NOTE TO USERS

Using *S. cerevisiae* Genetic Array Technologies to Understand Mode of Action of Ethnobotanical Mycotics

A Thesis

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By

Nadereh Hannah Mirrashed

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"The outcome of any serious research can only be to make two questions grow where only one grew before."

Thorstein Veblen (1857 - 1929).

Abstract

Using *S. cerevisiae* Genetic Array Technologies to Understand Mode of Action of Ethnobotanical Mycotics

Host toxicity, a limited spectrum of activity, and propensity to induce or select for resistant fungal pathogens by many current antifungals has created a need for additional agents to treat fungal infections. We have studied how *S. cerevisiae* responds to sub-lethal exposures of plant-derived compounds, including the alkaloid berberine and eight different Echinacea extracts. Microarray analysis revealed significant differential RNA transcript levels for ~106 genes when yeast cells were exposed to a minimum inhibitory concentration of berberine for two to six hours. These response genes were assigned to seven categories including cell cycle/division, metabolism, cell wall, gene expression, transport/secretion and unknown functions. Analysis of the microaaray data suggests that berberine (i) may not be effectively recognized by yeast as a xenobiotic and also (ii) it targets several proteins/pathways that provide insight into multiple modes of action of the compound. A yeast Gene Deletion Array (GDA, ~4600 gene deletion mutants) was also used to examine chemical-genetic interactions with yeast. Mutations in a subset of genes were identified in association with hypersensitivity to berberine and Echinacea extracts. Changes in berberine uptake/efflux as measured by HPLC were noted for several of these hypersensitive mutations. A significant pattern to emerge from the GDA analysis was that Echinacea extracts interfere with fungal cell wall functions. These experiments provide a template in medicinal plant research. The future direction of the study is to link the collected data to the antifungal drug discovery and development processes.

Acknowledgments

"Every time we remember to say "thank you," we experience nothing less than heaven on earth."

Sarah Ban Breathnach

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strength to find a way through the various challenges that life brings. A unique thank to my beloved sister for her invaluable advice in our last conversation that learning is worthless to those who lack insight. I know that this day would have meant a lot to all of you and I hope that you are looking down proudly. I am now at peace for having kept my promise to you, I wish I could kiss your hands for every thing that you have done for me, and I will do it and thank you in person when we meet again, until then your memories will always be with me.

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Finally, Thank you God for letting me finish, now I can watch TV and play with my daughter with no worries!!

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List of Abbreviations

ABC	ATP-binding Cassette
AIDS	Acquired Immune Deficiency Syndrome
anti-HIF	anti-Hypoxia Inducible Factor
ATP	Adenosine triphosphate
cDNA	complementary DNA
CFU	Colony-forming Unit
CsA	cyclosporine A
Cy3	Cyanine 3 (fluorescent dye)
Cy5	Cyanine 5 (fluorescent dye)
DNA	Deoxyribonucleic Acid
dATP	Deoxy-adenine triphosphate
dCTP	Deoxy-cytosine triphosphate
dGTP	Deoxy-guanine triphosphate
dNTP	Deoxy-nucleoside triphosphate
dTTP	Deoxy-thymine triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
5-FC	5-Fluorocytosine
FK506	Tacrolimus or Fujimycin
GD	Growth Detector
GDA	Gene Deletion Array
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatograghy
LOWESS	Locally Weighted Scatterplot Smoothing
MAT	Mating-type locus
MDR	Multi Drug Resistance
MFS	Major Facilitator Superfamily
MF	Major Facilitator
MIC	Minimum Inhibitory Concentration
MIPS	Munich Information Centre for Protein Sequences
MRP	Multi-drug Resistance Protein

NHP	Natural Health Product
OD	Optical Density
ORF	Open-Reading Frame
pARAB	Arabidopsis plasmid
PCR	Polymearse Chain Reaction
PDR	Pleiotropic Drug Resistance
RNA	Ribonucleic Acid
SAR	Systematic Acquired Resistance
SDS	Sodium dodecyl sulfate
SGD	Saccharomyces Genome Database
SOM	Self-Organizing Map
SSC	Sodium chloride/Sodium citrate
TIFF	Tagged Information File Format
UV	Ultra Violet light
WHO	World Health Organization
YAC	Yeast Artificial Chromosome
YF	Yeast Feature
YKO	Yeast Gene knockout
YPD	Yeast Protein Database
YPD medium	Yeast Peptone Dextrose medium
U	Unit
h	hour
°C	Degrees Celsuis
V	Voltage
HZ	Hertz (cycles per second)
µl	Micro-liters
µM	Micro-molar
nm	nano-metre
mM	milli-Molar
ml	milli-liters
mm	milli-metre

mg	milli-gram
min	minute
rpm	Revolutions per minute

This thesis is dedicated to the memory of my beloved father, mother and sister, in honour of their love.

CHAPTER 1

General Introduction and Literature Review

1.1 General Introduction

Fungi are morphologically and physiologically diverse eukaryotes, now usually classified in a separate kingdom. Among the attributes that distinguish the fungi are a hyphal or yeastlike form, a rigid, chitinous cell wall, often production of spores, and relatively small genome size (Hawksworth 2005; Dighton 2003; Alexopoulos and Mims, 1979). Fungi contribute to human well being in several ways. We consume fungi directly in the form of edible mushrooms and mycelium (e.g. Quorn) and fungi play important roles in biotechnological processes and research into genetic and other fundamental biological processes. More generally, fungi have valuable roles in environmental processes such as nutrient recycling and as mycorrhizal associates of plants.

Fungi also have negative impacts on human affaire. They can cause substantial material losses and destroy foodstuffs (Pitt and Hocking, 1997). For instance, certain *Aspergillus* species can spoil fruit juice concentrates, soft drinks and other pasteurized products due to contamination by their heat-resistance ascospores (Udagawa and Yaguchi, 2005). In addition, the spores of several fungal species are recognized as a major cause of human allergies, particularly in developed countries (Breitenbach *et al.* 2002). Many fungi produce mycotoxins that may contaminate our food and cause acute or delayed toxicity (Howard and Miller, 1996).

Fungi are also major pathogens of plants, and can result in economic crises and major human social problems. The role of fungal pathogens, particularly the rusts and powdery mildews, in agriculture has been the subject of considerable research efforts (Schumann, 1991). Another notable plant pathogen is *Fusarium graminearum*, which is capable of causing head blight or 'scab' on wheat, barley, rice and oats, and *Gibberella*

stalk and ear rot diseases on maize (Goswami and Kistler, 2004). The economic losses in crop yields and quality has resulted in the development of a large agrochemical industry that provides fungicides to combat these plant pathogens, however, fungi have developed resistance to many of these fungicides (Doohan 2005). Thus, intensified research towards the discovery of new antifungals, and into the molecular basis of both resistance mechanisms and pathogenicity by fungi is required to protect crop plants.

Recently, there has been increased interest in the biological control of fungal pathogens. Biological control of fungi is thought of by some as a more desirable approach to the control of fungi than the use of industrial-based pesticides. The idea is based on using microbes to inhibit fungal pathogens either (i) directly, such as through secretion of antifungal compounds or enzymes, or as infectious agents of the fungi, (ii) indirectly, by placing a barrier between the pathogen and the host, or (iii) through manipulating plant defenses by using specific microbes to induce systematic acquired resistance (SAR) to fungal pathogens (Doohan 2005).

In animal systems, many fungal species occur as members of the microflora (commensals), while fungal species of *Cryptococcus*, *Aspergillus* and *Candida*, as examples, can cause life threatening diseases, especially in individuals with compromised immune systems (Sullivan *et al.* 2005). These diseases in humans and animals associated with fungi are called "mycoses". Mycoses can be classified into two major groups, superficial and systematic infections (Pinner *et al.* 1996). Even if fungal diseases are not fatal, they can influence the host's health to reduce growth and fecundity with consequential effects on the population of the animals.

Antifungals used to treat mycoses are often referred to as "mycotics" and, just as with plants, there is a critical need for new mycotics due to a combination of several

factors (Lupetti *et al.* 2002; Georgopapadakou, 2002; White *et al.* 1998). These include host related factors such as invasive surgical procedures and an increasing frequency of patients who submit to immunosuppressive therapies. In addition, existing mycotics often have a narrow spectrum of antifungal activity and toxic side-effects. Finally, emergent fungal pathogens and genotypes of well established pathogenic species are turning up that are resistant to existing mycotics. Overall, the development of mycotics is impeded by a lack of selective toxicity. Fungi and animals are closely related and share many common cell physiology and biochemical features. Thus, antifungals may indiscriminately disrupt the normal metabolism of both fungal pathogen and host cells (Abu-Elteen and Hamad, 2005). The development of toxic side effects is therefore a major concern during chemotherapy treatments of fungal infections. Other problems associated with the available antifungal drugs include insolubility, instability and insufficient absorption through the gastrointestinal system (Hoffman *et al.* 2002; Ghannoum and Rice, 1999). Optimally, a good antifungal should exhibit selective toxicity at the infection site (Hoffman *et al.* 2002; Ghannoum and Rice, 1999).

1.2 Antifungal drugs in human therapy

With regard to fungal infections in animals, the first clinically used antifungal was potassium iodide in 1903, followed by Whitefield's ointment in 1907, and then undecylenic acid in 1940 (Abu-Elteen *et al.* 2005). These compounds were used for topical treatments of skin infections. Currently, there are five major classes of mycotics that are administered internally. These include polyenes, azoles, pyrimidines, candins and allylamines. The polyene, amphotericin B was discovered in 1957 from the actinomycete *Streptomyces nodosus* cultured from soil. It was the first commercially

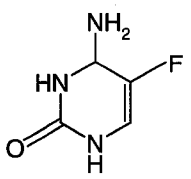
available systemic antifungal drug (Abu-Elteen and Hamad. 2005). Amphotericin B still is the gold standard for treatment of life-threating mycoses (Abu-Elteen and Hamad. 2005; Francois *et al.* 2005). The azole group is probably the most rapidly expanding and largest group of antifungal agents. From 1970s to 1980s, the number of available antifungals doubled with addition of azole derivatives such as miconazole, ketoconazole, fluconazole and intraconazoles for systemic treatment (Francois *et al.* 2005). 5-fluorocytosine (5-FC), a pyrimidine, was first synthesized in the 1950s and is currently recommended for use in combination with amphotericin B. Amphotericin B increases the fungal cell membrane permeability and increases the uptake of 5-FC by cells (Beggs *et al.* 1981). The candin, caspofungin was first used in 2001 for aspergillosis treatments and by 2003 for disseminated candidiosis (Francois *et al.* 2005). Finally, terbinafine is a major antifungal in the allylamines class which is now in clinical use (Abu-Elteen and Hamad, 2005). In addition to these five main classes, there are some miscellaneous agents such as ciclopirox and sordarines with antifungal activity that are now available for therapeutic use (Francois *et al.* 2005).

1.2.1 *Mechanism of action of antifungal drugs*

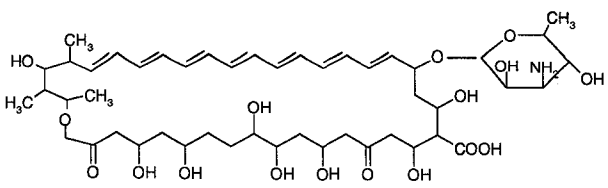
In a general view, antifungal compounds either kill fungal cells (fungicidal) or inhibit their growth without killing the cells (fungistatic). Further, antifungals may be prepared by fermentation or by chemical synthesis or semisynthesis. In the following section I review what is known about the modes of action for the most common currently available antifungal drugs within the five major classes.

(I) The polyenes. These are fungicidal compounds that selectively bind to ergosterol in the fungal cell membrane, and to cholesterol in host cells. Ergosterol is an analogue of

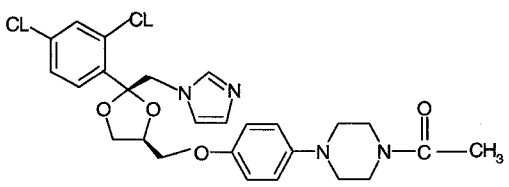
Figure 1.1. Chemical structures of some antifungal drugs.



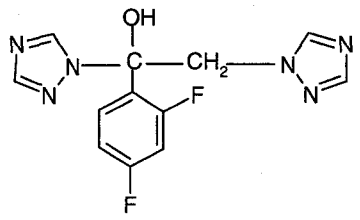
Flucytosine



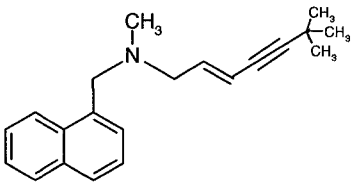
Amphotericin B



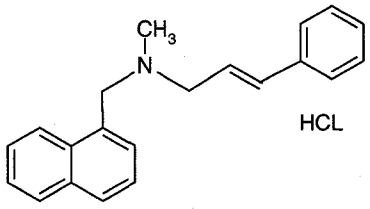
Ketoconazole



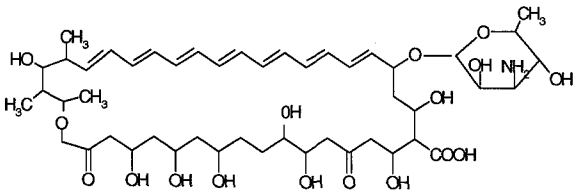
Fluconazole



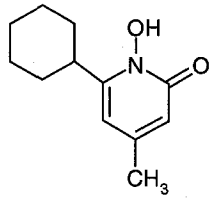
Terbinafine



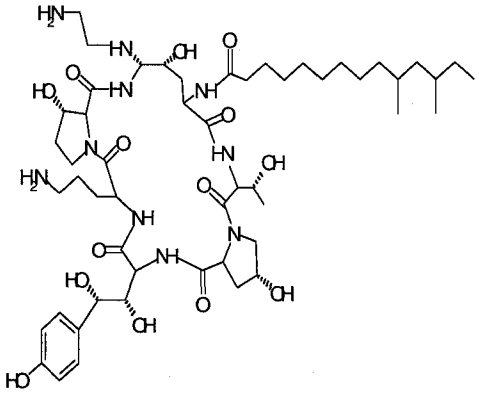
Naftifine



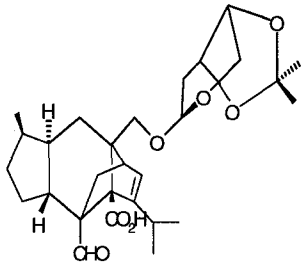
Nystatin



Ciclopirox



Caspofungin



Sordarin (GM193663)

the cholesterol in animal cell membranes and plays an essential role in fungal cell membrane fluidity and permeability. Being fungal-specific, ergosterol, and the enzymes involved in its biosysnthesis, are major targets for many current antifungal drug development projects. The interaction of polyenes with the fungal membrane results in the formation of pores with hydrophilic interiors. The formation of these pores causes leakage of cellular components, cell lysis and death (Matsumori *et al.* 2002; Vanden Bossche *et al.* 1987). The drugs in this class, such as amphotericin B and nystatin (Figure 1.1), show broad-spectrum antifungal activity. However, amphotericin B has both acute and chronic side effects in mammalian cells (DiDomenico 1999; Georgopapadakou, 1998; White *et al.* 1998).

(II) The azoles. The azoles are categorized into two groups (Francois *et al.* 2005; Abu-Elteen and Hamad, 2005). First are the N-substituted imidazoles, which contain two nitrogen atoms in the azole ring and have a complex side-chain attached to one of the nitrogen atoms (e.g. ketoconazole). The second group is the triazoles, with a similar structure but with three nitrogen atoms in the ring (e.g. fluconazole) (Figure 1.1). The azoles inhibit two cytochrome P450 enzymes involved in ergosterol biosynthesis (Kelly *et al.* 1997). The first step of inhibition is the cytochrome P450 that catalyzes the 14α-demethylation step, lanosterol 14α demethylase, encoded by *ERG11* (CYP51). The second inhibited step is the cytochrome P450 that catalyzes Δ22-desaturase encoded by *ERG5* (CYP61). Inhibition of these two enzymes leads to depletion of ergosterol, which is substituted by unusual sterols with consequences to membrane structure and activity of membrane-bound enzymes (Prasad *et al.* 2005; White *et al.* 1998; Vanden Bossche *et al.*

1987). Azoles are fungistatic which have no serious human host toxicities reported (DiDomenico, 1999).

(III) Fluorinated Pyrimidine. Compounds such as 5-fluorocytosine (5-FC or flucytosine) are synthetic compounds with pronounced antifungal activity (Figure 1.1). 5-FC inhibits nucleic acid synthesis and disrupts protein synthesis (Boucher *et al.* 2006; Abu-Elteen and Hamad, 2005). 5-FC can be fungicidal or fungistatic, depending on its concentration but has a more limited activity spectrum than do the polyenes (Georgopapadakou 1998). 5-FC is also used as an anticancer drug due to its activity against rapidly dividing cells (Francois *et al.* 2005).

(IV) Candins. Candins are amphophilic cyclic hexapeptides with an N-linked acyl side chain. Examples of this newest class of antifungals are Echinocandin B and aculeacin A, both of which are fungal secondary metabolites (Turner and Current, 1997). Both compounds were abandoned during clinical trials due to toxicity to patients (Francois *et al.* 2005). In this class, the fungicidal caspofungin is the only approved drug for clinical use (Figure 1.1). It interferes with fungal cell wall through inhibition of the synthesis of the structural ß-(1-3)-glucan component (Prasad *et al.* 2000) and has minimal host toxicity in clinical use (Georgopapadakou 2002).

(V) The allylamines and thiocarbamates. These two groups function as non-competitive inhibitors of squalene epoxidase (Figure 1.1). Naftifine and terbinafine are examples of allylamines in clinical use. Tolnaftate is the only available thiocarbamate for clinical use. Both allylamines and thiocarbamates block *ERG1* activity, a squalene epoxidase in the ergosterol biosynthetic pathway (Leber *et al.* 2003). The two synthetic compounds

naftifine and terbinafineare similar in chemical structure, having a naphthalene ring substituted at the 1-position with an aliphatic chain. Both are mainly used for topical fungal infections (Francois *et al.* 2005; Abu-Elteen and Hamad, 2005), are fungicidal and have broad-spectrum activity *in vitro* but poor pharmacokinetics (Cowen *et al.* 2002; Georgopapadakou 1998).

(VI) Other antifungal compounds. Examples of additional, miscellaneous antifungal agents include ciclopirox and sordarins (Abu-Elteen and Hamad, 2005). Ciclopirox (Figure 1.1) is a synthetic hydroxypyridone with broad-spectrum antifungal and antibacterial activities (Abu-Elteen *et al.* 2005). The compound inhibits nutrient uptake by fungi, resulting in a depletion of nucleotides and amino acids (Gupta *et al.* 2005). Sordarin has an alkyl substituted tetrahydrofuran ring (Figure 1.1). It was first discovered in the 1970s and revisited later as an inhibitor of *in vitro* protein synthesis in *C. albicans*. It appears to block the function of fungal elongation factor 2 (EF2) (Abu-Elteen and Hamad, 2005). It is worth noting that *C. albicans* EF2 is similar in amino acid sequence to the human equivalent and sordarin has toxic-side effects to host cells (Dominguez *et al.* 1998). However, due to its novel mode of action, analogs of sordarin may still hold promise for future development.

1.3 Antifungal resistance

Failures in fungal infection treatments have drawn attention recently to the problem of antifungal resistance and its underlying mechanisms. A brief and useful definition of resistance to antifungal compounds is proposed by Kerridge *et al.* (1988) as in a fungus, "... *that will continue to grow and to produce clinical symptoms of disease in*

the presence of the drug at maximum concentrations attainable at the site of infection." Therefore, to name a specific organism as resistant, one must know the *in vitro* susceptibility of the organism to a given drug, such as the minimum inhibitory concentration (MIC), and be aware of many other factors including possible drug interactions, immune response status, pharmacokinetics and the level of the antifungal agent that can be attained in the infected tissue (White *et al.* 1998; DeMuri and Hostetter, 1995). Lack of efficacy of a particular antifungal agent must then be verified in the patient and requires a means to assess a cure.

Studies so far suggest that there are various and complex mechanisms of antifungal resistance and these may be associated with multifactorial phenomena in both host and pathogen. The most studied and common mechanisms of antifungal resistance include the following. First, increased efflux of drugs due to overexpression of ABC transporters or major facilitator proteins (MSF) can result in resistance. For example, overexpression of a transcriptional regulator of efflux-encoding genes *PDR1* in *S. cerevisiae* and *CDR1* in *C. albicans*, was shown to be associated with resistance to fluconazole (Yang *et al.* 2006). Second, overexpression of a drug's target protein can result in sufficient activity by that protein in the presence of the drug. Examples of this include overexpression of *ERG11* in *C. albicans* in response to azole antifungals. Third, the loss of an enzyme function that prevents the accumulation of a toxic compound in the presence of the drug may result in resistance. Loss of function of *ERG3*, which is required for ergosterol biosynthesis in *S. cerevisiae*, provides an example of this mechanism (Smith and Parks, 1993). The resulting substitution of other sterols for ergosterol results in a loss of the drug target and, therefore, resistance. A fourth mechanism involves the overexpression of cell membrane efflux pumps that reduce

intracellular accumulation of the drug (Sanglard *et al.* 2002; Lupetti *et al.* 2002; Ghannoum and Rice, 1999; White *et al.* 1998; White 1997; Zaman *et al.* 1994). For instance, it was shown that the multidrug resistance protein, MRP, as a plasma membrane efflux pump decreases intracellular accumulation of a cytotoxic drug (e.g. daunorubicin) in human tumor cells (Zaman *et al.* 1994). In addition to the above four mechanisms, other mechanisms may be important contributors to antifungal resistance. For example, it was shown that in *Candida glabrata* the mitochondrial respiratory status displays azole resistance due to a loss of mitochondria and blockage of respiration. Subsequently, failure in mitochondrial function can cause up-regulation of *CgCDR1* and *CgCDR2*, genes encoding ABC pumps in *C. glabrata*, which can lead to reduced intracellular accumulation of azole drugs (Kaur *et al.* 2004, Brun *et al.* 2004). The role of Cytochrome P450 in detoxifying antifungal agents represents another important mechanism of resistance (Prasad *et al.* 2005). Panwar *et al.* 2001 observed that the expression of alkane-inducible cytochrome P450 gene, *CaALK8*, in *C. albicans* and *S. cerevisiae* is involved in multidrug resistance. Two classes of cytochrome P450 occur in yeast, P45014DM and P450alk (alkane-inducible) (Vanden Bossche and Koymans, 1998). Interestingly, Prasad *et al.* (2005) showed that the expression of *CaALK8* in *C. albicans* and *S. cerevisiae* confers resistance to many antifungal drugs, including fluconazole, cycloheximide, nitrosoquinoline, miconazole and itraconazole. They also investigated the possibility that CytP450 encoded by *CaALK8* modifies these incoming drugs as is the case for the alkanes. Kelly *et al.* (1997b) showed that the product of *ERG5,* a cytochrome P450 enzyme (CYP61) in *S. cerevisiae*, can also metabolize xenobiotic compounds. As discussed earlier, this is particularly interesting because *ERG5* is a possible target for azole antifungal drugs.

In more recent studies that look at the microevolution of resistance, it was shown that the different mechanisms of action by fungistatic and fungicidal antifungals can influence the development of resistance (Anderson 2005; St-Germain *et al.* 2001). For instance, the azoles are fungistatic and the overall rate of mutations is probably greater in populations of survivor cells exposed to these compounds than fungicidal compounds. This can lead to a resistance phenotype by strong natural selection for resistance (Anderson 2005). The selection process is fairly straightforward. When a group of cells is exposed to a specific antifungal, a subset of cells that are highly susceptible to the compound will die, but the cells that have some resistance from the start, or acquire it through mutation or gene exchange may survive. This happens especially if concentrations of the given drug are too low to overwhelm all cells present. The resistant cells will then go on to proliferate and will inevitably compete with each other. This scenario has been observed for fluconazole resistance among the opportunistic human pathogens *Candida albicans*, *C. krusei, C. glabrata* and *C. parapsilosis*, all of which pose serious problems with respect to chemotherapy resistance (Prasad *et al.* 2005). In contrast, treatments with the *fungicide* caspofungin results in a lower rate of mutation than fluconazole. However, this lower mutation rate, and the relative infrequency of acquired resistance to caspofungin, does not guarantee that resistance will never happen. Examples of resistance to caspofungin have been reported *in vivo* and *in vitro* (Hernandez *et al.* 2004). These mutations are likely promoted, again, by sub-MIC exposures to the compound.

Interestingly, Cruz *et al.* (2002) showed that the expression of the phosphatase calcineurin promotes survival of *C. albicans* cells treated with azole drugs. Therefore, it is not surprising that calcineurin inhibitors such as cyclosporine A (CsA) or tacrolimus

(FK506) (fungicidal) increase azole efficacy in *C. glabrata, C. krusei, C. albicans* and *S. cerevisiae.* This is an example of fungicidal synergism. Azole derivatives are substrates for Cdr1p, a multidrug efflux transporter. Immunosuppressant drugs such as CsA and FK506 directly block activity of *CDR1,* and thus increase the intracellular level of azoles (Marchetti *et al.* 2003; Maesaki *et al.* 1998). These observations indicate that the role of calcium homeostasis is also important in antifungal resistance (Kaur *et al.* 2004; Edlind *et al.* 2002).

The overall view is that the resistance mechanisms of fungi evolve through a variety different possible mechanisms. These processes may occur during the course of chemotherapeutic treatments and are not always easy to define or predict. In addition to the mechanisms briefly described above, it is likely that many additional resistance mechanisms await discovery.

1.4 Medicinal plants.

Plants have been the major source of food, fuel and fiber to human societies for centuries. Plants also provide humans with bioactive molecules for medicines (Cox and Balick, 1994). Increasingly, plant-derived compounds are the centre of both national and international interest, and the focus of searches for new therapeutic agents is based on traditional medicinal plant use. Lewis (1992) outlined several hundred traditionally used plants leading to medicinal natural products and their incorporation into modern pharmacopoeias. The structures of over 200,000 plant products have already been determined, but this is a small fraction of potential materials (Lewis 1992). The medicinal qualities of plants relate largely to their production of secondary metabolites, small molecular weight compounds that aid the plant in combating diseases caused by

microorganisms, competition by other plants, and herbivory by animals. Some of these compounds have recognized health improving effects on animals and are now commercially available as raw herbal medicines or refined drugs (Verpoorte *et al.* 2000).

A "medicinal plant" is one that contains a substance, or substances that can be used for therapeutical purposes or as precursors for the synthesis of functional drugs (Sofowora 1982). The World Health Organization (WHO) defined herbal medicines as follows (WHO 1996): "*Finished, labeled medicinal products that contain as active ingredients aerial or underground parts of plants, or other plant material, or combinations thereof, whether in the crude state or as plant preparations. Plant materials include juices, gums, fatty oils, essential oils, and any other substances of this nature. Herbal medicines may contain excipients in addition to the active ingredients. Medicines containing plant material combined with chemically defined active substances, including chemically defined, isolated constituents of plants, are not considered to be herbal medicines.*" This definition enables us to distinguish between medicinal plants whose thearpeutical properties have been established scientifically and plants that are regarded as medicinal but have not yet been subjected to scientific study.

1.4.1 *Medicinal plants and traditional medicine*

For 80 percent of humanity, traditional medicine is the primary source of health care according to WHO. Indigenous people use a wide range of plants to maintain their health. For instance, in south Asia 800 million people prefer to use herbal and traditional medicine rather than modern medicine (Sofowora 1982). Traditional medicine is cheaper than modern medicine and also more accessible to most of the population in developing and third world countries. The widespread use traditional or indigenous forms of

medicine could be due partly to inaccessibility of modern medicine, but the major reason is the fact that traditional medicine blends into the socio-cultural life of people and the community in whose culture it is deeply rooted (Sheldon *et al.* 1997). Traditional methods of therapy are the only form of health care available to many people today in countries such as Guatemala, Kenya, Nigeria and Ethiopia (Sheldon *et al.* 1997; Sofowora 1982). However, investigation of medicinal plants (ethnopharmacology) is not just a science of rediscovering traditional knowledge. It also constitutes a scientific base in the validation and improvement, or expansion, of the traditional medicine of different ethnic groups, either through the isolation of bioactive agents or through pharmacological research.

1.4.2 *Medicinal plants and modern medicine*

The percentage of modern medicines rooted in medicinal plants and natural products is estimated at 35%-50% (Small and Catling, 1999; Schultes 1994). Almost every class of drug includes a model structure derived from nature. In fact some 25 percent of the drugs prescribed by European and North America physicians are derived from plants and used either as pure compounds or as tea and extracts (Schultes 1994). For instance, one of the earliest plant-derived medicinal substances was the alkaloid morphine, extracted from opium poppy in 1806 (Small and Catling, 1999; Trease and Evans, 1978; Taylor, 1965). Many derivatives of the opium alkaloids such as pethidine, heroin and narlorphine have now been synthesized and these are still used in modern medicine (Sofowora, 1982). In general, plant-derived compounds can contribute to modern medicine and pharmaceutical research in three main ways: (1) as a continued supply of raw materials, (2) as chemical sources to create more complex compounds, and

(3) as a model of new types of pharmacological compounds that can be used as a template for the synthesis of novel analogous drugs with predicted biological activity. A recent example of a plant-derived natural product used in modern medicine is the anticancer alkaloid taxol. Taxol has been clinically available since 1994 and is derived from *Taxus brevifolia*, the pacific yew tree (Cox, 1990; Small and Catling, 1999).

In the last decade, there has been increasing interest in phytotherapy and medicinal plants in western medicine. Interestingly, in Germany all medical practitioners have to pass a phytotherapy examination before being allowed to practice. In the USA, the Dietary Supplement Health and Education Act of 1994 recognized the class of phytotherapeutic compounds derived from medicinal plants. In Canada, Natural Health Product (NHP) can be marketed as over-the counter drugs, if certain conditions are met such as scientific proof of efficacy and standardization of dosage or statement of contraindications is provided (Small and Catling, 1999).

1.4.3 *Medicinal plants and technologies*

"Natural product" does not necessarily mean safe product. For instance, there are a number of herbal products that are highly effective but can be poisonous for health purposes because natural, unrefined and crude plant extracts vary wildly in the concentration of active principles. Therefore, natural products should only be used by skilled medical practitioners. In the case of medicinal plants, usually two types of practice can be considered. First, aboriginal technology concerns the technological treatment of plants by indigenous peoples. For example, in Brazil, teas and extracts from *Pilocarpus* leaves have been used to induce sweating and salivation (Sheldon *et al.* 1997). Second, advanced technology of modern industries, academia and health sciences

(Schultes 1994) are used to facilitate laboratory evaluation of compounds from medicinal plants. Recently, tools from molecular biology have been introduced into the medicinal plant field. Application of these tools to the study of plant–derived compounds provides information such as effects on gene activation and gene expression in response to natural product exposure. These studies can provide insight into mechanisms of action, possible side effects and biological function(s) of novel compounds.

