGA2	orf19.4593	1.2	1.2	1.6	1.6	1.4	1.1	1.4	1.4	Rho GTPase activator activity (IDA) signal transducer activity (IEA)
RGS2	orf19.695	0.9	0.8	1.5	2.6	0.7	0.5	1.0	1.6	
RIB1	orf19.2862	1.3	1.3	1.8	1.5	1.3	1.1	1.7	1.4	cyclohydrolase activity (IEA)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
<i>RPN10</i>	orf19.4102	1.3	1.2	1.5	1.6	1.4	1.2	1.5	1.3	endopeptidase activity (IEA) protein binding, bridging (IEA)
<i>RVS162</i>	orf19.6349	1.1	1.7	1.8	1.5	1.2	0.9	1.7	1.3	cytoskeletal protein binding (IEA)
<i>SKN7</i>	orf19.971	1.2	1.5	1.7	1.7	1.1	1.0	1.3	1.5	two-component response regulator activity (ISS)
<i>SMP2</i>	orf19.1462	1.1	1.0	1.7	1.5	1.4	0.9	1.6	1.1	phosphatidate phosphatase activity (IEA)
<i>SNF3</i>	orf19.5962	1.5	1.1	1.9	1.5	1.5	1.1	1.9	1.4	glucose transmembrane transporter activity (ISS)
<i>SNZ1</i>	orf19.2947	1.0	1.6	1.7	1.9	1.3	1.4	1.3	1.6	protein binding (IEA)
<i>SSP96</i>	orf19.5145	0.9	1.9	1.5	2.3	1.3	1.2	1.3	2.1	
<i>SVF1</i>	orf19.6068	1.3	1.1	1.9	1.6	1.5	1.1	1.5	1.4	
<i>TUP1</i>	orf19.6109	1.2	1.7	1.7	1.6	1.0	1.3	1.3	1.4	transcription repressor activity (IGI, IMP, ISS)
<i>UBC15</i>	orf19.5337	1.4	1.4	1.8	1.5	1.3	1.0	1.7	1.4	
<i>UGA1</i>	orf19.802	1.2	1.8	1.8	1.5	1.1	1.1	1.4	1.3	4-aminobutyrate transaminase activity (IEA)
<i>VPS2</i>	orf19.945	1.3	1.7	2.1	1.9	1.6	1.3	1.8	1.4	
<i>XYL2</i>	orf19.7676	0.8	2.0	1.9	1.6	0.8	1.1	1.2	1.6	L-iditol 2-dehydrogenase activity (IEA)
<i>YAF9</i>	orf19.5501	0.9	1.5	1.5	1.5	0.7	1.1	1.7	1.3	contributes_to histone acetyltransferase activity (IC)
	orf19.1070	1.0	1.2	3.9	2.2	1.2	0.9	1.7	1.2	
	orf19.1191	1.1	1.3	1.6	1.5	1.1	1.0	1.5	1.4	contributes_to ubiquitin-protein ligase activity (IEA)
	orf19.1314	1.3	0.9	1.6	1.7	1.4	1.0	2.0	1.4	
	orf19.1364	1.2	1.4	2.0	1.6	1.1	0.9	1.5	1.2	
	orf19.1369	1.1	1.7	1.9	1.6	0.9	1.2	0.9	1.4	
	orf19.1392	1.5	1.2	1.5	1.7	1.9	1.0	2.0	1.3	chaperone binding (IEA) protein disulfide isomerase activity (IEA)
	orf19.1395	1.2	1.9	1.5	1.8	1.3	0.7	0.9	1.8	inorganic phosphate transmembrane transporter activity (IEA)
	orf19.1406	1.0	1.0	2.1	1.5	1.1	0.7	1.8	0.9	
	orf19.1421	1.0	1.8	1.7	1.5	1.1	1.0	1.2	1.3	ureidoglycolate hydrolase activity (IEA)
	orf19.1434	1.0	1.0	1.9	1.7	1.0	1.0	1.3	1.0	kinase activator activity (IEA) protein binding (IEA)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.1482	1.0	1.4	1.6	1.8	0.9	1.0	1.3	1.4	
	orf19.1496	1.3	1.1	1.6	1.6	1.2	0.8	1.3	1.4	transcription factor activity (ISS)
	orf19.1667	1.3	1.8	1.5	1.8	1.3	1.7	1.1	1.7	helicase activity (IEA)
	orf19.1723	0.9	1.4	1.5	1.5	1.0	1.1	1.3	1.4	
	orf19.1728	1.1	5.8	2.1	3.3	1.0	0.2	0.4	1.6	
	orf19.1730	1.1	1.0	1.6	1.7	1.5	1.0	1.4	1.3	
	orf19.1776	2.6	1.1	3.1	2.1	4.0	1.1	4.3	1.3	
	orf19.190	1.2	1.1	1.5	1.6	1.3	0.7	1.1	1.0	
	orf19.1917	1.3	1.5	1.9	1.5	1.2	1.2	1.4	1.5	
	orf19.192	1.3	1.4	1.6	1.8	1.2	0.9	1.4	1.4	
	orf19.2009	2.1	1.0	2.6	1.6	2.8	0.8	2.6	1.3	protein binding (IEA)
	orf19.2065	1.1	1.6	1.9	1.5	1.3	1.1	1.4	1.6	allantoicase activity (IEA)
	orf19.2204.2	1.5	1.2	2.4	1.5	1.3	1.3	1.9	1.4	GTPase activity (ISS) signal transducer activity (IDA)
	orf19.225	1.1	1.4	2.0	1.6	1.3	1.0	1.2	1.2	
	orf19.2257	1.2	1.3	1.5	1.6	1.3	1.2	1.4	1.3	
	orf19.2262	0.9	1.9	1.9	1.7	0.9	1.0	1.3	1.6	AU-rich element binding (IEA)
	orf19.2313	1.3	1.6	1.7	1.7	0.9	1.0	1.4	1.7	
	orf19.2346	1.6	1.0	2.0	1.5	1.7	0.8	1.7	1.2	
	orf19.2411	1.1	1.3	1.5	1.7	1.3	1.1	1.5	1.4	SNAP receptor activity (IEA)
	orf19.2496	1.0	1.3	1.7	1.7	0.8	1.6	1.4	1.4	
	orf19.2565	1.1	1.3	1.5	1.6	0.8	0.9	1.3	1.5	phosphatidylinositol 3-phosphate binding (IEA)
	orf19.268	0.9	1.4	1.8	1.5	1.0	0.7	1.3	1.5	
	orf19.2710	1.5	1.0	2.8	1.5	2.1	0.8	2.4	1.1	enzyme activator activity (IEA)
	orf19.273	1.2	1.2	1.8	1.5	1.2	1.3	1.4	1.4	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.2740	1.3	1.6	1.5	1.5	1.1	1.1	1.1	1.4	
	orf19.2755	1.6	0.9	2.0	1.6	2.0	0.9	1.6	0.7	endopeptidase activity (IEA)
	orf19.2791	1.1	1.6	1.7	1.5	1.0	1.0	1.3	1.4	myosin I binding (IEA)
	orf19.2888	1.4	0.7	1.8	1.6	2.2	0.8	2.6	1.0	
	orf19.2905	1.1	1.6	1.8	2.1	1.5	1.2	1.3	1.6	
	orf19.2913	1.1	1.1	1.6	1.5	1.1	1.0	1.4	1.3	
	orf19.2974	1.2	1.1	1.5	1.5	1.6	1.1	1.7	1.3	palmitoyltransferase activity (IEA)
	orf19.3151	0.8	1.4	1.5	1.5	1.1	0.9	1.6	1.1	oxidoreductase activity (NAS)
	orf19.3214	1.2	1.5	2.5	2.3	1.3	1.1	1.7	1.1	
	orf19.3215	1.3	1.6	1.8	1.5	1.2	1.0	1.5	1.2	
	orf19.3407	1.3	1.4	1.5	1.6	1.4	1.0	1.5	1.2	transcription factor activity (ISS)
	orf19.3411	1.4	1.1	1.7	1.5	1.3	1.0	1.8	1.4	
	orf19.345	1.6	1.3	1.5	1.7	1.4	1.6	1.4	1.3	succinate-semialdehyde dehydrogenase [NAD(P)+] activity (IEA)
	orf19.3536	1.4	1.2	1.7	1.7	1.4	1.2	1.6	1.4	acetylglucosaminyltransferase activity (IEA)
	orf19.3631	1.6	1.4	1.5	1.5	1.7	1.2	1.0	1.3	
	orf19.3658	1.0	1.5	1.5	1.8	1.0	1.3	1.4	1.5	
	orf19.3661	1.3	1.4	1.7	1.7	1.6	1.1	1.4	1.4	ubiquitin-specific protease activity (IEA)
	orf19.3679	1.0	1.7	2.2	1.5	1.0	0.9	1.5	1.4	
	orf19.3804	1.3	1.2	1.9	1.7	1.4	1.2	1.5	1.4	
	orf19.3806	1.4	1.2	2.0	1.5	2.0	0.9	2.0	1.4	
	orf19.3814	1.1	1.4	1.8	1.7	1.1	0.8	1.6	1.4	
	orf19.393	1.3	1.2	1.8	1.5	1.3	1.0	1.8	1.4	
	orf19.3939	2.2	1.1	2.1	1.5	2.5	1.1	2.4	1.2	
	orf19.3984	1.0	1.0	1.6	1.8	1.3	0.8	1.3	1.2	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.4061	1.5	1.2	1.8	1.5	1.2	1.0	2.1	1.3	
	orf19.4206	1.3	1.2	2.2	2.2	1.6	1.0	1.9	1.4	crossover junction endodeoxyribonuclease activity (IEA)
	orf19.4229	0.4	1.8	2.0	1.6	0.8	1.1	0.9	1.4	bis(5'-adenosyl)-hexaphosphatase activity (IEA)
	orf19.4246	1.4	1.3	2.2	1.5	1.6	1.2	1.9	1.4	
	orf19.4357	1.6	1.2	2.1	1.5	1.5	1.0	1.7	1.2	misfolded protein binding (IEA)
	orf19.4390	0.8	1.4	1.7	1.6	0.9	0.9	1.2	1.4	
	orf19.4503	1.4	1.1	1.8	1.5	1.5	1.3	2.0	1.3	
	orf19.4583	2.3	1.1	1.9	1.6	1.8	1.2	2.0	0.8	transporter activity (IEA)
	orf19.4691	1.2	1.4	2.5	2.0	0.9	0.8	1.0	1.3	
	orf19.4726	1.3	1.2	2.0	1.5	1.6	1.2	1.8	1.4	calcium ion binding (IEA) enzyme activator activity (IEA)
	orf19.4731	1.4	1.0	1.8	1.6	1.7	1.1	1.9	1.4	
	orf19.4791	1.2	1.8	2.3	1.6	1.0	0.7	1.5	1.1	
	orf19.4807	1.2	1.1	1.5	1.5	1.0	0.9	1.3	1.2	inorganic diphosphatase activity (IEA)
	orf19.4894	1.0	1.9	1.6	1.5	1.0	1.0	1.2	1.3	0.0
	orf19.4983	1.6	1.2	1.6	1.6	2.0	1.0	1.7	1.2	0.0
	orf19.5008.1	0.9	1.1	1.5	1.9	1.1	0.8	1.3	1.0	structural constituent of cytoskeleton (IEA)
	orf19.5014	1.1	1.1	1.5	1.6	1.0	0.9	1.2	1.2	0.0
	orf19.5095	1.1	1.4	2.2	1.9	1.1	1.0	1.4	1.4	oxysterol binding (IEA) sterol carrier activity (IEA)
	orf19.5169	0.8	1.9	2.4	2.0	1.5	1.4	0.6	1.6	amidase activity (IEA)
	orf19.52	1.0	1.3	1.5	1.6	1.1	1.1	1.4	1.3	0.0
	orf19.5236.1	1.2	1.0	1.5	1.5	1.5	1.0	1.7	1.3	dehydrodolichyl diphosphate synthase activity (IEA)
	orf19.5253	1.1	1.1	1.6	1.5	1.5	1.0	1.9	1.4	protein kinase activity (IEA)
	orf19.5270	1.1	1.4	2.0	1.5	0.8	0.4	1.9	1.3	
	orf19.5342	1.0	1.5	1.6	1.5	1.0	1.1	1.3	1.3	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.5345	1.3	1.4	1.6	1.6	1.2	1.2	1.6	1.3	protein binding, bridging (IEA)
	orf19.537	1.8	1.3	1.9	1.6	1.7	1.4	2.5	1.4	
	orf19.5547	3.1	0.9	1.7	1.6	5.7	1.3	3.4	0.9	
	orf19.5620	1.2	1.7	1.6	1.5	1.2	1.4	1.3	1.6	
	orf19.5773	1.4	1.5	2.0	1.5	1.4	1.3	1.6	1.4	dipeptidyl-peptidase activity (IEA)
	orf19.5784	1.0	1.8	1.7	1.5	0.9	0.7	1.1	1.7	
	orf19.5812	1.2	1.1	1.6	1.5	1.3	1.2	1.5	1.4	
	orf19.5862	0.5	2.8	2.4	1.7	0.9	0.9	1.7	0.7	arginase activity (IEA)
	orf19.5881	1.4	1.2	2.1	1.6	1.4	0.9	1.7	1.1	voltage-gated chloride channel activity (IEA)
	orf19.589	1.2	1.3	2.0	1.6	1.3	1.2	1.6	1.4	GTPase activity (ISS) GTP binding (IEA)
	orf19.5935	1.3	2.5	1.8	1.7	1.2	1.1	1.4	1.2	
	orf19.5952	1.1	1.0	1.6	1.6	1.1	0.9	1.1	1.6	
	orf19.5961	0.9	1.2	1.5	1.5	1.3	0.9	1.5	1.3	
	orf19.6045	1.2	1.2	1.6	1.5	1.1	1.1	1.4	1.4	phosphatidylserine decarboxylase activity (IEA)
	orf19.6187	1.3	1.6	1.7	1.6	1.4	1.2	1.4	1.3	
	orf19.6196	0.8	1.6	1.7	1.6	1.0	0.9	1.2	1.7	
	orf19.6200	1.3	1.3	2.7	1.7	1.6	0.8	2.1	1.4	
	orf19.6268	1.7	1.1	1.8	1.5	2.0	1.1	1.8	1.2	
	orf19.6357	2.2	1.2	2.0	1.6	2.1	1.2	2.6	1.4	
	orf19.6460	1.5	1.0	1.7	1.5	1.6	0.8	1.7	1.1	ATPase activity (IEA) protein heterodimerization activity (IEA)
	orf19.6462	0.9	1.0	1.5	2.1	1.2	1.1	1.2	1.4	
	orf19.6498	1.3	1.1	1.8	1.5	1.4	1.0	1.8	1.4	
	orf19.6552	1.4	1.2	1.5	1.5	1.5	1.1	1.6	1.2	thiol oxidase activity (IEA)
	orf19.6556	1.1	1.1	1.7	1.5	1.1	0.9	1.0	0.9	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.6569	1.9	1.1	2.1	1.7	1.5	1.1	2.6	1.3	cholesterol binding (IEA)
	orf19.6601	0.9	1.6	2.8	1.6	1.1	0.7	1.4	1.2	
	orf19.6709	1.0	1.4	1.6	1.6	0.8	1.1	1.3	1.3	
	orf19.6754	0.9	1.2	1.5	1.5	1.1	1.1	1.3	1.3	
	orf19.6867	1.2	1.5	1.7	1.7	1.5	1.2	1.5	1.4	
	orf19.6883	1.1	1.3	1.7	1.6	1.3	1.3	1.4	1.5	
	orf19.6900	1.2	1.5	1.7	1.7	1.4	1.1	1.4	1.3	
	orf19.6905	1.1	1.1	1.5	1.5	1.3	1.0	1.4	1.3	ATPase activity (IEA)
	orf19.7061	2.0	1.0	2.0	1.5	2.1	1.4	2.3	1.3	
	orf19.711	0.9	2.0	1.6	2.0	0.8	1.4	1.1	1.2	
	orf19.7179	1.3	1.1	1.5	1.5	1.4	1.0	1.7	1.4	
	orf19.7225	1.2	1.1	2.1	1.5	1.0	0.9	1.8	1.3	
	orf19.7229	1.1	1.5	1.8	1.8	1.2	1.1	1.4	1.5	
	orf19.7260	0.8	2.0	1.5	2.2	0.7	1.4	0.9	1.7	
	orf19.7265	1.1	1.6	1.6	1.7	1.2	1.3	1.2	1.5	
	orf19.7267	1.5	1.4	1.5	1.6	1.4	1.2	1.4	1.3	
	orf19.7288	1.1	1.4	1.6	1.5	1.1	0.9	1.4	1.4	
	orf19.7328	1.3	1.5	1.9	1.6	1.5	1.3	1.3	1.5	
	orf19.7365	1.2	1.0	1.7	1.5	1.1	0.9	1.1	0.9	
	orf19.7449	1.3	1.2	1.8	1.7	1.5	1.3	1.9	1.3	
	orf19.7507	0.9	1.3	1.6	1.7	1.3	0.8	1.1	1.3	
	orf19.751	1.2	2.6	1.6	1.9	1.0	1.7	1.3	2.6	DNA helicase activity (IEA)
	orf19.7538	1.2	1.5	1.5	1.7	1.2	1.2	1.4	1.5	
	orf19.834	1.5	1.1	1.9	1.5	1.5	1.2	1.7	1.0	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.91	1.2	0.9	1.5	1.5	1.2	1.0	1.5	1.3	
	orf19.6356	2.2	0.8	1.4	1.1	1.9	1.6	1.2	1.0	RNA splicing factor activity, transesterification mechanism (IEA)
<i>CMP1</i>	orf19.6033	1.4	1.2	1.2	1.8	1.7	1.5	1.1	1.3	calcium-dependent protein serine/threonine phosphatase activity (ISS)
<i>PHO13</i>	orf19.1405	1.4	0.8	1.3	1.5	1.6	1.5	1.4	1.4	4-nitrophenylphosphatase activity (IEA) alkaline phosphatase activity (IEA)
<i>YST1</i>	orf19.6975	2.1	1.2	1.5	0.4	2.4	1.5	0.8	1.6	structural constituent of ribosome (IGI)
	orf19.6589	2.2	0.9	1.4	1.3	2.1	1.5	1.7	1.2	rRNA binding (IEA)
	orf19.5291	1.5	0.9	1.7	1.2	1.6	1.5	1.3	1.4	
<i>FOX2</i>	orf19.1288	1.3	1.3	1.5	1.3	1.0	1.1	1.6	1.5	nucleotide binding
<i>ANT1</i>	orf19.6254	1.3	1.2	1.2	1.6	1.2	1.4	1.5	1.6	adenine nucleotide transmembrane transporter activity (IEA)
<i>BMT8</i>	orf19.860	1.0	1.4	1.6	1.3	1.0	0.8	1.5	2.0	
<i>CDC45</i>	orf19.1988	1.6	1.2	1.7	1.3	1.6	1.0	1.5	1.5	DNA replication origin binding (IEA) chromatin binding (IEA)
<i>DEF1</i>	orf19.7561	1.2	1.4	1.7	1.2	1.1	1.1	1.5	1.6	
<i>GAL1</i>	orf19.3670	1.3	1.1	1.2	1.4	1.5	1.0	2.0	1.6	galactokinase activity (IGI, NAS) transcription activator activity (IEA)
<i>GAL10</i>	orf19.3672	1.0	1.3	1.1	1.5	1.3	1.1	1.6	1.6	UDP-glucose 4-epimerase activity (IGI) aldose 1-epimerase activity (IEA)
<i>HAP31</i>	orf19.517	1.3	1.0	1.3	1.8	1.5	1.2	1.7	1.5	transcription factor activity (NAS) transcription activator activity (IEA)
<i>HGT17</i>	orf19.4682	0.7	0.8	1.5	1.2	0.8	0.9	2.3	2.2	glucose transmembrane transporter activity (ISS)
<i>HGT20</i>	orf19.1587	1.0	1.2	1.2	1.5	0.9	1.1	1.6	1.8	glucose transmembrane transporter activity (ISS)
<i>HOS1</i>	orf19.4411	1.1	0.9	1.5	1.4	1.3	1.2	1.7	1.5	histone deacetylase activity (ISS)
<i>INO1</i>	orf19.7585	1.2	0.6	1.4	1.5	1.7	1.2	2.5	1.6	inositol-3-phosphate synthase activity (IGI, ISS)
<i>PEX5</i>	orf19.5640	1.0	1.0	1.3	1.5	1.1	1.0	1.6	1.5	peroxisome matrix targeting signal-1 binding (IEA) protein binding, bridging (IEA)
<i>RBT5</i>	orf19.5636	1.3	1.5	1.2	1.8	1.4	1.8	1.8	2.0	heme binding (IDA, IMP)
<i>SOD6</i>	orf19.2108	1.2	0.9	1.2	1.5	1.6	0.8	2.4	1.6	superoxide dismutase activity (ISS)
<i>STF2</i>	orf19.2107.1	1.0	1.3	1.3	1.5	1.2	1.1	1.6	1.5	
<i>SUR2</i>	orf19.5818	1.0	1.1	1.4	1.1	1.2	1.1	1.5	1.6	sphingosine hydroxylase activity (IEA)

Appendix B. Continued.

		Change in fold expression ^a								
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
<i>VAM3</i>	orf19.5875	1.0	1.5	1.1	1.8	1.1	1.0	1.5	1.7	
<i>ZCF11</i>	orf19.2423	1.0	1.2	1.3	1.9	0.9	1.2	1.5	2.0	sequence-specific DNA binding (IEA)
<i>ZCF14</i>	orf19.2647	1.3	1.4	1.1	1.4	1.0	1.5	1.6	1.6	transcription factor activity (ISS)
	orf19.1412	1.4	1.1	1.4	1.5	1.4	1.0	1.8	1.5	
	orf19.1881	1.4	1.2	2.2	1.4	1.3	1.3	2.1	1.8	1-acylglycerol-3-phosphate O-acyltransferase activity (IEA)
	orf19.1940	1.0	1.2	1.3	1.9	1.2	1.2	1.5	1.7	
	orf19.2286	1.4	1.2	1.6	1.4	1.5	1.3	1.6	1.5	deoxyhypusine monooxygenase activity (IEA)
	orf19.2313	1.4	1.6	1.4	2.3	0.9	0.8	1.7	1.8	
	orf19.314	1.1	1.2	1.5	1.4	1.4	1.3	1.6	1.6	transcription repressor activity (IEA)
	orf19.3434	1.6	1.1	1.4	1.3	1.6	1.3	2.1	1.6	
	orf19.3877	1.3	1.4	1.5	1.0	1.1	1.2	1.6	1.6	
	orf19.3937	1.4	1.6	1.1	1.4	1.4	0.9	1.8	1.6	
	orf19.4174	1.1	1.3	1.4	1.5	0.9	1.1	1.5	1.6	nucleoside transmembrane transporter activity (IEA)
	orf19.4504	1.4	1.3	1.5	1.4	1.2	0.8	2.1	1.7	alcohol dehydrogenase (NAD) activity (IEA)
	orf19.4595	1.0	1.3	1.3	1.3	1.1	0.9	1.5	1.5	
	orf19.4726	1.4	1.1	2.4	1.3	1.9	0.8	2.4	1.6	calcium ion binding (IEA) enzyme activator activity (IEA)
	orf19.516	1.1	1.1	1.2	1.4	1.0	1.2	1.5	1.5	
	orf19.5237	1.2	1.2	1.4	1.7	1.6	1.1	1.7	1.8	
	orf19.5282	1.2	1.2	1.3	1.7	1.4	1.4	1.5	1.5	
	orf19.5319	1.7	1.4	1.6	1.1	2.1	0.9	1.7	1.6	single-stranded DNA binding (IEA)
	orf19.540	0.9	1.2	1.7	1.4	1.1	0.7	1.7	1.8	
	orf19.5523	1.0	1.0	1.2	1.2	1.3	0.9	1.6	1.5	
	orf19.6031	1.1	1.2	2.4	1.4	1.6	1.0	1.7	1.6	phosphatidylinositol 3-phosphate binding (IEA) protein binding (IEA)
	orf19.6393	1.0	1.2	1.4	1.2	1.3	1.0	1.9	1.5	ARF GTPase activator activity (IEA)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.6690	0.9	1.5	2.0	1.4	1.0	0.9	1.5	1.5	
	orf19.676	1.1	1.2	1.4	1.3	1.1	0.8	1.6	1.5	structural constituent of cytoskeleton (IEA)
	orf19.6816	1.0	1.0	1.3	1.6	1.2	1.1	1.6	1.5	aldehyde reductase activity (IEA)
	orf19.6852	1.0	1.1	1.4	1.6	1.3	1.2	1.6	1.5	
	orf19.6973	1.1	1.3	1.7	1.3	1.2	0.8	1.5	1.5	ATP-dependent peptidase activity (IEA)
	orf19.7444	1.2	1.6	1.3	1.2	2.0	0.9	2.4	1.6	
	orf19.7459	1.3	0.9	1.4	1.3	1.4	1.1	1.6	1.5	
	orf19.89	0.9	1.3	1.4	1.6	1.2	1.0	1.7	1.5	peroxisome matrix targeting signal-2 binding (IEA)
	orf19.897	1.1	1.2	1.5	1.4	1.1	1.0	1.8	1.5	protein binding (IEA)
	orf19.910	1.1	1.2	1.1	1.4	1.1	1.0	1.5	1.6	
<i>BNR1</i>	orf19.7537	1.6	1.5	2.3	1.5	1.3	1.2	1.8	1.4	cytoskeletal protein binding (IDA)
<i>MUC1</i>	orf19.4183	1.5	1.5	2.5	1.5	1.5	1.1	1.7	1.3	
<i>USO6</i>	orf19.6967	1.7	1.5	2.7	1.6	1.8	0.9	2.0	1.0	
	orf19.6148	1.5	17.0	3.5	19.0	1.9	7.7	0.9	5.5	structural constituent of cytoskeleton (IEA)
	orf19.6501	1.6	19.2	3.0	18.4	1.4	9.0	1.0	8.1	
	orf19.6790	1.5	1.5	2.8	1.5	1.6	0.7	1.3	0.9	mRNA binding (IEA)
	orf19.2400	1.5	1.9	1.6	2.0	1.8	1.3	1.4	1.4	
	orf19.3461	1.5	1.6	2.1	2.0	1.4	1.3	1.4	2.1	
	orf19.4330	1.7	2.0	2.3	1.9	1.7	1.5	1.4	1.8	
	orf19.6308	1.5	1.5	1.8	1.5	1.4	0.8	1.5	1.0	
	orf19.6920	2.0	1.8	2.7	2.1	1.6	1.4	1.5	0.9	
	orf19.2006	2.5	1.6	2.8	2.1	3.4	0.9	1.8	0.9	
	orf19.2204.2	1.5	1.5	2.1	1.8	1.7	1.4	1.8	1.4	* GTPase activity
	orf19.4168	1.6	1.7	1.6	1.7	2.3	1.4	1.9	1.1	ribonuclease MRP activity (IEA)

Appendix B. Continued.