1.4.4. *Canadian regulation for herbal medicines*

In Canada the production, processing, manufactures, and marketing of herbal products is regulated by Health Canada regulations. The Natural Health Products (NHP) are also governed by agencies such as the House of Commons Standing Committee and Canadian Food and Drug Administrations. The mission of these organizations is to ensure the highest level of responsibility with respect to the way herbs are manufactured, labeled and sold to Canadians. Based on Canadian Food and Drug Act regulations, herbal products can be classified as food or drugs depending on their purposes. For instance, Garlic can be be marketed either as a food, or as drug for lowering cholesterol (Small and Catling, 1999). On an ongoing basis, the regulations submit comments to a variety of provisions including product licensing, site licensing, good manufacturing practices, adverse reaction reporting, and clinical trials and labeling. The regulations came into force on January 1, 2004 and apply to all Natural Health Products as of this date. Information to register and produce a NHP is available from the Health Canada Drug Directorate (Tunney's Pasture, Address Locator #0702A, Ottawa, ON, K1A 0L2) and also from the Health Canada web site. Additional information about NHP

regulations can be found at http://canadagazette.gc.ca/partII/2003/20030618/html/sor196-e.html.

1.5 New antifungal agents and rationale for study

Biochemical and molecular biology investigations on constituent properties of herbal medicines have great potential in providing new insight into treatments of animal mycoses. Of particular interest, many plant compounds of extracts have antifungal activity, presumably reflecting a strong selection pressure on plant by fungal pathogens (Ficker *et al.* 2003; Jones *et al.* 2000; Omar *et al.* 2000). Since fungi are also a major cause of animal disease and material spoilage, there is a potential for many uses of such plant-derived antifungals. However, rational choices of natural products for development require information on mode of action. Recent developments in genomics provide new technologies for assigning mode of action of antifungals. In this thesis, one natural product (berberine) and one phytomedicine (Echinacea) are examined, as model antifungals. I describe how budding yeast *S. cerevisiae* responds to sub-lethal exposures of berberine by using DNA microarray techniques. I also provide insight into the modes of action of berberine and Echinacea using gene deletion arrays and thus add to the value of these bioactive herbal compounds. Furthermore, this study provides a template in medicinal plant research. The future direction of the study is to link the collected data to the drug discovery and development processes. In the following sections, I briefly describe what is known about these two phytomedicines and review how yeast is an ideal system for antifungal studies.

1.5.1 *Berberine*

The alkaloid Berberine (Figure 2.1) has demonstrated significant antimicrobial activity against bacteria, fungi, protozoa and viruses (Table 1.1). It is an alkaloid present in several clinically-important medicinal plants. Included in these are *Berberis vulgaris* (European barberry), and *Berberis aquifolium* (Oregon grape), derived from North American First Nations traditions, *Coptis chinensis* (Coptis or golden thread) from traditional Chinese medicine, and *Berberis thunbergii* (Japaness barbery) used in Ayurvedic medicine (Manske and Holmes, 1953) (Figure 1.2). In animals and humans, berberine inhibits intestinal ion secretion, smooth muscle contraction, ventricular tachyarrhythmias, but it stimulates bile and bilirubin secretion (Mahady and Chickwick, 2001). It also reduces inflammation and elevation of platelet count in patients with primary and secondary thrombocytopenia (Mahady and Chickwick, 2001). Berberine's most common clinical uses are in treating bacterial diarrhea, intestinal parasites, and ocular trachoma infections (Timothy *et al.* 1997). Amin *et al.* (1969) screened a total of 54 microorganisms for sensitivity to berberine, and found the alkaloid possesses antimicrobial activity against gram-positive and gram-negative bacteria, fungi, and protozoa (Table 1.2). In addition to those listed, *Mycobacterium smegmatis, Trichophyton mentagrophytes*, and some strains of *Escherichia coli, Klebsiella pneumoniae* and *Cryptococcus neoformans* exhibited moderate sensitivities to berberine (Mahady and Chicwick, 2001). Berberine has been also shown to inhibit HIV-1 reverse transcriptase (Gudima *et al.* 1994).

Much of the research on berberine has focused on its use in cases of diarrhea, including that caused by *Vibrio cholerae* and *Escherichia coli.* Studies have demonstrated a direct antibacterial effect of berberine against *V. cholerae*, and berberine

Table 1.1. Microorganisms sensitive *in vitro* to berberine. (Amin *et al.* 1969; Jones *et al.* 2000; Mahady *et al.* 2001; Ficker *et al.* 2003).

Bacteria	Fungi	Protozoa
Bacillus pumilus	*Candida utilis*	*Entamoeba histolytica*
Bacillus cereus	*Candida albicans*	*Giardia lamblia*
Bacillus subtilis	*Candida tropicalis*	*Trichomonas vaginalis*
Corynebacterium diphtheriae	*Candida glabrata*	*Leishmaniasis sp.*
Shigella boydii	*Sporotrichum schenkii*	
Staphylococcus aureus	*Saccharomyces cerevisiae*	
Staphylococcus albus	*Cryptococcus neoformans*	
Streptococcus pyogenes	*Aspergillus fumigatus*	
Vibrio cholerae	*Aspergillus alternata*	
Klebsiella pneumoniae	*Microsporum gypseum*	
Escherichia coli	*Wangiella dermatitidis*	
Mycobacterium smegmatis	*Fusarium oxysporum*	
	Pseudallescheria boydii	
	Rhizopus sp.	
	Trichophyton mentagrophytes	

has been shown to inhibit the intestinal secretory response caused by *E. coli* heat-stable enterotoxin (ST) and *V. cholerae* enterotoxins. Aqueous solutions of berberine have also been employed in cases of ocular infections, especially those resulting from *Chlamydia trachomatis* (Timothy *et al.* 1997). In addition to its direct antimicrobial action, berberine has been shown to block the adherence of *Streptococcus pyogenes* and *E. coli* to erythrocytes and epithelial cells. Thus, it is possible that berberine exerts an antibiotic effect even against organisms that do not exhibit *in vitro* sensitivity to the alkaloid (Mahady and Chickwick, 2001; Timothy *et al.* 1997).

Aside from antimicrobial activities, berberine has other physiological and biochemical effects. For example, berberine may have immunostimulatory effects, since Sabir *et al.* (1978) reported that berberine stimulates blood flow to the spleen. Further study showed that berberine also activates macrophages (Hamon 1990), thus providing further evidence of immunostimulation. *In vitro* studies indicate that berberine may inhibit growth of cancer cells by increasing the host macrophage activity. The kill rate of cancer cells such as SC-M1 cell line (gastric cancer cells) by berberine was estimated at 91% compared to 43% for anticancer drugs such as anti-HIF-1α based on an *in vitro* study (Lin *et al.* 2004; Li *et al.* 2000). In China, berberine is given after chemotherapy to aid restoration of white blood cell counts (Tyler and Brady, 1988). Berberine sulfate has also demonstrated antipyretic effects in experimentally induced fevers in rats. This effect has been found to be approximately three times greater than the antipyretic effect of sodium salicylate (Sabir *et al.* 1978). It is known that berberine inhibits the biosynthesis of DNA, RNA, proteins and lipids as well as the oxidation of glucose (Choi *et al.* 2001). This diversity of effects of berberine suggests that it has multiple and diverse targets within the cell.

Figure 1.2. Medicinal plants that contain berberine. Photos by Ivor Hughes (*B. aquifolium*), Bernd Liebermann (*B. vulgaris*), James Danoff-Burg (*B. thunbergii*) and Renée Ludec (*C. chinensis*) with written permission.

B. aquifolium

B. vulgaris

B. thunbergii

Coptis chinensis

Table 1.2. Minimum Inhibitory Concentration (MIC) of berberine against bacteria, fungi and protozoans.

Antibacterial	**MIC µg/ml**	**Reference.[a]**	**Antifungal**	**MIC µg/ml**	**Reference.[a]**	**Antiprotozoal**	**MIC µg/ml**	**Reference.[a]**
E.coli	50	1, 2	*C. albicans*	1000	3, 4	*Entamoeba bistolytica*	1000	1, 2
V. cholerae	50	1, 2	*C. neoformans*	1000	3, 4	*Giardia lamblia*	1000	1, 2
H. pylori	40	1, 2				*Tricomonas vaginalis*	1000	1, 2
S. aureus	25-50	1, 2						
B.subtilis	1000	1, 2						
M. smegmatis	25-50	1, 2						
St.pyogenes	12.5	1, 2						
C. perfringens	500	1, 2						

[a] 1. Mahady *et al.* 2001; 2. Knight 1999; 3. Mahajan *et al.* 1982; 4. Okunade *et al.* 1994.

1.5.2 *Echinacea*

Echinacea is a genus of the Asteraceae and the name was coined from Greek (*echinos*), a reference to the spiny floral bracts of these plants. The name Echinacea is used as a scientific plant name, a common plant name and also for drug preparations from the plants (Small and Catling, 1999). Echinacea species are purple coneflower where are native to the prairies of central and the southeastern United States, with one species extending to southeastern Saskatchewan and southern Manitoba in Canada (Small and Catling, 1999). This native North American flower was widely introduced as a decorative plant and has been used extensively for medicinal and clinical purposes. Echinacea is economically important and is one of the top ten herbal products in North America, representing 9.9% of sales (Brevoort, 1998). It has a rich tradition of use by First Nations groups who used it medicinally more than any other plant (Gilmore 1911, 1913; Hart 1981; Wacker and Hilbig, 1978). Its first recorded use by indigenous North Americans was in the 1600s and European settlers had adopted its use by the 1800s (Small and Catling, 1999; Tyler 1993). Today, it is widely held that the herb preparations of Echinacea are efficacious in preventing and treating colds, coughs, flu and other respiratory illnesses, in fighting infections (e.g. candidiasis) and generally used for immunostimulatory properties (Hudsen *et al.* 2005; Merali *et al.* 2003; Binns *et al.*, 2002b; Goel *et al.* 2002; WHO Herbal Monograph 1999; Hobbs 1994). In these respects, it is believed that Echinacea works through short-term stimulation of the immune system and it is not recommended for long-term use. A maximum period of continuous use is six to eight weeks (Wacker and Hilbig, 1978). With long-term use, Echinacea appears to lose effectiveness.

A taxonomic revision of the genus *Echinacea* was recently proposed (Binns *et al.* 2002a). In this revision the genus is split into two subgenera, four species and six varieties. These species, according to this new taxonomy, that are used medicinally are *Echinacea purpurea* (L.) Moench., *Echinacea pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist (syn. *E. angustifolia)* and *Echinacea pallida* (Nutt.) Nutt. var. *pallida* (syn. *E. pallida*) (Figure 1.3; Binns *et al.* 2002a, 2002b). The less complicated taxonomy of McGregor (1968) is still used by the medicinal plant industry and these species are *E. purpurea*, *E. angustifolia* and *E. pallida.*

Medicinal extracts of Echinacea are made from all parts of the plant, including the rhizomes (termed root in most references) and aerial parts such as leaves, flowerheads, and seeds. Many different formulations of Echinacea have been found in teas, tablets, tinctures or salves (Foster and Duke, 1990). A number of active ingredients, such as a volatile oil, alkamides, caffeic acid derivatives, and polysaccharides are considered important contributors to the therapeutic effects of Echinacea (Hudson *et al.* 2005, Vimalanathan *et al.* 2005; Merali *et al.* 2003, Binns *et al.* 2002a,b; Bradley *et al.* 1992; Bauer *et al.* 1991; Awang *et al.* 1991). The volatile oil contains, among other compounds, pentadeca-(1, 8-*Z*)-diene (44%), 1-pentadecene, ketoalkynes and ketoalkenes. The constituent alkamides, mostly isobutylamides of C11–C16 straight-chain fatty acids with olefinic or acetylenic bonds, or both, are found in the roots. Of the medicinally important species, the highest concentration of these compounds is in *E. angustifolia*, followed by *E. purpurea*, and the lowest is in *E. pallida* (Goel *et al.* 2002). The main alkamide is a mixture of isomeric dodeca-2,4,8,10-tetraenoic acid isobutylamides. The caffeic acid ester derivatives present include echinacoside, cynarin, and chicoric acid. Cynarin is present only in *E. angustifolia*, thus distinguishing it from the closely related *E. pallida*

Figure 1.3. The medicinal plants of *Echinacea* species. Photos by John T. Arnason (*E. purpurea* and *E. angustifolia*) and Shannon Binns (*E. pallida*) with written permission.

Echinacea purpurea

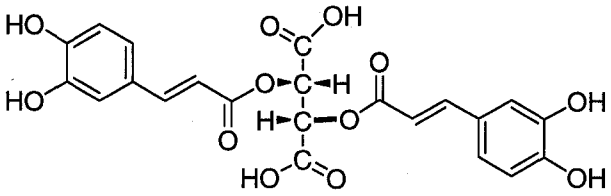
Echinacea pallida

Echinacea angustifolia

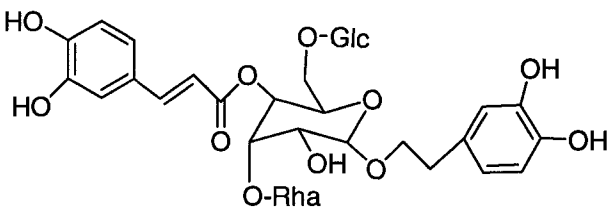
(Figure 1.4; Goel *et al.* 2002). Polysaccharide constituents are of two groups: a heteroxylan and an arabinorhamnogalactan. However, the various species of *Echinacea* have been characterized as possessing certain phenolic marker compounds. For example, cichoric and caftaric acids are phenols found within both the aerial and root portions of *E. purpurea*, while echinacoside is a phenolic found in higher levels specifically within *E. angustifolia* and *E. pallida* roots (Goel *et al.* 2002). These phenolics serve as markers to evaluate the quality of Echinacea products (Goel *et al.* 2002).

Echinacea has direct antimicrobial activity against bacteria, viruses and fungi (Hudson *et al.* 2005; Coker and Camper, 2004; Binns *et al.* 2000; Hobbs *et al.* 1999). The antimicrobial activities against some bacteria such as *E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus* are attributed to polysaccharides and alkylamides, however, the bioassay tests were not all done with sufficient quality control to be accepted in the U.S. (Coker and Camper, 2004; Hobbs 1990). Echinacea extracts also showed significant antiviral activity against *Herpes simplex* virus and influenza, correlated to the presence of alkamides (Hudson *et al.* 2005). In addition, light-mediated antimicrobial actions (phototoxicity) of Echinacea extracts is attributed to the ketoalkenes which are abundantly present in the roots (Binns *et al.* 2000). The antifungal activity of Echinacea was noted through significant *in vitro* antifungal activities against *S. cerevisiae* and various *Candida* species, including *C. albicans* strains that were azole resistant (Binns *et al.* 2000). Candidiasis is a fungal infection caused by *Candida* species that is common in immuno-compromised patients, pregnant women, diabetics and newborn children. The rate of recurrence is high for immuno-compromised individuals and clinical Candida isolates increasingly have resistance to current antifungal drugs (Cowen *et al.* 2002; Prasad *et al.* 2005).

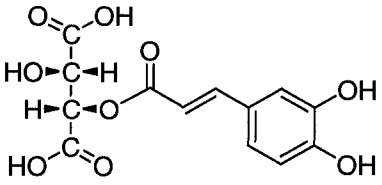
Figure 1.4. Chemical structures of *Echinacea* compounds.



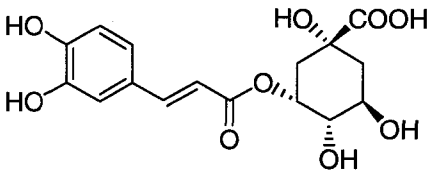
Cichoric acid
(2,3-*O*-dicaffeoyl tartaric acid)



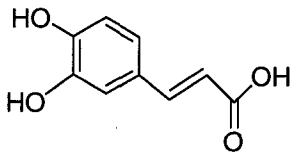
Echinacoside



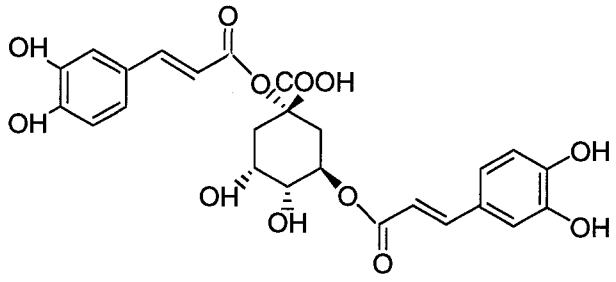
Caftaric acid
(2-*O*-caffeoyl tartaric acid)



Chlorogenic acid
(3-*O*-caffeoyl quinic acid)

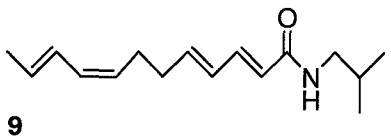


Caffeic acid

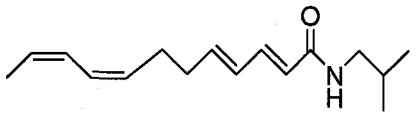


Cynarin
(1,3-dicaffeoyl quinic acid)

8



9



Alkamides 8/9

Furthermore, it was reported that the Echinacea constituents such as echinacosides, alkaloids and polysaccharides are antimicrobial, antiseptic, anti-inflammatory, tonic, detoxicant, antiparasitic, vasodilatory, vulnerary, and lymphatic (Merali *et al.* 2003; Bartram 1995). The British Herbal Compendium reported that Echinacea is an immunostimulant, anti-inflammatory, anti-bacterial, anti-viral and vulnerary (Vimalanathan *et al.* 2005; Binns *et al.* 2002a; Bradley 1992). Medicinal action of the herb activates the body's T-cells, when it does not target any specific viruses or bacteria, making it particularly effective to treat some chronic illnesses including AIDS and auto-immune disorders (Murray *et al.* 1998). Since the herb stimulates the body's own defense mechanisms against infection through non-specific and specific immunity, Echinacea would be a good choice of herb for people who suffer from common minor illnesses such as colds, sore throats or stress-exacerbated infections like ulcers, cold sores (herpes) and eczema which occur when the immune system is compromised (Bradley 1992). Cytokine, macrophages and natural killer cells are primary targets for action of phytomedicines in non-specific or innate immunity (early reaction), while lymphocytes are involved in specific or adaptive immune responses (later reaction) (Romani 2000; Lenschow *et al.* 1996). In addition, polyenes from the Asteraceae family are known to show phototoxicity (photo-activated toxins) against fungi (Towers *et al.* 1997) and other microorganisms (Hudson *et al.* 1991). Merali *et al.* (2003) also showed that quantity and diversity of polyenes in Echinacea species extracts may be responsible for enhanced phototoxicity and antifungal activity. Moreover, it was shown that the phototoxicity mechanism is involved with production of singlet oxygen and peroxidation as well as photoaddition to unsaturated lipids of cell membranes in target organisms (Towers *et al.* 1997).

1.6 Yeast as model organism

Saccharomyces cerevisiae, the common brewer's yeast, presents an excellent research system for the study of genetics and genetic environmental interactions. Some properties which make yeast a favorable model are; simple and economic growth requirements, a short generation time, single celled growth habit, a well-defined genetic system, thousands of available mutants, an easily manipulated sexual cycle, simple equipment requirements and a versatile DNA transformation system. Other important resources provided by the yeast system are Yeast Artificial Chromosomes (YACs). YACs enable the cloning and amplifications of large contiguous segments of DNA and are being used in many genome sequencing efforts, including that of the human. Additionally, many experiments are underway to analytically investigate the possible functions of all yeast genes by testing the phenotypes of strains that have disrupted genes. Normally, a disrupted gene is defined within a mutant strain within a single genetic background. Each strain carries only a single mutant gene at a time (Gene Deletion Array, GDA) or a systematic construction of double mutants (Systematic Genetic Array, SGA). The phenotypes arising after disruption of yeast genes has contributed significantly to understanding the function of certain proteins *in vivo*. Interestingly, many mutants with disrupted genes, which were previously assumed to be essential, are viable with little or no detrimental phenotype. Additionally, yeast is an ideal model for studies of protein-protein interactions and expression systems for the laboratory preparation of heterologous proteins (Sherman 1998).

1.6.1 *Yeast growth and life cycle*

Yeasts and yeast-like microorganisms are microfungi (single-celled/unicellular fungi), which live either as saprophytes or as parasites. Vegetative reproduction is by budding or by fission, although some yeast-like fungi, such as *Candida albicans*, may grow as simple irregular filaments (hyphae or pseudo-hyphae) (Byers 1981). Ascomycetous yeasts may also reproduce sexually, forming asci which contain 1-4 haploid ascospores or rarely 8-16 ascospores (e.g. in genus *Lipomyces*), or up to several hundred ascospores as in *Kluyveromyces polysporus* (Skinner *et al.* 1980). The basidomycete yeasts usually produce four spores on the outside of a specialized spore producing structure called a basidium (Skinner *et al.* 1980). The colony morphology of yeast-like fungi is diverse. They can be coloured with various shades of black, brown, red, pink, cream or orange and can have different textures including smooth, mucoid, and rough. The colour and texture of yeast colonies varies with the species, medium, nutrient status and growth temperature. The budding yeast *Saccharomyces cerevisiae*, better known as baker's or brewer's yeast, has thick-walled, oval cells and is ~10 μm long by ~5 μm wide (Fowell 1969). *S. cerevisiae* is an important model organism for understanding cellular and molecular processes in eukaryotes. *S. cerevisiae* was the first eukaryotic organism to have its genome completely sequenced, a project that was completed in 1996 (Goffeau *et al.* 1996; Dujon 1996). Being nonpathogenic, *S. cerevisiae* can be handled with minimal precautions. *S. cerevisiae* strains have both a stable haploid and diploid state. Therefore, recessive mutations can be easily isolated and observed in haploid strains, and complementation tests can be carried out in diploid strains. Lethal recessive mutations can also be maintained in heterozygous diploids. Haploid yeast strains normally divide by mitosis every ~90 minutes in complete YPD medium during

the exponential phase of growth at an optimum temperature of 30° C (Sherman 1998). *S. cerevisiae* can be maintained as either heterothallic or homothallic strains, both of which can be induced to enter the sexual stage and sporulate under well established environmental conditions such as nutrient deficiency (Sherman 1998).

1.6.2 *Yeast strains*

In fact, there are no wild-collected *Saccharomyces* strains that are commonly employed in genetic studies. Most current genetic studies are carried out with one of the following "standard" strains or their derivatives: S288C; W303; Σ1278B; AB972; SK1; FL100; D273–10B; X2180 and A364A. These strains have different properties that can significantly influence experimental outcomes. The haploid strain S288C (*MATα SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1*) is often used as a standard strain. It does not form pseudohyphae (Goffeau *et al.,* 1996). This is the strain for which the genome sequence of the species has been determined. In addition, a large set of isogenic mutant derivatives of S288C are available.

1.6.3 *Yeast genome information*

S. cerevisiae contains a haploid genome complement of 16 well-characterized chromosomes with sizes that range from 200 - 2,200 kb. Its genome constitutes 12,052 kb of chromosomal DNA sequences, a total of 6,183 Open Reading Frames (ORFs) of over 100 codons long, and almost 5,800 of these are predicated to match actual protein-encoding genes (Sherman 1998; Mewes *et al.* 1997; Goffeau *et al.* 1996). About 25 – 30% of the ORFs are of unknown function as of 2006 (SGD web source, see below). The general form YALXXW is now used to designate genes uncovered by systematically sequencing the yeast genome, where Y designates yeast; A, B, C, etc. designates the

chromosome I, II, III, etc.; R or L designates the right or left arm of the chromosome; XX designates the relative position of the start of the open-reading frame from the centromere toward the telomere; and W or C designates the Watson or Crick strand. For example, YGL014W denotes *PUF4*, a previously known but unmapped gene that is located on the left arm of chromosome VII, fourteen open reading-frames from the centromere on the Watson strand. These ORF numbers are designated by MIPS and Max Planck Institute for Biochemistry; www.mips.biochem.mpg.de/yeast/. In addition, any useful information or database entries concerning yeast such as DNA sequences, list of genes and protein information can be conveniently retrieved on different websites including the Saccharomyces Genome Database (SGD) (http://www.stanford.edu/Saccharomyces/), the "Yeast Protein Database (YPD)" (http://www.proteome.com/YPDhome.html), or the Munich Information Centre for Protein Sequences (MIPS) (http://www.mips.biochem.mpg.de/).

1.6.4 *Yeast and genomic analysis*

Since the completion of the *S. cerevisiae* genome sequence, efforts have concentrated on comprehensive studies addressing key aspects of yeast cell biology on a system-wide level. These include genome–wide analyses of gene expression (microarray); biochemical activity, protein-protein interaction and gene disruption phenotypes (Tong *et al.* 2001, 2004; Hughes *et al.* 2000; Martzen *et al.* 1999; Winzeler *et al.* 1999). In the present study two genomic techniques were employed as follows.

(I) DNA Microarray: Gene expression profiling by DNA microarrays represents a major advance in genome functional analysis and most of these studies have focused on genes that respond to a treatment of interest. For example, microarray experiments using

pharmacologic agents help to identify mechanisms for drug action as well as identification of potential drug targets. This method might improve the efficiency of drug discovery by a rapid identification of potential gene or protein targets, or by identification of compounds likely to be toxic. For this purpose, cells are treated with a drug and the gene expression profile that is determined will be compared to one based on non-treated control cells. The assumption is that gene transcript level changes occur in response to a given treatment (stress or inhibitor), and may be involved in protecting the cells from the treatment. The yeast cDNA microarray contains 6,183 ORFs from *S. cerevisiae* spotted onto the glass slide in duplicate. In a typical microarray experiment two RNA samples, one derived from treated and one from untreated (control) cell cultures, are reverse transcribed into cDNA, labeled using different fluorochrome-labelled (Cyanine) dyes (usually a red fluorescent dye, Cy5, and a green fluorescent dye, Cy3) and then hybridized to the DNA microarray. The array slide is scanned fluorometrically and fluorescent intensity values of the individual DNA spots are indicative of transcript levels. Comparisons in gene expression levels between the two samples, RNA from treated cells and control RNA, are derived from the resulting intensity ratios (Yang *et al.* 2002; DeRisi *et al.* 1997). The hypothesis underlying microarray analysis is that the measurement of intensities for each arrayed gene represents its relative expression level. A ratio of relative gene expression is measured for each individual gene on the array. A particular gene is designated as differentially regulated using an arbitrary cutoff of greater than, or less than 2-fold difference (either increased or decreased expression) (Yang *et al.* 2002). The statistical analysis of the data is the most important and complex step in the microarray experiment. For example, normalization is the first transformation applied to microarray data to remove any systematic variation (e.g. unequal quantities of RNA or

differences in labeling between fluorescent dyes) rather than biological differences between the samples (Yang *et al.* 2002). Due to statistical issues related to the microarray techniques, it is important to validate the microarray findings using independent and alternate methods such as Northern blot or real-time PCR techniques for confirmation of differential gene expression to guard against technical errors. Genes chosen for this purpose should show significant differences between two or more experimental samples (Nair *et al.* 2003).

(II) Gene Deletion Array (GDA): A second high-throughput experimental method used in this thesis is the Gene Deletion Array (GDA) method. GDA analysis has already provided useful information on the molecular mechanisms of action of several bioactive chemicals (Parsons *et al.* 2004; Tong *et al.* 2001). For GDA analysis, a set of approximately 4700 viable haploid yeast gene-deletion mutants were generated in the background of *S. cerevisiae* strain S288C (Tong *et al.* 2001). This method is based on quantitative phenotypic sensitivity profiles, generated from inactivation of two target genes. The first gene is disabled by a deletion mutation and the second gene, or gene-product, is inactivated by the target compound. If the combination results in an altered growth rate by a specific deletion mutant, it is possible that the chemical target is in a pathway that is parallel to that of the first mutation. This phenotypic screening is a valuable tool for the elucidation of the mode of action of compound. In some cases, phenotypic screening elucidate cellular interactions of the compound with a specific gene product and results in attractive candidates for drug development (Baetz *et al.* 2004). In addition, loss of function caused by mutation in a gene, which encodes a target for an inhibitory compound, can model the primary effect of the compound.

In the present study we have done comprehensive profiles of *S. cerevisiae* gene deletion arrays to provide clues about the mechanism of action(s) and pathway(s) of plant-derived antifungal compounds. For example, 23 deletion mutants were identified from GDA data that were consistently sensitive to eight different ethanol extracts of roots, flowers or leaves of *E. purpurea* and *E. angustifolia* plants. The significant pattern to emerge from these GDA analyses was that Echinacea extracts interfere with fungal cell wall function. Therefore, the identification of compounds with specific bioactivities like Echinacea will allow us to derive a more suitable chemical-genetic basis for standardization of Echinacea preparation and also to improve commercial formulation of Echinacea as an antifungal compound. In the context of treating fungal infections in human subjects, knowledge from *in vitro* studies of this type can help us to determine how antifungal candidates work *in vivo* and what gene products might be suitable targets for further therapeutic intervention.

1.7 Purpose and Objectives

It was hypothesized that DNA microarray and gene deletion array with *S. cerevisiae* would provide insight into the antifungal modes of action of the model natural product berberine and phytomedicine Echinacea. The objectives for this study are as follows:

(1) To investigate gene expression profiles in *S. cerevisiae* to sub-lethal exposures of berberine using DNA microarray methods (Chapter 2).

(2) To investigate the sensitivities within the complete set of viable haploid deletion mutants of *S. cerevisiae* to sub-lethal exposures of berberine (Chapter 3) and Echinacea extracts (Chapter 4).

(3) To compare the efficacy of microarray and GDA analyses in elucidating mode of action of new antifungals (Chapter 3 and 5).

(4) To develop "genome-wide" methods as a template in medicinal plant research.

CHAPTER 2

Identification of Differentially Expressed Genes in Yeast Exposed to the Antifungal Berberine.

2.1 Introduction

Many current antifungal drugs are limited by host toxicity, a limited spectrum of activity, and propensity to induce or select for resistant fungal pathogens. Therefore, a need has been created for additional agents to treat fungal infections. Of particular interest is the observation that many extracts of traditional medicinal plants have antifungal activity (Ficker *et al.* 2003; Jones *et al.* 2000; Omar *et al.* 2000), presumably reflecting a strong selection pressure by fungal pathogens on plant hosts. Since fungi are a major cause of plant and animal diseases and materials spoilage, there are many potential uses for such plant-derived antifungals.

Among plant-derived compounds, the alkaloid berberine (Figure 2.1) is a component of a number of important medicinal plants such as *Berberis vulgaris* (barberry), and *Berberis aquifolium* (Oregon grape), used from North American First Nations people, *Coptis chinensis* (Coptis or golden thread) from traditional Chinese medicine and *Berberis thunbergii* (Japaness barbery) used in Ayurvedic medicine (Manske *et al.* 1953). Berberine has demonstrated significant antimicrobial activity against different organisms including fungi (Ficker *et al.* 2003; Mahady *et al.* 2001; Timothy *et al.* 1997; Gudima *et al.* 1994; Tyler *et al.* 1988) and is relatively nontoxic to humans (Rabbani *et al.* 1987). In this study we used DNA microarrays to examine changes in transcript levels in the fungus *Saccharomyces cerevisiae* after exposure to a Minimum Inhibitory Concentration (MIC) of berberine. We hypothesized that changes in yeast transcription profiles could provide insights into the mode of antifungal action of berberine and fungal resistance mechanisms, two critical considerations in developing and optimizing antifungal compounds.