		Change in fold expression ^a								
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
	orf19.5772	1.8	1.5	1.7	1.6	1.9	1.2	1.9	1.1	
	orf19.5295	1.6	2.1	2.5	1.6	0.9	0.9	1.9	1.3	
	orf19.641	1.8	1.5	2.2	1.6	1.7	1.3	2.0	1.3	
	orf19.1297	2.2	1.6	2.5	1.8	1.3	1.5	2.2	1.4	
	orf19.5426	1.7	1.9	2.6	1.9	1.5	0.9	2.3	1.4	triglyceride lipase activity (IEA)
	orf19.3437	2.5	2.3	2.5	1.9	0.2	1.0	2.3	1.3	
	orf19.1444	2.0	1.7	4.4	1.9	2.9	1.6	2.6	1.4	phosphatidylinositol-4,5-bisphosphate binding
	orf19.3607	3.8	10.1	3.2	15.1	1.4	6.4	0.4	13.9	
ARG81	orf19.4766	1.7	1.5	1.9	1.6	2.0	1.0	1.7	1.2	transcription factor activity (ISS) transcription cofactor activity (IEA)
BIO2	orf19.2593	1.6	1.8	3.0	1.6	1.3	1.0	1.5	1.3	biotin synthase activity (NAS)
BNR1	orf19.7537	1.6	1.5	2.3	1.5	1.3	1.2	1.8	1.4	cytoskeletal protein binding (IDA) cytoskeletal regulatory protein binding (IEA)
USO6	orf19.6967	1.7	1.5	2.7	1.6	1.8	0.9	2.0	1.0	
	orf19.6501	1.6	19.2	3.0	18.4	1.4	9.0	1.0	8.1	
	orf19.3461	1.5	1.6	2.1	2.0	1.4	1.3	1.4	2.1	
	orf19.2400	1.5	1.9	1.6	2.0	1.8	1.3	1.4	1.4	
	orf19.5295	1.6	2.1	2.5	1.6	0.9	0.9	1.9	1.3	
	orf19.641	1.8	1.5	2.2	1.6	1.7	1.3	2.0	1.3	
	orf19.5772	1.8	1.5	1.7	1.6	1.9	1.2	1.9	1.1	
	orf19.4168	1.6	1.7	1.6	1.7	2.3	1.4	1.9	1.1	ribonuclease MRP activity (IEA)
	orf19.3998	1.5	1.6	3.7	1.6	2.0	0.6	1.9	1.1	
	orf19.6308	1.5	1.5	1.8	1.5	1.4	0.8	1.5	1.0	
	orf19.2006	2.5	1.6	2.8	2.1	3.4	0.9	1.8	0.9	
	orf19.6920	2.0	1.8	2.7	2.1	1.6	1.4	1.5	0.9	
	orf19.6790	1.5	1.5	2.8	1.5	1.6	0.7	1.3	0.9	mRNA binding (IEA)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
<i>RBT1</i>	orf19.1327	1.9	1.6	1.2	1.1	1.6	1.9	1.1	1.2	aldehyde dehydrogenase (NAD) activity (IEA)
	orf19.3851	1.5	1.6	2.1	1.1	0.9	0.9	1.6	1.6	
<i>ALD6</i>	orf19.742	2.6	3.2	1.4	1.2	1.7	0.7	1.8	1.5	
	orf19.136	1.6	1.5	1.3	1.3	1.0	1.1	1.6	1.5	drug transporter activity (ISS) multidrug efflux pump activity (IEA)
<i>CIRT4B</i>	orf19.2839	1.9	1.7	2.9	1.3	1.4	0.5	3.4	1.5	
<i>CAG1</i>	orf19.4015	1.2	2.1	1.7	1.5	1.7	1.7	1.2	1.4	GTPase activity (IGI) guanyl nucleotide binding (ISS)
<i>FGR23</i>	orf19.1616	0.7	2.5	1.5	2.4	1.7	1.5	1.2	2.3	
	orf19.345	2.1	1.4	1.6	1.8	2.3	1.5	1.9	1.3	succinate-semialdehyde dehydrogenase [NAD(P)+] activity (IEA)
	orf19.6805	1.8	1.3	2.8	1.6	2.1	1.5	1.5	1.0	
	orf19.2650.1	1.4	1.2	1.7	1.6	1.8	1.6	1.3	1.1	structural constituent of ribosome (IEA)
	orf19.6023	1.3	1.6	2.2	1.5	1.7	1.8	1.3	1.5	drug transmembrane transport
	orf19.6311	1.0	4.2	3.9	4.5	2.1	1.7	1.4	1.2	
<i>HGT20</i>	orf19.1587	1.3	1.1	1.5	1.6	1.0	1.0	1.8	1.8	glucose transmembrane transporter activity (ISS)
<i>HGT3</i>	orf19.4356	1.1	1.5	2.5	1.7	1.0	1.0	1.9	1.7	glucose transmembrane transporter activity (ISS)
<i>HYR1</i>	orf19.4975	1.3	1.3	1.7	1.5	1.2	1.0	1.8	1.5	
<i>IFC3</i>	orf19.3749	1.1	1.7	5.9	3.6	1.3	1.2	3.6	3.4	oligopeptide transmembrane transporter activity
<i>IHD1</i>	orf19.5760	1.3	1.8	1.7	1.9	1.2	1.7	1.7	2.0	
<i>IRS4</i>	orf19.6953	1.3	1.4	1.9	1.6	1.5	1.3	1.7	1.5	
<i>LAP3</i>	orf19.539	1.1	2.0	2.6	2.0	1.2	1.0	1.8	2.0	aminopeptidase activity (NAS)
<i>MEP1</i>	orf19.1614	1.1	2.2	6.6	4.5	1.2	1.1	2.9	3.5	ammonium transmembrane transporter activity (IGI)
<i>MET13</i>	orf19.2887	1.4	1.3	1.8	1.6	1.3	1.3	1.6	1.5	
<i>MSO1</i>	orf19.5248	1.3	1.3	3.0	2.9	2.1	1.3	2.9	2.3	
<i>ORC3</i>	orf19.6942	1.4	1.2	1.8	1.6	1.4	1.2	1.8	1.6	DNA replication origin binding (IEA) chromatin binding (IEA)
<i>PGA10</i>	orf19.5674	1.3	2.2	2.1	2.8	1.4	2.1	1.7	2.6	heme binding (IDA, IGI)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
<i>PLB4</i>	orf19.1443	1.4	1.9	2.0	1.9	1.2	1.3	1.5	1.5	lysophospholipase activity (ISS)
<i>POS5</i>	orf19.6800	1.1	1.5	2.2	2.1	1.4	0.9	1.9	1.9	NADH kinase activity (IEA)
<i>PTC4</i>	orf19.6638	0.9	1.1	1.6	1.7	1.2	1.0	1.5	1.5	protein serine/threonine phosphatase activity (IEA)
<i>PTC6</i>	orf19.3705	1.7	1.3	2.1	2.1	1.7	1.2	2.3	1.9	protein serine/threonine phosphatase activity (IDA)
<i>RAD57</i>	orf19.2174	1.3	1.3	2.3	1.7	1.6	1.1	1.9	1.5	protein heterodimerization activity (IEA)
<i>RPN4</i>	orf19.1069	1.4	1.1	2.0	2.2	1.5	1.3	1.7	1.6	sequence-specific DNA binding (IDA) transcription factor activity (ISS)
<i>SAP5</i>	orf19.5585	1.0	2.3	1.9	2.9	0.9	0.8	1.9	2.8	aspartic-type endopeptidase activity (IDA, ISS) drug binding (IDA,ISS)
<i>SCT2</i>	orf19.5815	1.5	1.1	2.5	1.7	1.3	1.2	1.8	1.5	
<i>SOD6</i>	orf19.2108	1.9	1.2	1.9	2.1	1.6	1.1	2.6	1.7	superoxide dismutase activity (ISS)
<i>UBA4</i>	orf19.2324	1.5	1.0	1.5	1.7	1.6	1.1	1.9	1.6	URM1 activating enzyme activity (IEA)
<i>VAM3</i>	orf19.5875	0.9	1.5	1.6	1.7	1.5	1.0	1.5	2.0	
<i>VPS51</i>	orf19.5568	1.2	1.3	2.0	1.7	1.9	1.0	2.0	1.5	
<i>VRP1</i>	orf19.2190	1.0	2.3	2.2	2.4	1.2	1.2	1.6	2.1	actin binding (IEA)
<i>YPT31</i>	orf19.2622	1.5	1.2	2.0	1.6	1.7	1.4	1.9	1.5	GTPase activity (IEA)
<i>ZCF21</i>	orf19.4166	1.8	1.2	2.2	1.9	1.6	1.3	2.1	1.6	
	orf19.90	1.3	1.1	4.9	3.4	1.9	1.1	3.9	2.3	
	orf19.2515	1.4	4.0	9.4	4.5	1.8	1.3	3.6	3.2	
	orf19.5205	1.3	1.6	2.5	2.7	1.5	0.9	3.4	2.5	
	orf19.4031	1.6	1.4	3.3	2.4	2.0	1.2	3.4	2.2	
	orf19.6027	1.3	1.3	1.5	1.6	2.1	1.0	3.3	1.5	
	orf19.7578	1.2	1.7	2.7	2.2	1.5	1.0	3.1	2.3	protein transporter activity (IEA)
	orf19.7457	1.4	1.6	2.3	2.7	1.7	1.2	2.7	1.8	
	orf19.4897	1.9	1.3	2.7	2.2	2.3	1.4	2.7	1.8	phosphatidylinositol transporter activity (IEA)
	orf19.3318	1.5	1.2	3.1	2.0	1.6	1.0	2.6	1.6	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.5777	1.2	2.1	3.5	3.6	1.3	1.6	2.6	2.9	
	orf19.5535	1.4	1.7	2.2	2.6	1.4	1.9	2.5	2.9	anion:cation symporter activity (ISS) pantothenate transporter activity (IEA)
	orf19.5727	1.2	1.5	2.7	2.4	1.4	1.2	2.5	2.0	
	orf19.3544	1.1	1.9	3.5	1.6	1.1	1.0	2.4	1.9	
	orf19.5275	1.4	1.3	3.0	2.2	2.7	1.4	2.4	1.8	ATPase activator activity (IEA)
	orf19.951	1.3	1.4	2.1	2.6	1.8	1.1	2.4	1.5	
	orf19.288	1.6	1.2	2.7	2.4	1.7	1.4	2.4	1.8	methylenetetrahydrofolate reductase (NADPH) activity (IEA)
	orf19.1985	1.6	1.4	2.4	2.1	1.6	1.3	2.3	2.2	* protein phosphorylation
	orf19.4287	1.1	2.1	3.2	3.7	1.1	1.3	2.3	3.0	(R,R)-butanediol dehydrogenase activity (IEA)
	orf19.910	1.7	1.2	1.7	1.7	1.9	1.3	2.3	1.7	
	orf19.1277	1.2	2.1	2.2	2.3	1.5	1.3	2.3	1.9	
	orf19.2943	1.5	1.0	2.4	2.3	1.7	0.9	2.3	1.7	amino acid transmembrane transporter activity (IEA)
	orf19.1592	1.9	1.2	2.1	1.6	2.0	1.3	2.2	1.5	
	orf19.996	1.3	1.3	2.5	1.9	1.9	1.1	2.2	1.7	adenylate cyclase activity (IEA)
	orf19.1698	1.7	1.3	2.3	1.5	1.6	1.0	2.2	1.7	
	orf19.4723	1.5	1.2	1.7	1.6	1.4	1.2	2.2	1.5	FMN adenylyltransferase activity (IEA)
	orf19.2691	1.0	2.8	3.8	4.1	0.9	1.3	2.2	3.2	
	orf19.7347	1.5	1.1	1.9	1.6	1.6	1.2	2.1	1.7	ubiquitin-protein ligase activity (ISS)
	orf19.35	1.3	1.1	2.1	1.9	1.4	1.1	2.1	1.6	
	orf19.89	1.2	1.0	1.6	1.9	1.3	1.0	2.1	1.6	peroxisome matrix targeting signal-2 binding (IEA)
	orf19.4711	1.2	1.3	2.1	2.1	1.4	1.2	2.1	1.8	
	orf19.3135	1.3	1.2	2.0	1.8	1.3	1.0	2.1	1.7	protein binding, bridging (IEA)
	orf19.296	0.9	2.4	3.4	2.3	0.9	1.0	2.1	2.2	
	orf19.6912	1.4	1.1	2.5	1.6	1.5	1.1	2.1	1.7	choline kinase activity (IEA)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.1857	1.7	1.2	2.2	1.6	1.9	1.0	2.1	1.5	histone acetyltransferase activity (IEA)
	orf19.2026	1.2	1.1	1.7	1.7	1.5	1.0	2.1	1.6	ubiquitin-specific protease activity (IEA)
	orf19.2703	1.2	1.1	2.1	1.7	1.1	1.1	2.1	1.6	
	orf19.6624	1.6	1.3	1.9	1.8	1.2	1.2	2.1	1.5	
	orf19.5601	1.2	1.5	2.9	2.4	1.4	1.0	2.1	1.7	
	orf19.5492	1.2	1.0	2.0	1.8	1.4	1.2	2.1	1.5	mRNA binding (IEA)
	orf19.7131	1.4	1.8	2.9	1.9	1.3	1.3	2.0	1.6	gamma-butyrobetaine dioxygenase activity (IMP)
	orf19.6557	1.8	1.4	2.4	1.5	1.7	1.1	2.0	1.9	
	orf19.7567	1.4	1.8	3.7	2.7	1.7	1.2	2.0	2.0	
	orf19.3881	1.2	1.3	2.2	2.0	1.2	1.0	2.0	1.7	
	orf19.5249	1.2	1.4	2.6	1.9	1.6	1.1	2.0	1.5	
	orf19.3734	1.2	1.3	2.0	1.9	1.4	1.1	2.0	1.7	
	orf19.5114	1.0	1.4	2.0	2.2	1.1	1.1	2.0	1.6	phosphatidylinositol 3-phosphate binding (IEA) protein binding (IEA)
	orf19.4658	1.6	1.4	3.3	2.6	1.7	1.3	2.0	1.5	
	orf19.6057	1.4	1.3	1.9	2.2	1.5	1.4	2.0	1.9	3-methyl-2-oxobutanoate hydroxymethyltransferase activity (IEA)
	orf19.1162.1	1.1	1.3	2.4	1.6	1.3	1.1	1.9	1.7	
	orf19.719	1.1	1.5	1.7	2.4	1.3	1.2	1.9	1.8	ubiquitin-protein ligase activity (IEA)
	orf19.6148	1.4	1.9	4.5	3.0	1.8	1.4	1.9	1.8	structural constituent of cytoskeleton (IEA)
	orf19.6626	1.2	1.3	1.9	2.2	1.4	1.4	1.9	1.8	
	orf19.6411	1.3	1.4	2.4	2.3	1.3	1.3	1.9	1.8	enzyme activator activity (IEA)
	orf19.4292	1.2	1.1	1.8	1.8	1.4	1.1	1.9	1.5	SNAP receptor activity (IEA)
	orf19.6832	1.2	1.3	1.8	1.8	1.4	1.1	1.9	1.6	
	orf19.1306	1.2	0.9	2.2	2.1	1.4	1.3	1.9	1.8	
	orf19.775	1.1	1.6	2.4	2.1	1.3	1.1	1.8	1.6	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.2278	1.4	1.6	2.5	2.2	1.6	1.4	1.8	1.7	phosphatidylinositol transporter activity (IEA) oxalate transmembrane transporter activity (ISS) phospholipase activity (ISS) triglyceride lipase activity (IEA)
	orf19.5125	1.1	1.8	2.4	1.6	1.1	0.8	1.8	1.5	
	orf19.5711	1.4	1.4	2.1	1.5	1.4	1.2	1.8	1.7	
	orf19.1424	1.1	1.6	2.1	2.2	1.1	1.5	1.8	1.9	
	orf19.4699	1.0	2.2	2.1	2.5	1.2	1.3	1.8	2.0	
	orf19.5925	1.3	1.1	1.5	1.6	1.7	1.0	1.8	1.8	
	orf19.577	1.2	1.4	2.3	1.8	1.2	1.1	1.8	1.7	
	orf19.6194	1.1	1.5	1.8	1.7	1.3	1.1	1.8	1.5	
	orf19.5459	1.3	2.0	1.9	1.6	1.2	1.8	1.7	1.8	
	orf19.7092	1.3	1.2	2.0	1.7	1.4	1.1	1.7	1.5	
	orf19.346	1.2	1.6	2.0	1.8	1.1	1.0	1.7	1.6	L-alanine:2-oxoglutarate aminotransferase activity (IEA)
	orf19.5751	1.2	1.2	2.0	2.4	1.4	1.3	1.7	1.5	
	orf19.3058	1.3	1.4	1.9	2.0	1.3	1.3	1.7	1.9	aldehyde reductase activity (IEA) contributes_to ubiquitin-protein ligase activity (IEA) D-lactate dehydrogenase (cytochrome) activity (IEA)
	orf19.6816	1.0	1.1	1.5	1.7	1.2	1.1	1.7	1.6	
	orf19.5752	1.1	1.3	1.8	2.0	1.2	1.2	1.7	1.7	
	orf19.6043	1.3	1.5	2.5	1.9	1.7	1.3	1.7	1.8	sterol esterase activity (IEA)
	orf19.2558	1.3	1.3	1.5	2.1	1.4	1.6	1.7	1.9	
	orf19.2050	0.9	1.6	1.9	1.9	1.0	1.1	1.7	2.0	transcription factor activity (ISS) ubiquitin-protein ligase activity (IEA)
	orf19.3615	1.3	1.6	2.3	2.9	1.5	1.3	1.7	1.9	
	orf19.3991	1.3	1.2	1.7	1.6	1.3	1.3	1.7	1.5	
	orf19.6850	1.1	1.3	1.7	1.7	1.2	1.1	1.7	1.7	protein binding (IEA) ubiquitin-specific protease activity (IEA)
	orf19.3508	0.8	2.0	2.2	2.0	1.0	1.2	1.7	2.3	
	orf19.135	1.4	1.2	2.1	1.6	1.7	1.0	1.7	1.5	
	orf19.3815	1.4	1.1	1.5	1.8	1.4	1.0	1.7	1.6	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.1630	1.5	1.1	1.6	1.6	1.3	1.3	1.7	1.5	specific RNA polymerase II transcription factor activity (IEA)
	orf19.3910	0.9	2.2	2.9	2.4	0.8	0.6	1.7	1.7	
	orf19.1433	1.1	1.4	1.8	2.0	1.3	1.1	1.6	1.6	
	orf19.6102	1.2	1.4	1.9	1.8	1.3	1.3	1.6	1.8	
	orf19.467	1.3	2.0	2.8	1.7	1.3	1.1	1.6	1.6	CDP-diacylglycerol-inositol 3-phosphatidyltransferase activity (IEA)
	orf19.6860	1.2	1.4	1.6	1.8	1.2	1.3	1.6	1.6	
	orf19.1307	1.0	2.9	3.2	1.7	0.8	1.2	1.6	2.1	2,4-dienoyl-CoA reductase (NADPH) activity (IEA)
	orf19.6838	1.1	1.8	1.9	2.0	1.1	1.3	1.6	2.0	
	orf19.1162	1.2	1.5	1.7	1.6	1.3	1.4	1.6	1.7	
	orf19.1940	1.0	1.4	1.6	2.1	1.2	1.3	1.6	1.8	phosphatidylinositol-3,5-bisphosphate 5-phosphatase activity (IEA)
	orf19.4865	1.2	1.2	1.5	1.6	1.2	1.3	1.6	1.7	
	orf19.6003	1.6	1.3	2.2	1.7	1.6	0.8	1.6	1.5	
	orf19.3659	1.1	1.2	1.6	1.7	1.3	1.2	1.6	1.6	
	orf19.5614	0.9	2.6	2.4	3.1	0.9	2.0	1.6	2.3	ribonuclease H activity (IEA)
	orf19.7459	1.2	0.9	1.5	1.5	1.3	1.2	1.6	1.6	metallochaperone activity (IEA) transporter activity (IEA)
	orf19.2838	0.7	1.5	1.5	1.6	0.8	0.8	1.6	1.9	
	orf19.2163	1.0	1.7	1.8	1.9	1.1	1.2	1.6	1.6	
	orf19.417	1.4	1.4	1.6	1.8	1.3	1.5	1.6	1.6	
	orf19.3973	0.9	2.0	2.1	2.1	1.0	1.1	1.6	2.0	sequence-specific DNA binding (IEA) transcription factor activity (IEA)
	orf19.5953	1.4	1.2	1.5	1.9	1.3	1.4	1.5	1.7	
	orf19.4127	1.1	1.2	1.5	1.7	1.3	1.2	1.5	1.5	
	orf19.4055	0.7	1.1	1.5	1.5	0.9	0.7	1.5	1.6	
	orf19.3021	0.8	1.5	1.9	2.0	1.4	1.0	1.5	1.7	
	orf19.5943	1.3	1.2	1.6	1.6	1.2	1.0	1.5	1.5	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.7038	1.1	1.4	1.8	1.8	1.1	1.0	1.5	1.5	phosphatidylinositol 3-phosphate binding (IEA)
	orf19.6443	1.2	1.9	2.1	1.9	1.2	1.2	1.5	1.7	dodecenoyl-CoA delta-isomerase activity (IEA)
	orf19.3233	1.2	1.2	1.9	1.5	1.2	1.2	1.5	1.6	protein binding (IEA)
	orf19.6995	1.0	1.1	1.9	1.6	0.6	0.9	1.5	1.6	
	orf19.5236	1.2	1.4	1.5	1.5	1.3	1.0	1.5	1.7	dehydrodolichyl diphosphate synthase activity (IEA)
	orf19.6469	1.1	1.5	1.7	1.7	1.1	1.2	1.5	1.7	* RNA-directed DNA polymerase activity
	orf19.6440	1.1	1.2	1.7	1.5	1.0	0.9	1.5	1.5	ubiquitin-protein ligase activity (IEA)
	orf19.278	1.4	1.6	1.6	1.9	1.4	1.2	1.5	1.7	
	orf19.827	1.4	1.9	1.9	2.9	1.3	1.4	1.5	1.8	
	orf19.4070	1.2	1.6	1.7	1.6	1.3	1.2	1.5	1.7	
	orf19.5239	1.0	1.8	2.4	2.1	1.8	1.0	1.5	1.5	
	orf19.6048	0.8	1.3	1.5	1.5	0.8	1.0	1.5	1.6	dolichyl-phosphate-mannose-protein mannosyltransferase activity (IEA)
ALS1	orf19.5741	2.6	0.9	1.3	2.2	2.8	2.3	3.4	2.1	protein binding (IDA) cell adhesion molecule binding (IEA)
IFM3	orf19.2176	1.6	1.4	1.4	1.4	1.6	1.7	1.8	1.8	
MSO1	orf19.5248	1.3	1.4	2.7	3.0	1.6	1.5	2.9	2.2	
	orf19.4041	1.8	1.3	1.2	2.4	1.9	1.5	3.2	2.3	ubiquitin-protein ligase activity (IEA)
	orf19.1050	1.3	2.5	0.8	3.2	1.9	2.9	1.6	1.9	
	orf19.2016	1.1	1.5	1.3	2.1	1.5	1.7	1.5	1.6	protein binding, bridging (IEA)
COQ4	orf19.3008	1.7	1.4	2.6	2.8	1.8	1.5	2.9	2.3	
DAL1	orf19.5454	2.1	1.3	1.8	2.3	2.8	2.4	2.8	1.9	
DAP1	orf19.489	1.3	2.0	3.0	3.9	1.8	2.0	2.7	2.8	heme binding (IEA)
ERG11	orf19.922	1.6	1.4	1.8	1.6	1.7	1.6	1.7	1.7	drug binding (IDA, ISS) sterol 14-demethylase activity (IDA)
ERG6	orf19.1631	1.6	1.4	2.1	1.5	1.8	1.8	1.7	1.9	sterol 24-C-methyltransferase activity (IGI, IMP, ISS)
ERG7	orf19.1570	1.4	1.6	2.4	2.1	1.6	1.7	2.4	2.7	lanosterol synthase activity (IGI, ISS)