Figure 2.1. Chemical structure of berberine.

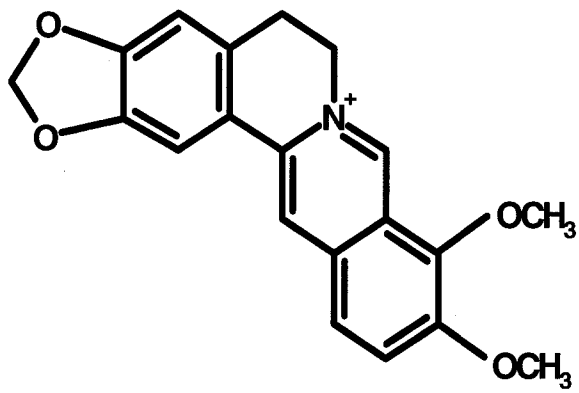


Table 2.1. *S. cerevisiae* strains used.

Strain	Background	Genotype	Source
YSec4	S288C	*MATα SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1*	J. Anderson
Y800	-	*MATa leu2-Δ98cry1^{R}/ MATα leu--Δ98CRY1 ade2-101 HIS3/ ade2- 101his3-Δ200ura3-52cani^{R}/ura3-52CAN1lys2-801/ lys2-801CYH2/cyh2^{R} trp1-1/TRP1Cir^{0}*	M. Snyder
YLR162W	Y800	with transposon insertion into YLR162W	Open-Biosystem
YHR071W	"	with transposon insertion into YHR071W	"
YPR080W	"	with transposon insertion into YPR080W	"
BY4741	-	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	"
YDR4541W	BY4741	*YLR454W::kanMX4*	"
YGL014W	"	*YLR014W::kanMX4*	"
YDR035W	"	*YDR035W::kanMX4*	"
YFL021W	"	*::YFL021W*	A. Golshani
YDR010C	"	*::YDR010C*	A. Golshani

S. cerevisiae has been used extensively to address the mechanisms underlying cellular responses to osmotic, chemical and oxidative stresses. For instance, the ATP-binding-cassette (ABC) homolog in yeast, *YAP1/SNQ3,* whose function is well documented in stress response, is also implicated in drug resistance (Bauer *et al.*1999). Bauer *et al.* (1999) also showed that *YAP1* induces expression of genes that are important for cell viability under stress conditions. In other study, *PDR15* mRNA and protein levels are significantly increased by various stress conditions including heat shock, weak acid and osmotic stress (Wolfger *et al.* 2004). In the present study, *S. cerevisiae* was subjected to MIC levels of berberine for 2, 4, 5 and 6 hours and transcript levels were subsequently monitored with DNA microarrays. We then focused on genes that showed a consistent and significant increase or decrease in transcript level across these exposure times and evaluated MIC and berberine uptake in strains with mutations in some of the response genes. The study illustrates that DNA microarrays provide insight into the mode of action of berberine and can serve as an exploratory step toward analyzing promising antifungal agents from medicinal plants.

2.2 Methods and Materials

2.2.1 *Growth conditions and strains*

Berberine chloride was obtained from E. Merck (batch# 119H0687, Darmstadt, Germany) and purity was determined to be >95% by HPLC. Berberine solutions were prepared using warm distilled water, and subsequent dilutions were made in Yeast Peptone Dextrose medium (YPD), consisting of 2% bactopeptone, 2% dextrose and 1% yeast extract (Difco, Detroit, MI), with or without 1.7% bacto-agar (Difco), as

appropriate. The yeast strains used in this study are described in Table 2.1. The Synthetic Defined Dropout medium (Q-Biogene, Krackeler Scientific, Albany, NY) was used for yeast insertional mutant strains. Yeast Knock-Out (YKO) strains produced by the Saccharomyces Genome Deletion Project (SGDP, http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html) were initially grown in YPD broth including G418 (200 μg/ml, Biosciences, La Jolla, CA). All strains were grown at 30°C.

2.2.2 *Minimum Inhibitory Concentration (MIC) assays*

Yeast cells were grown in YPD overnight at 30°C with shaking at 200 rpm to an OD_{600} of ~0.9. Cultures were diluted ~500-fold in YPD medium, and distributed at 100 μl/well into disposable 96-well microtiter plates (Costar, round bottom, Broadway, MA). Each well contained ~1000 yeast cells as estimated by hemocytometer counting. A berberine concentration series was established between 6.7 to 1 x 10^{-5} mg/ml within the microtiter plates. A drug-free control was also included for each experiment. The plates were incubated at 30°C for 48 h before determining MIC by visual inspection and by reading optical densities at 490, 600 and 750 nm in a microplate reader (Spectramax 340PC, Molecular Devices Corp., Sunnyvale, CA). MIC in these experiments was defined as the lowest drug concentration that gave ~80% inhibition of growth compared with the growth of drug-free controls.

2.2.3 *RNA isolation and DNA microarray analysis*

All microarray work was done with *S. cerevisiae* strain YSec4 derived from *S. cerevisiae* S288C (Table 2.1). Yeast cells were grown in YPD medium to an OD_{600} of 0.6-1 and exposed to an initial concentration of 0.07 mg/ml berberine. Total RNA was

extracted from yeast cells using hot acidic phenol (Ausubel *et al.* 2000) and purified using the RNeasy Mini kit (Qiagen, Mississauga, ON). The concentration of purified RNA was determined by spectrophotometry (Sambrook *et al.* 1989) and adjusted to 10-20 μg/μl. Total RNA (20 μg) was labeled with either Cy3 or Cy5 (Amersham Pharmacia, Piscataway, NJ) by standard protocols (Ontario Cancer Institute, Toronto, ON). The reverse transcription cocktail included 100 pmol/μl 5' Biotin AncT mRNA primer (5'-T_{20}VN), 6.67 mM each of dATP, dGTP and dTTP, 2 mM dCTP and 0.1 mM Cy3- or Cy5- labeled dCTP. Reactions were incubated at 65°C for 5 min, then 42°C for 5 min, before the addition of 400U reverse transcriptase (Superscript II, Life Technologies) and 30U RNAse Inhibitor (MBI Fermentas). The reaction was then incubated at 42°C for another 2 hours. The experimental cDNA labeled with Cy5 and control cDNA labeled with Cy3 were mixed together and incubated at 65°C for 2 minutes before the addition of 4 μl of 50 mM EDTA and 2 μl of 10 N NaOH followed by a further incubation at 65°C for 20 minutes. Four μl of 5 M acetic acid and 50 μl of 100% isopropanol were added to precipitate the cDNA samples at -20 °C for 10 minutes. The samples were then washed with cold 70% ethanol and resuspended in 5 μl of TE or UV treated dH_2O.

Yeast Y6.5k ORFs Microarray slides were purchased from University Health Network Microarray Center (UHN, Toronto, ON). The 80 μl hybridization solution consisted of 100 μl DIG Easy Hyb solution (Boehringer Mannheim, Lancaster County, PA), 0.05 mg/μl of yeast tRNA (Life Technologies, Rockville, MD) and 0.05 mg/μl of denatured calf thymus DNA (Sigma, ON, Canada). Sixty μl of hybridization solution was added to each pooled pair of Cy3-labeled control and Cy5-labeled experimental cDNA and incubated at 65°C for 2 min. After cooling to room temperature, the cDNA mixture was placed on the microarray slide, covered by a 24 x 50 mm coverslip and

incubated at 37°C for 18 h. The slide was then washed 3 times for 10 minutes each at 50°C in pre-warmed 1 x SSC, then three times for 10 min each in 0.1% SDS and then 4-6 times in 1 x SSC with gentle, occasional agitation. Slides were dried at 500 rpm (Centrifuge 5804R, Eppendorf, Hamburg, Germany) for 5 min and scanned with a GSI Lumonics ScanArray 3000 Biochip Analysis System (G.S.I., Watertown, MA). Scanned images were analyzed using the GenePix software package (version IV, Axon Instruments, Foster City, CA).

We obtained expression profiles for samples harvested at 2, 4, 5, and 6 hours following exposure to the 0.07 mg/ml berberine treatment. Each of these conditions (designated T2, T4, T5, and T6) was sampled from a separate yeast culture, and compared to a control condition at time zero (no treatment) using a two-color cDNA microarray hybridization assay. For each expression profile, total RNA from experimental yeast cells (grown in YPD + berberine) labeled with Cy5 (red color) and total RNA from control yeast cells (grown in YPD) labeled with Cy3 (green color) were used. The large expression data set enabled identification of genes that were up- or down-regulated relative to the control, as well as groups of genes showing parallel expression patterns that might indicate co-regulation in response to berberine.

2.2.4 *Calibration across microarray experiments*

Variation among experiments was monitored using the chlorophyll synthetase gene from *Arabidopsis*. The *Arabidopsis* plasmid, pARAB (0.5 μg/μl), (UHN, Toronto, ON) contained a fragment of the *Arabidopsis* chlorophyll synthetase gene with no homology to yeast sequences. The plasmid insert (~1.4 kb) was identical to the one spotted on the cDNA microarray slide. After purifying plasmid DNA, an *in vitro*

transcription reaction was done according to recommendations (T7 kit, Promega, Madison, WI) and DNA template was removed (Technical Manual No. 016, Promega Corp.). The RNA was then reverse transcribed (Superscript II reverse transcriptase, Life Technologies) and cDNA was separately labeled with Cy3 and Cy5, mixed and used to hybridize to the same slide in each microarray experiment.

2.2.5 *Northern-blotting*

Northern blots were prepared and hybridized with selected genes observed by microarray analyses to be up-regulated (2 genes: *PCL5, ARO3*) or down-regulated (3 genes: *FMP27, PUF4*, YLR162W) in response to berberine treatments. Twelve μg of total RNA from cells exposed to berberine for each of 2, 4, 5 and 6 hr was subjected to electrophoresis on a denaturing 1.3% agarose gel by standard protocols (Sambrook *et al.* 1989), stained with ethidium bromide, photographed over UV light and transferred to Hybond N membranes (Amersham Pharmacia, Buckinghamshire, UK). Ribosomal RNA band intensities were analyzed to ensure relatively even loading across all lanes. PCR using genomic DNA template was used to make a DNA probe for each selected ORF used in northern hybridizations. *S. cerevisiae* genomic DNA was isolated by the bead-beater method (Adams *et al.* 1997) using 0.5 mm glass beads (BioSpec Products, Bartlesville, OK) and STET buffer (8% sucrose in 50 mM Tris pH 8.0, 5% TritonX-100 and 50 mM EDTA). PCR reactions (25 μl total) contained 1 μl of DNA (100 ng/ μl), 2.5 μl PCR buffer (10X), 2.5 μl reverse primer (10 μM), 1 μl forward primer (10 μM), 1.5 mM $MgCl_2$, 0.5 μM dNTPs, 0.5 U/ μl Taq DNA polymerase (Invitrogen Inc., Burlington, ON). PCR forward and reverse (respectively) primer pairs were (5'→3'):

PCL5: TTGGCGGGAGTTCCGACGC and CAGAAATCCGTATCAGACTCCG;

ARO3: TCACCCTCCGTCAGCTGGTC and ACGCCGGTGACAGGAAACGC;

FMP27: TCCGGATGTCCACGTCCTGG and GCCCAGAACAATGACTAGCGC; PUF4: GGCTTACCACCATCATTCTTCA and GATGATGTACCATCAGTAGACC; YLR162W: AGCAACGGTGCTCTTGGCGG and TGCAGCACACGCTTACCCGG. PCR reactions were done in a Biometra T-Gradient thermocycler (Montréal Biotech, Montréal, QC) programmed for 35 cycles of; 30 seconds at 95°C (denaturation), 45 seconds at 63°C (annealing) and 3.5 minutes at 72°C (extension), followed by a final extension of 5 minutes at 72°C. Amplicons were labeled with α^{32}P-dCTP (Amersham Pharmacia) according to standard protocols for use as hybridization probes (Sambrook *et al.* 1989). Hybridizing band intensities were analyzed with the AlphaImage 2200 scanner and software (Alpha Innotech Corp, San Leandro, CA).

2.2.6 *Mutant strains*

The annotated ORF list was obtained from the *S. cerevisiae* genome database (SGD, May 2006, http://genome-www.stanford.edu/Saccharomyces). From our microarray dataset, we selected eight genes that were consistently up- or down-regulated at all exposure times and examined the effects of berberine on strains with mutations in these genes. We obtained three strains with transposon-based insertion mutations (mTn) (Open Biosystems, Huntsville, AL), three Yeast Gene Knockout (YKO) *MATa* haploid deletion mutants (Open Biosystems), and two single haploid deletion mutant strains (Table 2.1). For comparisons, we used BY4741, the strain from which knockout and deletion mutants were obtained, and Y800, from which insertion mutants were derived.

2.2.7 *Berberine uptake experiments*

Yeast cells were grown at 30°C to obtain an OD_{600} of 0.9 (~1 x 10^7 cells/ml). The cultures were exposed to 0.07 mg/ml berberine for 0.5, 1.0, 2, 4 and 6 hrs, harvested by centrifugation, washed twice with sterile YPD sonicated at 115V, 60Hz for 20 minutes (Bransonic Ultrasonic, Danbury, CT) and extracted with methanol. Extracts were filtered and subjected to HPLC analysis using a method adapted for alkamide analysis (Bergeron *et al.* 2000). Using this program the berberine reference standard had a retention time of ~7 min and gave a linear peak area versus concentration response. The method gave baseline separation of the berberine peak in cell extracts and berberine was quantified as the area under the peak using the reference standard (Bauer *et al.* 1988).

2.3 Results

2.3.1 *Microarrays and data normalization*

To examine the sensitivity of *S. cerevisiae* S288C to berberine hydrochloride, yeast cells in the exponential growth phase were exposed to berberine concentrations that ranged from 0 to 6.7 mg/ml. MIC, taken as the berberine concentration that caused ~80% growth reduction over controls without berberine, was found to be ~0.07 mg/ml. This berberine concentration was used in subsequent microarray experiments.

Systematic bias and variation can arise within and among microarrays for a number of reasons, including spot inconsistency, differences in RNA concentration or quality, unequal dye incorporation and fluctuation in scanner strength (Smyth *et al.* 2003; Yang *et al.* 2002). We used widely-accepted methods to identify and remove biases that could affect the measured expression levels, starting with image processing. Each

hybridization produced a pair of 16-bit TIFF (Tagged Information File Format) images. These images were analyzed to measure the relative fluorescence intensities for each spot. We selected median pixel intensity as an indicator of expression level because medians are less affected by outlying pixel values that are often caused by inclusion of background pixels in a digitized spot. We did not subtract background values from these measurements, as experience in the laboratory has consistently indicated that this reduces the correlation among replicate spots. We used the $\log_2$ ratio of median spot intensities as an indication of expression level in a treated sample (T) relative to the control (C). Due to known biases in scanned dye ratios that are dependent on label intensity, we used the LOWESS (Locally Weighted Scatterplot Smoothing) method of ratio centering (Yang *et al.* 2002). In this procedure, intensity-dependent effects are visualized by plotting M *vs.* A, where $M = \log_2 (T/C)$ and $A = \log_2 (T*C)/2$. Since the scatter-plot for all substances revealed trends away from the origin that were dependent on "A" (an estimate of average intensity), a LOWESS curve was fitted for each microarray, and the M value for each point was adjusted toward the origin based on the local regression value. We used LOWESS parameters 0.4 for smoothing and 0.01 for delta, and applied three iterations of the smoothing algorithm. The Arabidopsis chlorophyll synthetase spiking controls confirmed that the LOWESS normalization achieved or maintained a value of M for each spiking controls that was consistently close to the origin (M=0). The result of these corrections is shown in Figure 2.2. To verify the quantitative results of DNA microarrays, we compared microarray data with those of northern blot analyses for a selected set of 5 genes. We chose three genes, YGL014W, YLR454W and YLR162W, that were consistently down-regulated by berberine treatment and two genes, YDR035W and YHR071W that were up-regulated. In all cases, quantitative northern blot analyses

Figure 2.2. Analysis of differential regulation of 6,183 genes in *S. cerevisiae* strain S288C after exposure to berberine hydrochloride for 2, 4, 5 and 6 hours. Median pixel intensities from paired treatment *(T)* and control *(C)* samples are presented as adjusted value of $M = \ln (T/C)$ *vs.* $A = \ln (T*C)/2$, where M is normalized using the LOWESS transformation to remove intensity-dependent bias (Yang *et al.* 2002). Threefold cutoffs (yellow lines) are shown.

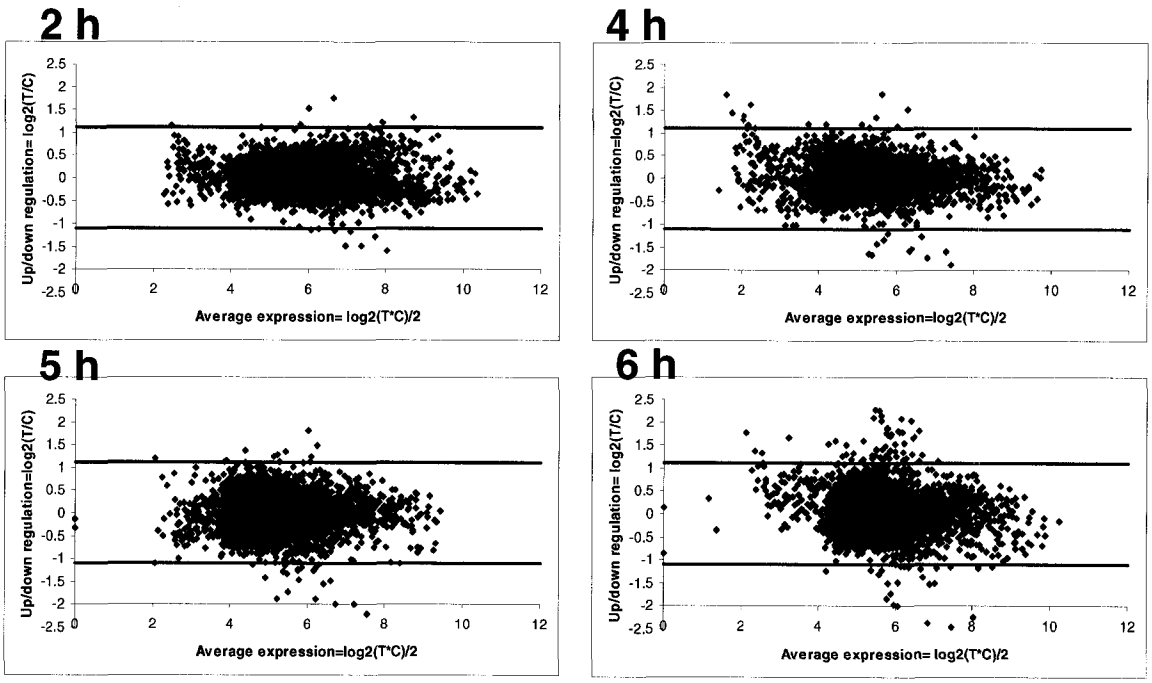
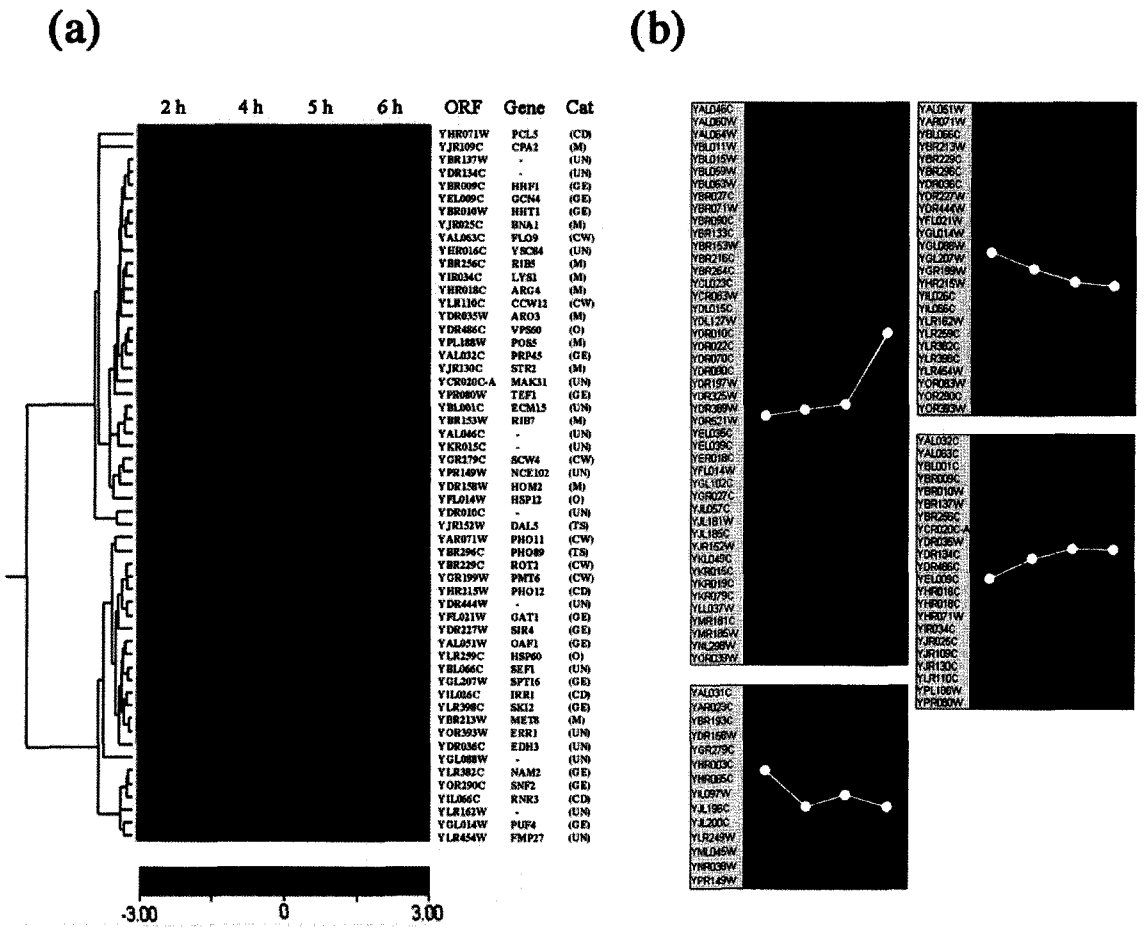


Figure 2.3. Color coded ratios of transcript levels for genes that were up- (red) or down-regulated (green) in yeast samples exposed for 2, 4, 5 or 6 h to 0.07 mg/ml of berberine relative to a control. Data for each gene are given in Appendix **I**. Numbers at top represent berberine exposure times. **(a)** Hierarchical clustering of a set of 55 genes that showed three-fold or greater change in expression at any one time, and two-fold expression change for at least two time points. On the right are ORF identities, gene names and functional categories; UN=Unknown function, TS=Transport/Secretion, CW=Cell Wall, M=Metabolism, O=Other, CD=Cell cycle/Division and GE=Gene Expression. At bottom is color key to expression level change. **(b)** A set of 106 genes that showed 3-fold or greater expression for any time point. Genes in this set are shown in four clusters formed by Self-Organizing Maps (SOM) based on their degree of similarity in gene expression patterns. The line graphs (in white) represent the average gene expression plot for each panel.



of these genes corroborated changes in expression levels as determined by microarray experiments (data not shown) indicating that DNA microarrays provided accurate expression level data in our study.

2.3.2 *Identifying differentially expressed genes and hierarchical analyses*

A preliminary data set was constructed containing all genes with non-missing values in at least three experiments. This set was then restricted to 106 genes that showed a greater than 3-fold up- or down-regulation based on median pixel values in at least one experiment. The selected genes were then further restricted to a set of 55 genes showing consistent 2-fold up- or down-regulation in at least two experiments and/or those that had been tested or validated by other experimental methods such as MIC or HPLC. Expression profiles for each of these 55 genes were generated from yeast treated with berberine for 2, 4, 5 and 6 hours and compared to controls (no berberine profiles) at each time point. The methods of hierarchical clustering and non-hierarchical clustering (Self-Organizing Maps, SOMs) were used to find differential gene expression and coordinated gene expression at the four different time points (Zhang and Zhao, 2000) and to visualize gene expression. The non-centered hierarchical cluster analysis (Figure 2.3a) shows that each of the 55 genes falls into one of two major groups, one that is consistently up-regulated and the other consistently down-regulated. The SOMs analysis shows that the 106 genes identified as highly regulated also form a relatively small number of groups based on patterns of gene expression (Figure 2.3b). Significantly, with few exceptions, all 106 genes had a consistent expression pattern at different exposure times, with variations that tended to differ only in the degree of change. For example, the gene YGL014W exhibited -1.726, -2.157, -2.736 and -3.415-fold changes in experiments T2,

T4, T5 and T6, respectively. This gene appears to be gradually down-regulated with increases in berberine exposure time.

These consistent regulation patterns across multiple exposure times, together with the northern analyses, provide validation of expression changes relative to untreated controls. Although care must be exercised in the interpretation of substances that changed in only one exposure time (Figure 2.3b), the clusters illustrated in both Figures 2.3a and b also provide interesting information on potentially co-regulated groups of genes. For example, one cluster in Figure 2.3a consists of four genes, three of which are of unknown function (YBL001C, YAL046C and YKR015C) and one (YBR153W), which is involved in metabolism. Such clusters may help to predict possible functions for genes with previously unknown functions.

2.3.3 *Analyses of the functions of the genes regulated upon berberine exposure*

The Saccharomyces genome database was used to extract, where possible, the gene functions of the 55 ORFs that were differentially expressed during berberine exposure. Based on this list, the ORFs were assigned to seven functional categories (Figure 2.4) as follows;

Gene expression: YAL032C, YAL051W, YBR009C, YBR010W, YDR227, YEL009C, YFL021W, YGL014W, YGL207W, YLR382C, YLR398C, YOR290C and YPR080W.

Transport and secretion: YBR296C and YJR152W.

Cell wall: YAL063C, YAR071W, YBL001C, YBR229C, YGR199W, YGR279C and YLR110C.

Metabolism: YBR153W, YBR213W, YBR256C, YDR035W, YDR158W, YHR018C, YIR034C, YJR025C, YJR109C, YJR130C and YPL188W.

Cell cycle/division: YHR071W, YHR215W, YIL026C and YIL066C

Other: YDR486C, YFL014W and YLR259C

Unknown: YAL046C, YBL066C, YBR137W, YCR020C-A, YDR010C, YDR036C, YDR134C, YDR444W, YGL088W, YHR016C, YKR015C, YLR162W, YLR454W, YOR393W and YPR149W.

The "other" category includes genes that function in filament formation and heat shock response. Interestingly, the unknown category has the largest number of genes, indicating several genes that responded to berberine have no previously characterized cellular function.

The fact that berberine exposure appears to regulate genes from several distinct functional categories suggests that the compound may have a complex mode of action involving multiple targets. One target of interest appears to be the cell wall. At least three genes that are highly regulated upon berberine exposure are involved in cell wall synthesis, YAL063C (encodes mannose binding protein), YBR229C (encodes alpha-glucosidase) and YGR199W (encodes protein O-mannosyltransferase). Additional regulated genes that were assigned to other functional groups are also likely involved in cell wall function. For example, YDR158W, assigned to the metabolism group, has o-linked glycosylation function that may be involved indirectly in cell wall integrity. Compounds that affect the fungal cell wall are especially interesting since the cell wall is a defining and critical feature of most fungi, and these compounds may offer a high degree of specificity when dealing with pathogenic fungi in animal or plant hosts. Producing multiple antifungals that target different pathways, or, as is apparent in the case of berberine, having an inhibitor with multiple cellular targets, may be two ways whereby plants avoid pathogen resistance mechanisms. Presumably, it would be difficult

Figure 2.4. Functional categories of the 55 genes identified by microarray analysis to be up- or down-regulated upon exposure to berberine. Functional category abbreviations given in parentheses are cross-listed in Figure 2.3a.

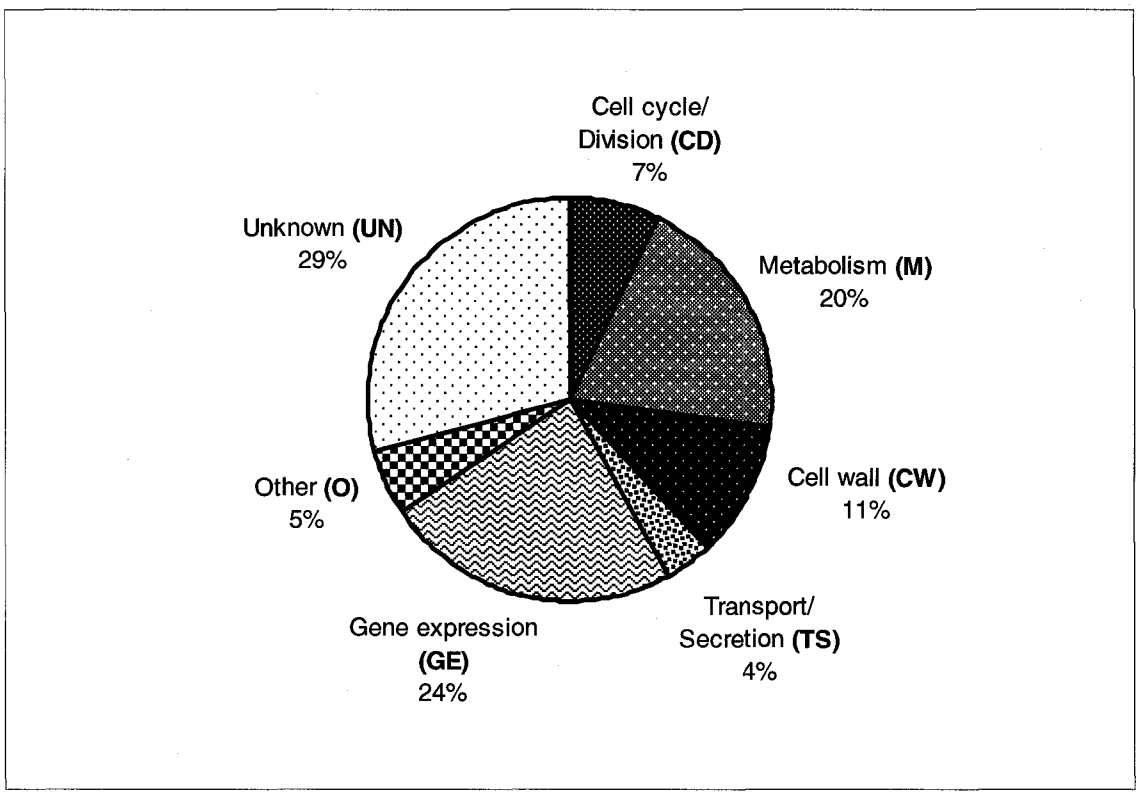
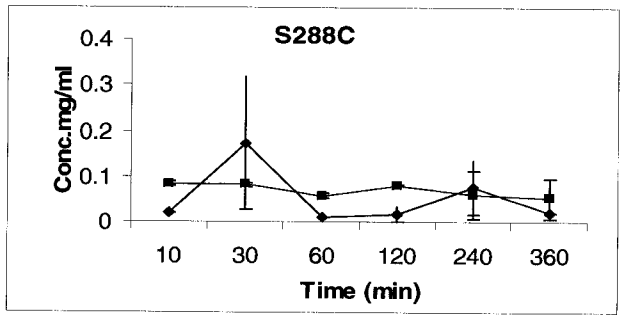


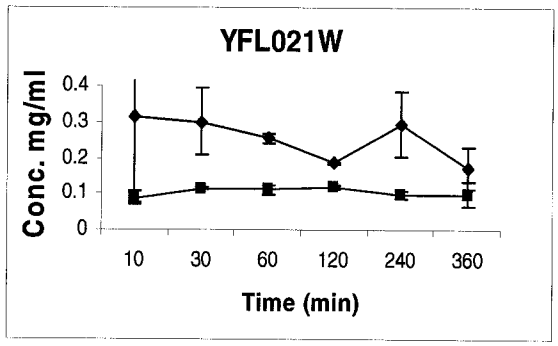
Table 2.2. Susceptibilities of *S. cerevisiae* strains to berberine.