Appendix B. Continued.

		Change in fold expression ^a								
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
<i>ERG9</i>	orf19.3616	1.5	1.3	2.0	1.7	1.7	1.5	1.8	1.8	farnesyl-diphosphate farnesyltransferase activity (ISS)
<i>EXG2</i>	orf19.2952	2.0	1.4	2.4	2.4	2.0	1.5	2.4	1.9	glucan 1,3-beta-glucosidase activity (IDA, IGI, IMP)
<i>IPK2</i>	orf19.1377	2.1	1.2	3.0	2.2	2.5	1.5	3.7	2.1	inositol 1,3,4,5,6-pentakisphosphate kinase activity (IEA)
<i>PGA10</i>	orf19.5674	1.4	2.3	1.8	2.4	1.6	2.2	1.6	2.5	heme binding (IDA, IGI)
<i>SLD1</i>	orf19.260	1.6	1.4	1.9	2.0	1.7	1.6	1.9	1.8	
<i>SNO1</i>	orf19.2948	1.4	1.4	2.4	2.0	1.8	1.5	2.3	1.9	imidazoleglycerol-phosphate synthase activity (IEA)
	orf19.692	1.3	2.7	2.2	3.4	1.7	1.8	3.0	4.1	
	orf19.1344	4.7	1.3	2.9	1.8	3.2	2.5	3.5	2.1	
	orf19.7504	2.2	1.4	4.0	2.4	2.4	1.8	2.6	1.8	
	orf19.2175	1.5	1.3	1.5	1.6	1.8	1.6	1.8	1.7	
	orf19.2452	2.2	1.3	2.1	1.9	2.2	1.5	2.7	1.7	
	orf19.4167	1.9	0.7	2.2	2.2	2.8	1.7	2.9	1.7	
	orf19.4690	1.2	5.0	11.3	10.3	1.9	2.4	3.4	6.7	manganese ion transmembrane transporter activity (IEA)
	orf19.3921	1.6	1.2	2.8	2.2	1.6	1.5	2.0	1.7	
	orf19.633	1.7	1.0	2.3	2.3	1.9	1.6	2.8	1.9	
	orf19.4459	1.6	1.2	3.5	3.5	2.1	1.7	2.4	2.0	
	orf19.6753	1.4	1.5	3.3	1.5	1.8	1.5	2.9	2.0	
	orf19.6558	1.7	1.4	1.7	1.7	1.7	1.5	1.8	1.5	GTPase activator activity (IEA)
	orf19.633	1.7	1.0	2.3	2.3	1.9	1.6	2.8	1.9	
	orf19.6558	1.7	1.4	1.7	1.7	1.7	1.5	1.8	1.5	GTPase activator activity (IEA)
	orf19.2296	2.2	2.4	3.6	1.2	1.7	1.9	2.7	1.9	
	orf19.6342	1.9	3.9	3.3	9.3	0.6	4.7	2.5	9.9	
<i>ARO9</i>	orf19.1237	1.5	3.4	5.8	4.2	1.5	1.9	2.1	2.8	aromatic-amino-acid:2-oxoglutarate aminotransferase activity (ISS)
<i>CAG1</i>	orf19.4015	2.4	1.6	2.7	1.5	1.4	0.9	2.1	1.5	GTPase activity (IGI) guanyl nucleotide binding (ISS)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
<i>CAN1</i>	orf19.97	1.6	1.7	5.5	3.2	1.2	1.1	2.5	2.4	amino acid binding
<i>CDR11</i>	orf19.919	1.8	2.1	4.1	4.9	1.4	1.5	2.3	4.2	ATP binding (ISS)
<i>CNH1</i>	orf19.367	2.3	1.9	2.8	2.3	1.9	1.3	2.4	1.8	Candida albicans Na+/H+ antiporter
<i>ENT3</i>	orf19.1553	2.1	1.9	2.2	2.1	2.0	1.4	1.5	1.9	
<i>HAP31</i>	orf19.517	1.5	1.7	2.3	2.4	1.8	1.4	2.0	1.9	transcription factor activity (NAS) transcription activator activity (IEA)
<i>HAP42</i>	orf19.1481	1.6	1.5	3.6	2.3	2.2	1.0	2.1	1.5	
<i>IFR1</i>	orf19.1763	1.6	3.3	6.8	2.9	1.9	1.1	2.6	2.4	
<i>LIG4</i>	orf19.5798	1.7	1.5	3.3	2.4	1.9	1.2	2.6	1.9	DNA ligase activity (IGI, ISS) DNA ligase (ATP) activity (IEA)
<i>PXP2</i>	orf19.1655	1.5	2.5	3.5	3.3	0.8	1.3	2.0	2.2	acyl-CoA oxidase activity (ISS)
<i>UGA6</i>	orf19.5820	1.7	1.9	3.7	1.5	1.7	1.0	3.0	1.8	amino acid transmembrane transporter activity
<i>ZCF11</i>	orf19.2423	1.7	1.5	2.8	2.5	2.1	1.4	2.9	2.3	sequence-specific DNA binding (IEA)
	orf19.4191.1	1.5	1.5	3.2	2.9	1.9	1.4	3.5	3.1	ubiquitin-ubiquitin ligase activity (IEA)
	orf19.2836	2.5	1.7	5.3	3.4	2.0	1.3	5.7	3.0	
	orf19.6970	1.8	1.9	3.8	3.8	2.6	1.4	4.7	2.8	
	orf19.3848	2.4	1.8	6.3	3.5	2.0	1.1	6.4	2.8	
	orf19.5799	1.7	2.0	3.4	2.4	1.5	1.5	2.4	2.6	
	orf19.6986	2.4	1.8	2.5	2.2	2.8	1.2	2.6	2.2	
	orf19.1881	1.5	1.6	2.4	2.2	1.5	1.5	2.4	2.1	1-acylglycerol-3-phosphate O-acyltransferase activity (IEA)
	orf19.4763	1.6	2.0	2.7	3.1	1.7	1.1	2.3	2.0	
	orf19.1964	1.5	2.0	1.6	2.1	1.2	1.9	1.6	2.0	
	orf19.5644	1.9	1.6	3.1	2.2	1.7	1.2	2.6	1.9	
	orf19.4982	1.7	1.6	3.0	2.0	1.9	1.1	2.7	1.8	
	orf19.5037	3.1	1.5	2.3	2.0	2.0	1.2	2.7	1.8	
	orf19.541	1.6	1.6	1.7	1.9	1.6	1.2	2.2	1.7	

Appendix B. Continued.

		Change in fold expression ^a								
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
	orf19.4170	2.3	1.7	2.8	2.5	2.9	1.3	4.2	1.7	structural constituent of cell wall (IEA)
	orf19.5426	2.0	1.9	3.0	1.8	1.6	1.2	2.1	1.7	triglyceride lipase activity (IEA)
	orf19.7380	1.5	2.1	2.4	2.3	1.3	1.2	1.6	1.6	
	orf19.6797	1.5	2.1	1.5	1.8	1.1	1.7	1.8	1.8	
	orf19.4657	2.1	1.5	2.8	2.0	2.1	1.4	2.5	1.6	contributes_to phosphoprotein phosphatase activity (IEA)
	orf19.1504	1.6	1.6	2.7	1.6	1.8	1.0	3.0	1.6	
	orf19.7345	1.7	1.6	2.1	2.0	1.8	1.1	2.6	1.6	ATP binding (IEA)
	orf19.4346	1.6	1.6	2.4	1.8	1.6	0.9	2.1	1.5	protein anchor
	orf19.4589	1.5	1.5	1.8	1.6	1.3	1.3	1.7	1.5	amine oxidase activity (IEA)
	orf19.2607	1.7	1.5	2.2	1.7	2.0	1.3	1.8	1.5	
	orf19.6348	1.7	2.0	2.0	2.2	1.5	1.2	2.4	1.5	
PRM9	orf19.2508	14.1	2.9	15.1	1.6	13.3	2.6	13.7	1.2	
	orf19.1444	2.0	1.7	4.4	1.9	2.9	1.6	2.6	1.4	phosphatidylinositol-4,5-bisphosphate binding
	orf19.4330	1.7	2.0	2.3	1.9	1.7	1.5	1.4	1.8	
ARO9	orf19.1237	1.5	3.4	5.8	4.2	1.5	1.9	2.1	2.8	aromatic-amino-acid:2-oxoglutarate aminotransferase activity (ISS)
BTS1	orf19.6674	2.0	2.5	6.8	6.4	2.6	2.0	5.0	4.1	farnesyltranstransferase activity (IEA)
BUL1	orf19.5094	1.9	1.9	4.1	3.1	2.5	1.8	2.1	1.9	
COQ4	orf19.3008	1.5	1.5	2.3	2.8	1.9	1.6	2.6	2.5	
DDR48	orf19.4082	2.2	4.3	5.8	4.7	2.7	2.9	3.4	4.3	
ERG1	orf19.406	1.8	1.8	2.7	2.3	2.2	2.0	2.3	2.3	squalene monooxygenase activity (IDA, IMP) drug binding (IEA)
ERG2	orf19.6026	1.7	1.5	2.3	1.9	1.9	1.9	2.1	2.0	C-8 sterol isomerase activity (IGI, IMP)
ERG24	orf19.1598	1.7	1.7	2.3	2.1	1.7	1.8	2.1	2.3	delta14-sterol reductase activity (IGI, ISS)
ERG26	orf19.2909	1.7	1.6	2.7	2.6	2.2	2.0	2.7	2.7	C-3 sterol dehydrogenase (C-4 sterol decarboxylase) activity (IGI, ISS)
ERG27	orf19.3240	1.8	2.1	3.3	3.4	2.2	2.2	3.1	3.2	3-keto sterol reductase activity (IGI, IMP)

Appendix B. Continued.