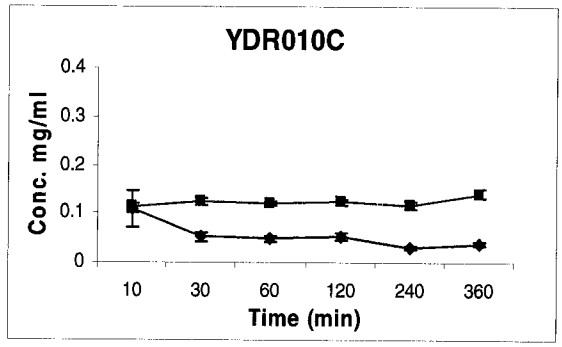
Yeast Strain	Progenitor strain	Description of mutation	Regulation pattern	MIC±SD (mg/ml) (n≥4)
BY4741	BY4741	wt	-	0.42 ± 0.11
YDR035W[a]	"	ARO3	Up-	0.41 ± 0.20
YLR454W[a]	"	FMP27	Down-	0.27 ± 0.10*
YGL014W[a]	"	PUF4	Down-	0.26 ± 0.10*
YDR010C[b]	"	Hypothetical ORF	Up-	0.17 ± 0.04*
YFL021W[b]	"	GAT1	Down-	0.05 ± 0.03*
Y800	Y800	wt	-	0.24 ± 0.07
YLR162W[c]	"	Hypothetical ORF	Down-	0.40 ± 0.20
YHR071W[c]	"	PLC5	Up-	0.40 ± 0.20
YPR080W[c]	"	TEF1	No change	0.35 ± 0.20

[a] Yeast knockout strains.
[b] Yeast deletion strain.
[c] Yeast insertional mutant strains.
* Significantly different MIC from progenitor strain ($P<0.05$, 2-tailed T-test).

Figure 2.5. HPLC-based measurements of berberine concentration (mg extract /ml) inside (closed diamonds) and outside (open squares) of treated *S. cerevisiae* wildtype S288C and deletion mutant strains, YDR010C and YFL021W, at various exposure times. Means and standard deviation bars are shown for each data point based on three independent trials.







to overcome multi-target inhibitors with a simple mutation and the likelihood of developing resistance may thus be reduced.

2.3.4 *MIC of yeast gene knock-out and insertional mutant strains*

If the genes that are strongly regulated by exposure to berberine are involved in the mechanism by which berberine affects the cells, then it might be expected that mutation in these genes would affect the sensitivity of the cells to berberine. To test this hypothesis, we randomly selected eight strains with mutations in genes that had been identified as significantly up- or down-regulated in microarray assays. MICs of the *S. cerevisiae* mutants used were compared to that of their progenitor wild-type strain, BY4741 or Y800, as indicated in Table 2.2. We found that four of the eight mutant strains tested, YLR454W (encodes *FMP27*), YGL014W (encodes *PUF4*), YDR010C (unknown function) and YFL021W (encodes *GAT1*), had relatively high sensitivities to berberine compared to the respective progenitor wild-type strain, indicating potential target specificities to berberine. The remaining four mutants did not significantly differ in MIC from their respective progenitor strain.

2.3.5 *Berberine concentration inside/outside of wildtype and mutant strains*

We compared uptake and efflux of berberine in wildtype S288C and two mutant strains, YDR010C (up-regulated) and YFL021W (down-regulated). These two mutants were found to be the most susceptible mutant strains tested by MIC assays. Mid log-phase yeast cells were exposed to 0.07 mg/ml berberine and the concentration of berberine inside and outside of *S. cerevisiae* cells was measured by HPLC at each of 0, 0.5, 1, 2, 4 and 6 hours (Figure 2.5). Uptake of berberine was evident in cultures of wildtype S288C within 30 minutes after addition of berberine. Previous work with

Staphylococcus aureus indicated that rapid uptake of berberine into bacterial cells is driven by membrane potential (Stermitz *et al.* 2000). At ~1 hour exposure, a drop in intracellular berberine was observed with S288C, to levels that are below extracellular berberine concentrations. Lower concentrations of berberine in yeast cells after ~1 hour exposure times may be associated with increased activity by multidrug resistance pumps (MDRs). It was demonstrated in other studies that either synthetic hydrophobic cations such as quaternary ammonium antiseptics (Hsieh *et al.* 1998) or natural substrates such as cationic berberine alkaloids (Lewis 1999) are preferred substrates for MDR pumps. Berberine and its derivatives are also regarded as substrates for the P-glycoprotein multidrug resistance pump, which is a member of the ABC family that is responsible for multidrug resistance by some human tumors (Yang *et al.* 2003; Gros *et al.* 1992). A study on a human intestinal epithelial cell line indicated that berberine can up-regulate the expression of P-glycoprotein pumps that are associated with a number of MDR homologous in *S. cerevisiae* (Yang *et al.* 2003). In our study, up-regulation of three transporter genes YKR104W (encodes *NFT1*), YAL026C (encodes *DRS2*) (both moderately up-regulated, data not shown) and YJR152W (encodes *DAL5*) may explain the decrease in intracellular berberine concentrations observed in S288C at ~1 hour. Notably, in *S. cerevisiae* the *DAL5* gene encodes a hydrophobic protein similar to P-glycoprotein that mediates multidrug resistance in mammalian cells (Chen *et al.* 1986). Therefore, efflux pumps may be transcriptionally up-regulated to help fungi adapt to berberine (Amin *et al.* 1969).

As shown in Figure 2.5, the uptake/efflux pattern of berberine is very different in the two mutant strains when compared to S288C. While YDR010C is up-regulated upon exposure to berberine, and a strain with this gene mutated is significantly more sensitive

to berberine, intracellular berberine concentrations are consistently below those of the medium for all measurements after 10 minutes. The absence of an initial berberine influx in YDR010C may therefore indicate a role for this gene of "unknown function" in establishing or maintaining membrane potential. Notably, in Figure 2.3a, YDR010C and *DAL5* are co-regulated based on cluster analysis and this also suggests that YDR010C may be involved in transport and secretion. In contrast, whereas YFL021W is significantly down-regulated at all berberine exposure time points based on microarrays, and the strain that is mutant for this gene has increased sensitivity to berberine, this mutant has consistently higher intracellular concentrations compared to berberine levels in the medium. YFL021W encodes *GAT1*, a member of the GATA family of DNA binding proteins and a positive regulator of transcription. Therefore it is possible that the down regulation of *GAT1* in the presence of berberine may reduce the expression of gene(s) involved in drug efflux, in turn leading to higher intracellular berberine concentrations and increased yeast sensitivity.

2.3.6 *Comparison of gene regulation of other antifungals and berberine*

Finally, we compared our results to similar microarray data showing differential expression caused by the antifungals, ketoconazole, amphotericin B, caspofungin and 5-fluorocytosine (Table 2.3). Expression levels of most of the selected genes were not significantly differentially regulated in response to berberine. At the two hour exposure time only three genes out of 20 genes, which are significantly regulated during exposure to other inhibitors, had a 2-fold or greater change in expression level. These genes include *ELO1* (down-regulated with ketoconazole, up-regulated with berberine), *RNR3* (up-regulated with amphotericin B and 5-FC, down-regulated with berberine) and *HSP12*

Table 2.3. Comparison of berberine-induced regulatory changes of *S. cerevisiae* genes with significantly altered transcript levels with exposure to other antifungals.

		Gene Expression Responses after 2 hours				
Function ORF	**GENE**	**Ketoconazol[1]**	**Amphotericin B[1]**	**Caspofungin[1]**	**5-fluorocytosine[1]**	**berberine[2]**
Cell wall maintenance						
YHL028W	*WSC4*	-2.2	-3.5	-4.9	-2.8	1.4
Transport						
YKR039W	*GAP1*	-2.2	-3.7	-2.4	-16	-1.1
YNL142W	*MEP2*	-2.6	-2.2	-3.5	-34.1	-1.3
YBR068C	*BAP2*	nt	4.1	3.9	5	1.2
YDR046C	*BAP3*	nt	8.3	4.5	7	1.0
Lipid, fatty acid and sterol metabolism						
YJL153C	*INO1*	-2.1	2.3	2.9	-3.7	1.1
YML008C	*ERG6*	2.4	nt	nt	nt	1.1
YJL196C	***ELO1***	-3.9	nt	nt	nt	**6.8**
Unknown						
YLR267W	*BOP2*	nt	8.2	3.1	3.5	-1.2
YKR091W	*SRL3*	3.1	3.5	9.5	2	1.3
Transcription						
YFL021W	***GAT1***	nt	nt	nt	-2.3	**-4.1***
Nucleotide metabolism						
YIL066C	***RNR3***	nt	2.8	nt	47.1	**-4.9**
Cell cycle control						
YJL157C	*FAR1*	nt	-2.9	-4	-2.3	1.1
Cell stress						
YFL014W	***HSP12***	nt	38.2	18.3	3.2	**3.4**
YOL053C-A	*DDR2*	nt	9.2	2.8	3.3	1.6
YPL223C	*GRE1*	nt	93.9	15	9.2	-1.3
Protein degradation						
YLR121C	*YPS3*	nt	4.1	8.9	2.2	1.9
Mating response						
YML047C	*PRM6*	nt	-2.6	-7.4	-3.6	-1.1

[1] Values obtained from (59) Agarwal *et al.* 2003; [2] Values obtained in this study from microarray analysis after 2 (or 4 where indicated by *) hours exposure to berberine; nt = not tested.

Table 2.4. Transcript levels of transporter genes and genes involving in cytochrome P450 enzyme activity in *S. cerevisiae* exposed to berberine for 2, 4, 5 and 6 hours.

ORF	Gene product	2 hrs	4 hrs	5 hrs	6 hrs	Molecular function*
YOR153W	*PDR5*	0.202	0.325	0.137	-1.173	Xenobiotic-transport ATPase activity
YDR011W	*SNQ2*	0.307	0.075	0.102	-0.619	Xenobiotic-transport ATPase activity
YOR328W	*PDR10*	0.417	0.515	-0.75	-0.54	ABC transporter activity
YIL013C	*PDR11*	0.235	0.264	0.383	-0.121	ABC transporter activity
YDR406W	*PDR15*	0.547	0.189	0.475	-0.303	ABC transporter activity
YPL058C	*PDR12*	-0.36	0.125	0.216	-0.25	Xenobiotic-transport ATPase activity
YGR281W	*YOR1*	0.637	0.316	0.062	-0,634	Xenobiotic-transport ATPase activity
YOR011W	*AUS1*	0.097	0.117	0.337	-0.284	ABC transporter activity
YNR070W	YNR070W	0.189	0.146	0.062	-0.508	ABC transporter activity
YCR011C	*ADP1*	-0.355	0.109	0.047	-0.295	ABC transporter activity
YJR152W	*DAL5*	0.145	0.28	0.566	1.727	Allantoate transporter activity
YDR402C	*DIT1/CYP56*	0.681	0.059	1.054	-0.346	Catalytic activity
YMR015C	*ERG5/CYP61*	0.886	0.245	0.597	-0.024	Cytochrome P450 involved in C-22 sterol desaturase activity
YLR216C	*CPR6/CYP40*	0.319	0.042	0.047	-0.122	Chaperone activity
YGR234W	*YHB1/CYP55*	1.455	0.154	0.003	-0.012	Flavohemoglobin
YHR007C	*ERG11/CYP51*	0.163	0.455	0.916	0.129	Cytochrome P450 lanosterol 14a-demethylase
YDR155C	*CPR1/CYP1*	0.034	0.302	0.169	0.169	Peptidyl-prolyl cis-trans isomerase activity
YHR057C	*CPR2/CYP2*	0.377	0.166	0.36	-0.026	Peptidyl-prolyl cis-trans isomerase activity

* Based on Saccharomyces Genome Database (SGD).

(up-regulated after exposure to amphotericin B, Caspofungin, 5-fluorocytosine and berberine). However, if we take into account results obtained for longer berberine exposure times, we find another gene that also shows comparable changes in expression levels (boldface in Table 2.3). The gene *GAT1* (down-regulated with both 5-fluorocytosine and berberine) shows a greater than two fold change in expression level after 4 hours of exposure to berberine. This comparative analysis suggests that yeast responds in a specific manner to berberine and/or that different pathways are involved in berberine activity as compared to the other inhibitors. The differential regulation by genes *ELO1, GAT1, RNR3* and *HSP12* suggests that these four genes may have more general functions in stress response, inhibitor sensitivity and/or resistance.

2.4 Discussion

We investigated the use of DNA microarrays to identify genes in *S. cerevisiae* that are transcriptionally regulated in response to berberine. The rationale for this approach is that the identification of genes that have altered transcript levels may provide insights into the target proteins and/or pathways associated with berberine toxicity and into the defensive response of yeast to this plant derived toxin. It is assumed that yeast cells will specifically modify the transcription levels of some genes in response to deficiencies in metabolic processes brought about by berberine toxicity. This approach is applicable to any compound that inhibits the growth of fungi and may provide a way to study therapeutic targets and molecular mechanisms of antifungal activity. We chose to do microarray analyses on yeast that had been exposed to berberine for 2, 4, 5 and 6 hours to assess the change in transcript profiles over short time exposures. Relatively few cell divisions would occur during our experiments given that the yeast doubling time

of ~90 minutes is lengthened in the presence of MIC levels of berberine. The analysis of multiple exposure times provided a form of replication to the study while also giving information about transcript level changes through time.

From the microarray analyses, 55 genes were identified having at least a three-fold difference in one experiment and consistent two-fold up- or down-regulation in transcript level for at least two experiments compared to no-berberine controls. The observed genes and their related functions (Figure 2.3a) are of considerable interest since they demonstrate that the mechanism(s) of berberine action is complex and varied. As shown in Figure 2.4, genes involved in at least seven general cellular functions appear to respond to berberine exposure. This may be significant since a plant-derived inhibitor that targets multiple pathways would likely be difficult to overcome by resistance mechanisms, unlike the situation with the azoles where a single gene mutation in the ergosterol biosynthetic pathway can result in resistance (Alexander *et al.* 1997; Anderson 2005; Prasad *et al.* 2005; Cowen *et al.* 2002). That berberine may target multiple biochemical pathways could also explain its multiple pharmacological activities that include antiprotozoal (Bova *et al.* 1992), antibacterial (Amin *et al.* 1969), anti-inflammatory (Tsai *et al.* 1991), and antiarrhythmic activities (Wang *et al.* 1997).

Of particular interest, there is some evidence that berberine may interfere with fungal cell wall functions. The cell wall is essential for survival for most fungi and it is an attractive target for antifungal agents since it is a defining feature of fungi. As with other fungi, the *S. cerevisiae* cell wall consists mainly of the polysaccharides glucose, mannose and N-acetylglucosamine (Cabib *et al.* 1989). The mannose polysaccharides are linked to proteins to form a layer of mannoprotein at the external surface of the cell wall

and act as a filter for large molecules (Cabib *et al.* 2001). We are currently testing whether berberine-treated fungi are compromised for cell wall function.

Using analysis methods of hierarchical clustering and self-organizing maps, we identified groups of genes expressed in a coordinated manner across exposure times. With hierarchical clustering (Figure 2.3a) we found two different expression patterns: consistently down-regulated and consistently up-regulated. SOM analysis supports these general trends (Figure 2.3b). Although we present only hierarchical clustering and SOM results, the k-means method showed similar trends and can also be used to prioritize genes within clusters for further biological studies of the function of these genes.

We examined the microarray data set for evidence of responses by transporter genes to berberine (Table 2.4). It was previously shown that there is a correlation between drug uptake by the strain and its sensitivity to that specific drug (Vazquez 1964). For example, Egner *et al.* (1998) showed that, in comparison to wild-type strains, *S. cerevisiae* strains with deficiencies in transporter genes were more sensitive to growth inhibitory substances. In *S. cerevisiae, PDR5,* the cell-surface ABC-like transporter has been linked to cycloheximide and mycotoxin resistance, and has also been found to transport steroid compounds and many structurally and functionally unrelated compounds through an ATP-dependent drug efflux mechanism (Emerson *et al.* 2004; Wolfger *et al.* 2001; Bauer *et al.* 1999). Also, in the filamentous fungus *Aspergillus nidulans,* four ABC transporters, *atrA, B, C* and *D* have been characterized (Wolfger *et al.* 2001; Andrade *et al.* 2000a; Andrade *et al.* 2000b). The two transporters encoded by *atrA* and *atrB* are similar to yeast Pdr5p and are also over-expressed upon treatment with azoles and plant toxins. Interestingly, deletion of *atrD* resulted in increased sensitivity to cycloheximide, while deletion of *atrC* did not show any change in cycloheximide

sensitivity. On the other hand, loss of *atrB* function seems to increase sensitivity to a number of substances (such as azoles), while its over-expression renders cells resistant to azoles and decreases cellular accumulation of radio-labelled fungicide, fenarimol. In the present study, we observed PDR (Pleiotropic Drug Resistance) genes were repressed by berberine (Table 2.4). The PDR family is the largest group in ABC drug efflux pumps, encompassing a number of P-glycoprotein homologs (Taglight & Michaelis 1998), and is directly associated with modulation of resistance to xenobiotics in *S. cerevisiae* (Bauer *et al.* 1999). Furthermore, we observed a significant reduction in cytochrome P450 enzyme activity in cells following berberine treatment. Genes repressed in this category included *ERG11, ERG5, YHB1, DIT1, CPR1* and *CPR2*. The cytochrome P450 (CYP) genes are involved in metabolism of xenobiotics such as drugs, plant toxins and endogenous compounds such as steroids (Scott 1999). The observed repression of PDR genes and CYP enzymes in cells exposed to berberine suggests that not only is berberine not effectively recognized as a xenobiotic by cells (Maeng *et al.*2002; Bauer *et al.* 1999), but it may also influence the cells ability to detoxify and transport xenobiotics out of the cell. On the other hand, the induction of the allantoate transporter, *DAL5*, observed in this study (Table 2.3) suggests that this transporter may have substrate specificity for berberine. Allantoate is the end product of purine metabolism in mammals and some fishes, and is widely distributed in plants as an important source of stored nitrogen (Piedras *et al.*, 1998). In previous studies it was observed that berberine inhibits uptake of purines (Modak *et al.* 1970). Berberine may act as a substrate for *DAL5* and thus may interfere with purine uptake by cells. In addition, as discussed earlier, the *DAL5* gene in *S. cerevisiae* encodes a hydrophobic protein similar to the P-glycoprotein that mediates multidrug resistance in mammalian cells (Chen *et al.* 1986).

To validate potential berberine targets, we assessed MICs and uptake/efflux of berberine in a subset of strains that had mutations in genes with significantly altered transcript levels in our microarray studies. Each mutant strain was defective in a gene that was observed to be significantly up- or down-regulated in response to berberine exposure. It was determined that mutants for four of the eight genes examined had significantly increased sensitivity to berberine based on MIC in comparison to progenitor strains. This indicates that a subset of the regulated genes we uncovered are involved in ameliorating berberine toxicity. HPLC methods were used to investigate the relationship between intracellular and extracellular berberine levels with increasing exposure. The characteristic pattern of uptake/efflux of berberine over time for wildtype S288C was significantly altered in the two mutants tested. The YDR010C insertion mutant did not exhibit the uptake/efflux response within the first 30 minutes of exposure that was evident for the wildtype S288C. Of interest, YDR010C is of unknown function but clusters in our analysis together with *DAL5*, which is involved in transport. The combined evidence of up-regulation of YDR010C, loss of berberine uptake/efflux in cells when this gene is mutated and co-regulation with *DAL5,* suggests that this gene encodes a protein that is involved in transport. The greater berberine sensitivity of the strain mutated at YDR010C is consistent with over-expression of this gene upon treatment with berberine. The low relative intracellular concentrations observed in this mutant may be the result of leakage of the intracellular pool of the organism after its exposure to berberine. This effect may be similar to that of the polyene antibiotic amphotericin B, which is thought to induce permeability changes in the cell membrane in fungi, causing leakage of intracellular molecules (Alexander *et al.* 1997). The leakage of the intracellular pool may depend on adsorption of the drug by the cell membrane of the

sensitive strain. It is clear, however, that berberine has a different target(s) than azoles since Ficker *et al.* (2003) showed that berberine exhibited antifungal activities against several clinical azole- and amphotericin B-resistant fungi.

In a different response pattern from that of the YDR010C mutant, the YFL021W mutant takes up berberine rapidly at the initial time of exposure and appears to be unable to pump it out, resulting in an intracellular accumulation of berberine. Apparently, a functional YFL021W (encodes *GAT1*, a transcriptional activator) is important for berberine resistance, possibly through either drug efflux or cell membrane permeability alteration, two major factors that represent important molecular mechanisms of antifungal resistance. In support of the former possibility, transcriptional activation of efflux pump-encoding genes in *A. nidulans* and *Cryptococcus neoformans* is often accompanied by reduced intracellular accumulation of drugs (Posteraro *et al.* 2003; Semighini *et al.* 2002). As well, in *C. albicans*, over-expression of genes encoding ABC transporters and MFS (major facilitator) proteins is associated with reduced intracellular accumulation (Prasad *et al.* 2005). However, in the case of *GAT1*, reduced transcription (via knockout) apparently increases intracellular concentrations of berberine. The gene *GAT1* is a member of GATA family whose expression is regulated by *SSY1* a component of yeast plasma membrane SPS nutrient sensor (Forsberg *et al.* 2001). *GAT1* encodes a transcriptional activator that is involved in nitrogen regulation and its activity is regulated by the quality of environmental nitrogen (Forsberg *et al.* 2001). In a previous study, it was demonstrated that the treatment of *S. cerevisiae* cells with the immunosuppressive drug rapamycin results in alteration of many genes in nutrient pathways (Saxena *et al.* 2003). It is also known that Gat1p regulates some genes such as the transporter gene *GAP1* (general amino acid permease) and *GLN1* (glutamine synthetase) (Stanbrough *et*

al. 1995) in addition to *CAR1*, *ASP3*, *PUT1* and *PUT2* (enzymes involved in amino acid degradation) (Oliveira *et al.* 2003; Saxena *et al.* 2003). The observed down regulation of *GAT1* after berberine treatment suggests that the uptake of certain essential cell metabolites might be inhibited by berberine and this could affect various cellular biosynthetic pathways

Significantly, we obtained leads on mutants with different uptake/efflux patterns and berberine sensitivities through microarray analysis. This shows that the microarray data can be a reliable predictor of genes that effect berberine sensitivity. The relationship between sensitivity (MIC differences) and transcriptional regulation of genes identified by microarray analysis suggests that the physiological functions of many of the 55 genes are associated with a berberine-specific response. In support of a berberine-specific response, a comparison between genes that have altered transcript levels in response to four different antifungals and berberine showed that the transcriptional response to berberine in yeast is very distinct (Table 2.3). Nonetheless, four genes, *ELO1*, *GAT1*, *RNR3* and *HSP12* displayed similar expression patterns regulated in response to berberine and to some of the other antifungals. Of interest here, *S. cerevisiae* has two genes that encode distinct forms of the ribonucleotide reductase large subunit, *RNR1* and *RNR3*. Ribonucleotide reductase converts NDPs into dNDPs and is thus essential for DNA synthesis and repair. *RNR1* is thought to function mainly during DNA replication and is under cell cycle regulation whereas up-regulation of *RNR3* is a key response to DNA damage (Yao *et al.* 2003). Because ribonucleotide reductases are essential for the survival of all living organisms, the enzyme is a potential target for anticancer, antibacterial and antiviral drug development. For instance, hydroxyurea, an anticancer drug, blocks DNA replication by inhibition of the ribonuclease reductase activity

(Lubelsky *et al.* 2005). The down-regulation of *RNR3* in our study suggests that berberine may arrest DNA repair processes in yeast cells, although, to our knowledge, increased mutation frequencies associated with berberine exposure have not been evaluated. These commonly regulated genes may also be associated with adaptive or non-adaptive stress responses by yeast to xenobiotics. However, to date, the exact mode of action of stress-induced genes and the relevance of variations in their expression levels are not fully understood (Wolfger *et al.* 2004; Alarco *et al.* 1999).

This study enhances our understanding of the relationship between yeast gene expression changes and both antifungal sensitivity and uptake/efflux characteristics. Our data indicates that DNA microarray is useful in monitoring the expression of large numbers of genes in response to new pharmaceutical compounds. The microarray studies reported here support the view that the mechanism(s) of action of berberine is complex and that multiple biochemical pathways and cellular functions are affected by berberine treatment. These multiple targets may be significant for berberine to overcome fungal resistance mechanisms or delay onset of resistance by selection, unlike the situation with certain antifungals, such as azoles. The reduction of cytochrome P450 enzyme activity and repression of PDR transporter genes also suggests that berberine is not effectively recognized as a xenobiotic by yeast. Such characteristics may encourage complementary and alternative antifungal therapies based on berberine-containing phytomedicines.

CHAPTER 3

Use of Gene Deletion Array to Identify Mode of Action of Berberine in *S. cerevisiae.*

3.1 Introduction

Berberine is a medically important isoquinoline alkaloid compound produced by plant species in the *Berberidaceae* (Simeon *et al.* 1989; Manske *et al.* 1953). Berberine has a long history in herbal medicine and has been shown to possess significant antimicrobial activity against different organisms, including fungi (Ficker *et al.* 2003; Mahady *et al.* 2001), and is relatively nontoxic to humans (Rabbani *et al.* 1987).

Here, we investigate the activity of berberine by performing susceptibility tests with an array of yeast gene deletion mutants, and compare these results to the DNA microarray analysis that was covered in Chapter 2. In our previous microarray study, we hypothesized that genes that are transcriptionally regulated would provide insight into berberine's mode of action and help identify possible resistance mechanisms by yeast to the antifungal. The results revealed significant differential RNA transcript levels for ~106 genes which were assigned to seven categories including cell cycle/division, metabolism, cell wall, gene expression, transport/secretion and unknown functions. Furthermore, based on this transcriptional response, we hypothesized that berberine is an effective antifungal because, at least in part, it is not apparently recognized nor degraded as a xenobiotic. It was previously shown that transcriptional differences are not only responsible for phenotypic differences but they are also indicative of cellular interactions of the compound and its corresponding gene products (Baetz *et al.* 2004). Indeed, increased sensitivity was noted in MIC tests with four of seven selected strains that had berberine response genes deleted (Table 2.2, Chapter 2). However, a systematic analysis of susceptibility to berberine has not been carried out with the comprehensive set of *S. cerevisiae* deletion strains.

Here, we develop a chemical-genetic profile for the antifungal berberine in order to identify possible target pathways. We used ~4600 *S. cerevisiae* strains, each with a nonessential gene deleted, called a Gene Deletion Array (GDA). This technology holds promise as a powerful and versatile tool for discovery of the target(s), or mode of action, of antifungal drugs and to study the pathways affected by them at a genome-wide level. The concept is based on quantitative phenotypic sensitivity profiles of strains with two target genes inactivated. However, in this case the first gene is disabled by a deletion mutation and the second gene, or gene product, is deactivated by the use of the compound under investigation. If the combination results in a significantly altered growth rate, measured through colony size, we predict that the chemical target is in a parallel pathway to the first mutation. Such studies can lead to the determination of attractive candidates for drug development. In particular, inhibited growth, resulting from perturbations in specific biochemical pathways, provide clues for regulatory mechanisms and broader cellular functions of the studied compounds. Also, in the context of treating fungal infections, knowledge of this kind of *in vitro* study can help us to determine how antifungals and antifungal candidates work *in vivo* and which gene products might be suitable targets for further therapeutic intervention.

Using gene deletion arrays in the present study, we identify a set of sensitive mutants and carry out a bioinformatics analysis of the deleted gene functions. We also compare minimal inhibitory concentration (MIC) values for some of the sensitive mutants and examine their berberine intracellular accumulations in yeast cells by HPLC methods. Finally, we compare the genes associated with sensitivity in these GDA experiments to those identified in Chapter 2 that were significantly differentially regulated upon berberine exposure. Our findings from these two studies suggest that

there is a weak relationship between a differentially regulated gene and the impact of the loss of that specific gene on the sensitivity of the cell to berberine.

3.2 Methods & Materials

3.2.1 *Yeast strains and media*

The wildtype strain YSec4 derived from *S. cerevisiae* S288C (*MATα SUC2 mal 2mel gal2 CUP1 flo1 flo8-1 hap1)* was used in this study. The mutant yeast strains used for MIC and HPLC experiments are listed in Table 3.1. In addition, YNL108C was included in these experiments to represent a mutant strain from the array set that was not more or less sensitive to berberine than wildtype strains (Table 3.2). Standard procedures for yeast media preparation were followed as described in Chapter 2. Berberine chloride was obtained from E. Merck (99%, batch# 119H0687, Darmstadt, Germany). Berberine solutions were prepared using warm distilled water, and subsequent dilutions were made in Yeast Peptone Dextrose medium (YPD), with or without 1.7% bacto-agar (Difco), as appropriate.

3.2.2 *MIC Determination*

A single colony of *S. cerevisiae* was grown overnight to mid-log in YPD at 30°C with shaking at 200 rpm. The culture was diluted to obtain ~1000 CFU/ml (OD_{600} of ~0.9, Spectra Max 340PC, Molecular Devices, Sunnyvale CA) in YPD broth, and distributed at 100 µl/well into disposable 96-well microtiter plates (Costar, round bottom, Broadway, MA). Minimum inhibitory concentration (MIC) assays were prepared as described in Chapter 2. All quantitative MIC experiments and sensitivity assays were

Table 3.1. Deletion mutants identified as most sensitive to berberine.

ORF	Gene	Biological process	Molecular Function
YML008C	*ERG6*	ergosterol biosynthesis	sterol 24-C-methyltransferase activity
YMR166C	YMR166C	transport	transporter activity
YLR320W	*MMS22*	double-strand break repair	unknown
YGL013C	*PDR1*	regulation of transcription from Pol II promoter in response to drug	DNA binding,transcriptional activator activity
YGR281W	*YOR1*	involved in export of proteins, RNAs, and ribosomal subunits from the nucleus	xenobiotic-transporting ATPase activity
YBR195C	*MSI1*	DNA repair	transcription regulator activity
YJL181W	YJL181W	Unknown	Unknown
YIL097W	*FYV10*	negative regulation of gluconeogenesis	Unknown
YBL042C	*FUI1*	uridine transport	uridine transporter activity
YDR197W	*CBS2*	protein biosynthesis	Unknown
YPR191W	*QCR2*	aerobic respiration, mitochondrial inner membrane electron transport	ubiquinol-cytochrome-c reductase activity
YDR010C	YDR010C	Unknown	Unknown
YFL021W	*GAT1*	transcription initiation from RNA polymerase II promoter	transcriptional activator activity

done independently for each strain under two conditions: following UV light irradiation (10 W/m^2 for two hours using three 20 W black-light blue tubes, 320-400 nm range) and without UV irradiation (dark treatment), for which plates were wrapped in aluminum foil. The MIC value is taken in this study as the concentration that produced 80% growth inhibition in the presence of berberine in comparison to drug free controls. Berberine sensitivity for each strain was scored visually and through optical density readings after incubation for 48 hours at 30°C.

3.2.3 *Gene Deletion Array (GDA) experiment*

A set of ~4700 viable haploid *S. cerevisiae* gene-deletion mutants were kindly provided through Dr. Golshani (Carleton University). The mutants were generated in the background strain BY4741, a derivative of S288C (Tong *et al.* 2001). The GDA strains were transferred by hand with a 384-floating pin replicator (16 X 24, VP384F) into a control plate (no berberine) and an experimental plate (with berberine) (Omni Tray, Nalge Nunc International, Rochester, NY, USA). The replicator was sterilized between transfers by agitating for 30 seconds in 10% bleach and washing with sterile water for 1 min, followed by 95% ethanol exposure for 1 min and drying with warm air for 30 seconds. The control plate contained 50 ml of liquid YPD. The experimental plates contained 50 ml YPD agar medium with sub-MIC levels of berberine (~0.05 mg/ml). The plates were incubated for 1-2 days at 30 °C in the dark and digitally photographed with a Hewlett Packard PhotoSmart 735 digital camera. The growth rate of each mutant strain was then estimated by determining colony size on both the control plate (no drug) and the experimental plates with the computerized image system called Growth Detector (GD) (Memarian *et al.* in press). The GD system calculated the difference in colony area

in both control and experimental plates. For both control and experimental plates the size of each colony was compared to the average colony size for all colonies on the plate. The normalized difference in colony size for each strain was then saved to a Microsoft Excel spread sheet. A set of the 195 (5% of total mutants examined) of the most inhibited mutants were identified and verified by visual inspection of plate images.

3.2.4 *Sample preparation for berberine uptake using HPLC*

S. cerevisiae wildtype strain S288C and experimental mutant strains were grown overnight at 30°C with shaking at 200 rpm to an OD_{600} of ~0.9. After addition of berberine to an initial concentration of 0.07 mg/ml, cells were harvested by centrifugation at 10 and 30 min, and at 1, 2, 4 and 6 hours. The supernatant was removed and saved, and cells were washed twice with sterile YPD followed by centrifugation. Pelleted cells and supernatant were separately frozen at -80°C until analyzed. The experiments were done in triplicate for each exposure time and curves shown are averages of three experiments with error bars representing 1 SD. Berberine uptake was evaluated for both intercellular (using supernatant) and intracellular (using pellet) by HPLC assays. Prior to breaking open the cells, the cell pellets as mentioned above, were resuspended in 80% methanol. Berberine was extracted from cells using two 15 minute bath sonication steps (Branson 5510 Ultrasonic Corp, Danbury, CT) followed by centrifugation at 13,000 rpm for 10 minutes at room temperature. Supernatant from both extractions were recovered and pooled and the volume adjusted to 2.5 ml with 80% methanol. Samples were filtered through 0.2 μm PTFE membranes (Chromatographic Specialities, Brockville, Canada) before injection of 5 μl into HPLC column.

The HPLC was performed on an Agilent Technologies Inc. system (Palo Alto, CA, USA) composed by Degasser G1322A, Quat Pump G1311A, Autosampler G1313A and DAD G1315A. Separations were performed with a YMC ODS-AM S-3 120°A, 5 µl, 2.0 x 100 mm column (Waters Inc., Milford, MA, USA). The mobile phases were (A) acetonitrile and (B) 0.05 % trifluroacetic acid (TFA) in water, at a flow rate of 0.3 ml/min and a column temperature of 50°C. The gradient was 15 – 70 % A in 12 min; 70 – 15 % A in 2.5 min; hold 15 % A for 0.5 min and a post time of 1 min. Five µl of each sample was injected and eluting compounds were detected at 350 nm. The berberine retention time was verified by comparison with a reference standard and quantified on the basis of the area under the peak (Bauer *et al.* 1988). To evaluate whether recoveries were linear using this method, known amounts of purified berberine standard were included among of samples. The reproducibility of the applied method was assessed by testing triplicate aliquots for each sample and determination of the coefficient of variation (Figure 3.1). In order to confirm the identity of berberine, the UV spectrum for the peak in the sample was compared with that of the standard peak.

3.3 Results

3.3.1 Identification of sensitive mutants

To examine the antifungal mode of action of berberine, colony sizes were monitored for ~ 4700 strains, each with a different deletion mutation, that were grown on media with and without ~0.05 mg/ml of berberine. The most sensitive mutants were identified using a threshold of 5%, to obtain 195 most sensitive mutants. The products of the deleted genes in these 195 mutant strains were assigned to ten functional categories;

Figure 3.1. HPLC chromatogram of berberine. **(a)** Standard mix (includes 100 µl of 0.12 mg/ml berberine chloride in MeOH). **(b)** A representative chromatogram of the tested samples. Arrow indicates berberine peaks detected at 350nm.

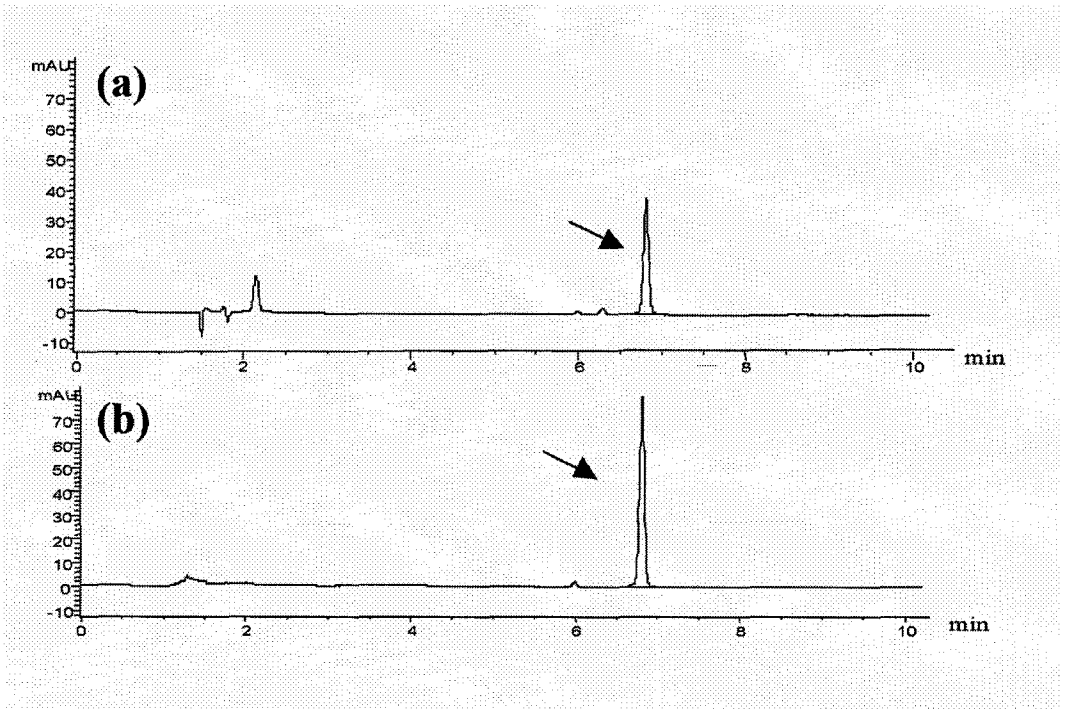
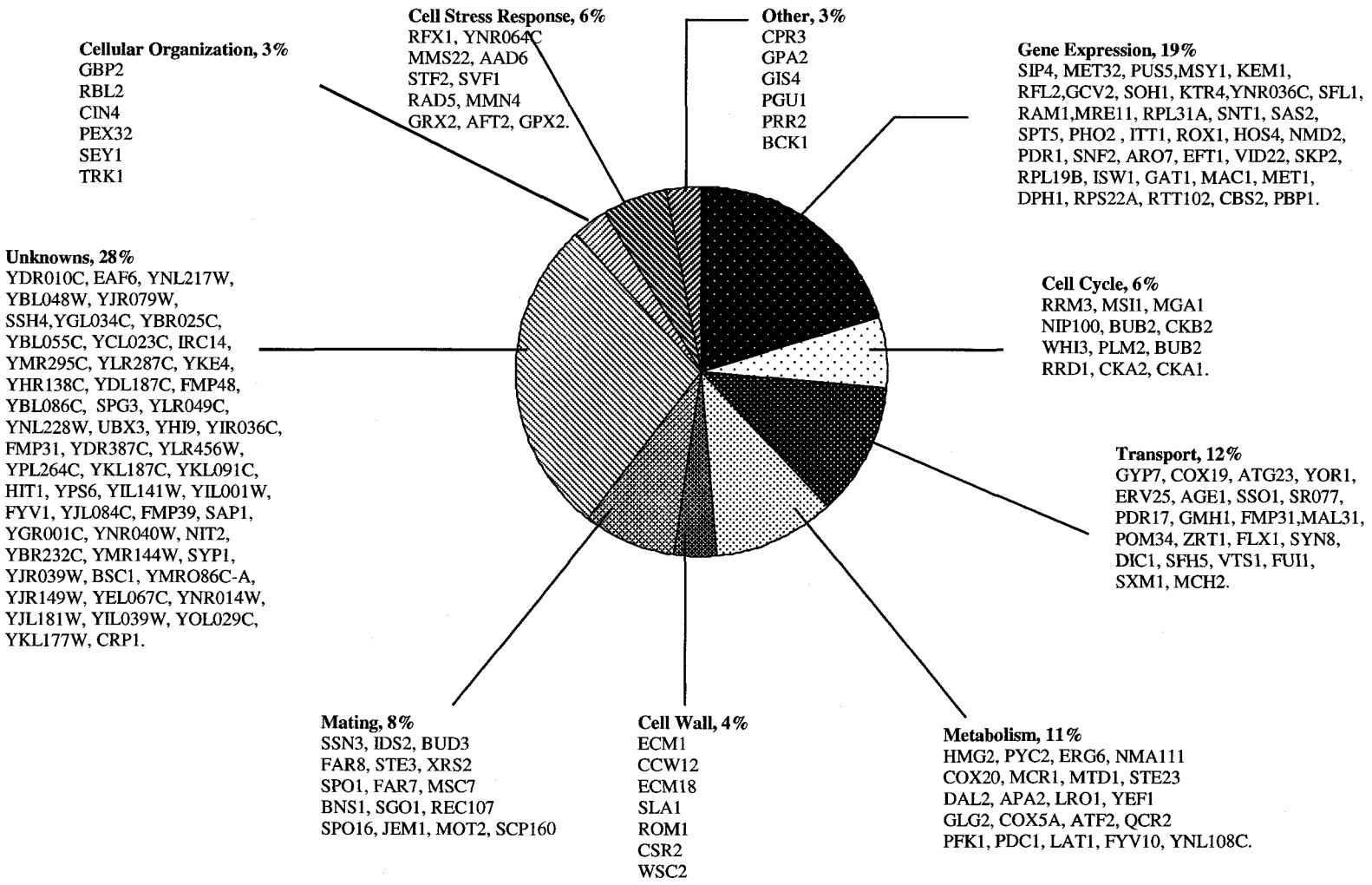


Figure 3.2. Functional catergories of the 195 genes identified by GDA upon exposure to berberine in *S. cerevisiae*. Data for each gene are given in Appendix **II**.



gene expression (19%), transport (12%), cell wall (4%), metabolism (11%), mating (8%), cellular organization (3%), cell cycle (6%), cell stress response (6%), unknown function (29%) (Figure 3.2). The "other" category includes genes that function in pseudohyphal growth (*GPA2* and *PGU1*) and signal transduction (*PRR2, GIS4, CPR3* and *BCK1*). The unknown category has the largest number of genes, indicating several genes that responded to berberine have no previously characterized cellular function.

3.3.2 *MIC of mutant strains*

To test the accuracy of the GDA dataset, we determined MIC for 13 deletion mutant strains that were found to be sensitive to berberine based on GDA analysis. MIC values of the selected strains were compared to the wildtype, S288C, and YNL108C, a deletion mutant that showed no significant reduction in size in GDA analysis (Table 3.2). Overall, the MIC values were correlated to GDA analysis as evidenced by a significant negative slope ($P = 0.002$) in Figure 3.3. Those mutants that had the smallest colony size on GDA plates also had the lowest MIC values. YNL108C, a mutant that had no significant reduction in size on GDA plates, also had a MIC value that was very close to S288C. As indicated in Table 3.2, twelve mutant strains (*ERG6,* YMR166C, *MMS22, PDR1, YOR1, YJL181W, FYV10, FUI1, CBS1, QCR2,* YDR010C and *GAT1*) out of the 13 deletion strains selected as sensitive had significantly lower MIC values than S288C. Of those strains that were identified by GDA to be sensitive, only the YBR195C strain (deleted for *MSI1*) did not show a low MIC value. Overall then, increased sensitivity of this subset of deletion strains to berberine verifies the GDA dataset. The potential insight gained from the GDA dataset is that pathways that are parallel to the deleted gene are possible targets for antifungal action of berberine.

3.3.3 *Berberine uptake and efflux by sensitive mutants*

We employed HPLC to quantify berberine concentration inside and outside of cells of the 13 deletion strains listed in Table 3.1 and wildtype S288C, to find out whether there is a correlation between sensitivity and uptake patterns. Mid log-phase yeast cells were exposed to 0.07 mg/ml berberine and the concentration of berberine inside and outside of *S. cerevisiae* cells was measured by HPLC at each of 10 min, 30 min, 1, 2, 4 and 6 hours (Figure 3.4).

Examining wildtype S288C, an initial uptake of berberine was evident in cells of samples within ~30 minutes after addition of berberine. This is followed by efflux of berberine to levels below that of the medium time point of 60 minutes and longer. As shown in Figure 3.4, comparative analyses of all 14 mutant strains to wildtype S288C revealed striking differences to this wildtype pattern of berberine uptake/efflux. Based on these patterns, three major groups were identified; *a)* consistently higher intracellular concentrations compared to berberine levels in the medium, *b)* consistently lower intracellular concentration to those of the medium for all measurements after 10 minutes, and *c)* variable and time-dependent intracellular concentrations. In the first group, YML008C and YFL021W showed very high intracellular berberine concentrations at 10, 30 and 240 minutes resulting in higher overall intercellular levels (Figure 3.4). Apparently, an active efflux in the second group, YMR166C, YIL097W, YGR281W, YGL013C, YPR191W and YDR010C, consistently decreased berberine intracellular accumulation. Finally, deletion mutants YBL042C, YBR195C, YLR320W, YJL181W and YDR197W were assigned to the third group. As clearly shown in Figure 3.4C, the accumulation of berberine in cells of these strains was time-dependent and peaked at various times after addition of berberine. The mean (±S.D.) intra- and intercellular

Table 3.2. Minimum Inhibitory Concentration (MIC, mg/ml) of selected deletion mutants to berberine. The growth reductions demonstrate the degree of inhibition of mutant strains by berberine.

ORF	Gene	Gene regulation	MIC ± SD (n≥4)	GDA % growth reduction
YML008C	*ERG6*	Down-reg.	0.004 ± 0.002	52
YMR166C	YMR166C	Down-reg.	0.041 ± 0.02	34
YLR320W	*MMS22*	[a]	0.032 ± 0.02	36
YGL013C	*PDR1*	Up-reg.	0.03 ± 0.024	48
YGR281W	*YOR1*	Down-reg.	0.046 ± 0.028	45
YBR195C	*MSI1*	Down-reg.	0.064± 0.057 [b]	14
YJL181W	YJL181W	Up-reg	0.041 ± 0.023	15
YIL097W	*FYV10*	Up-reg.	0.042 ± 0.023	27
YBL042C	*FUI1*	Up-reg	0.025 ± 0.021	55
YDR197W	*CBS2*	Up-reg.	0.037 ± 0.03	25
YPR191W	*QCR2*	[a]	0.048± 0.027	33
YDR010C	YDR010C	Up-reg.	0.04 ± 0.028	23
YFL021W	*GAT1*	Down-reg.	0.036 ± 0.020	20
YNL108C [c]	YNL108C	[a]	0.065 ± 0.058 [b]	8

[a] Variable (Down or Up) regulation at different time of berberine exposure, adapted from the microarray assay (Chapter 2). [b] Not significantly different MIC from S288C strain (2-tailed T-test). [c] Not sensitive to berberine based on GDA analysis.

berberine concentrations were calculated across all time points and are presented in Figure 3.5. Each strain was designated as accumulator, neutral or excluder in this figure, based on the intracellular concentrations.

3.3.4 *Genetic interaction profile by GDA*

Figure 3.6A shows a genetic interaction network representing protein-protein interactions of deleted genes in berberine-sensitive mutants. In this network, every module is linked to its cognate partners in the yeast genome (external). We chose BioGRID database as a reference for the set of protein-protein interactions reported here. The network of these interactions connects subcellular regions that are functionally related, as well as indicating dynamic interchange of proteins between the target genes. For this analysis, the 195 genes deleted in the most sensitive mutants were first imported into the BioGRID (version 2.0.20) database SGD, then formatted and exported for visualization in graphviz (version 2.8.1) to build the graph (http://www.graphviz.org/). The genes are represented as nodes and the interactions are represented as edges connecting the nodes in the figure. The genes are associated with one another by multiple interactions, as may occur in a complex. The most highly connected network among the 195 genes is shown in Figure 3.6B in which each gene has at least one interaction with another gene in the subgraph (internal). This network demonstrates both the complexity of cell response to berberine treatment and the relevant protein-protein interaction between all identified genes in our study. The identification of more than one distinct protein interaction networks encompassing genes identified by GDA further supports the view that berberine has multiple targets.

Figure 3.3. A significant negative slope to the correlation between MIC and GDA for berberine dark treatments with P value of 0.002.

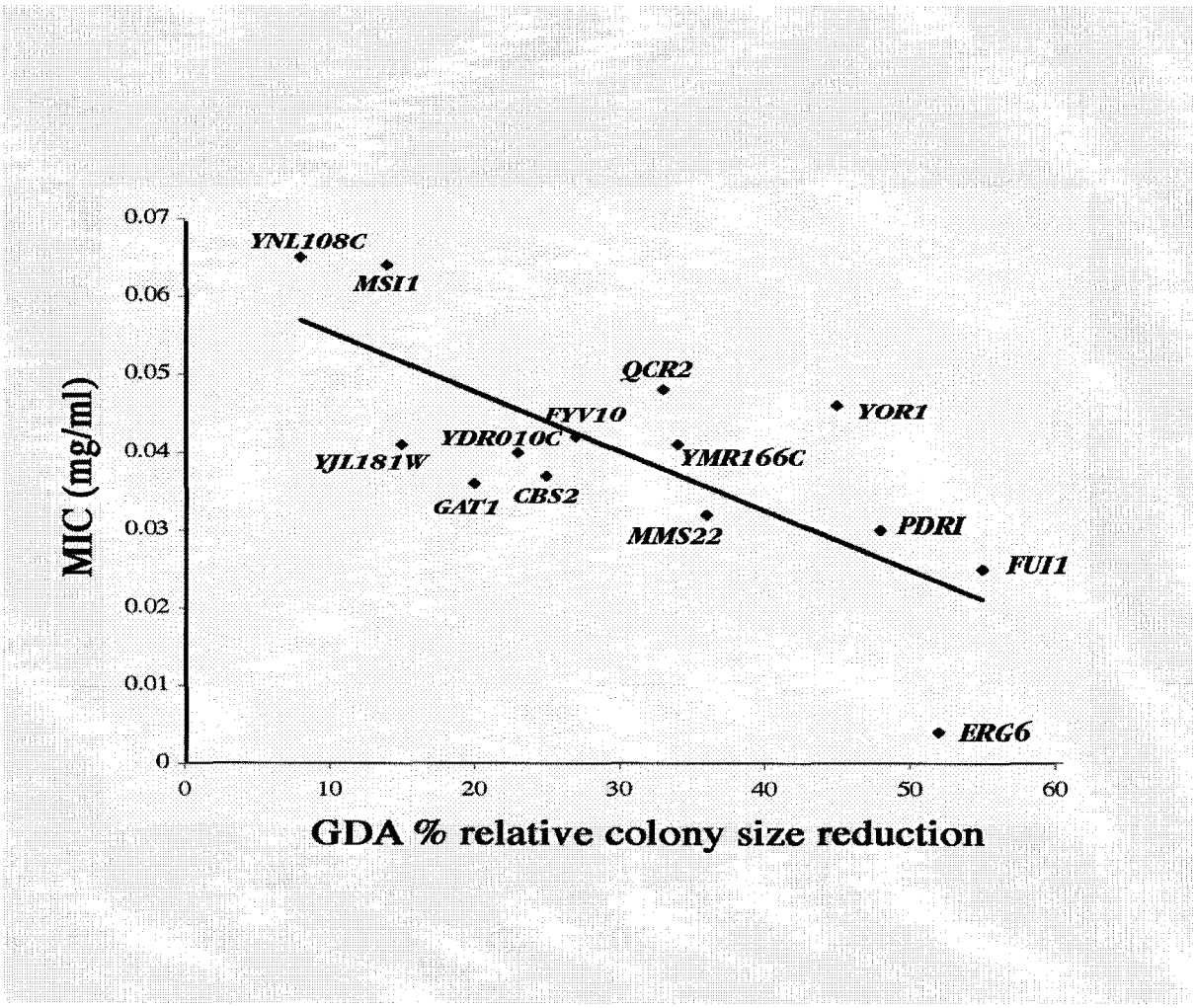
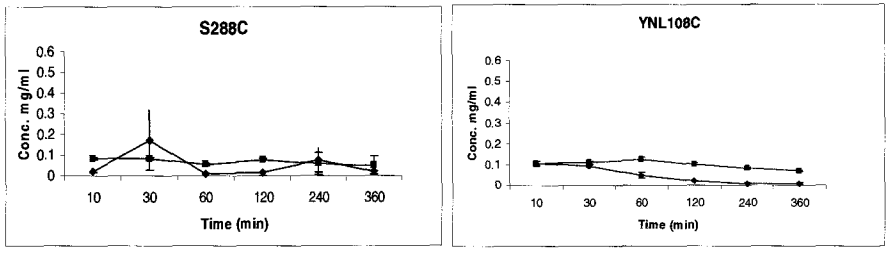
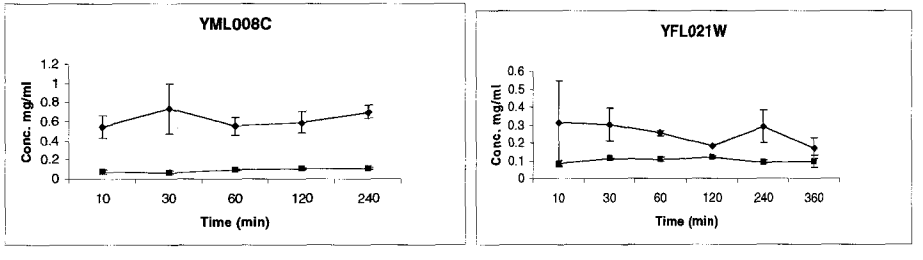


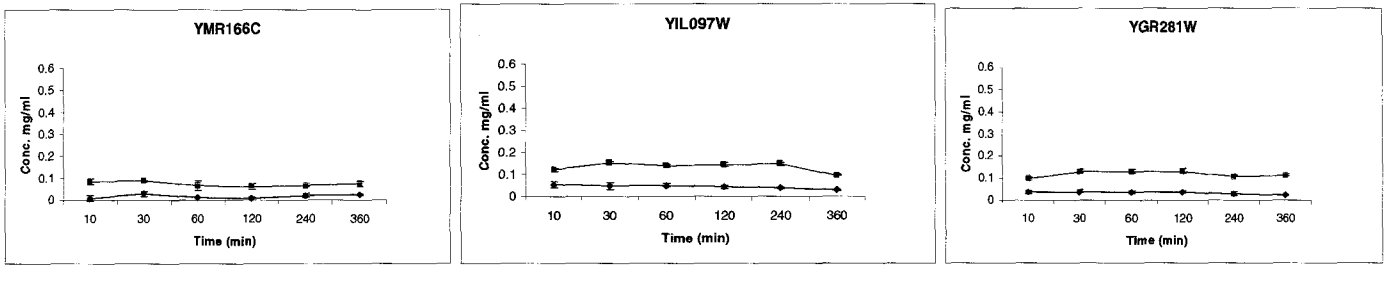
Figure 3.4. HPLC-based measurements of berberine concentration (mg extract /ml) inside (blue) and outside (red) of treated *S. cerevisiae* wildtype S288C and 14 deletion mutant strains at various exposure times. Means and standard deviation bars are shown for each data point based on three independent trials.

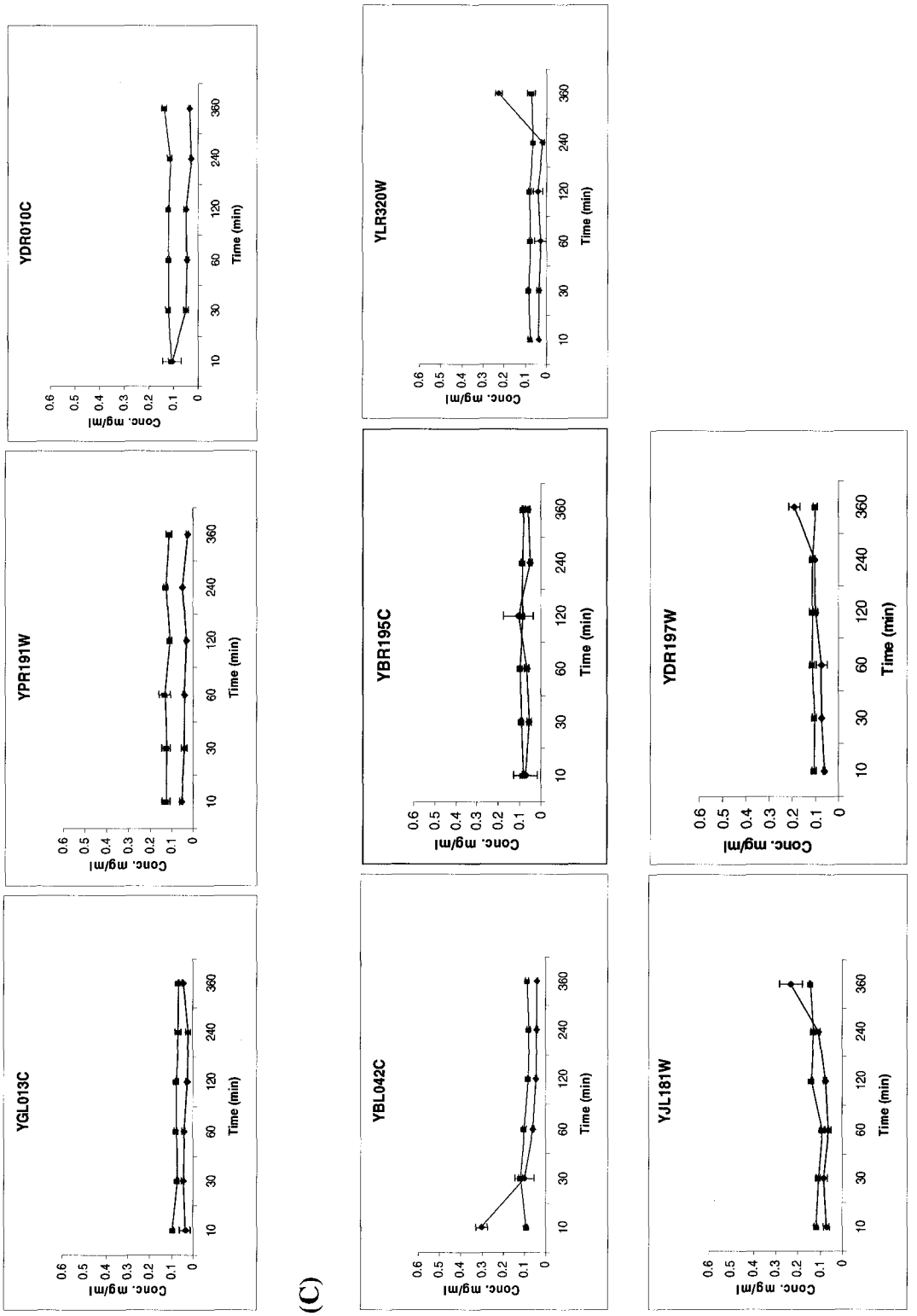


(A)



(B)





(C)

Figure 3.5. Comparison of berberine concentrations inside and outside of yeast cells among 14 deletion mutants with wildtype *S. cerevisiae* (S288C). Berberine concentrations inside (blue) and outside (red) of cells were calculated based on average berberine concentration values at each of 10 min, 30 min, 1, 2, 4 and 6 hours. Three major groups were identified as follows; (i) accumulator (high intracellular berberine concentrations), (ii) neutral and (iii) excluder (low intracellular berberine concentration). Mean and standard deviation bars are shown for each data point based on three independent trials.