		Change in fold expression ^a								
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
<i>ERG7</i>	orf19.1570	1.5	1.6	2.8	2.1	1.8	1.6	2.7	2.4	lanosterol synthase activity (IGI, ISS)
<i>FMA1</i>	orf19.6837	1.8	3.2	2.7	5.3	1.5	3.3	1.7	3.0	nucleotide binding, oxidoreductase activity
<i>FRP1</i>	orf19.5634	5.4	5.7	17.3	17.5	8.1	5.9	16.3	14.7	ferric-chelate reductase activity (IEA, IMP)
<i>FTH1</i>	orf19.4802	2.6	2.6	5.3	5.2	2.3	1.9	6.6	5.2	
<i>GYP1</i>	orf19.3811	1.6	1.6	3.7	3.5	1.8	1.5	3.7	3.4	Rab GTPase activator activity (IEA)
<i>HEM14</i>	orf19.4747	2.1	1.6	3.6	3.0	2.2	1.8	2.9	2.3	oxygen-dependent protoporphyrinogen oxidase activity (ISS)
<i>HXT5</i>	orf19.4384	1.8	2.1	2.6	2.0	1.8	1.5	2.3	1.7	hexose transmembrane transporter activity (IEP)
<i>IDP2</i>	orf19.3733	2.2	1.6	3.3	2.8	2.6	2.0	3.2	2.5	isocitrate dehydrogenase (NADP+) activity (IEA)
<i>IFD7</i>	orf19.629	1.7	3.2	4.6	6.8	1.7	2.7	1.7	4.3	
<i>MDR1</i>	orf19.5604	5.9	5.5	15.1	8.0	6.6	6.1	5.9	5.7	drug transporter activity (ISS) fluconazole transporter activity (IDA)
<i>NCP1</i>	orf19.2672	1.6	1.7	2.6	2.0	1.7	1.9	1.9	1.9	NADPH-hemoprotein reductase activity (ISS) electron carrier activity (IEA)
<i>PGA45</i>	orf19.2451	1.7	1.5	2.1	2.2	1.7	1.9	2.0	2.3	
<i>PGA7</i>	orf19.5635	2.3	3.7	5.7	7.4	2.7	3.8	4.8	6.1	
<i>PHO100</i>	orf19.4424	1.5	3.4	4.8	2.7	1.6	1.5	2.0	2.3	acid phosphatase activity (NAS)
<i>SET3</i>	orf19.7221	2.8	2.7	6.3	5.7	4.4	3.6	4.8	4.8	NAD-dependent histone deacetylase activity (IEA)
<i>SLD1</i>	orf19.260	1.8	1.5	2.2	1.8	1.7	1.6	2.0	1.9	oxidoreductase activity
<i>TEF4</i>	orf19.2652	16.4	16.6	55.8	90.2	29.9	32.7	65.5	67.8	translation elongation factor activity (IEA)
<i>YMX6</i>	orf19.5713	1.6	2.6	2.9	2.0	1.6	1.6	1.7	1.8	NADH dehydrogenase activity (IEA)
	orf19.1801	1.7	1.6	2.4	2.0	1.9	1.6	2.0	1.8	cytochrome-b5 reductase activity (IEA)
	orf19.5535	1.5	1.6	2.2	2.7	1.5	1.9	2.7	3.2	anion:cation symporter activity (ISS) pantothenate transporter activity (IEA)
	orf19.6169.2	1.7	1.8	2.6	2.5	1.8	1.7	2.0	1.5	
	orf19.288	1.8	1.5	3.0	2.5	2.1	1.5	2.5	2.0	methylenetetrahydrofolate reductase (NADPH) activity (IEA)
	orf19.329	2.1	2.3	2.4	2.6	2.0	1.6	2.5	2.5	RNA binding (IEA)
	orf19.7547	1.8	1.5	2.8	2.7	2.0	1.5	3.1	2.3	phosphatidylinositol 3-phosphate binding (IEA) ubiquitin-protein ligase activity (IEA)

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.7329	1.6	2.1	3.1	3.0	1.7	1.8	2.5	2.3	ubiquitin-protein ligase activity (IEA)
	orf19.3627	4.0	1.9	7.0	4.0	4.4	1.9	5.1	1.9	
	orf19.5210	1.5	2.1	2.7	2.7	1.8	1.5	2.9	2.3	transcription factor activity (IEA)
	orf19.2350	1.7	1.7	3.4	4.4	2.0	1.9	3.2	3.0	drug transporter activity (ISS)
	orf19.3483	2.0	2.4	5.3	4.7	2.9	2.2	3.5	3.3	phosphatidylglycerol phospholipase C activity (IEA)
	orf19.344	8.2	9.0	25.0	20.7	9.7	7.3	13.3	10.2	
	orf19.5777	1.7	2.4	4.8	3.8	1.9	1.5	3.1	2.5	
	orf19.5799	1.7	2.0	3.4	2.4	1.5	1.5	2.4	2.6	
	orf19.3261	1.8	2.2	2.7	3.4	1.6	2.3	4.5	4.5	
	orf19.7456	1.7	2.6	4.4	4.1	2.2	2.1	3.9	3.5	
	orf19.7043	2.5	1.9	5.2	3.6	2.8	2.3	4.9	3.0	
	orf19.4013	2.6	2.2	4.2	3.5	3.1	2.8	4.8	3.8	
	orf19.4014	1.5	2.1	2.3	2.4	1.7	1.6	2.6	2.6	
	orf19.4031	1.8	1.8	2.5	2.5	1.6	1.5	2.9	2.5	
	orf19.6840	2.0	2.1	4.1	4.4	2.6	2.4	3.1	2.6	
	orf19.286	2.1	2.5	2.3	2.2	1.9	1.9	3.5	1.9	
	orf19.1865	2.4	2.2	3.7	2.9	2.4	2.2	3.4	3.1	
	orf19.7504	2.1	1.8	3.0	3.3	1.9	2.0	2.2	2.1	
	orf19.3737	1.6	1.9	2.9	3.4	2.0	1.8	3.1	2.6	
	orf19.7263	1.8	1.9	2.9	2.8	1.6	1.7	2.1	1.9	
	orf19.1964	2.0	1.8	2.5	2.2	1.7	1.6	2.2	1.6	
	orf19.1800	2.3	2.2	4.6	3.9	2.5	2.2	4.5	3.4	
	orf19.6025	2.2	2.1	3.9	5.2	3.8	3.1	5.2	5.2	N-acetylglucosaminylidiphosphodolichol N-acetylglucosaminyltransferase activity
	orf19.2691	1.5	5.4	5.4	9.1	1.6	2.3	4.4	6.5	

Appendix B. Continued.

CGD Name	orf19 Designation	Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.496	2.4	2.3	4.4	3.8	2.5	2.0	3.6	3.1	DNA-dependent ATPase activity (IEA)
	orf19.1881	1.5	1.6	2.4	2.2	1.5	1.5	2.4	2.1	1-acylglycerol-3-phosphate O-acyltransferase activity (IEA)
	orf19.2285	1.7	2.5	1.8	1.5	1.8	2.4	2.1	2.1	

^a Fold change defined as the average ratio of gene expression levels in the isolates compared in two independent microarray experiments.

Bold indicates “real” result as defined in Materials and Methods

Red shading indicates ratio values above 1.5.

APPENDIX C. GENES DOWN-REGULATED IN STRAINS CARRYING *UPC2* GOF ALLELE BY AT LEAST 1.5 FOLD

		Change in fold expression ^a								
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
<i>ABP140</i>	orf19.3676	1.6	0.4	0.5	0.5	1.7	1.0	0.8	0.5	
<i>ADH3</i>	orf19.4505	0.5	1.8	0.6	0.6	0.7	1.4	0.8	0.8	alcohol dehydrogenase (NAD) activity (IEA)
<i>AGP2</i>	orf19.4679	1.1	0.8	0.6	0.6	0.8	0.7	0.9	0.6	amino acid transmembrane transporter activity (IEA)
<i>ARX1</i>	orf19.3015	1.2	0.5	0.4	0.6	1.2	1.0	0.7	0.6	
<i>AYR2</i>	orf19.5615	0.7	1.1	0.6	0.6	0.6	0.7	0.7	0.9	acylglycerone-phosphate reductase activity (IEA)
<i>BUD21</i>	orf19.5430	1.1	0.6	0.4	0.4	1.2	1.2	0.7	0.5	snoRNA binding (IEA)
<i>BUD22</i>	orf19.3287	1.5	0.6	0.4	0.5	1.3	0.9	0.7	0.4	
<i>CAN2</i>	orf19.111	0.8	0.8	0.6	0.6	0.6	0.7	0.7	0.7	arginine transmembrane transporter activity
<i>CCW14</i>	orf19.1969	0.8	0.6	0.4	0.6	0.8	0.5	0.9	0.5	structural constituent of cell wall (IEA)
<i>CDR1</i>	orf19.6000	1.0	0.7	0.5	0.6	0.9	0.8	0.8	0.8	ATP binding (IDA, IGI, IMP, ISS)
<i>CDR3</i>	orf19.1313	0.9	0.8	0.6	0.6	0.8	0.8	0.8	0.7	
<i>CHR1</i>	orf19.3756	2.0	0.5	0.6	0.5	2.3	0.8	0.8	0.3	ATP-dependent RNA helicase activity (ISS)
<i>CHS6</i>	orf19.5155	0.9	0.7	0.6	0.4	0.7	0.5	0.8	0.4	
<i>CIC1</i>	orf19.124	1.9	0.5	0.5	0.5	1.8	1.1	0.9	0.5	protein binding, bridging (IEA)
<i>CNS1</i>	orf19.6052	1.2	0.7	0.6	0.6	1.3	1.0	0.7	0.6	Hsp70 protein binding (IEA) Hsp90 protein binding (IEA)
<i>CSI2</i>	orf19.5232	1.5	0.5	0.5	0.6	1.7	1.0	1.0	0.5	5S rRNA binding (IEA) rRNA primary transcript binding (IEA)
<i>DBP7</i>	orf19.6902	1.6	0.5	0.5	0.6	1.5	1.1	0.9	0.6	ATP-dependent RNA helicase activity (IEA)
<i>DBP8</i>	orf19.6652	1.6	0.5	0.5	0.6	1.8	0.9	0.7	0.3	ATP-dependent RNA helicase activity (IEA)
<i>DFI1</i>	orf19.7084	0.9	0.8	0.5	0.6	0.8	1.0	0.7	0.8	
<i>DIP2</i>	orf19.5106	1.5	0.6	0.5	0.5	1.4	1.1	0.8	0.5	snoRNA binding (IEA)
<i>DLD1</i>	orf19.5805	0.8	1.0	0.6	0.6	0.6	0.8	0.5	0.7	D-lactate dehydrogenase (cytochrome) activity (IEA)
<i>ECM1</i>	orf19.5299	1.9	0.6	0.5	0.6	1.6	0.9	0.8	0.4	

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
<i>ENP1</i>	orf19.5507	1.5	0.6	0.5	0.4	1.3	1.1	0.7	0.4	snoRNA binding (IEA)	
<i>ENP2</i>	orf19.6686	1.5	0.5	0.3	0.5	1.6	1.0	0.9	0.3		
<i>FAD3</i>	orf19.4933	1.1	0.5	0.3	0.5	1.0	1.0	0.8	0.6	omega-3 fatty acid desaturase activity (IMP)	
<i>FCR1</i>	orf19.6817	0.7	0.9	0.6	0.6	0.6	0.7	0.7	0.7	purine nucleotide binding (ISS) transcription factor activity (IGI, ISS)	
<i>FCY21</i>	orf19.1357	1.2	1.0	0.6	0.6	1.0	0.4	1.0	0.3		
<i>GAL102</i>	orf19.3674	0.7	0.5	0.6	0.4	1.1	0.4	0.8	0.5	UDP-glucose 4-epimerase activity (IEA)	
<i>GAP4</i>	orf19.4456	0.9	0.8	0.6	0.6	0.8	0.9	0.7	0.7		
<i>GAT2</i>	orf19.4056	1.2	1.4	0.4	0.5	0.8	1.0	0.6	0.4	transcription factor activity (IEA)	
<i>HBR3</i>	orf19.6955	1.3	0.6	0.5	0.5	1.5	1.3	0.7	0.5	SSU rRNA binding (IEA) endoribonuclease activity (IEA)	
<i>HCA4</i>	orf19.2712	1.5	0.5	0.6	0.5	1.8	1.0	0.8	0.5		
<i>HGT8</i>	orf19.2021	0.8	0.8	0.5	0.5	0.7	0.7	0.6	0.7	glucose transmembrane transporter activity (ISS)	
<i>IFD6</i>	orf19.1048	0.7	0.7	0.6	0.4	0.9	0.6	0.8	0.6	aryl-alcohol dehydrogenase activity (NAS)	
<i>IPT1</i>	orf19.4769	0.8	0.8	0.5	0.6	0.6	0.8	0.7	0.6	transferase activity, transferring phosphorus-containing groups (IMP, ISS)	
<i>KTI12</i>	orf19.2385	1.2	0.7	0.5	0.5	1.5	0.9	0.7	0.5	chromatin binding (IEA) enzyme regulator activity (IEA)	
<i>MDN1</i>	orf19.4697	1.4	1.1	0.6	0.3	1.1	1.1	0.8	0.5	ATPase activity (IEA)	
<i>MET10</i>	orf19.4076	0.9	0.7	0.5	0.6	0.7	0.8	0.7	0.7	sulfite reductase (NADPH) activity (IEA) contributes_to sulfite reductase (NADPH) activity (IEA)	
<i>MET14</i>	orf19.946	0.9	0.8	0.6	0.5	0.9	1.0	1.0	0.9	adenylylsulfate kinase activity (IEA)	
<i>MET3</i>	orf19.5025	1.4	0.9	0.4	0.4	1.1	0.8	1.3	0.8	sulfate adenylyltransferase (ATP) activity (ISS)	
<i>NGT1</i>	orf19.5392	1.6	0.4	0.5	0.6	1.0	0.8	1.1	0.7	N-acetylglucosamine transmembrane transporter activity (IDA, IMP, ISS)	
<i>NIP7</i>	orf19.3478	1.4	0.5	0.4	0.5	1.2	1.0	0.7	0.4	protein binding (IEA)	
<i>NOC4</i>	orf19.1902	1.4	0.5	0.4	0.4	1.4	1.0	0.7	0.5		
<i>NSA1</i>	orf19.2185	1.3	0.5	0.5	0.6	1.7	1.1	0.7	0.5		
<i>PGA26</i>	orf19.2475	0.6	0.8	0.5	0.5	0.5	0.7	0.6	0.7		

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
<i>PGA32</i>	orf19.6784	0.9	0.9	0.6	0.5	0.8	0.8	0.7	0.6		
<i>PHO113</i>	orf19.2619	0.8	0.7	0.6	0.6	0.8	0.8	0.7	0.7	acid phosphatase activity (IEA)	
<i>POL93</i>	orf19.6078	1.2	0.7	0.6	0.6	1.0	0.8	0.9	0.6	DNA binding, RNA binding, RNA directed DNA polymerase activity	
<i>POL93</i>	orf19.6078	1.0	0.5	0.3	0.4	0.8	0.8	0.8	0.7		
<i>POP3</i>	orf19.7657	1.2	0.7	0.5	0.5	1.3	1.1	0.7	0.5	ribonuclease MRP activity (IEA)	
<i>POP4</i>	orf19.5567	1.2	0.7	0.6	0.6	1.3	0.9	0.7	0.6	ribonuclease MRP activity (IEA)	
<i>PRN4</i>	orf19.2461	0.9	0.7	0.6	0.5	0.9	0.6	0.7	0.6		
<i>PUT1</i>	orf19.4274	1.2	0.8	0.6	0.5	1.0	0.7	0.7	0.6	proline dehydrogenase activity (IEA)	
<i>PUT2</i>	orf19.3974	1.2	0.9	0.6	0.6	1.0	0.8	0.8	0.8	1-pyrroline-5-carboxylate dehydrogenase activity (IEA)	
<i>PWP2</i>	orf19.3276	1.4	0.5	0.5	0.5	1.4	1.0	0.7	0.5	snoRNA binding (IEA)	
<i>RCL1</i>	orf19.1886	1.4	0.7	0.6	0.5	1.3	0.9	0.8	0.6	RNA-3'-phosphate cyclase activity (IEA)	
<i>REI1</i>	orf19.59	1.8	0.5	0.5	0.5	1.7	1.0	0.8	0.4	sequence-specific DNA binding (IEA)	
<i>RHD3</i>	orf19.5305	0.8	0.9	0.4	0.5	0.7	0.8	0.6	0.8		
<i>RIM2</i>	orf19.4499	1.1	0.7	0.6	0.6	0.9	1.0	0.7	0.6	pyrimidine nucleotide transmembrane transporter activity (IEA)	
<i>RLM1</i>	orf19.4662	1.2	0.8	0.5	0.6	1.0	0.8	0.9	0.5	transcription regulator activity (IMP) DNA bending activity (IEA)	
<i>RPA12</i>	orf19.2287	1.8	0.5	0.5	0.5	1.7	1.0	1.0	0.4	DNA-directed RNA polymerase activity (IEA) exoribonuclease activity (IEA)	
<i>RPF1</i>	orf19.2667	1.5	0.5	0.5	0.6	1.4	1.1	1.0	0.6	rRNA primary transcript binding (IEA)	
<i>RRN11</i>	orf19.718	1.3	0.5	0.6	0.6	1.4	0.9	0.9	0.4	transcription factor activity (ISS) RNA polymerase I transcription factor activity (IEA)	
<i>RRP15</i>	orf19.563	1.6	0.5	0.5	0.5	1.6	1.0	0.7	0.5		
<i>RTA2</i>	orf19.24	0.9	1.0	0.6	0.5	0.7	0.7	0.8	0.7	phospholipid-translocating ATPase activity (IEA)	
<i>SDA1</i>	orf19.6648	1.4	0.5	0.4	0.5	1.4	1.0	0.8	0.5		
<i>SKN1</i>	orf19.7362	1.5	0.4	0.3	0.3	1.2	0.7	0.8	0.3		
<i>SNG3</i>	orf19.1333	1.0	0.8	0.6	0.6	0.8	0.5	0.8	0.6		

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
<i>SPC98</i>	orf19.2600	0.9	0.9	0.4	0.6	1.2	0.7	0.8	0.6	structural constituent of cytoskeleton (IEA)	
<i>SYG1</i>	orf19.768	0.8	0.9	0.5	0.6	0.8	0.7	0.7	0.6		
<i>THI4</i>	orf19.5986	0.9	1.0	0.6	0.5	0.7	0.9	0.6	0.7		
<i>TRM2</i>	orf19.3327	1.4	0.7	0.5	0.5	1.5	1.0	0.7	0.4	double-stranded DNA specific 5'-3' exodeoxyribonuclease activity (IEA)	
<i>TSR2</i>	orf19.2998	1.4	0.5	0.5	0.6	1.6	1.0	0.8	0.5		
<i>ULP1</i>	orf19.353	0.8	0.6	0.6	0.6	1.0	0.8	0.8	0.6	SUMO-specific protease activity (IDA)	
<i>UTP15</i>	orf19.3609	1.1	0.6	0.6	0.6	1.3	0.9	0.7	0.6	snoRNA binding (IEA)	
<i>UTP21</i>	orf19.1566	1.6	0.5	0.6	0.5	1.4	0.8	0.7	0.4		
<i>UTP4</i>	orf19.1633	1.4	0.5	0.5	0.6	1.3	1.1	1.1	0.6	snoRNA binding (IEA)	
<i>UTP5</i>	orf19.7599	1.5	0.6	0.6	0.5	1.5	1.0	0.8	0.5		
<i>UTP8</i>	orf19.5436	1.1	0.7	0.5	0.6	1.0	1.1	0.7	0.6	tRNA binding (ISO) snoRNA binding (IEA) tRNA binding (IEA)	
<i>UTP9</i>	orf19.6710	1.1	0.6	0.5	0.6	1.2	1.0	0.7	0.5	snoRNA binding (IEA)	
<i>YHB4</i>	orf19.7637	0.7	0.8	0.6	0.6	0.8	0.8	0.7	0.7	nitric oxide dioxygenase activity (IMP, ISS)	
<i>YTM1</i>	orf19.4815	1.1	0.6	0.4	0.6	1.3	1.0	0.7	0.5	cytoskeletal protein binding (IEA)	
<i>YVH1</i>	orf19.4401	1.3	0.6	0.6	0.5	1.6	1.0	0.8	0.4	protein tyrosine/serine/threonine phosphatase activity (ISS) protein tyrosine phosphatase activity (IEA)	
<i>ZRT2</i>	orf19.1585	0.7	0.8	0.5	0.6	0.6	1.0	0.6	0.8	low-affinity zinc ion transmembrane transporter activity (IEA)	
	orf19.5905	1.1	0.5	0.5	0.4	1.6	0.5	0.9	0.2		
	orf19.2384	1.5	0.8	0.6	0.4	1.2	0.8	0.7	0.3		
	orf19.1642	2.6	0.5	0.6	0.3	2.5	0.9	0.9	0.3	mRNA binding (IEA)	
	orf19.4760	1.6	0.5	0.4	0.4	2.0	0.8	0.8	0.4		
	orf19.2547	1.3	0.7	0.5	0.4	1.1	0.7	0.7	0.4		
	orf19.5353	1.8	0.7	0.6	0.5	1.4	0.8	0.9	0.4		
	orf19.6886	2.1	0.5	0.6	0.4	2.0	0.9	1.0	0.4	rRNA binding (IEA)	

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
	orf19.5704	1.2	0.6	0.6	0.5	1.1	0.9	0.7	0.4		
	orf19.1708	2.3	0.5	0.5	0.4	2.1	1.1	0.9	0.4		
	orf19.6862	1.8	0.7	0.5	0.4	1.7	0.9	0.8	0.4		
	orf19.2386	1.7	0.6	0.6	0.4	2.0	1.1	1.0	0.4		0
	orf19.2320	1.7	0.4	0.5	0.3	1.7	0.9	0.7	0.4	amino acid kinase activity (IEA) protein kinase activity (IEA)	
	orf19.6828	2.0	0.3	0.4	0.4	2.0	1.1	0.9	0.4	RNA binding (IEA)	
	orf19.107	1.5	0.7	0.6	0.5	1.4	1.0	0.7	0.4	RNA helicase activity (IEA)	
	orf19.6234	1.8	0.5	0.6	0.4	1.5	0.9	0.9	0.4		
	orf19.2167	1.2	0.7	0.6	0.6	1.5	0.6	0.8	0.4		
	orf19.6297	1.5	0.7	0.6	0.6	1.4	0.9	0.7	0.4	pseudouridine synthase activity (IEA) pseudouridylate synthase activity (IEA)	
	orf19.6014	2.4	0.5	0.6	0.5	2.4	0.9	1.2	0.4		
	orf19.1082	1.7	0.5	0.5	0.5	1.2	1.0	0.7	0.4		
	orf19.5038	1.3	0.6	0.5	0.5	1.1	1.1	0.7	0.4	tRNA (guanine) methyltransferase activity (IEA)	
	orf19.2830	1.6	0.6	0.5	0.4	1.7	1.0	0.7	0.4	snoRNA binding (IEA)	
	orf19.7468	1.1	0.9	0.5	0.6	1.2	0.9	0.7	0.4		
	orf19.2870	0.7	1.3	0.6	0.4	0.7	0.4	0.7	0.5		
	orf19.2319	2.0	0.6	0.6	0.6	1.6	1.0	0.9	0.5	RNA binding (IEA)	
	orf19.3393	1.4	0.5	0.5	0.5	1.5	1.1	0.7	0.5	ATP-dependent RNA helicase activity (IEA)	
	orf19.7650	1.4	0.7	0.6	0.6	1.5	0.9	0.8	0.5		
	orf19.4793	1.4	0.5	0.5	0.5	1.4	1.1	0.8	0.5		
	orf19.1360	0.9	1.1	0.4	0.6	0.8	1.1	0.7	0.5		
	orf19.7384	1.5	0.6	0.5	0.5	1.5	0.9	0.9	0.5	GTPase activity (IEA)	
	orf19.2362	1.3	0.7	0.5	0.6	1.5	0.9	0.9	0.5		

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
	orf19.3463	1.6	0.6	0.6	0.5	1.6	0.9	0.8	0.5		
	orf19.4792	1.4	0.8	0.5	0.6	1.2	0.9	0.8	0.5		
	orf19.5039	1.5	0.8	0.6	0.6	1.6	0.9	0.7	0.5	3'-5'-exoribonuclease activity (IEA)	
	orf19.715	0.8	0.5	0.4	0.4	0.8	0.7	0.8	0.5		
	orf19.3252	0.9	0.7	0.5	0.5	1.0	0.8	0.8	0.5	specific RNA polymerase II transcription factor activity (IEA)	
	orf19.4479	1.6	0.5	0.6	0.6	1.6	1.0	0.9	0.5	RNA binding (IEA)	
	orf19.6007	0.9	0.7	0.5	0.5	0.7	0.8	0.7	0.5		
	orf19.3089	1.0	0.9	0.5	0.6	0.8	0.8	0.6	0.5		
	orf19.603	1.2	0.7	0.6	0.6	1.2	1.1	0.7	0.5	rRNA primary transcript binding (IEA) single-stranded telomeric DNA binding (IEA)	
	orf19.4342	1.1	0.7	0.6	0.6	0.9	0.8	0.7	0.5	specific RNA polymerase II transcription factor activity (IEA) transcription factor activity (IEA)	
	orf19.1150.1	0.9	0.9	0.6	0.5	0.8	0.7	0.7	0.5		
	orf19.6431	1.5	0.7	0.6	0.6	1.5	0.8	0.9	0.5		
	orf19.2090	1.4	0.6	0.6	0.5	1.4	1.0	0.8	0.5	RNA helicase activity (IEA) snoRNA binding (IEA)	
	orf19.2306	1.0	0.7	0.6	0.6	1.0	0.8	0.7	0.5	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	
	orf19.1404	1.3	0.7	0.6	0.6	1.5	0.8	0.7	0.5	tRNA dihydrouridine synthase activity (IEA)	
	orf19.7296	1.1	1.0	0.6	0.4	0.8	0.4	0.9	0.5		
	orf19.1687	1.3	0.6	0.5	0.6	1.3	1.0	0.8	0.5	ATP-dependent RNA helicase activity (IEA) RNA splicing factor activity, transesterification mechanism (IEA)	
	orf19.3539	1.1	0.6	0.5	0.5	1.5	1.1	0.7	0.5		
	orf19.6874	1.0	0.9	0.6	0.6	0.8	0.8	0.7	0.5		
	orf19.3704	1.1	0.6	0.6	0.6	1.1	0.9	0.7	0.6	ATP-dependent RNA helicase activity (IEA)	
	orf19.1959	1.1	0.7	0.6	0.6	1.1	0.8	0.7	0.6		
	orf19.962	1.3	0.6	0.4	0.6	1.5	0.9	0.9	0.6		

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
	orf19.3303	1.1	0.6	0.6	0.5	1.1	0.9	0.7	0.6	tRNA methyltransferase activity (IEA)	
	orf19.6137	1.0	0.6	0.6	0.6	1.0	0.7	0.7	0.6		
	orf19.7190	1.3	0.8	0.4	0.6	1.2	0.8	0.7	0.6	oxidized purine base lesion DNA N-glycosylase activity (ISA, IEA)	
	orf19.7593	1.3	0.7	0.6	0.5	1.2	0.8	0.8	0.6	asparaginase activity (IEA)	
	orf19.4940	1.2	0.7	0.6	0.6	1.2	1.0	0.7	0.6	L-histidine transmembrane transporter activity (IEA)	
	orf19.4835	1.2	0.7	0.6	0.6	1.7	0.8	0.8	0.6	snoRNA binding (IEA)	
	orf19.5802	1.3	0.6	0.6	0.5	1.2	0.7	0.8	0.6		
	orf19.4375	1.0	0.7	0.6	0.6	1.1	0.9	0.8	0.6		
	orf19.6723	1.3	0.7	0.6	0.6	1.1	0.7	0.8	0.6		
	orf19.2527	1.4	0.5	0.6	0.6	1.6	0.9	0.8	0.6		
	orf19.6580	0.9	0.7	0.6	0.5	1.0	0.7	0.8	0.6		
	orf19.6730	1.8	0.5	0.6	0.6	1.4	0.9	1.1	0.6		
	orf19.6578	0.9	0.9	0.5	0.5	0.8	0.8	0.7	0.6	neurotransmitter transporter activity (ISS) inorganic phosphate transmembrane transporter activity (IEA)	
	orf19.7422	1.4	0.6	0.6	0.5	1.5	0.8	0.8	0.6	RNA binding (IEA)	
	orf19.2319	1.4	1.0	0.5	0.4	2.0	0.9	1.3	0.6	RNA binding	
	orf19.6766	1.3	0.5	0.5	0.6	1.3	1.0	0.8	0.6	RNA binding (IEA)	
	orf19.1697	1.2	0.8	0.6	0.5	1.3	0.8	0.8	0.6		
	orf19.254	1.0	0.9	0.5	0.5	1.1	0.8	0.7	0.6		
	orf19.443	1.3	0.6	0.6	0.6	1.1	0.9	0.9	0.6	DNA-directed RNA polymerase activity (IEA)	
	orf19.7107	1.3	0.5	0.6	0.6	1.5	0.9	1.0	0.6		
	orf19.4666	0.9	0.8	0.6	0.5	0.7	0.8	0.7	0.6		
	orf19.3270	0.9	0.9	0.6	0.6	0.7	0.8	0.7	0.6	1,3-beta-glucan synthase activity (IEA)	
	orf19.2045	1.0	0.7	0.6	0.6	1.0	0.9	0.8	0.6	diacylglycerol kinase activity (IEA)	