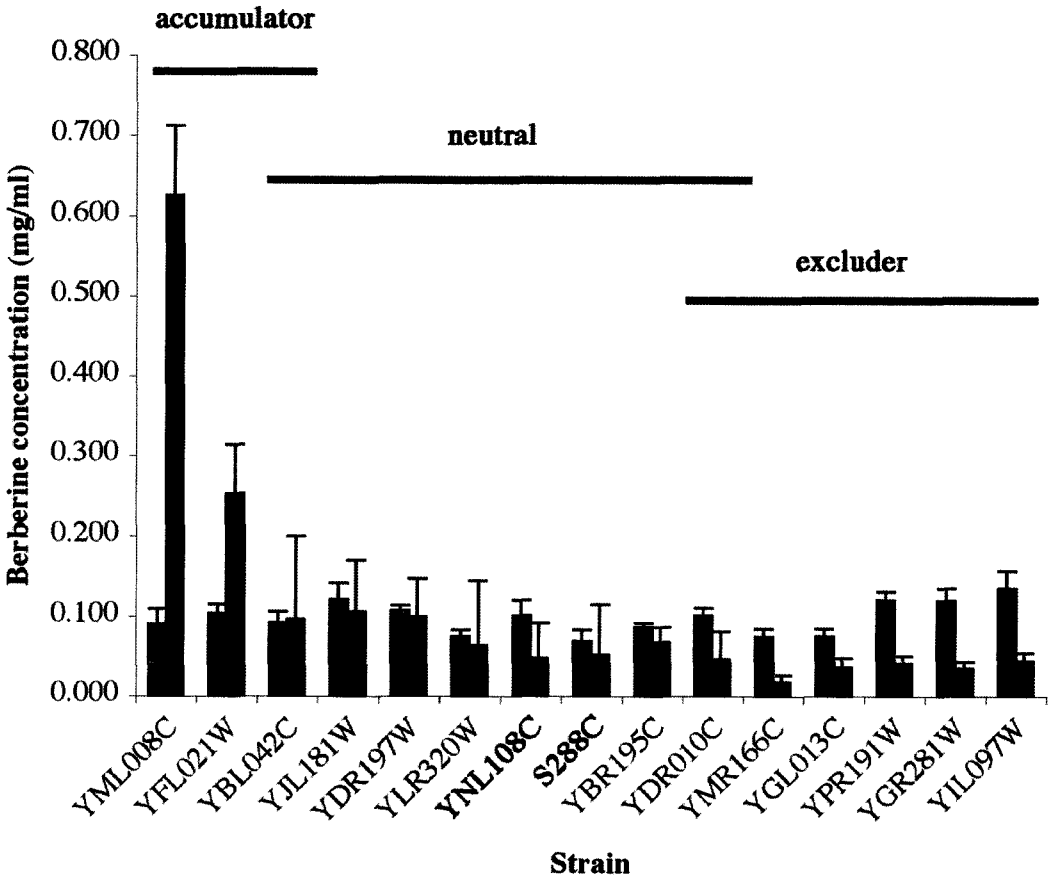
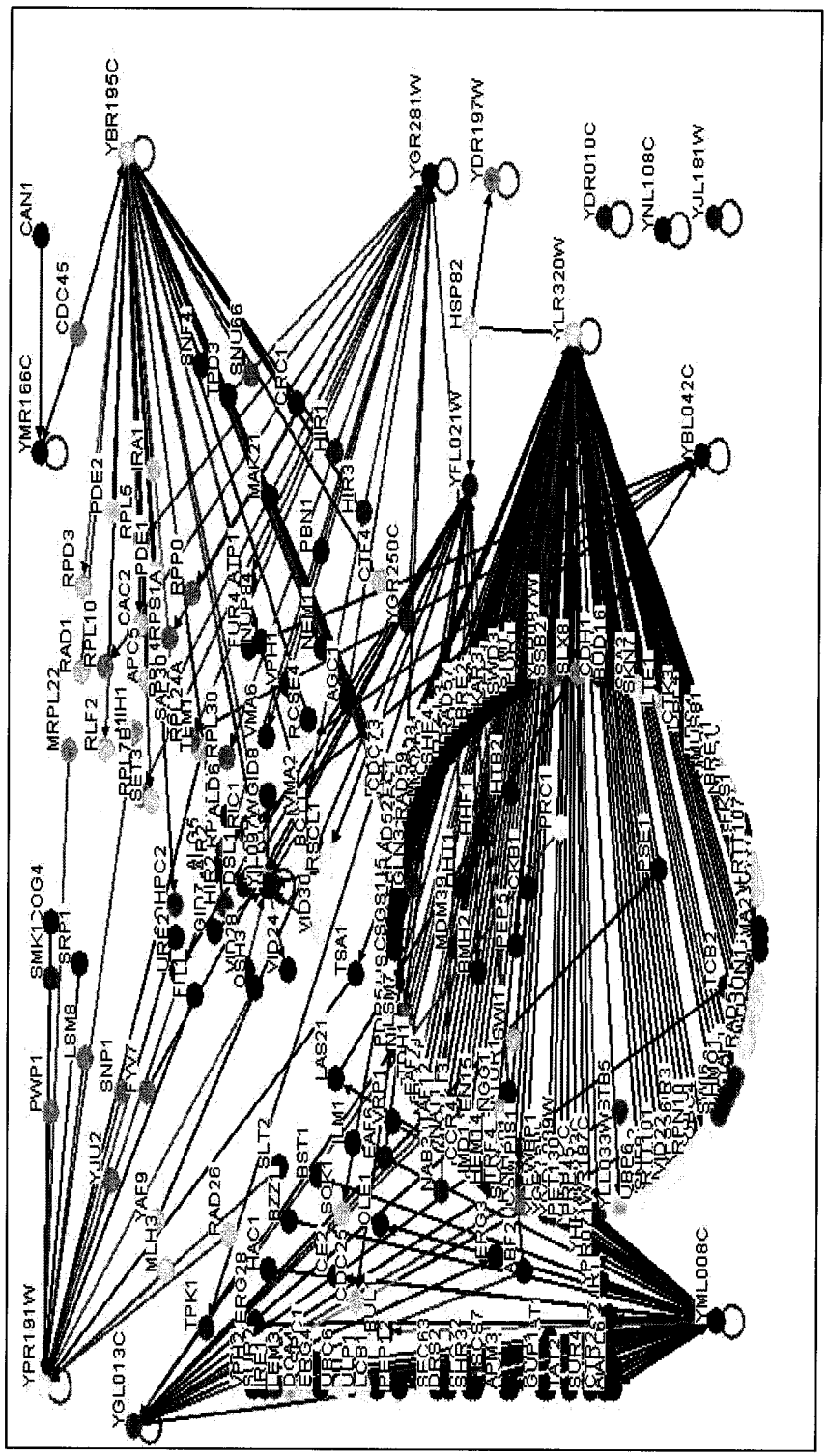


Figure 3.6. Protein-protein interaction networks of genetically connected gene functions. Proteins are represented as full nodes, and interactions are represented as edges that connect the nodes. **(A)** *External interaction:* protein-protein interaction network between 10 sensitive mutant strains (YML008C, YFL021W, YLR320W, YGL013C, YGR281W, YBR195C, YIL097W, YBL042C, YDR010C and YPR191W) and *S. cerevisiae* genome using BioGRID (in total 5299 nodes and 51100 edges) and the SGD database, containing 288 proteins and 303 interactions. **(B)** *Internal interaction:* The protein-protein interaction network, containing 55 proteins and 75 interactions, demonstrates the interconnected proteins among 195 identified genes in this study, in which each protein has at least one interaction. All these interactions representing the lethal/sick interactions determined by GDA analysis. The empty oval-shape represents protein self-interaction.

(A)



(B)

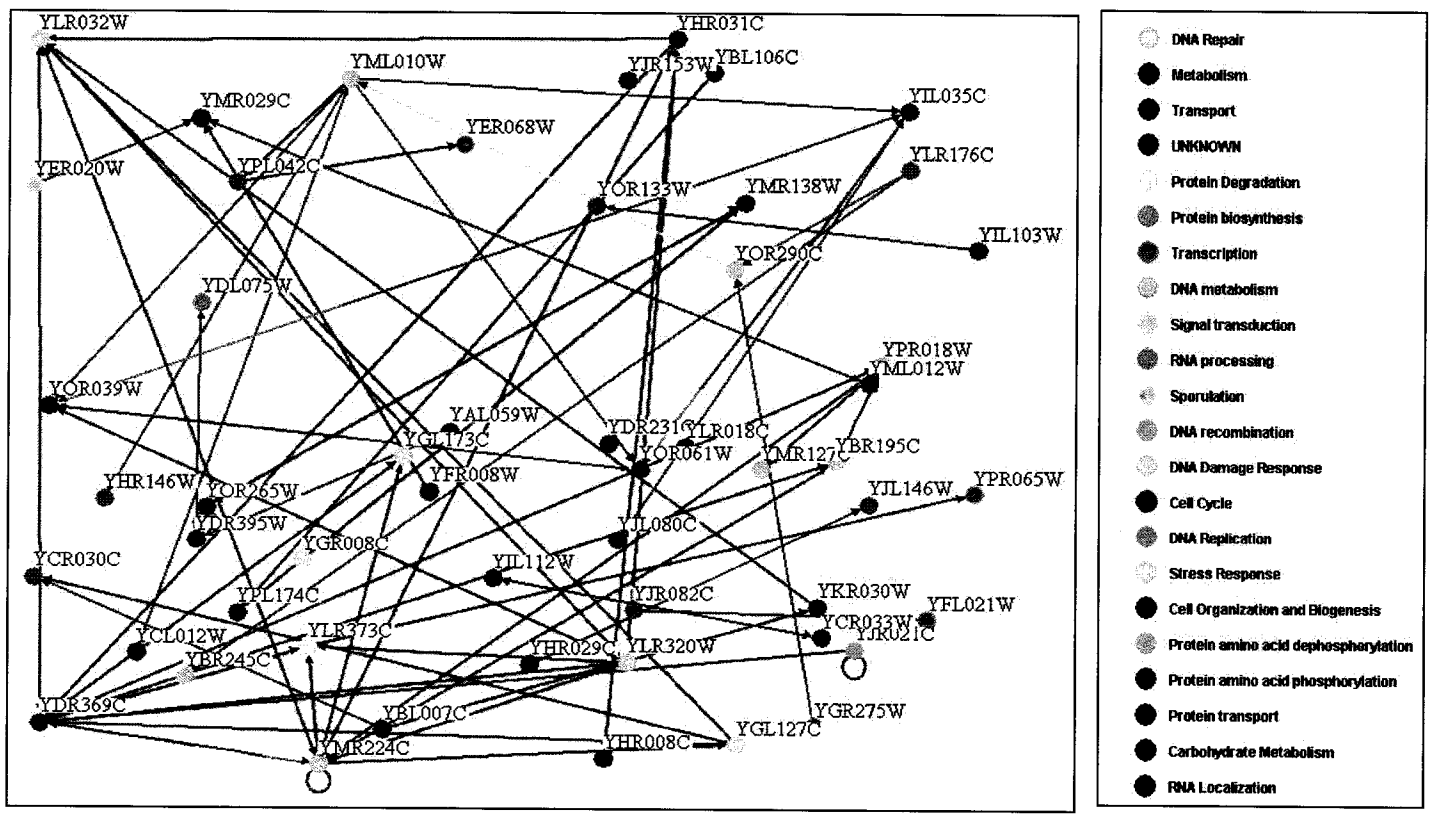
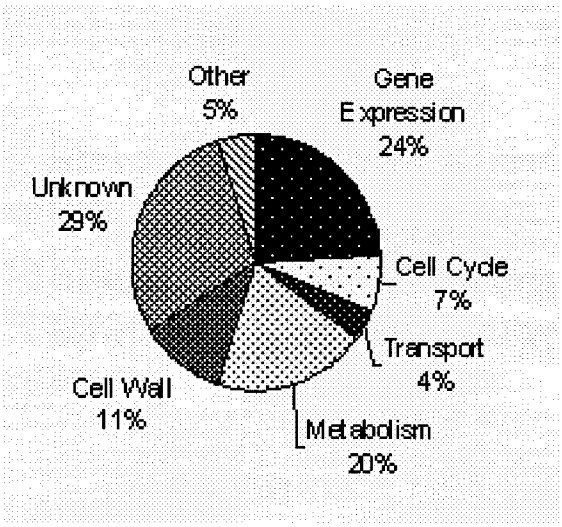
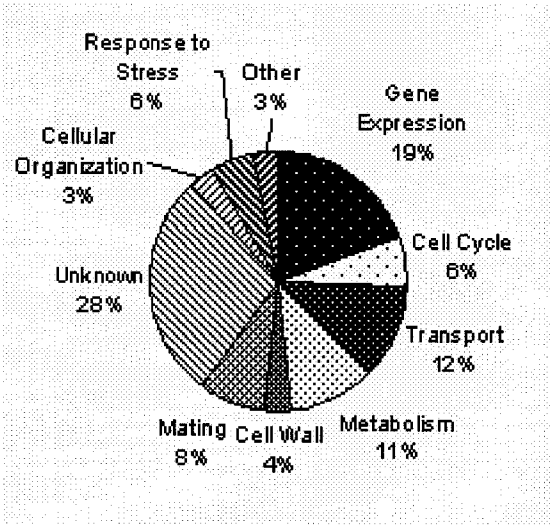
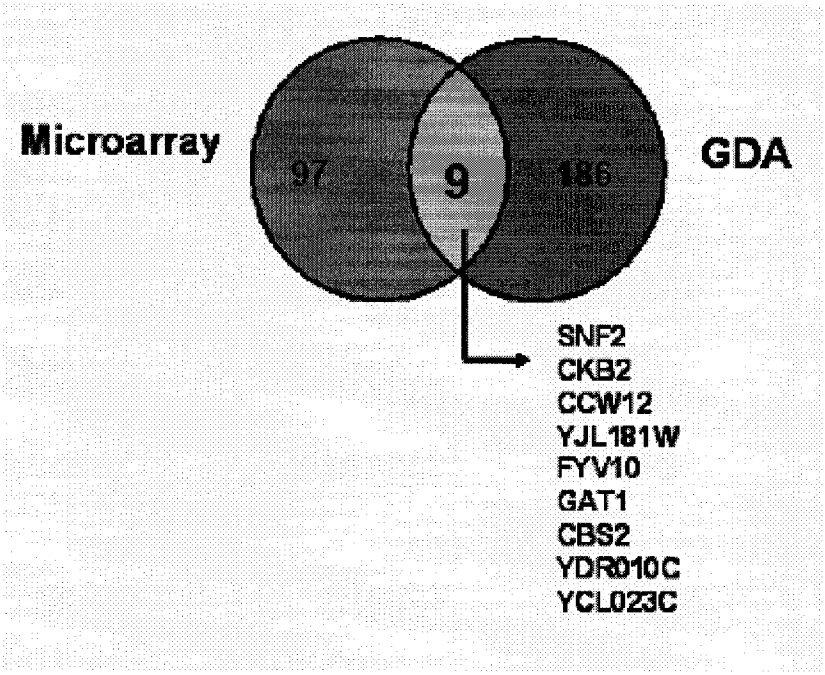


Figure 3.7. Comparison of the microarray *vs* GDA data. The pie graphs **(A)** represents seven functional categories of 55 identified genes in DNA microarray assay (Chapter 2), and **(B)** represents ten functional categories of 195 genes identified by GDA analysis. The identified nine common genes between DNA microarray and GDA analysis are listed at bottom.

(A) **(B)**







3.3.5 *Comparison of microarray and GDA analysis for berberine targets*

To compare data derived from microarray and GDA analysis for genes encoding berberine targets, we looked at genes common to the two dataset (Figure 3.7). Among, the total of 106 genes that were identified as significantly differentially regulated in microarray (Chapter 2), only nine were identified as among the most sensitive to berberine in GDA analysis. These nine genes are involved in cell wall (*CCW12*), gene expression (*GAT1* and *SNF2*), cell cycle (*CKB2*) and unknown function (YJL181W, *FYV10*, *CBS2*, YDR010C and YCL023C). In particular, the functional analysis of these nine mutants may provide insight into possible mechanisms involved in cellular sensitivity to berberine and its target pathways.

3.4 Discussion

We used a gene deletion array (GDA) method to explore the mode of action of berberine in terms of identification of possible targets and/or target pathways in yeast cells. In this method we assume that a mutation leading to modification of a gene function will result in detection of chemical-genetic interaction of a compound of interest. In addition, this leads us to study the sensitivity or resistance of a specific defective pathway(s) to a compound such as berberine. The method consisted of the following stages; a) treatment of each deleted strains with sub-MIC levels of berberine under identical conditions, b) phenotypic screening to score all significant growth reduction of treated strains in comparison to untreated strains, c) statistical analysis to identify the most sensitive mutants, d) selection of potential pathways as targets based on ranking of berberine sensitivity, and finally, e) assessments of selected candidates and evaluation of their sensitivity to berberine using secondary assays. This approach is

applicable to the study of mode of action of any compound that inhibits the growth of fungi.

In our study, we scored each of the mutants for significant growth reduction using Growth Detector (GD) analysis system (Memarian *et al.* 2006, in press). Although we may have missed some strains with partial sensitivity due to errors in reading the colony sizes using this automated approach, it is likely that at least a subset of deletion strains of any given pathways that are inhibited by berberine will be detected with our methods. Supporting our selection of sensitive strains based on GDA, 12 of 13 sensitive strains identified by GDA also had significantly lower MIC values than strain with wildtype levels of sensitivity. Therefore, increased sensitivity of this subset of deletion strains to berberine and more importantly a correlation between MIC and GDA, verified the quantitative results of GDA

Berberine is a weak photosensitizer. It produces both singlet oxygen ($^1O_2$) and radical species in a nonpolar environment (Inbaraj *et al.* 2001). Inbaraj *et al.* showed that berberine photochemistry is governed by the subcellular location of the alkaloid which tends to be in the mitochondria. In addition, in a previous study it was suggested that based on the polarity of mitochondrial membrane potential, berberine moves into the inner membrane of mitochondria toward the less polar region (Mikes and Dadak, 1983). With respect to phototoxicity of berberine, of the 14 mutants listed in Table 3.2, we found that only the gene mutation in YMR166C showed enhanced sensitivity to berberine with UV light exposure (data not shown). It is interesting that YMR166C possesses a transport function in mitochondria (Table 3.1; Belenkiy *et al.* 2000). The transport of various ions across mitochondrial membranes is necessary to maintain the proton gradient that drives oxidative phosphorylation (Belenkiy *et al.* 2000). Therefore, the

increased photosensitivity of this strain in the presence of berberine suggests a connection between mitochondrial function in berberine toxicity. Further studies are necessary to investigate the photodynamic properties of berberine in view of these findings.

3.4.1 *Accumulation and efflux-mediated sensitivity to berberine*

HPLC was employed to investigate the relationship between berberine intracellular accumulation and relative sensitivity to berberine. The characteristic overall intracellular concentration of berberine over all time points was significantly altered in eight out of 13 mutant strains tested as compared to wildtype S288C (Figure 3.5). Thus, it appears that the uptake/efflux of berberine plays a significant role in mutant sensitivity in the GDA assay. Berberine transport mechanisms have not been elucidated clearly, however for cationic compounds, such as berberine, a passive diffusion and participation of carrier-mediated transport have been suggested (Stermitz *et al.* 2000; Terao *et al.* 1996). A recent study demonstrated that the rapid uptake of berberine, as a hydrophobic cation, into *Staphylococcus aureus* cells is driven by membrane potential (Stermitz *et al.* 2000). Furthermore, Lewis (1999) showed that a positive charge on a molecule, such as a cationic antimicrobial compound like berberine, will increase its accumulation in bacterial cells. Lewis suggested that positively charged antibiotics such as berberine will accumulate 2-3 fold in cells, thus enhancing potency over similar but neutral molecules.

In addition, in yeast, a plasma membrane H^+-ATPase plays a key role in creating a proton gradient to provide a driving force for the transport of alkali cations by different transporters (Welihinda *et al.* 1994). For instance, in *S. cerevisiae* two genes *TRK1* (Gaber *et al.* 1988) and *TRK2* (Ko *et al.* 1990) code for two different K+ transporters and *HOL1* also encodes a non-specific cation transporter (Gaber *et al.* 1990). We observed a

low uptake of berberine in some of the susceptible mutant strains, including YDR010C, YMR166C, YGL013C, YPR191W, YGR281W and YIL097W (called "excluder" in Figure 3.5). These "excluder" characteristics may be explained by increased activity by drug efflux pumps or decreased cell permeability. Previously, we demonstrated that transcription of the PDR genes is repressed by berberine (Chapter 2), suggesting that berberine may influence the cells ability to transport xenobiotics out of the cell. In this study, the strain with a mutation in *pdr1* exhibited a low level of berberine accumulation (Figures 3.4 and 3.5), providing more evidence that PDR does not function in berberine efflux. The *PDR1* (encoded by YGL013C) is a master PDR regulator involved in regulating multidrug resistance genes such as *YOR1*, *PDR5* and *SNQ2* (DeRisi *et al.* 2000). Similarly, the observed low uptake of berberine by the strain defective in *yor1* can further explain the possible increased sensitivity to berberine by the both mutants. More interestingly, *YOR1* (encoded by YGR281W) gene product is a member of the xenobiotic ATP-binding cassette transporter family across cell membranes (Katzmann *et al.* 1995). Katzmann *et al.* (1995) also showed that overexpression of *YOR1* provides a dramatic elevation in oligomycin resistance in *S. cerevisiae.* In contrast, the suppression of *YOR1* during exposure to berberine and low levels of berberine accumulation by *yor1* strongly suggests that not only do the cells fail to recognize berberine as a xenobiotic, but also that *YOR1* may not play a role in berberine resistance. In *S. cerevisiae*, there is a complex network of various transcription factors that in regulate PDR genes. For instance, *YOR1* expression is responsive to *PDR1* and *PDR3*, while *PDR5* expression depends on presence of both genes *PDR1* and *PDR3.* The *PDR5* transporter plays a critical role for resistance to several compounds, including cycloheximide and chloramphenicol (Balzi *et al.* 1994; Hirata *et al.* 1994). The sensitivity of *YOR1* (yeast

oligomycin resistance 1) to berberine and its regulation on PDR genes prompt us to conclude that berberine may thus produce a synergistic effect by blocking *YOR1* downstream from PDR gene functions and, accordingly, increase cell sensitivity to other antifungals.

The gene YPR191W encodes *QCR2*, subunit II of cytochrome c which plays an important role in cell death in *S. cerevisiae* (Kluck *et al.* 1997; Yang *et al.* 1997). Previous studies have demonstrated that cationic drugs such as gentamycin and lidocaine can detach cytochrome c from membranes containing acidic phospholipids by binding to liposomes (Jutila *et al.* 1998). All of these drugs have positive charges and bind to membranes containing acidic phospholipids. This suggests that berberine, as a cationic compound, may also interfere with the lipid/protein interactions of integral membrane proteins. Therefore, berberine may play a role in the initial cell defense response by changing mitochondrial permeability and proceed to release cytochrome *c* from mitochondria, causing plasma membrane disruption and eventually cell death. Similarly, it was shown that anticancer drugs can trigger extensive efflux of cytochrome c, from mitochondria and cell death through the formation of membrane channels by overexpression of the pro-apoptotic protein Bax (Rosse *et al.* 1998). The detection of sensitivity with mutant YMR166C, a mitochondrial transporter, in our study can provide further evidence for berberine interference with mitochondrial permeability. It has been also shown that deletion in YMR166C increases rapamycin and wortmannin resistance (SGD, July 2006, http://genome-www.stanford.edu/Saccharomyces), quite opposite to what we found with respect to berberine sensitivity.

On the other hand, as shown in Figure 3.5, the mutant strains *gat1* and *erg6,* called "accumulators", showed sensitivity to berberine, possibly because of defective

efflux of berberine and high intracellular accumulation of the compound. We previously discussed that *GAT1* (encoded by YFL021W, a transcriptional activator) is important for berberine resistance, possibly through roles of either drug efflux or cell membrane permeability alteration (Chapter 2). Accordingly, the observed down-regulation of *GAT1* expression when yeast is exposed to berberine is suggestive in this respect. In addition, the sterol methyltransferase gene, *ERG6*, converts zymosterol to fecosterol in the ergosterol biosysnthesis pathway by methylating position C-24 (SGD, July 2006, http://genome-www.stanford.edu/Saccharomyces). Since membrane sterol can change the activity of cation transporters, the alteration of sterol composition in *erg6* affects the overall arrangement of the proton gradient across the membrane, hyperactivation of the pump and cation uptake (Welihinda *et al.* 1994). Interestingly, Welininda *et al.* (1994) also showed that deletion of *ERG6* results in a growth defect in addition to cation hypersensitivity. Moreover, several lines of evidence have indicated that *erg6* mutants have severely altered membrane permeability (Bard *et al.* 1978) and structural features (Lees *et al.* 1979), as well as growth rate and limitation on utilizable energy sources (Lees *et al.* 1980).

The mutant *erg6* susceptibility to berberine provides important applicability in antifungal drug development because the C-24 methyl group is found in fungal but not in human sterol. The results predict that increased permeability in *erg6* mutant cells make them sensitive to known antifungal compounds or might even make them sensitive to new antifungal compounds to which the cell is impermeable when ergosterol is present in the cells. This phenomenon suggests that treatment of cells with berberine and inhibitors of *ERG6* such as azole antifungal compounds would thus produce a synergistic effect to combat fungal infections. Previously, in a study of a synergic effect in a medicinal plant,

Stermitz and colleagues showed antimicrobial activity of berberine can be potentiated by an inhibitor of NorA MDR pump of *Staphylococcus aureus* (Stermitz *et al.* 2000). Similarly, it was shown that deletion strain *ERG6* in *C. albicans* provides drug susceptibilities as compared to wildtype to a number of antifungals and metabolic inhibitors such as terbinafine and cycloheximide (Jensen-Pergakes *et al.* 1998). Notably, it was previously shown that a possible cellular target of protoberberines is sterol 24-methyl transferase (Park *et al.* 1999) which is compatible with our finding that ergosterol biosynthesis pathway may be a target of berberine toxicity.

Efflux mechanisms often account for resistance to a wide range of chemically unrelated compounds. Reduced intracellular accumulation may be correlated with overexpression of multidrug resistance pumps (MDRs), efflux transporter genes of the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) classes (Lupetti *et al.* 2002). For example, in yeast, overexpression of pdr5p results in multiple, or pleiotropic drug resistance (PDR) (Balzi *et al.* 1994). There is a similar effect of increased gene numbers of ABC pumps in mammalian cells (Egner *et al.* 1995). In previous studies it was observed that hydrophobic cations, such as berberine alkaloids and its derivatives, are the preferred substrates for most multidrug resistance pumps of the MF and ABC families (Lewis and Klibanov, 2005), such as P-glycoprotein multidrug resistance pumps that are responsible for multidrug resistance by some human tumors (Yang *et al.* 2003; Gros *et al.* 1992). This leads to the deduction that berberine may be rapidly extruded from yeast cells by the MDR pumps. From this perspective, the activity of MDRs may explain the similar pattern and level of intra- and intercellular berberine accumulations in mutant strains including YBL042C, YJL181W, YDR197W, YLR320W and YBR195C in comparison to wildtype S288C and the mutant control YNL108C

(Figures 3.4 and 3.5). For example, uridine transporter, *FUI1* (encoded by YBL042C), tested in this study (Table 3.2) provides further evidence that, like *DAL5* (allantoate transporter, Chapter 2), this transporter may have substrate specificity for berberine. Previously, it was shown that *fui1*-disruption mutant increased resistance to 5-fluorouridine suggesting that *FUI1* mediates intracellular nucleoside transporter and nucleoside analog drugs across cell membranes in *S. cerevisiae* (Vickers *et al.* 2000; Wagner *et al.* 1998). The gene *FUI1* is placed in "uracil/allantoin" permease family and is highly selective for uridine (Nelissen *et al.* 1997). As shown in Figure 3.4 **(C)**, rapid uptake of berberine was observed at 10 minutes of sampling time, indicating time-dependent uptake followed by a quick drop in intracellular berberine to levels below that of extracellular berberine concentrations. The lower concentration of berberine in mutant culture of *fui1* is perhaps associated with disruption of *FUI1* function.

In conclusion, the GDA results reported here have revealed a subset of the genes, that when deleted, result in increased berberine toxicity. The observed genes and their related functions are of considerable interest since they confirm further that the mechanism(s) of berberine is complex and varied. Interestingly, deleted genes of the most sensitive mutants were mostly in the unknown category. This indicates that we still have much to investigate about the cellular effects of plant-derived compounds on microbial systems. It may be noted that a comparison of DNA microarray (Chapter 2) and GDA yields relatively little overlap in the datasets. Possibly, this lack of congruence is due to differences in exposure times in the two experimental designs. In Chapter 2, microarray analyses were done on cells exposed to berberine for up to 6 hours, whereas GDA experiments involved 24 - 48 hour exposures. It is also important to note that only a subset of genes were selected for further investigation in both studies. It should also be

pointed out, however, that the two experimental procedures examine very distinct processes. DNA microarrays can reveal transcriptional response patterns, whereas GDA analysis is a more direct examination of pathway interactions. In fact, some genes with altered transcription under berberine exposure, are identified in Chapter 2, may encode products that are in pathway parallel to those affected in the sensitive deletions identified in Chapter 3. Taken together, our study showed a potential role for integration of GDA as a genomic tool to screen gene targets of bioactive compounds such as berberine.

CHAPTER 4

Mode of Action of Mycotic Extracts of *Echinacea* through GDA Analyses.

4.1 Introduction

Echinacea is native to the prairies of central and the southeastern United States, with one species extending to southeastern Saskatchewan and southern Manitoba in Canada (Small and Catling, 1999). The genus has a rich tradition of use by First Nations of the North American prairies who used it medicinally more than any other plant (Shemluck 1982). Its use was quickly adapted by European settlers (Small and Catling, 1999) and it is now one of the top-selling herbs in natural products market surveys in North America (Brevoort 1998). Medicinal Extracts of Echinacea are made from all parts of the plant, including the root, leaves, flowerheads, and seeds. The herb is used to prevent, treat and reduce the symptoms and duration of colds, coughs, flu and other upper respiratory illnesses (Merali *et al.* 2003; Binns *et al.* 2002b; Hobbs 1994). In Europe, it is also used to treat infections such as systemic candidiosis, inflammations caused by a variety of infectious bacteria, viruses and other pathogens associated with wounds, and in relief of swelling and pains (Hudsen *et al.* 2005; Merali *et al.* 2003; Goel *et al.* 2002; Hobbs 1994; Wacker and Hilbig, 1978). *In vivo* and *in vitro* pharmacological investigations have shown immunomodulatory effects by Echinacea with short term use (Wacker and Hilbig, 1978). In particular, phagocytosis by granulocytes is induced by Echinacea extracts (Bauer 1998; Bauer 1991).

Recently, the genus *Echinacea* was partitioned into four species and six varieties (Binns *et al.* 2002b). The two species commonly used as medicine are *Echinacea purpurea* (L.) Moench and *Echinacea pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist (syn. *E. angustifolia)* (Binns *et al.* 2002a,b). A number of chemical entities are considered important contributors to the therapeutic effect of Echinacea. These include a volatile oil, alkamides, alkaloids, caffeic acid derivatives, flavonoids, essential oils,

polyacetylenes and polysaccharides (Percival 2000; Letchamo *et al.* 1999; Bauer *et al.* 1998; Bradley *et al.* 1992; Awang *et al.* 1991). Like many herbal remedies, it is not clearly understood which of Echinacea's chemical components are responsible for its effects and the mode(s) of action of Echinacea has not been sufficiently investigated.

The aim of this study was to evaluate the antifungal activity of eight different ethanolic extracts of *E. purpurea* (L.) Moench and *E. pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist (syn. *E. angustifolia)* using genetic mutants of *Saccharomyces cerevisiae.* We examined potential phototoxicity and light-independent toxicity for each extract for a total 16 treatments. To study the possible mode(s) of action of Echinacea, we used a set of ~ 4700 viable gene deletion mutants of *S. cerevisiae.* Each mutant carries a defined deletion of a characterized or putative open reading frame. The mutants were grown with and without each treatment to provide a view of the chemical-genetic interactions and identify pathways affected by the *Echinacea* extracts. The notable trend in these data sets was that several of the most sensitive mutants were defective in cell wall-associated functions. We therefore tested whether cell wall function was compromised in yeast cells exposed to Echinacea extracts.

4.2 Methods and Materials

4.2.1 *Echinacea source*

Echinacea purpurea (L.) Moench and *Echinacea pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist (syn. *E. angustifolia)* (Heliantheae: Asteraceae) plant materials were commercially grown and classified according to the most recent taxonomic revision (Binns *et al.* 2002b). Voucher specimens were deposited in the

herbarium at the University of Ottawa, Canada. Voucher numbers, plant parts used and extract codes for each are provided in Table 4.1. Ethanol extracts (55% and 70%) were prepared as described previously (Hudson *et al.* 2005; Vimalanathan *et al.* 2005) using an Accelerated Solvent Extraction system (DIONEX). These were fractionated according to Binns *et al.* (2002a) three times with equal proportions of n-hexane and distilled water, resulting in three n-hexane fractions (pooled into one) and three hydroalcoholic portions (pooled). All fractions were concentrated at 30°C in a rotary-evaporator and adjusted to 50 ml in the appropriate solvent. HPLC characterization was carried out on each fraction and is presented in Table 4.2.

4.2.2 *Fungal strains and MIC assays*

The wildtype (S288C, *MATα SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1)* and mutant strains of *S. cerevisiae* and *Cryptococcus neoformans* (OMH # FR2704) used in this study are maintained at Carleton University. Yeast cells were grown with YPD media with and without 2% agar. Minimum Inhibitory Concentration (MIC) assays were done in sterile 96-well plates. *S. cerevisiae* strain S288C was grown to mid-log phase in YPD broth, adjusted to OD_{600} ~0.8 and diluted 1:500 (to obtain ~10^3 CFU/ml). Echinacea extracts were serially diluted across wells containing ~100 cells/100 µl YPD/well with the last column serving as a drug-free control. Each fraction was tested under two conditions: following UV light irradiation (10 W/m^2 for two hours using three 20 W black-light blue tubes, 320-400 nm range) and without UV light irradiation (dark treatment), for which plates were wrapped in aluminum foil. The microtiter plates were incubated in the dark at 30°C and monitored at 48 hours. Activity of each extract was based on the concentration of each fraction (data not shown). An average MIC value was

Table 4.1. *Echinacea* sp. samples voucher numbers, codes for extracts and ethanol extraction.

Echinacea samples	UO voucher # *	Fraction	Ethanol (55%)	Ethanol (70%)
E. purpurea	UO # 010502-18	Root	SG 1	SG EPR
	UO # 010410-15	Herb[b]		SG EPH
	UO # 19182	Flower[c]		SG EPF
	UO # 010625-20	Leaves & stem		SGEPLS
E. pallida var. *angustifolia*				
	UO # 010410-12	Root	SG 7a	SG EARa
	UO # 020607-01	Root		SG EARb

* University of Ottawa voucher number; [b] Source: EPH-5, Sunfirst Herbs (2000); [c] Field K6, Stage flower III (1999).

Table 4.2. Phytochemical profiling of *E. purpurea* and *E. angustifolia* extracts by HPLC. The mean concentration of the compounds are presented with Relative Standard Deviation of analyses <%5.

Plant Type	Extract Type	Sample	Caftaric ug/ml*	Chlorogenic ug/ml*	Caffeic ug/ml*	Cynarin ug/ml*	Cichoric ug/ml*	Echinacoside ug/ml*	PID 8/9 TET ug/ml*
E. purpurea root	EtOH 70%	SG-EPR	895	61	28	0	4861	0	0
E. purpurea root	EtOH 55%	SG-1	781	55	27	0	4774	0	0
E. angustifolia root "A"	EtOH 55%	SG-7A	20	282	15	238	46	1859	483
E. angustifolia root "A"	EtOH 70%	SG-EAR-A	23	286	16	207	34	1831	488
E. angustifolia root "B"	EtOH 70%	SG-EAR-B	60	91	4	462	36	2426	342
E. purpurea herb	EtOH 70%	SG-EPH	767	45	220	0	2879	0	0
E. purpurea flower	EtOH 70%	SG-EPF	919	208	160	0	7340	0	39
E. purpurea leaf & stem	EtOH 70%	SG-EPLS	913	45	421	5	6001	0	19

* Data are means with CV<5%

calculated from concentrations in multiple wells (≥3) that depicted similar growth inhibition. MICs were recorded as the concentration at which there was ~80% reduction in growth in comparison to wells with no inhibitor present as determined by eye and optical density readings (OD_{600}, Spectra Max 340PC, Molecular Devices, Sunnyvale CA). Relative sensitivity for selected mutants was calculated based on MIC of Control-Mutant over MIC of control.

4.2.3 *Gene deletion array (GDA) analysis*

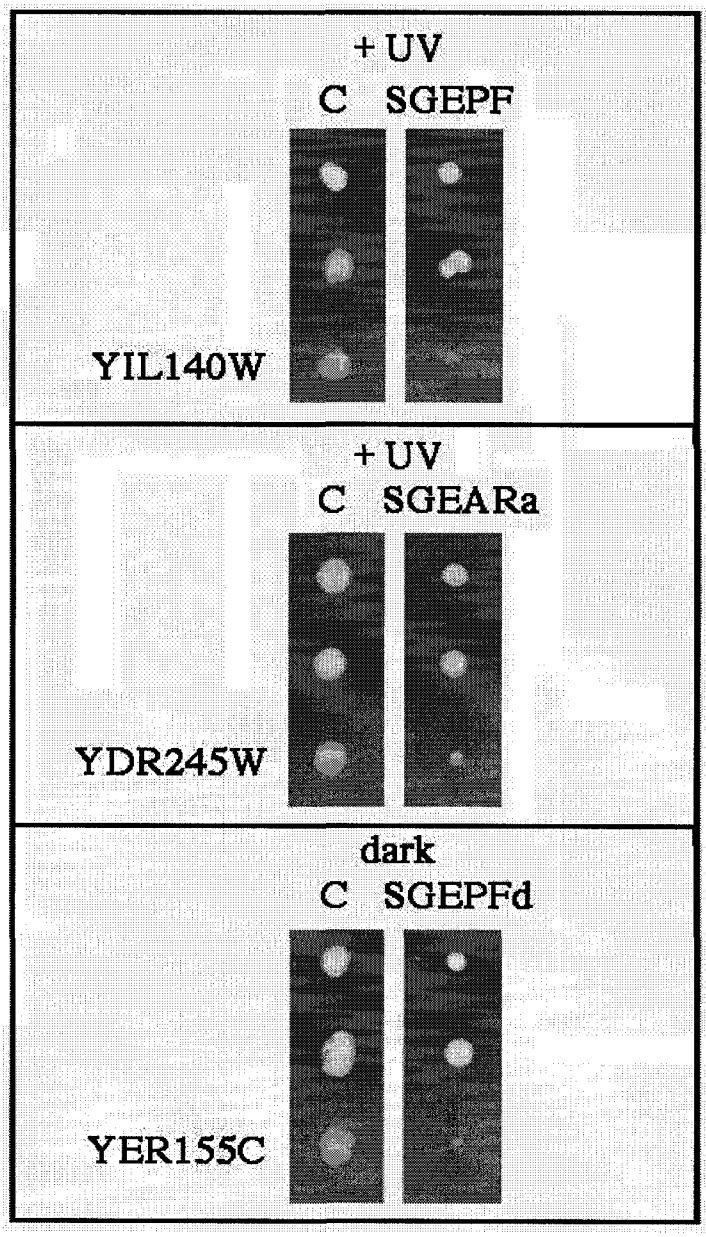
The chemogenomic profiling of Echinacea extracts were done using yeast gene deletion array (GDA) analysis as described by Parsons *et al.* (2004). This method is based on the size of the colonies formed by yeast gene deletion strains in the presence of the semi-inhibitory concentration of the target drug. We used a set of haploid *S. cerevisiae* strains representing ~4700 gene mutants in the background strain BY4741, a derivative of S288C (Tong *et al.* 2001). Echinacea extracts were added to ~55 °C molten YPD agar medium (2% peptone, 1% yeast extract, 2% glucose and 2% agar) at a concentration that was ~80% of MIC. YPD agar plates (Omni Tray, Nalge Nunc international company, Rochester, NY, USA) without (control) and with Echinacea extract (experimental), were inoculated by hand pinning with a 384-floating pin replicator (VP384F) sets of 384 (16 X 24) mutant strains per plate. GDA analyses were done with each extract following UV light irradiation and with no UV light exposure (dark). After pinning, plates were incubated for 1 - 2 days at 30 °C, photographed with a high-resolution digital camera (Hewlett Packard PhotoSmart 735 digital camera), and analyzed as described in Memarian *et al.* (2006). The colony areas were determined by digital analysis and saved to a Microsoft Excel spread sheet. For both experimental and control

plates, the size of each colony was compared to the average colony size for all colonies on the plate. Potentially interesting "supersensitive" mutants were identified as those colonies that were, a) significantly smaller than average colony size on the experimental plate, and b) not significantly different in colony size on the control plate. After normalizing for mutation-associated colony size differences and overall growth inhibition due to the treatment, a set of 5% (~230) of the most sensitive mutants were selected from each of the 16 *Echinacea* extract treatments (light and dark treatments with 8 extracts). Examples of these most sensitive mutants with and without various Echinacea treatments are shown in Figure 4.1. We then selected the subset of mutants that were supersensitive to five or more extract treatments.

4.2.4 *Cell wall disruption assays*

Strains were grown overnight in ambient light at 30° C with shaking in 1 ml YPD broth without (control) and with (experimental) *E. purpurea* 70% EtOH root extract added. For S288C and *C. neoformans*, 6.4 mg/ml of extract was added, whereas for YLR338W, 4.2 mg/ml extract was added. These extract concentrations were selected to cause a ~20% reduction in growth rate for the respective strain. The overnight cultures were diluted by addition of 4 ml of YPD (control) or YPD + extract and incubated for an additional ~1 hour at 30°C or until OD_{600} ~0.8. Cultures were adjusted to ~1.0 x 10^7 cells/ml, aliquoted into 1.5 ml epitubes and placed on ice. Aliquots (1 ml) of control and experimental cultures were sonicated at 3 μm amplitude for 0 and 2.15 minutes at room temperature and then placed back on ice. Cultures were vortexed briefly and then 10 times serial diluted to ~10^3 cells/ml. Ten μl of each dilution was spotted onto YPD agar plates, incubated for 48 h at 30° C before colony counts were performed.

Figure 4.1. Examples of nonsensitive and sensitive colonies from GDA experiments. In each panel, three strains are shown on control plate (left) and treatment plate (right) for Echinacea treatments, SGEPF (+UV), SGEARa (+UV) and SGEPFd (dark). The top two strains in each panel are not sensitive, while sensitivity of the lower strain in each panel, designated YIL140W, YDR245W and YER155C, is evident from the relatively small colony size on treatment plate.



4.3 Results

4.3.1 *Antifungal activities of Echinacea extracts*

We determined MIC values for *S. cerevisiae* S288C exposed to each extract with and without UV-light. As shown in Table 4.3, all extracts had antifungal activity, although the MIC values differ depending on source species, plant part and extract preparation. The results also reveal light-mediated antifungal activity (phototoxicity) for *E. purpurea* root and herb extracts. Previous study showed that ethanol fractions of *E. purpurea* contained a strong phototoxicity due to the presence of polyacetylenes and alkamide compounds, some of which are known to possess antifungal activity (Vimalanathan *et al.* 2005; Merali *et al.* 2003). The observed differences in MIC values between extracts may indicate variation of these polyacetylenic compounds between *Echinacea* species and plant parts. Additionally, we compared the response of 231 mutant strains of *S. cerevisiae* to the eight different extracts of Echinacea (data not shown). The cumulative results of these experiments showed that the 70% ethanol root extract of *E. purpurea*, SGEPR, had the greatest antifungal activity by inhibiting the largest number of mutant strains of all extracts tested.

4.3.2 *Gene Deletion Array data analysis*

Genetic profiles for the 16 different *Echinacea* ethanolic extract treatments were generated. The effect of each treatment on gene deletion strains was determined by measuring colony sizes (Figure 4.1). Mutants were ordered based on percent reduction in colony size and the 5% (~230) of ~4700 mutants that showed greatest reduction in growth on experimental plates as compared to no-compound control plates were

Table 4.3. Minimum Inhibitory Concentration (MIC) of *Echinacea* extracts with and without exposure to UV-light.

Echinacea species extract code	*S. cerevisiae* S288C MIC (mg/ml)	
	+ UV	Dark
E. purpurea		
SG1	4.5 ± 2.6	5.16 ± 2.6
SGEPR	2.28 ± 0.65	4.5 ± 2.6
SGEPH	0.84 ± 0.7	1.3 ± 0
SGEPF	2.12 ± 0.57	0.84 ± 0.7
SGEPLS	2.15 ± 0.69	4.0 ± 2.17
E. pallida* var. *angustifolia		
SG7a	1.76 ± 0	1.76 ± 0
SGEARa	0.55 ± 0.52	0.55 ± 0.52
SGEARb	1.3 ± 0	0.32 ± 0.03

designated as sensitive mutant strains for each treatment. We next compared these most sensitive mutants from each treatment and selected those mutants that were among the most sensitive in five or more treatments. Based on binomial proportions, it is unlikely ($P<0.05$) that a given mutant within this most sensitive 5% group would occur in five or more treatments by chance. This "consensus" set encompasses the 23 mutants listed in Table 4.4.

As shown in Table 4.4, these 23 consensus mutants are involved in cell wall, Golgi, transcription and other functions. The "other" category includes genes that function in protein tagging and amino acid biosynthesis. Additional mutants in this set are of unknown function. The significant pattern to emerge from this analysis is that at least ten of these 23 most sensitive deletion mutants appear to have impaired fungal cell wall function. We used the Yeast Features (YF) software tool (Dumontier *et al.* submitted) to assess the statistical significance of shared features among the set of 23 yeast proteins. Based on YF, the probability that these common cell wall "features" would occur together in our data set by chance is unlikely (P values are less than 4×10^{-3} for all 5 cell wall-related features; Table 4.6). Therefore, it appears that Echinacea extracts are interacting with proteins involved in cell wall function.

4.3.3 *MIC of mutant strains*

We hypothesized that mutants that were sensitive to Echinacea extracts in our GDA experiments would have relatively lower MIC values when exposed to the same extract. To test this hypothesis, MICs of the 23 sensitive mutants were compared to the wild-type strain, S288C, following exposure to SGEPR in the presence and absence (dark treatment) of UV light (Figure 4.2). The results indicated that deletion of any one of

Table 4.3. Minimum Inhibitory Concentration (MIC) of *Echinacea* extracts with and without exposure to UV-light.

Echinacea species extract code	*S. cerevisiae* S288C MIC (mg/ml)	
	+ UV	Dark
E. purpurea		
SG1	4.5 ± 2.6	5.16 ± 2.6
SGEPR	2.28 ± 0.65	4.5 ± 2.6
SGEPH	0.84 ± 0.7	1.3 ± 0
SGEPF	2.12 ± 0.57	0.84 ± 0.7
SGEPLS	2.15 ± 0.69	4.0 ± 2.17
E. pallida* var. *angustifolia		
SG7a	1.76 ± 0	1.76 ± 0
SGEARa	0.55 ± 0.52	0.55 ± 0.52
SGEARb	1.3 ± 0	0.32 ± 0.03

Table 4.4. Mutants identified as sensitive to five or more Echinacea treatments.

([1] YPD, [2] SGD).

Gene Standard Name / Alias	Gene ID	Gene Function Description
1- GAS1 / *CWH52, GGP1*	YMR307W	1,3-β-glucanosyltransferase activity[1,2], cell wall assembly[2], deletion leads to increase chitin & calcofluor white sensitivity[2]
2- *KRE6 / CWH48*	YPR159W	β-1,6-glucan biosynthesis[1,2], integral to membrane[2]
3- *MNN10 / BED1, SLC2, REC41*	YDR245W	α-1,6-mannosyltransferase activity[1,2], deletion leads to increase chitin & calcofluor white sensitivity[2]
4- *HOC1*	YJR075W	α-1,6-mannosyltransferase activity[1,2], cell wall mannon integrity[1]
5- *BEM2 / IPL2, SUP9, TSL1*	YER155C	Rho GTPase activator activity[2], required for bud emergence[1,2] and cell cycle for cytoskeletal organization[1]
6- *OPI3 / PEM2*	YJR073C	Phospholipid methyltransferase[1,2], deletion leads to normal level chitin & calcofluor white sensitivity[2]
7- *CAX4 / CWH8*	YGR036C	Generation of cell wall mannoprotein[1], Pyrophosphatase activity intermediate synthesis and protein N-glycosylation[2]
8- *URM1*	YIL008W	Ubiquitin[1], protein tagging activity and required for normal growth
9- *AXL2*	YIL140W	Unknown, required for axial budding in haploid cells membrane[1,2]
10- *OPI9*	YLR338W	Unknown[1,2], deletion leads to increase chitin & calcofluor white sensitivity[2]
11- YPL264C	YPL264C	Unknown[1,2], integral to membrane[2]
12- YPR071W	YPR071W	Unknown[1,2], integral to membrane[2]
13- YDR455C	YDR455C	Unknown[1,2]
14- YLR402W	YLR402W	Unknown[1,2]
15- YPL182C	YPL182C	Unknown[1,2]
16- *WSS1*	YHR134W	Unknown, UV-sensitive mutant phenotype and possible DNA damage response element[2]
17- YAL056C-A/ YAL058C-A	YAL056C-A	Unknown
18- ARF1	YDL192W	GTPase activity, Golgi trafficking
19- SPT20 / ADA5	YOL148C	Subunit of the SAGA transcriptional regulatory complex[1,2],
20- PIG2	YIL045W	Protein phosphatase type 1 regulator activity[1,2]
21- SIN4 / BEL2, GAL22, SDI3,	YNL236W	RNA polymerase II transcription mediator activity[1,2]
22- PHO2/ BAS2, GRF10	YDL106C	Transcription factor of phosphate metabolism[1,2]
23- PRO2	YOR323C	Catalyzes the second step in proline biosynthesis[1,2]

Table 4.5. Selected deletion strains sensitive to *Echinacea* extracts.

ORFs	Cell wall org & syn [a]	Calc Wt sensitive [b]	Mannosyl transferase [c]	chitin [d]	budding [e]
YPR159W	+				
YER155C	+				
YJR075W	+		+		
YDR245W	+	+	+	+	
YMR307W	+	+		+	
YLR338W		+		+	
YJR073C		+			
YIL140W					+
YIL008W					+
P-value	6.9×10^{-5}	8.2×10^{-5}	2.2×10^{-4}	1.6×10^{-3}	4.2×10^{-3}
P-value rank	1	2	3	5	8

[a] cell wall organization and biogenesis
[b] decreased resistance to calcofluor white (systematic deletion)
[c] α-1, 6-mannosyltransferase complex
[d] increased levels of chitin (systematic deletion)
[e] cell budding

Table 4.6. Effects of Echinacea extract on colony numbers before and after sonication of *S. cerevisiae* strains and *Cryptococcus neoformans*. *S. cerevisiae* strains used S288C (wildtype) and YLR338W, a putative cell wall mutant that also shown sensitivity reduced growth in the presence of Echinacea. The *C. neoformans* strain used is a clinical isolate associated with mycotic infections.

Strain	Echinacea Conc. mg/ml	cells/ml ($x10^6$) at specified sonication times 0 min	2.15 min
S. cerevisiae	0	11.0	2.0
S. cerevisiae	6.4	2.0	0.01
YLR338W	0	3.0	0.2
YLR338W	4.2	2.0	< 0.001
C. neoformans	0	2.0	0.2
C. neoformans	6.4	1.0	0.002

these 23 genes tends to increase susceptibility to *E. purpurea* root extracts in dark or light treatments, or both. Furthermore, the MIC values were correlated with colony size reductions obtained from GDA analyses. This is evident by a significant negative slope (P =0.015) of the regression line, showing that the mutants with greater growth reduction in GDA experiments also tend to have the lowest MIC values (Figure 4.3). Interestingly, a tight correlation is observed for the subset of consensus mutants that were in the top 5% of genes inhibited based on GDA with SGEPR dark treatment (triangles in Figure 4.3). Figure 4.3 also shows that the cell wall defective mutants (numbered 1-10) have the lowest MIC values, further indicating that the cell wall may be highly susceptible to the inhibitory effects of Echinacea. The above MIC determinations also served to substantiate the quantitative results of GDA analyses.

4.3.4 *Sonication experiments to test cell wall effects of Echinacea extracts*

We assessed whether *Echinacea purpurea* 70% root extract perturbs yeast cell wall function using a sonication assay. For this purpose, we subjected yeast cells grown with or without SGEPR extract to sonication and subsequently monitored cell survival. These assays were done with *S. cerevisiae* wild type and a sensitive mutant, YLR338W, and *C. neoformans.* As shown in Table 4.6, all strains were ~100x more sensitive to sonication when grown with *E. purpurea* extract. These data support the hypothesis that yeast cell wall functions are compromised by Echinacea extracts.

Figure 4.2. Relative sensitivity to SGEPR based on MIC values of twenty-two out of 23 consensus mutants (YPR071W was not tested) strains identified as sensitive to Echinacea extracts in GDA screens. Strains are number as in Table 4.4 and the wildtype S288C strain is circled in green MICs were determined with (UV, triangles), and without (dark, circles) exposure to UV light. Plotted for each strain is the value, 1-(MIC mut – MICcon), where MICmut and MICcon are the mutant and S288C MIC values, respectively. Sensitive mutant strains, relative to S288C, are positioned above the horizontal line. Strains with normal sensitivity are on, or below the horizontal line.

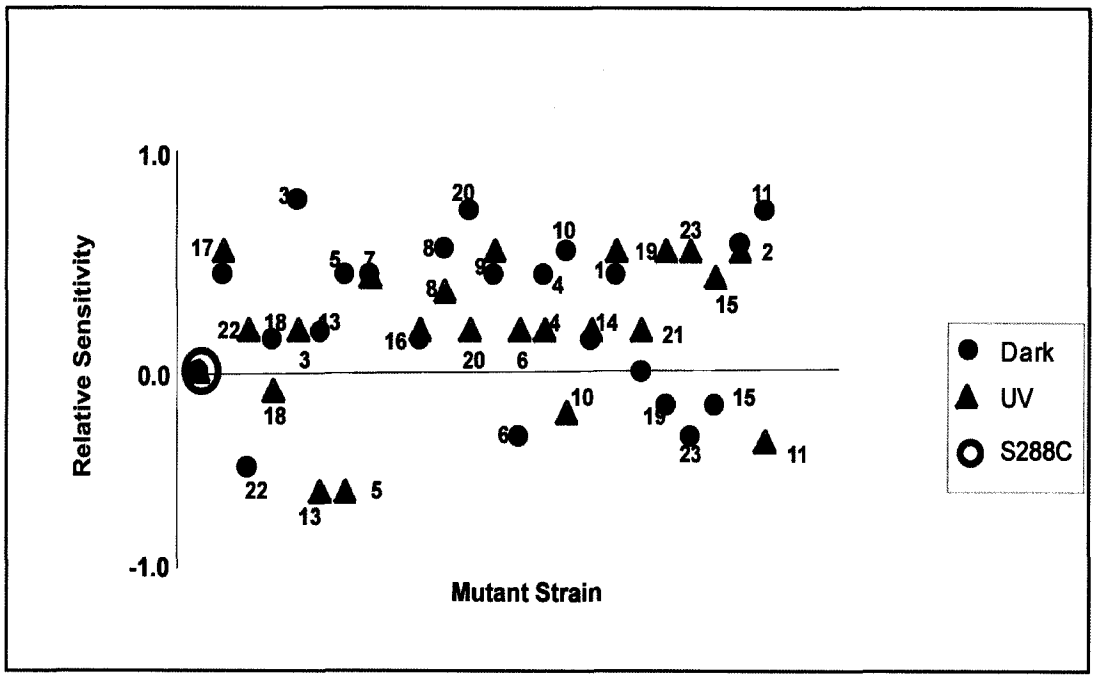
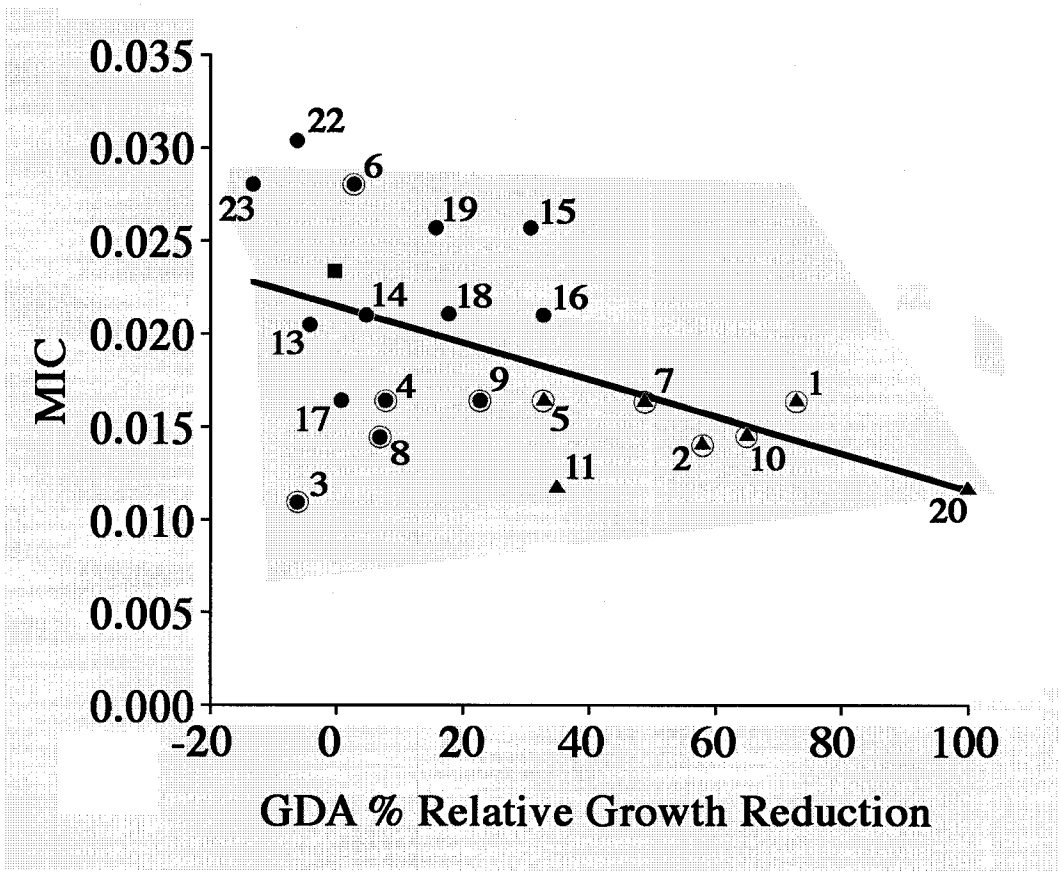


Figure 4.3. There is a significant negative slope to the correlation between MIC and GDA data for Echinacea extract SGEPR dark treatments (P = 0.015). Triangles represent mutants in the top 5% inhibited mutants based on GDA with SGEPR dark treatment. Circles are strains from the consensus group based on GDA but are not among those sensitive to SGEPR dark treatments. Numbers refer to mutants as listed in Table 4 and unlabeled square is S288C. Circled points are mutants deleted for cell wall associated genes. Twenty-one of 23 consensus mutants are shown (two, YPR071W and YNL236W were not tested).



4.4 Discussion

In this study we demonstrate antifungal activities in diverse ethanol extracts from *E. purpurea* and *E. pallida* var. *angustifolia*. A comparison of MIC assays conducted in the presence and absence of light supports earlier observations of phototoxic compounds in some these extracts (Merali *et al.* 2003; Hudson *et al.* 2005).

Previously, Towers *et al.* (1997) suggested that the increased UV-light-mediated inhibitory activity of such compounds is attributed to the addition of a singlet oxygen molecule and peroxidation of cell membrane in the target organism in the presence of UVA light. The herb fractions of *E. purpurea* also show significant antifungal activity in dark that may be attributed to the presence of chicoric acid, caffeic acid derivatives, and other compounds that do not require photo-induction (Vimalanathan *et al.* 2005). Previously, it was observed that ethanol extracts of *E. purpurea* flowers contained very low photosensitizing activity (Vimalanathan *et al.* 2005). It should be noted, however, that our studies indicate that antifungal activity of Echinacea preparations decreases within about 3 months after extraction as evident by increased concentrations required to inhibit growth in MIC assays, for example.

Using an ordered set of *S. cerevisiae* gene deletion mutants we also explored the molecular mechanism of antifungal activity of *Echinacea* in terms of identification of potential target proteins and pathways in yeast cells. From the susceptible mutants identified by GDA analyses, we selected a set of 23 mutants that were sensitive to five or more Echinacea extract treatments. The most significant pattern that was observed in this analysis was that at least nine of these supersensitive deletion mutants appear to have impaired cell wall functions. The fungal cell wall is a dynamic structure that plays an important role in protecting the cell from environmental stresses such as osmotic shock

and noxious chemicals. The cell wall is comprised mainly of four components; mannose covalently bound to protein components, together known as mannoproteins, ß1,3-glucans, ß1,6-glucans, and N-acetylglucosamine which forms chitin (Aguilar-Uscanga *et al.* 2003; Dallies *et al.* 1998; Cabib *et al.* 1991). The ß1,3- and ß1,6-glucans and chitin form the internal "skeletal" layer that is responsible for the rigid structure of the cell wall and mannoproteins form the outer protein layer that protects the fungal cell against enzymatic damage and acts as a barrier in response to environmental changes (Ram *et al.* 1994). Chitin and ß1,3-glucan are synthesized in the plasma membrane, whearas ß1,6-glucan is synthesized in the endoplasmic reticulum and golgi apparatus, with final processing accomplished extra-cellularly (Bussey 1991). Fungal cell wall synthesis mechanisms are tightly regulated at various stages of the cell cycle and are specifically activated in response to cell wall perturbing agents or mutation (Aguilar-Uscanga *et al.* 2003).