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
	orf19.6418	1.2	0.6	0.6	0.6	1.2	1.0	0.8	0.6		
	orf19.58	1.3	0.6	0.6	0.6	1.2	1.1	0.8	0.6	3'-5'-exoribonuclease activity (IEA)	
	orf19.4896	1.3	0.7	0.6	0.6	1.3	1.1	0.8	0.7	DNA-directed RNA polymerase activity (IEA)	
	orf19.7618	1.7	0.5	0.4	0.6	1.3	1.2	0.8	0.7	unfolded protein binding (IEA)	
	orf19.7495	1.2	0.6	0.6	0.6	1.2	0.8	0.9	0.7	NADPH dehydrogenase activity (IEA)	
	orf19.3887	1.0	0.6	0.4	0.6	1.2	1.0	0.7	0.7		
	orf19.2263	0.9	0.6	0.6	0.6	0.9	0.9	0.9	0.7		
	orf19.6938	1.1	0.7	0.6	0.6	1.0	0.9	0.7	0.7	S-methyl-5-thioadenosine phosphorylase activity (IEA)	
	orf19.7011	1.2	0.5	0.5	0.6	1.1	0.9	0.8	0.7		
	orf19.7502	1.0	0.7	0.4	0.5	0.9	0.8	0.9	0.7		
	orf19.3778	1.3	0.6	0.5	0.6	1.2	1.0	0.8	0.7		
	orf19.3475	0.7	0.8	0.4	0.5	0.7	0.8	0.6	0.7		
	orf19.1646	1.4	0.6	0.6	0.5	1.4	1.0	0.7	0.7		
	orf19.409	0.8	0.8	0.6	0.6	0.8	0.9	0.6	0.7		
	orf19.2246	0.8	0.7	0.6	0.5	0.8	0.8	0.7	0.7		
	orf19.6770	0.7	0.9	0.6	0.6	0.6	0.9	0.6	0.7	cytoskeletal adaptor activity (IEA)	
	orf19.7566	1.1	0.7	0.6	0.6	0.8	0.9	0.9	0.7	amino acid transmembrane transporter activity (IEA)	
	orf19.5017	0.8	1.0	0.5	0.5	0.6	0.8	0.4	0.7	urea transmembrane transporter activity (IEA)	
	orf19.6996	1.7	0.5	0.6	0.6	1.5	0.9	1.1	0.7	alpha-1,3-mannosyltransferase activity (IEA)	
	orf19.6679	0.9	0.9	0.6	0.5	0.8	0.7	0.8	0.7		
	orf19.1363	0.7	0.8	0.0	0.4	0.5	1.1	0.6	0.8		
	orf19.1449	1.2	0.3	0.4	0.4	1.6	0.9	1.1	0.8		
	orf19.1179	2.4	0.5	0.6	0.6	2.4	0.6	2.0	0.8		

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
	orf19.4266	1.0	0.8	0.6	0.3	1.1	0.8	0.4	0.8		
	orf19.2959.1	0.6	0.9	0.5	0.6	0.6	0.8	0.6	0.8		
	orf19.3894	0.9	0.9	0.5	0.6	1.0	1.1	0.9	0.9		
	orf19.670.2	0.8	0.4	0.3	0.4	0.7	0.5	1.5	0.9		
<i>ARA1</i>	orf19.2172	0.6	1.2	1.3	1.0	0.6	0.4	0.8	0.7	D-arabinose 1-dehydrogenase [NAD(P)+] activity (IDA)	
<i>CIP1</i>	orf19.113	0.7	2.3	1.1	0.7	0.5	0.5	0.7	0.8		
<i>CIRT4B</i>	orf19.2839	0.8	1.4	1.6	1.3	0.6	0.6	1.4	1.3		
<i>GAC1</i>	orf19.7053	0.7	1.9	1.2	0.7	0.6	0.6	0.6	0.6	protein phosphatase type 1 regulator activity (IEA)	
<i>IFC3</i>	orf19.3749	0.6	1.7	1.4	1.1	0.6	0.6	1.0	1.1	oligopeptide transporter activity (IGI, ISS)	
<i>IFE1</i>	orf19.769	0.5	1.7	0.8	0.8	0.4	0.6	0.4	0.8		
<i>LIP1</i>	orf19.4821	0.3	0.9	1.0	0.8	0.6	0.4	0.9	0.6	lipase activity (IDA)	
<i>MIH1</i>	orf19.3071	0.7	0.9	0.7	0.6	0.6	0.6	0.6	0.8	protein tyrosine phosphatase activity (IEA)	
<i>OPT7</i>	orf19.5673	0.7	1.4	0.4	0.4	0.3	0.6	0.6	0.7	oligopeptide transporter activity (ISS)	
<i>PRC2</i>	orf19.4135	0.6	2.2	0.9	0.7	0.4	0.5	0.6	0.8		
<i>PSA2</i>	orf19.4943	0.6	1.4	0.9	0.7	0.5	0.5	0.8	1.0	mannose-1-phosphate guanylyltransferase activity (IDA, IGI, ISS)	
<i>RBT7</i>	orf19.2681	0.7	1.2	0.8	0.4	0.4	0.4	0.5	0.7	ribonuclease activity (ISS)	
<i>SAP10</i>	orf19.3839	0.5	1.3	0.8	0.5	0.5	0.5	0.6	0.7		
<i>SKN2</i>	orf19.348	0.7	1.1	0.6	0.7	0.5	0.6	0.8	1.0		
	orf19.5775	0.9	1.2	1.1	0.8	0.6	0.4	0.9	0.6		
	orf19.7311	0.6	1.0	0.7	0.8	0.6	0.6	0.6	0.6		
	orf19.6795	0.7	0.8	0.5	0.7	0.5	0.5	0.7	0.7	transcription regulator activity (IEA)	
	orf19.3758	0.4	0.8	0.1	0.8	0.6	0.5	0.3	0.7		
	orf19.1484	0.7	0.8	0.9	1.2	0.6	0.5	1.0	0.7		

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
	orf19.1258	0.5	1.4	1.0	0.6	0.4	0.5	0.6	0.7		
	orf19.5449	0.8	1.0	0.8	0.6	0.6	0.6	0.7	0.7		
	orf19.1600	0.7	0.9	0.9	0.5	0.5	0.6	0.6	0.7		
	orf19.4768	1.0	0.5	0.9	1.8	0.4	0.5	0.7	0.8		
	orf19.2737	0.5	1.2	0.8	0.7	0.5	0.6	0.5	0.8		
	orf19.4349	0.9	0.7	0.5	0.9	0.5	0.6	0.4	0.8		
	orf19.2030	0.7	1.2	0.8	0.7	0.6	0.6	0.8	0.8		
	orf19.1887	0.6	1.1	0.9	0.7	0.6	0.6	0.6	0.8	sterol esterase activity (IEA)	
	orf19.764	0.1	1.0	0.6	0.8	0.6	0.1	0.6	0.8		
	orf19.6017	1.2	0.9	0.8	0.7	0.6	0.6	1.0	0.9		
	orf19.1159	0.8	0.9	0.6	0.7	0.6	0.6	1.0	0.9	homoserine O-acetyltransferase activity (IEA)	
	orf19.4530.1	0.4	2.1	0.9	0.9	0.4	0.6	0.5	0.9		
	orf19.6307	0.6	1.1	0.5	1.2	0.4	0.6	0.8	1.1		
	orf19.5527	0.6	0.8	1.0	0.9	0.6	0.5	1.4	1.2	endoribonuclease activity (IEA)	
	orf19.247	0.7	0.7	1.1	1.0	0.6	0.4	0.7	1.4		
	orf19.4055	0.5	0.8	0.6	1.0	0.3	0.4	1.7	1.8		
<i>ASR3</i>	orf19.842	0.8	1.2	0.8	0.5	0.8	0.5	0.6	0.5		
<i>BMT4</i>	orf19.5612	0.8	1.4	1.0	0.4	0.7	0.6	0.6	0.6		
<i>CHT3</i>	orf19.7586	0.7	0.8	0.6	0.7	0.6	0.7	0.6	0.6	chitinase activity (IDA, IGI, ISS) endochitinase activity (IEA)	
<i>CLA4</i>	orf19.4890	0.7	0.8	0.5	0.8	0.8	0.6	0.5	0.4	protein serine/threonine kinase activity (ISS)	
<i>CRZ2</i>	orf19.2356	0.8	1.9	1.0	0.5	0.8	0.7	0.3	0.4	transcription factor activity (ISS)	
<i>CSP37</i>	orf19.2531	0.6	0.9	0.7	0.6	0.7	0.5	0.6	0.6		
<i>FCY21</i>	orf19.1357	1.2	1.1	0.8	0.8	0.8	0.7	0.6	0.6	drug membrane transporter activity	

Appendix C. Continued.

		Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
<i>FGR6</i>	orf19.5315	0.8	0.9	0.8	0.6	0.7	0.5	0.6	0.5	
<i>GIT1</i>	orf19.1979	0.8	1.3	0.9	0.7	0.8	0.8	0.6	0.6	inorganic phosphate transmembrane transporter activity (ISS) phospholipid transporter activity (IEA)
<i>GNP3</i>	orf19.7565	1.0	0.8	1.0	0.7	0.8	0.8	0.6	0.6	amino acid transmembrane transporter activity (IEA)
<i>KRE1</i>	orf19.4377	1.2	1.2	0.7	0.6	1.0	1.0	0.6	0.5	
<i>MEA1</i>	orf19.5919	1.1	1.0	0.7	0.6	0.9	0.8	0.6	0.6	
<i>MRR1</i>	orf19.7372	1.1	1.0	0.7	0.6	1.1	0.9	0.6	0.5	specific RNA polymerase II transcription factor activity (IMP) sequence-specific DNA binding (IEA)
<i>PHO84</i>	orf19.655	0.8	1.2	0.9	0.6	0.8	0.9	0.6	0.6	inorganic phosphate transmembrane transporter activity (ISS)
<i>RCL1</i>	orf19.1886	0.9	0.7	0.5	0.7	0.8	1.0	0.6	0.6	RNA-3'-phosphate cyclase activity (IEA) enzyme activator activity (IEA)
<i>SFUI</i>	orf19.4869	1.1	1.1	0.8	0.6	0.9	0.9	0.6	0.6	DNA binding (IGI) transcription factor activity (IGI, IMP, ISS)
<i>TEC1</i>	orf19.5908	1.0	0.9	0.7	0.4	0.9	0.8	0.5	0.6	specific RNA polymerase II transcription factor activity (IMP, ISS) transcription factor activity (ISS)
<i>XUT1</i>	orf19.2882	0.9	1.1	1.0	0.4	0.9	0.5	0.5	0.5	nucleobase:cation symporter activity (ISS)
	orf19.5826	0.5	0.8	0.6	0.7	0.6	0.4	0.5	0.4	L-gamma-aminobutyric acid transmembrane transporter activity (IEA)
	orf19.6277	1.0	1.6	0.8	0.3	0.9	0.5	0.3	0.4	
	orf19.7590	0.9	0.9	0.7	0.5	1.1	0.5	0.6	0.5	
	orf19.5775	0.8	0.9	1.2	0.4	1.0	0.5	0.6	0.6	
	orf19.3470	1.4	0.6	0.9	0.3	1.5	0.5	0.3	0.3	nitrogenase (flavodoxin) activity (ISS)
	orf19.5070	0.8	1.2	0.7	0.3	0.7	0.6	0.5	0.5	
	orf19.3988	0.9	1.1	0.4	0.7	1.1	0.6	0.5	0.6	
	orf19.750	0.7	1.4	0.6	0.7	0.7	0.6	0.6	0.6	
	orf19.5872	1.2	1.0	0.9	0.7	0.4	0.7	0.6	0.4	general RNA polymerase II transcription factor activity (IEA)
	orf19.48	1.8	0.9	1.0	0.8	1.4	0.7	0.4	0.4	ribonuclease P activity (IEA)
	orf19.5910	1.0	0.8	1.0	0.6	1.0	0.7	0.6	0.6	histone acetyltransferase activity (IEA) methylated histone residue binding (IEA)
	orf19.1656	0.4	1.5	1.3	0.7	0.6	0.7	0.6	0.6	phosphatidylinositol binding (IEA)

Appendix C. Continued.

		Change in fold expression ^a								Molecular function
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.2824	0.8	0.9	0.7	0.7	0.8	0.7	0.6	0.6	double-stranded DNA binding (IDA) specific transcriptional repressor activity (IGI)
	orf19.2604	1.3	0.7	0.7	0.4	1.3	0.7	0.5	0.4	
	orf19.2231	1.1	0.9	0.8	0.8	1.2	0.7	0.6	0.5	
	orf19.2547	1.3	0.6	0.7	0.5	1.1	0.8	0.6	0.4	
	orf19.4143	1.5	0.6	0.8	0.4	1.2	0.8	0.6	0.4	
	orf19.3170	1.5	0.6	0.8	0.7	1.5	0.8	0.6	0.5	
	orf19.6976	0.9	1.0	0.8	0.7	0.9	0.8	0.6	0.6	folic acid transporter activity (ISS)
	orf19.5326	1.3	1.0	0.7	0.6	1.0	0.9	0.6	0.6	transcription factor activity (ISS) transcription corepressor activity (IEA)
	orf19.3481	1.0	0.7	0.5	0.7	0.9	0.9	0.6	0.6	
	orf19.3831	1.7	1.2	0.9	0.7	1.5	1.0	0.6	0.6	
	orf19.6416	1.0	1.2	0.7	0.8	0.8	1.0	0.6	0.6	
	orf19.6662	1.1	0.9	0.9	0.7	1.2	1.0	0.6	0.6	ubiquinone binding (IEA)
	orf19.5128	1.0	1.1	0.9	1.2	1.1	1.0	0.6	0.6	RNA binding (IEA)
	orf19.1772	1.8	1.1	0.6	0.8	1.7	1.2	0.6	0.5	
	orf19.69	0.6	1.0	0.7	0.9	0.5	1.3	0.5	0.5	
	orf19.6586	0.9	1.7	0.7	0.6	1.0	1.3	0.4	0.4	
	orf19.4563	1.1	0.6	0.5	0.8	1.0	1.5	0.5	0.6	
	orf19.1117	1.1	5.5	0.1	0.7	0.8	2.1	0.4	0.1	formate dehydrogenase activity (ISS)
	orf19.2442	0.5	0.5	0.3	0.5	0.5	0.7	0.5	1.0	
	orf19.2163	0.3	0.5	0.6	0.7	0.4	0.3	0.7	1.1	
HAL21	orf19.99	0.4	0.4	0.4	1.2	0.2	0.4	0.4	0.6	3'(2'),5'-bisphosphate nucleotidase activity (IEA)
AFG1	orf19.4743	0.8	0.6	0.5	0.5	0.6	0.5	0.9	0.5	ATPase activity (IEA)
HGT1	orf19.4527	0.9	0.5	0.2	0.5	0.6	0.5	0.8	0.6	glucose transmembrane transporter activity (IGI, ISS) sugar transmembrane transporter activity (ISS)

Appendix C. Continued.

		Change in fold expression ^a									
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>			
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function	
<i>HSP30</i>	orf19.4526	0.6	2.3	0.3	0.4	0.4	0.4	0.5	0.7		
<i>IFF11</i>	orf19.5399	0.6	0.8	0.6	0.5	0.5	0.6	0.8	1.0		
<i>OPT7</i>	orf19.5673	0.7	1.4	0.4	0.4	0.3	0.6	0.6	0.7	oligopeptide transporter activity (ISS)	
<i>SAP3</i>	orf19.6001	0.6	1.4	0.6	0.6	0.5	0.6	0.6	1.1	aspartic-type endopeptidase activity (IDA, ISS) drug binding (IDA,ISS)	
<i>SIT1</i>	orf19.2179	1.0	0.7	0.4	0.5	0.6	0.6	0.8	0.5	ferrichrome transporter activity (IGI, IMP) siderophore-iron transmembrane transporter activity (ISS)	
	orf19.675	0.5	1.5	0.6	0.6	0.4	0.6	0.4	0.7		
<i>ARG11</i>	orf19.5926	1.0	0.9	0.4	0.5	1.3	0.8	0.5	0.3	L-ornithine transmembrane transporter activity (IEA)	
<i>CCC1</i>	orf19.6948	0.8	0.9	0.4	0.4	0.7	0.9	0.3	0.4	ferrous iron transmembrane transporter activity (IEA)	
<i>CHO2</i>	orf19.169	1.1	0.7	0.6	0.5	0.9	0.8	0.6	0.5	methylene-fatty-acyl-phospholipid synthase activity (IEA)	
<i>CUP9</i>	orf19.6514	1.0	1.1	0.6	0.5	0.9	0.9	0.6	0.5	chromatin binding (IEA) sequence-specific DNA binding (IEA)	
<i>DIM1</i>	orf19.5010	1.6	0.6	0.4	0.4	1.9	1.2	0.6	0.3	rRNA (adenine-N6,N6-)-dimethyltransferase activity (IEA)	
<i>ELP3</i>	orf19.7387	1.1	0.7	0.6	0.6	1.1	1.0	0.6	0.5	RNA polymerase II transcription elongation factor activity (IEA)	
<i>FDH1</i>	orf19.638	0.8	1.4	0.6	0.4	0.7	0.7	0.5	0.5	formate dehydrogenase activity (ISS)	
<i>FET3</i>	orf19.4211	0.9	0.5	0.3	0.4	0.7	0.7	0.6	0.5	ferroxidase activity (IGI, IMP)	
<i>FRE30</i>	orf19.6140	1.4	0.5	0.3	0.3	1.2	0.6	0.6	0.3		
<i>FRE7</i>	orf19.6139	1.1	0.6	0.2	0.4	0.8	0.9	0.5	0.5	ferric-chelate reductase activity (IEA)	
<i>HCM1</i>	orf19.4853	0.8	0.8	0.5	0.6	0.6	0.8	0.6	0.6	sequence-specific DNA binding (IEA)	
<i>HMX1</i>	orf19.6073	1.1	0.8	0.3	0.5	1.0	1.1	0.4	0.4	heme oxygenase (decyclizing) activity (IDA, IMP, ISS)	
<i>HSP12</i>	orf19.3160	0.6	1.2	0.6	0.4	0.6	0.7	0.5	0.6		
<i>NOG2</i>	orf19.5732	1.4	0.6	0.6	0.4	1.4	0.7	0.6	0.2		
<i>NOP14</i>	orf19.5959	2.2	0.6	0.6	0.4	2.9	0.7	0.6	0.2	snoRNA binding (IEA)	
<i>PGA55</i>	orf19.207	0.9	0.7	0.5	0.5	0.6	0.8	0.5	0.5		
<i>RLI1</i>	orf19.3034	1.1	0.7	0.6	0.5	1.1	0.9	0.6	0.5	ATP binding (ISS) iron ion binding (IEA)	

Appendix C. Continued.

		Change in fold expression ^a								
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
<i>SEN2</i>	orf19.2735	1.3	0.5	0.4	0.3	1.2	1.1	0.5	0.3	
<i>SMM1</i>	orf19.1362	1.4	0.6	0.5	0.3	1.4	0.7	0.5	0.3	tRNA dihydrouridine synthase activity (IEA)
<i>SPB4</i>	orf19.6298	1.5	0.6	0.5	0.5	1.6	1.1	0.6	0.4	ATP-dependent RNA helicase activity (IEA)
<i>UTP18</i>	orf19.7154	1.2	0.6	0.4	0.5	1.1	1.1	0.6	0.6	
	orf19.4445	1.0	0.4	0.2	0.1	1.2	0.5	0.3	0.1	
	orf19.6873.1	2.2	0.8	0.3	0.2	1.6	0.9	0.5	0.2	electron carrier activity (IEA) iron ion binding (IEA)
	orf19.1091	1.5	0.4	0.4	0.3	1.9	0.5	0.6	0.2	RNA binding (IEA)
	orf19.5049	1.9	0.5	0.5	0.5	1.4	0.9	0.4	0.2	
	orf19.5207	1.2	0.6	0.4	0.5	1.1	1.1	0.3	0.2	
	orf19.5500	1.7	0.6	0.5	0.4	1.9	1.2	0.5	0.3	
	orf19.2934	1.6	0.5	0.4	0.4	1.8	0.7	0.6	0.3	
	orf19.341	1.0	1.0	0.2	0.2	0.6	0.7	0.2	0.3	drug transporter activity (ISS)
	orf19.7077	1.0	0.4	0.2	0.3	0.7	0.6	0.4	0.3	ferric-chelate reductase activity (IEA)
	orf19.5843	0.7	0.7	0.3	0.4	0.7	0.8	0.4	0.3	
	orf19.5020	1.2	0.7	0.4	0.3	1.2	0.9	0.5	0.3	
	orf19.1774	1.0	1.0	0.3	0.3	0.5	0.9	0.3	0.3	
	orf19.4273	1.6	0.7	0.5	0.4	1.3	1.1	0.5	0.3	
	orf19.3448	0.9	1.0	0.2	0.2	0.9	0.5	0.4	0.3	
	orf19.2724	0.6	1.3	0.4	0.3	0.6	0.7	0.3	0.3	
	orf19.494	1.3	0.5	0.5	0.5	1.5	0.9	0.6	0.4	RNA binding (IEA)
	orf19.3088	1.3	0.5	0.4	0.4	1.3	1.0	0.6	0.4	transcription factor activity (IEA)
	orf19.2604	1.3	0.5	0.4	0.4	1.4	0.8	0.6	0.4	
	orf19.1771	1.3	0.8	0.4	0.4	1.3	0.6	0.4	0.4	

Appendix C. Continued.

		Change in fold expression ^a								
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
	orf19.6660	0.6	1.2	0.5	0.3	0.7	0.6	0.3	0.4	
	orf19.2018	1.1	0.6	0.5	0.4	1.0	0.8	0.5	0.4	
	orf19.22	0.8	0.7	0.4	0.3	0.6	0.7	0.4	0.4	
	orf19.6899	1.0	2.7	0.4	0.5	0.6	0.9	0.2	0.4	
	orf19.1287	1.7	0.8	0.6	0.5	1.5	1.1	0.6	0.4	
	orf19.3759	0.9	0.8	0.4	0.4	1.3	0.7	0.5	0.4	RNA polymerase II transcription elongation factor activity (IEA)
	orf19.4634	1.6	0.6	0.6	0.6	1.2	1.0	0.6	0.4	
	orf19.5431	0.7	0.7	0.3	0.3	0.5	0.7	0.4	0.4	
	orf19.3048	0.6	0.8	0.6	0.5	0.8	0.5	0.6	0.5	
	orf19.1388	1.5	0.5	0.5	0.5	1.6	1.1	0.5	0.5	
	orf19.2330	1.2	0.5	0.4	0.5	1.2	0.9	0.5	0.5	snoRNA binding (IEA)
	orf19.7088	1.3	0.7	0.5	0.6	1.4	1.1	0.6	0.5	single-stranded telomeric DNA binding (IEA)
	orf19.4161	0.9	0.8	0.5	0.6	1.4	0.6	0.6	0.5	
	orf19.4900	0.8	0.7	0.5	0.4	0.7	0.5	0.6	0.5	alpha-1,3-mannosyltransferase activity (IEA)
	orf19.7085	0.6	1.0	0.5	0.4	0.8	0.6	0.5	0.5	
	orf19.3335	0.7	0.8	0.6	0.6	0.9	0.7	0.6	0.5	
	orf19.6751	1.2	0.8	0.6	0.6	1.3	1.0	0.6	0.5	tRNA methyltransferase activity (IEA)
	orf19.3869	0.9	0.8	0.4	0.5	0.8	0.7	0.6	0.5	
	orf19.5626	0.8	0.8	0.4	0.5	0.9	0.8	0.6	0.6	
	orf19.28	0.8	0.7	0.5	0.6	1.0	0.9	0.6	0.6	thiamin transmembrane transporter activity (IEA)
	orf19.4889	1.0	0.7	0.6	0.4	0.9	0.8	0.6	0.6	drug transporter activity (ISS)
	orf19.3854	1.1	0.9	0.6	0.5	1.1	0.9	0.6	0.6	protein kinase activity (IEA)
	orf19.2022	0.8	0.8	0.5	0.5	0.7	0.7	0.6	0.6	glucose transmembrane transporter activity (ISS)

Appendix C. Continued.

		Change in fold expression ^a								
		G648S		G648D		Y642F		A643V		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Molecular function
	orf19.6691	0.9	0.8	0.5	0.4	0.8	0.9	0.6	0.6	
	orf19.4282	1.3	0.9	0.5	0.6	0.8	0.9	0.6	0.6	
	orf19.4959	0.5	0.9	0.4	0.6	0.5	0.9	0.5	0.6	
	orf19.3110	0.6	0.8	0.6	0.5	0.6	0.7	0.5	0.6	
	orf19.2726	0.8	1.2	0.6	0.5	0.7	0.9	0.6	0.6	
	orf19.6245	0.6	1.0	0.6	0.6	0.7	0.7	0.6	0.6	
	orf19.6077	0.8	0.9	0.6	0.5	0.6	0.7	0.6	0.6	
MOH1	orf19.3369	0.4	2.1	0.8	0.3	0.4	0.4	0.4	0.4	
GAC1	orf19.7053	0.7	1.9	1.2	0.7	0.6	0.6	0.6	0.6	protein phosphatase type 1 regulator activity (IEA)
	orf19.251	0.6	1.4	0.8	0.4	0.5	0.6	0.6	0.6	
	orf19.7311	0.6	1.0	0.7	0.8	0.6	0.6	0.6	0.6	
	orf19.1368	0.7	1.0	0.7	0.4	0.6	0.6	0.6	0.6	
AOX1	orf19.4774	0.5	0.8	0.4	0.3	0.4	0.4	0.3	0.3	alternative oxidase activity (IMP, ISS)
ASR2	orf19.7284	0.4	1.2	0.6	0.4	0.4	0.6	0.4	0.4	
CDG1	orf19.7314	1.2	0.6	0.6	0.5	0.6	0.5	0.6	0.5	
CFL2	orf19.1264	0.7	0.3	0.3	0.2	0.5	0.4	0.5	0.4	ferric-chelate reductase activity (IEA)
DAL8	orf19.5859	0.7	0.8	0.6	0.6	0.5	0.5	0.5	0.4	anion:cation symporter activity (ISS)
FMP45	orf19.6489	0.4	1.7	0.5	0.4	0.2	0.3	0.3	0.5	
FRE10	orf19.1415	1.0	0.6	0.4	0.4	0.6	0.4	0.5	0.4	ferric-chelate reductase activity (IDA, IGI, IMP)
HSP31	orf19.3664	0.7	0.7	0.2	0.3	0.4	0.5	0.3	0.2	
RTA4	orf19.6595	0.9	0.7	0.3	0.3	0.5	0.5	0.5	0.5	phospholipid-translocating ATPase activity (IEA)
SOU1	orf19.2896	0.5	1.3	0.5	0.4	0.4	0.6	0.5	0.6	mannitol 2-dehydrogenase (NADP+) activity (IDA)
	orf19.3107	0.2	0.9	0.6	0.0	0.4	0.5	0.1	0.4	

Appendix C. Continued.

		Change in fold expression ^a								Molecular function
		<u>G648S</u>		<u>G648D</u>		<u>Y642F</u>		<u>A643V</u>		
CGD Name	orf19 Designation	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	
	orf19.7310	0.4	1.5	0.6	0.3	0.4	0.5	0.5	0.4	
	orf19.822	0.3	1.4	0.3	0.3	0.4	0.5	0.3	0.4	
	orf19.3439	0.6	1.0	0.1	0.2	0.5	0.5	0.4	0.5	
	orf19.6741	0.7	0.8	0.4	0.4	0.5	0.6	0.4	0.5	
	orf19.1368	0.5	0.8	0.6	0.4	0.5	0.5	0.5	0.5	
	orf19.2846	0.5	1.9	0.5	0.3	0.5	0.5	0.5	0.6	
	orf19.3932	0.4	1.7	0.5	0.6	0.4	0.5	0.4	0.6	
	orf19.94	0.5	0.7	0.4	0.4	0.5	0.5	0.4	0.6	
<i>HAL21</i>	orf19.99	0.4	0.4	0.4	1.2	0.2	0.4	0.4	0.6	3'(2'),5'-bisphosphate nucleotidase activity (IEA)
<i>SUL2</i>	orf19.2738	0.6	0.6	0.2	0.3	0.4	0.5	0.7	0.7	sulfate transmembrane transporter activity (NAS)
	orf19.2163	0.3	0.5	0.5	0.6	0.4	0.4	0.9	1.0	peptidase activity
<i>HGT10</i>	orf19.5753	0.6	0.5	0.2	0.2	0.5	0.6	0.2	0.3	glucose transmembrane transporter activity (ISS)
<i>SAP7</i>	orf19.756	0.4	0.4	0.3	0.2	0.4	0.4	0.3	0.4	aspartic-type endopeptidase activity (IDA, ISS)
	orf19.716	0.4	0.5	0.4	0.4	0.6	0.6	0.4	0.4	
	orf19.3621	0.3	0.5	0.3	0.3	0.6	0.4	0.5	0.5	
	orf19.4873	0.4	0.5	0.6	0.5	0.6	0.6	0.6	0.6	

^a Fold change defined as the average ratio of gene expression levels in the isolates compared in two independent microarray experiments

Bold indicates real result as defined in Materials and Methods

Green shading indicates ratio values of 0.6 or less.

VITA

Stephanie A. Flowers was born in 1978 in Oceanside, California, to Allan and Marcia Flowers. She graduated from Fallbrook Union High School in 1996 and then attended the University of California at Davis, graduating with a BS in Neurobiology, Physiology and Behavioral Sciences and a minor in Japanese. In 2003, she enrolled in pharmacy school at the University of Southern California where she graduated with honors and moved to Memphis, Tennessee, to complete her Pharmacy Practice Residency at the Veterans Affairs Medical Center in Memphis. After residency, she started her fellowship and graduate training at the University of Tennessee, Health Science Center and anticipates completing her Doctor of Philosophy in December of 2013.