Table 4.4 lists five functions in order of *P*-value rank that, when compromised by mutation, result in increased sensitivity to Echinacea extracts. These likely involve different cell wall-associated processes such as organization and synthesis of the α-1,6-mannosyltransferase complex. The deletion strain *KRE6* is defective in β-1,6 glucan biosynthesis, a major structural component of cell wall, and was shown to have significantly reduced growth with at least 6 of the 16 Echinacea treatments. Kre6p is a golgi membrane protein and required for proper response to changing metabolic conditions (Bowen *et al.* 2004; Roemer *et al.* 1993). It was recently reported that strains with a Kre6p deficiency show reduction in ß-1,6-glucan synthase and decreased levels of alkaline soluble proteins in the *S. cerevisiae* cell wall (Bowen *et al.* 2004). Also, it was shown that mutations affecting Kre6p cause synthetic lethality when expressed in *gas1*

mutant cells (Tomishige *et al.* 2003). Note that *GAS1* was also identified in our set of 23 mutants. This synthetic lethality means that *KRE6* function is essential in providing normal ß-1,6-glucan synthesis when *gas1* mutant cells are defective in the processing of ß-1,3-glucan. *GAS1* encodes a *g*lycosyl*p*hosphatidyl*i*nositol (GPI)-anchored protein that is located in yeast plasma membrane (Vai *et al.* 1991). The disruption in *GAS1* shows leakage of ß-1,3-glucan into the medium, a decrease in growth rate, hypersensitivity to calcofluor white and an increase chitin content in the cell wall (Ram *et al.* 1998; Popolo *et al.* 1993; Vai *et al.* 1991). Our identification of both *GAS1* and *KRE6* mutants as sensitive suggests that a parallel cell wall associated pathway is effected by Echinacea extracts.

Mutant *cax4* was previously shown to have an anomalous cell wall defect in dolichyl pyrophosphate (Dol-P-P)-linked oligosaccharide intermediate synthesis and transmembrane protein *N*-glycosylation (van Berkel *et al.* 1999). *N*-linked glycosylation is a crucial modification for secretory proteins in eukaryotic cells and it was shown that the cell walls of most fungi contain *N*-glycosylated proteins (Ballou, 1990). In *S. cerevisiae*, Cax4p is required for addition of *N*-linked oligosaccharides to secretory proteins in the lumen of the rough endoplasmic reticulum (Fernandez *et al.* 2001). Also, studies by Ram *et al.* (1994) showed that *cax4* mutant cells grow slowly and have an alteration in the cell wall in which the amount of mannan is reduced to 50% of the wildtype level. In *S. cerevisiae,* most of the mannan in cell wall is *N*-glycosidically linked to wall protein (Klis 1994).

The identification of the two genes *MNN10* and *HOC1* among the most sensitive strains in the present study provides additional evidence that the fungal cell wall is one of the targets of Echinacea compounds. In yeast, the synthesis of the mannon backbone is

performed by two protein complexes in the golgi apparatus (Markovich *et al.* 2004). Mnn10p and Hoc1p and three other characterized proteins (Mnn11p, Mnn9p and Anp1p) are all components of the same structure (Jungmann *et al.* 1999). Deletion of Mnn10p results in defective mannan biosynthesis *in vivo* and up-regulation of other cell wall components, especially chitin (Markovich *et al.* 2004; Jungmann *et al.* 1999; Dallies *et al.* 1998). It has also been shown that *HOC1* encodes a subunit of a Golgi-localized complex in mannosyltranferase and Hoc1p has a regulatory function on mannan size (Neiman *et al.* 1997).

Cell wall integrity is likely to be critical during budding. Previous studies suggested that Bem2p, also listed in Table 4.4, is important for cytoskeleton organization as well as cell wall maintenance in yeast (Wang and Bretscher, 1995). In yeast, genes encoding Rho GTPases such as *RHO1*, which is activated by Bem2p, play an essential role in the regulation of cell wall synthesis and cytoskeleton organization including bud emergence and growth (Cabib *et al.* 1998; Yamochi *et al.* 1994). Interestingly, *BEM2* is involved in bud emergence and there is also a direct genetic interaction between *BEM2* and *RHO1* in the same pathway that regulates microfilament-mediated polarized cell growth (Wang and Bretscher, 1995).

Another important consideration is that Echinacea extracts affect some other functions that may indirectly cause alterations in cell wall synthesis. Of particular interest is the finding that deletion of the ubiquitin gene, *URM1,* results in increased sensitivity to Echinacea. Ubiquitin is a small modifier protein, which occurs in most eukaryotic cells, whose main function is to mark other proteins for proteolysis. It can also mark transmembrane proteins for removal from the membrane (Kawakami *et al.* 2001; Liakopoulos *et al.* 1998). In *S. cerevisiae*, ubiquitin-related protein conjugation is

essential for viability and plays a role in regulating septin ring formation on the mother cell side of the bud neck involved in cytokinesis (Johnson and Blobel, 1999). Intriguingly, it has also been shown that ubiquitin genes are involved in the regulation of IκBα, a regulatory protein in inflammatory response in both human and yeast cells (Desterro *et al.* 1998). Recently, Merali *et al.* (2003) showed that *Echinacea angustifolia* root extract has anti-inflammatory activity by inhibition of the 5-LOX enzyme. The 5-LOX enzyme is an inactive enzyme unless stimulate by proper cues (Bossu *et al.* 1999). This suggests a possible role for Echinacea may involve ubiquitination providing the required stimulation for the 5-LOX enzyme activation.

In addition to budding, it was shown that the Urm1p conjugation pathway (urmylation) has a potential role in oxidative stress response in yeast. Goehring *et al.* (2003) showed that the antioxidant protein Ahp1p is the first target for urmylation pathway through Urm1p conjugation to Ahp1p. Ahp1p (alkyl hydroperoxide reductase) is a thioredoxin peroxidase that plays an important role in protection of yeast cells against metal toxicity and glutathione depletion by reducing hydrogen peroxide and alkyl hydroperoxides (Nguyen-Nhu and Knoops, 2002). Oxidative stress is generated by toxic compounds and other reactive oxygen species that can damage cell constituents (Nguyen-Nhu and Knoops, 2002; Jamieson 1998). Therefore, our identification of the URM1 mutant as sensitive to Echinacea could relate to a compromised stress response, or to its association with budding.

Further investigation of the possibility that Echinacea extracts impair fungal cell wall functions was carried out on wild type S288C and the hypersensitive mutant, YLR338W, strains of yeast. The results provided evidence that significantly increased sonication-associated cell death occurred when either strain was grown in the presence of

Echinacea purpurea 70% root extract (Table 4.6). Similar results were obtained with a clinical isolate of the human pathogen *Cryptococcus neoformans*, a basidiomycete, yeast-like fungus. This suggests a direct effect by Echinacea extracts on the fungal cell wall in general. Ruiz *et al.* (1999) showed the usefulness of the sonication method in determining loss of cell wall integrity, and to isolate cell wall mutants and cell wall related genes on a large scale. Expanded use of this sonication approach may identify additional genes that contribute to cell wall function in the set of 9 "unknown functions mutants in Table 4.4. For example, at least two of these, YPL264C and YPR071W, are similar to integral membrane proteins and may therefore also be involved in cell wall processes.

These studies demonstrate the potential for the integration of GDA as a tool to screen for gene targets affected by natural-product-based bioactive compounds. Fungi are recognized as a sister taxon to animals. Being eukaryotes, they also share many biochemical and structural cellular features with plants. It is difficult to develop compounds that inhibit fungal growth that do not harm the plant or animal host. One of the defining characteristics of fungi, however, is that of the structure and makeup of the cell wall. The development of compounds that target fungal cell walls are attractive since these may offer a high degree of specificity to fungal pathogens. The indications from this work that the fungal cell wall is a major target of Echinacea is therefore of considerable interest.

CHAPTER 5

Summary and Conclusion

5.1 General Conclusion

In this thesis, we studied the mode of action of plant-derived antifungal compounds, the alkaloid berberine and those in Echinacea, by using DNA microarray and Gene Deletion Array (GDA) in *Saccharomyces cerevisiae.*

In Chapter 2, we studied the transcriptional regulation of yeast cells in response to berberine. The isoquinoline alkaloid berberine is a component of many important medicinal plants species such as *Berberidaceae* (Simeon *et al.* 1989; Manske *et al.* 1953). From this approach, we identified 106 yeast genes that showed a greater than 3-fold up- or down-regulation at the transcript levels upon exposure to minimum inhibitory concentration (MIC) of berberine for 2, 4, 5 and 6 hours. The 106 selected genes were then further restricted to a set of 55 genes by showing a constant 2-fold up- or down-regulation in at least two experiments, and were assigned to seven functional categories. In addition, we compared our gene regulation data to that with other antifungals, ketoconazole, amphotericin B, caspofungin and 5-fluorocytosine. This comparison suggested that the berberine response gene profile is distinct from differential gene expression patterns in yeast exposed to other antifungals. This indicates the berberine response and that specificity to different pathways are involved in berberine activity as compared to the other inhibitors. Taken together, the DNA microarray data predict several target genes are regulated upon berberine exposure and provide insights into what appear to be, multiple modes of action by the compound. The reduction of cytochrome P450 enzyme activity and repression of PDR genes suggests that berberine is an effective antifungal because, at least in part, it is not apparently recognized as a xenobiotic. Such characteristics may encourage complementary and alternative antifungal therapies based on berberine-containing phytomedicines.

In Chapter 3, we used a set of all nonessential *S. cerevisiae* strains with deletions, to investigate the chemical-genetic interaction profile of berberine. We hypothesized that in a chemical-genetic interaction a deletion mutant show hypersensitivity to sub-MIC levels of inhibitory compound and thus significantly reduced growth rate. In particular, chemical-genetic interactions can identify the pathway and targets inhibited by drug treatment at a genome-wide level. We identified a set of 195 sensitive mutant strains which were assigned in ten functional categories.

We employed HPLC to measure the intra- and intercellular berberine concentration in 14 of these sensitive mutant strains to find out whether there is a correlation between sensitivity and uptake patterns. The comparative analyses between 14 mutant strains to wildtype showed that (i) there are significant differences to patterns of berberine uptake/efflux and (ii) there is a correlation between sensitivity and uptake patterns. Also, in comparison with data derived from DNA microarrays only a group of nine genes were identified as most sensitive to berberine in GDA analysis. This lack of overlap in the two data sets can be due to the fact that the response of some of the 195 identified genes may be regulated post-transcriptionally. In addition, it is possible that differences in the experimental procedures (e.g. exposure times) in these two studies may account for some differences. Mostly, the lack of overlap in this data sets reflects the different, but complementary, aspects of these two arrays assays, as was discussed at the end of Chapter 3. Interestingly, the unknown function category in both, DNA microarray and GDA analysis assays, has the largest number of genes, indicating that a great deal of insight into functions of genes can be gained by these types of studies. In summary, the GDA analysis is a powerful genomic tool in screening potential targets of inhibitory compounds such as berberine like the microarray analysis, GDA revealed that berberine

has a complex and diverse mode of action and the results from these two genomic methods are comparable in this respect.

In Chapter 4, we examined the antifungal activity of eight different ethanolic extracts *Echinacea purpurea* (L.) Moench., *Echinacea pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist (syn. *E. angustifolia)* and *Echinacea pallida* (Nutt.) Nutt. var. *pallida* (syn. *E. pallida*) using GDA analysis. We also evaluated potential phototoxicity and light-independent toxicity for each extract for a total 16 treatments. A subset of 23 consensus mutants were identified from GDA data that were consistently sensitive to five or more different Echinacea treatments. These 23 consensus mutants are involved in 4 functional categories including cell wall function. In support of the hypothesis that Echinacea extracts interfere with fungal cell wall function, a follow-up experiment was done. The cell wall assay showed that yeast cells cultured in the presence of sub-inhibitory levels of Echinacea extracts were more sensitive to sonication than control cells. In conclusion, the overall profile of Echinacea mode of action indicates that fungal cell wall is one the major target of Echinacea. This is of particular interest, since development of compounds that target fungal cell walls are attractive because these may offer a high degree of specificity to fungal pathogens. This study provides a powerful means for integration of GDA as a genomic tool to screen for possible gene targets and inferring mechanism of action of Echinacea in order to add to the value of these bioactive compounds.

In summary, this thesis has provided a broad-based research into use of genomics and proteomics tools to investigate ethnopharmacology. This study also provides a template in medicinal plant research selected to the study of antimicrobial mode of action. DNA microarray and GDA analysis are complementary techniques that can be

used for the identification of possible mechanisms of action of herbal medicines and natural compounds. The future direction of genomic profiling of herbal products would greatly facilitate access to new antifungal compounds in natural environment and speed up the drug discovery process.

References

Abu-Elteen, KH. and Hamad, M. (2005). Antifungals agents for use in human therapy. Fungi, biology and applications. Editor, Kevin Kavanagh. John Wiley & Sons, Ltd. pp.171-190

Adams, A., Gottschling, DE., Kaiser, CA., and Stearns, T. (1997). Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 112

Agarwal, AK., Rogers, PD., Baerson, SR., Jacob, MR., Barker, KS., Cleary, JD., Walker, LA., Nagle, DG. And Clark, AM. (2003). Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifungal agents in *Saccharomyces cerevisiae*. *J Biol Chem.* 278(37):34998-35015

Alarco, AM. and Raymond M. (1999). The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J Bacteriol.* 181(3):700-708

Alexander, BD. and Perfect, JR. (1997). Antifungal resistance trends towards the year 2000. Implications for therapy and new approaches. Drugs. 54(5):667-678

Alexopoulos, CJ. And Mims, CW. (1979). Introductory Mycology. 3rd edition. John Wiley & Sons. pp. 1-5

Ames, BN., Shigenaga, MK. and Gold, LS. (1993). DNA lesions, inducible DNA repair, and cell division: three key factors in mutagenesis and carcinogenesis. Environ Health Perspect. 101 Suppl. 5:35-44

Amin, AH., Subbaish, TV., and Abbasi, KM. (1969). Berberine sulfate, Antimicrobial activity, bioassay, and mode of action. *Can J. Microbiol.* 15:1067-1076

Anderson, JB. (2005). Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nat Rev Microbiol.* 3(7):547-556

Andrade, AC., Del Sorbo, G., Van Nistelrooy, JG., De Waard, MA. (2000a). The role of ABC transporters from *Aspergillus nidulans* mediates resistance to all major classes of fungicides and some natural toxic compounds. Microbiology. 146(Pt 8):1987-1997

Andrade, AC., Van Nistelrooy, JG., Peery, RB., Skatrud, PL. and De Waard, MA. (2000b). The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. *Mol. Gen. Genet.* 263(6):966-977

Ausubel, FM., Brent, R., Kingston, RE., Moore, DD., Seidman, JG., Smith, JA. and Struhl, K. (2000). Current Protocol in Molecular Biology. Wiley & Sons, Inc. Vol.II

Awang, DVC. and Kindack, DG. (1991). Herbal medicine, Echinacea. Canadian Pharmaceutical Journal. 124:512–515

Baetz, K., McHardy, L., Gable, K., Tarling. T., Reberioux, D., Bryan, J., Anderson, RJ., Dunn, T., Hiete, P. and Roberge, M. (2004). Yeast genome-wide drug-induced haploinsufficiency screen to determine drug mode of action. *Proc Natl Acad Sci U S A.* 101(13):4525-4530

Ballou, C. (1990). Isolation, characterization, and properties of *Saccharomyces cerevisiae mnn* mutants with nonconditional protein glycosylation defects. *Methods Enzymol.* 185: 440-470

Balzi, F., Wang, M., Leterme, S., Van Dyck, L. and Goffeau A. (1994). *PDR5*, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator *PDR1*. *J Biol Chem.* 269(3):2206-2214

Bard, M., Lees, ND., Burrows, LA., and Kleinhans, FW. (1978). Differences in crystal violet uptake and cation-induced death among yeast sterol mutants. *J Bacteriol.* 135(3):1146-1148

Bauer, BE., Wolfger, H. and Kuchler, K. (1999). Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. *Biochimica et biophysica Acta.* 1461(2):217-236

Bauer, R. (1998). *Echinacea*: Biological effects and active principles. Phtomedicines of Europe: Chemistry and biological activity. In: Lawson, LD, Bauer, R. eds., *ACS Symposium Series* 691. Washington, DC, American Chemical Society. pp. 140-157

Bauer, R. and Wagner, H. (1991). *Echinacea* species as potential immunostimulatory drugs. Economic and Medicinal plant Research, 5th ed., Academic Press, London, pp. 253-321

Bauer, VR., Jurcic, K., Puhlmann, J. and Wagner, H. (1988). Immunologic *in vivo* and *in vitro* studies on Echinacea extracts. Arzneimittelforschung. 38(2):276-281

Bartram T. (1995). Encyclopedia of Herbal Medicine. (1st Edition) Grace Publishers, 161

Beggs, WH., Andrews, FW. and Sarosi, GA. (1981). Combined action of amphotericin B and 5-fluorocytosine on pathogenic yeasts susceptible to either drug alone. Chemotherapy. 27(4):247-251

Belenkiy, R., Haefele, A., Eisen, MB. and Wohlrab, H. (2000). The yeast mitochondrial transport proteins: new sequences and consensus residues, lack of direct relation between consensus residues and transmembrane helices, expression patterns of the transport protein genes, and protein-protein interactions with other proteins. *Biochim Biophys Acta.* 1467(1):207-218

Benzi G. and Moretti, A. (1995). Are reactive oxygen species involved in Alzheimer's disease? *Neurobiol Aging.* 16(4):661-674

Bergeron, C., Livesey, JF., Awang, DVC., Arnason, JT., Rana, J., Baum, BR. and Letchamo, WA. (2000). A quantitative HPLC method for the quality assurance of *Echinacea* products on the North America market. Phytochemical Analysis. 11:1-9

Binns, SE., Hudson, J., Merali, S. and Arnason, JT. (2002a). Anti-viral activity of characterized extracts from *Echinacea* spp. (Heliantheae: Asteraceae) against herpes simplex virus (HSV-1). *Planta Med.* 68:780-783

Binns, SE., Baum, BR. and Arnason, JT. (2002b). A taxonomic revision of the genus Echinacea (Heliantheae: Asteraceae). *Syst Bot.* 27:610-632

Binns, SE., Purgina, B., Bergeron, C., Smith, ML., Ball, L., Baum, BR. and Arnason, JT. (2000). Light-mediated antifungal activity of *Echinacea* extracts. *Planta Med.* 66(3):241-244

Birdsall, TC., and Kelly, GS. (1997). Berberine: Therapeutic potential of an alkaloid found in several medicinal plants. *Altern Med Rev.* 2(2):94-103

Boucher, PD., Im, MM., Freytag, SO. and Shewach, DS. (2006). A novel mechanism of synergistic cytotoxicity with 5-fluorocytosine and ganciclovir in double suicide gene therapy. *Cancer Res.* 66(6):3230-3237

Bova, S., Padrini, R., Goldman, WF., Berman, DM. and Cargnelli, G. (1992). On the mechanism of vasodilating action of berberine: possible role of inositol lipid signaling system. *J Pharmacol Exp Ther.* 261:318-323

Bowen, S. and Wheals, AE. (2004). Incorporation of sed1p into the cell wall of *Saccharomyces cerevisiae* involves *KRE6. FEMS Yeast Research.* 4:731-735

Bradley, PR. (1992). British herbal Compendium. British Herbal Medicine Association. Vol. 1. pp. 81.

Breitenbach, M., Crameri, R. and Lehrer, SB. (eds). (2002). Fungal Allergy and pathogenicity. Chemical Immunology. Basel: Karger. Vol. 81. pp. 310

Brevoort, P. (1998). The booming US botanical market. *HerbalGram* (American Herbal Council). 44:33-48

Broach, JR. and Deschenes RJ. (1990). The function of RAS genes in *Saccharomyces cerevisiae. Adv. Cancer Res.* 54:79-139

Brun, S., Berges, T., Poupard, P., Vauzelle-Moreau, C., Renier, G., Chabasse, D., and Bouchara, JP. (2004). Mechanisms of azole resistance in petite mutants of *Candida glabrata. Antimicrob Agents Chemother.* 48(5):1788-1796

Burnett, JH. (1987). Evolutionary biology of the fungi: Aspects of the Macro and Microevolution of the fungi. University press, Cambridge. pp. 340-341

Byers, B. (1981). Cytology of the Yeast Life Cycle. Strathern, JN., Jones, EW., and Broach, JR. (Eds.). The Molecular Biology of the Yeast Saccharomyces. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. pp. 59-96

Cabib, E., Roh, DH., Schmidt, M., Crotti, LB. and Varma, A. (2001). The yeast cell wall and septum as paradigms of cell growth and morphogenesis. Journal of Biological Chemistry. 276(23):19679-19682

Cabib, E., Drgonova, J. and Drgon, T. (1998). Role of small G proteins in yeast cell polarization and wall biosynthesis. *Annu Rev Biochem.* 67:307-333

Cabib, E., Sburlati, A., Bowers, B. and Silverman, SJ. (1989). Chitin synthase 1, an auxiliary enzyme for chitin sysnthesis in *Saccharomyces cerevisiae. J. Cell Biol.* 108:1665-1672

Chen, C., Chin, JE., Ueda, K., Clark, DP., Pastan, I., Gottesman, M. and Roninson, IB. (1986). Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. Cell. 47(3):381-389

Choi, D., Kim, S., Jung, MY. (2001). Inhibitory activity of berberine on DNA strand cleavage induced by hydrogen peroxide and cytochrome c. *Biosci Biotechnol Bichem.* 65(2): 452-455

Coker, PS. and Camper, D. (2004). Bioassays of *Echinacea* extracts and commercial products. Echinacea, the genus *Echinacea*. Medicinal and Aromatic Plants-industrial profiles. CRC press. New York. pp. 181-187

Cowen, LE., Anderson, JB. and Kohn, LM. (2002). Evolution of drug resistance in *Candida albicans*. *Annu Rev Microbiol.* 56:139-165.

Cox, PA. and Balick, MJ. (1994). The ethnobotanical approach to drug discovery. *Sci Am.* 270(6):82-87.

Cronquist, A. (1980). Vascular flora of the southeastern United States. Vol. 1. Asteraceae. The University of North Carolina Press, Chapel hill, NC. pp. 26

Cruz, MC., Goldstein, AL., Blankenship, JR., Del Poeta, M., Davis, D., Cardenas, ME., Perfect, JR., McCusker, JH. and Heitman, J. (2002). Calcineurin is essential for survival during membrane stress in *Candida albicans*. *EMBO J.* 21(4):546-559

Dalby-Brown, L., Barsett, H., Landbo, AK., Meyer, AS., Molgaard, P. (2005). Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on in vitro oxidation of human low-density lipoproteins. *J Agric Food Chem.* 53(24):9413-9423

Dallies, N., Francois, J. and Paquet, V. (1998). A new method for quantitative determination of polysaccharides in the yeast cell wall. Application to the cell wall defective mutants of *Saccharomyces cerevisiae*. Yeast. 14(14):1297-1306

DeMuri, GP. and Hostetter, MK. (1995). Resistance to antifungal agents. *Pediatr Clin North Am.* 42(3):665-685

DeRisi, J., van den Hazel, B., Marc, P., Balzi, E., Brown, P., Jacq, C. and Goffeau, A. (2000). Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. *FEBS Lett.* 470(2):156-160

DeRisi, JL., Iyer, VR., and Brown, PO. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. Science. 278(5338):680-686

Desterro, JM., Rodriguez, MS. and Hay, RT. (1998). SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Mol Cell.* 2(2):233-239

DiDomenico, B. (1999). Novel antifungal drugs. *Curr Opin Microbiol.* 2(5):509-515

Dighton, J. (2003). Fungi in Ecosystem Processes. Mycology series. Marcel Dekker Inc. Vol. 17. pp. 1-20

Dominguez, JM., Kelly, VA., Kinsman, OS., Marriott, MS., Gomez de las Heras, F., and Martin, JJ. (1998). Sordarins: A new class of antifungals with selective inhibition of the protein synthesis elongation cycle in yeasts. *Antimicrob Agents Chemother.* 42(9):2274-2278

Doorhan, F. (2003). Fungal pathogens of plants. Fungi, biology and applications. Editor, Kevin Kavanagh. John Wiley & Sons,Ltd. pp. 219-235

Dujon B. (1996). The yeast genome project: what did we learn? *Trends Genet.* 12:263-270

Dumontier, M., Green, JR., Golshani, A., Smith, ML., Mir-Rashed, N., Alamgir, MD., Eroukova,V., Dehne, F. and Cheetham, J. (2006). Identifying Significant Features Shared Among Yeast Proteins for Functional Genomics. Genome Biology. *(In press)*

Edlind, T., Smith, L., Henry, K., Katiyar, S. and Nickels, J. (2002). Antifungal activity in *Saccharomyces cerevisiae* is modulated by calcium signalling. *Mol Microbiol.* 46(1):257-268

Egner, R., Rosenthal, FE., Kralli, A., Sanglard, D. and Kuchler, K. (1998). Genetic separation of FK506 susceptibility and drug transport in the yeast Pdr5 ATP-binding cassette multidrug resistance transporter. *Mol Biol Cell.* 9(2):523–543

Emerson, LR., Skillman, BC., Wolfger, H., Kuchler, K. and Wirth, DF. (2004). The sensitivities of yeast strains deficient in PDR ABC transporters, to quinoline-ring antimalarial drugs. *Ann Trop Med Parasitol.* 98(6):643-649

Fernandez, F., Rush, JS., Toke, DA., Han, GS., Quinn, JE., Carman, GM., Choi, JY., Voelker, DR., Aebi, M. and Waechter, CJ. (2001). The *CWH8* gene encodes a dolichyl pyrophosphate phosphatase with a luminally oriented active site in the endoplasmic reticulum of *Saccharomyces cerevisiae*. *J Biol Chem.* 276(44):41455-41464

Ficker, CE., Arnason, JT., Vindas, PS., Alvarez, LP., Akpagana, K., Gbéassor, M., De Souza, C. and Smith, ML. (2003). Inhibition of human pathogenic fungi by ethnobotanically selected plant extracts. Mycoses. 46:29-37

Forsberg, H., Gilstring, CF., Zargari, A., Martinez, P. and Ljungdahl, PO. (2001). The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. Molecular Microbiology. 42(1):215-228

Foster, S. and Duke, J. (1990). A field guide to medicinal plants. Houghton Mifflin, Boston.

Fowell, RR. (1969). Sporulation and Hybridization of Yeasts. In Rose, AH. and Harrison, JS. (Eds.), London: Academic Press Inc. Ltd. pp. 303-383

Francois, IE., Aerts, AM., , BP. and Thevissen, K. (2005). Currently used antimycotics: spectrum, mode of action and resistance occurrence. *Curr Drug Targets.* 6(8):895-907

Gaber, RF., Kielland-Brandt, MD. and Fink, GR. (1990). *HOL1* mutations confer novel ion transport in *Saccharomyces cerevisiae. Mol Cell Biol.* 10(2):643-652

Gaber, RF., Styles, IA. and Fink, GR. (1988). *TRK1* encodes a plasma membrane protein required for high-affinity potassium transport in *Saccharomyces cerevisiae Mol Cell Biol.* 8(7):2848-2859

Gilmore, A. (1911). Uses of plants by the Indians of Missouri River region. *Bur Am Ethnol Annu Rep.* 33:368

Gilmore, A. (1913). A study in the ethnobotany of the Omaha Indians. *Coll Neb State Hist Soc.* 17:314-357

Georgopapadakou, NH. (2002). Infectious disease 2001: drug resistance, new drugs. *Drug Resist Updat.* (5):181-191

Georgopapadakou, NH. (1998). Antifungals: mechanism of action and resistance, established and novel drugs. *Curr Opin Microbiol.* 1(5):547-557

Georgopapadakou, NH. and Walsh, TJ. (1994). Human mycoses: drugs and targets for emerging pathogens. Science. 264:371-373

Ghannoum, MA. and Rice, LB. (1999). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clinical Microbiology reviews. 12(4):501-517

Goel, V., Chang, C., Slama, J., Barton, R., Bauer, R., Gahler R. and Basu, T. (2002). Echinacea stimulates macrophage function in the lung and spleen of normal rats. *J Nutr Biochem.* 13(8):487

Goehring, AS., Rivers, DM. and Sprague, GF. Jr. (2003). Urmylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Mol Biol Cell.* 14(11):4329-4341

Goehring, AS., Rivers, DM., Sprague and GF, Jr. (2003). Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. *Eukaryot Cell.* 2(5):930-936

Goffeau, A., Barrell, BG., Bussey, H., Davis, RW., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, JD., Jacq, C., Johnston, M., Louis, EJ., Mewes, HW., Murakami, Y., Philippsen, P., Tettelin, H. and Oliver, SG. (1996). Life with 6000 genes. Science. 274(5287):546, 563-567

Goswami, RS. and Corby Kistler, H. (2004). Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology. 5(6):515-525

Gros, P., Talbot, F., Tang-Wai, D., Bibi, E. and kaback, HR. (1992). Lipophilic Cations: a group of model substrates for the mulidrug-resistance transporter. Biochemistry. 3(7):1992-1998

Gudima, SO., Memelova, LV., Borodulin, VB., Pokholok, DK., Mednikov, BM., Tolkachev, ON. and Kochetkov, SN. (1994). Kinetic analysis of interaction of human immunodeficiency virus reverse transcriptase with alkaloids. *Mol Biol* (Mosk). 28(6):1308-1314

Grover, A. and Gowthaman, G. (2003). Strategies for development of fungus-resistant transgenic plants. Current Science. 84(3):330-340

Gupta, AK., Schouten, JR. and Lynch, LE. (2005). Ciclopirox nail lacquer 8% for the treatment of onychomycosis: a Canadian perspective. *Skin Therapy Lett.* 10(7):1-3

Hamon, NW. (1990). Herbal medicine: Goldenseal. Canadian Pharmaceutical Journal 123:508-510

Hart, JA. (1981). The ethnobotany of the Northern Cheyenne Indians of Montana *J Ethnopharmacol.* 4(1):1-55

Hawksworth, DL. (2005). The biodiversity of fungi and its human relevance. Biodiversity of fungi, their role in human life. Science Publishers, Inc. pp. 3-11

Hernandez, S., Lopez-Ribot, JL., Najvar, LK., McCarthy, DI., Bocanegra, R. and Graybill, JR. (2004). Caspofungin resistance in *Candida albicans*: correlating clinical outcome with laboratory susceptibility testing of three isogenic isolates serially obtained from a patient with progressive *Candida esophagitis*. *Antimicrob Agents Chemother.* 48(4):1382-1383

Hirata, D., Yano, K., Miyahara, K. and Miyakawa, T. (1994). *Saccharomyces cerevisiae YOR1,* which encodes a member of the ATP-binding cassette (ABC) superfamily, is required for multidrug resistance. *Curr Genet.* 26(4):285-294.

Hobbs, C. (1994). HerbalGram. 30:34-47

Hobbs, C. (1990). Echinacea: The immune herb, Botanica Press, Santa Cruz, CA.

Hoffman, HL. And Rathbun, RC. (2002). Review of the safety and efficacy of voriconazole. *Expert Opinion Investig Drugs.* 11:409-429

Howard, DH. and Miller, JD. (eds). (1996). Human and animal relationships. *Mycota.* Berlin: Springer. Vol. VI. pp. 399

Hsieh, P., Siegel, SA., Rogers, B., Davis, D. and Lewis, K. (1998). Bacteria lacking a multidrug pump: a sensitive tool for drug discovery. *Proc Natl Acad Sci USA.* 95(12):6602-6606

Hudson, J., Vimalanathan, S., Kang, L., Treyvaud Amiguet, V., Livesey, J. and Arnason, JT. (2005). Characterization of antiviral activities in *Echinacea* root preparations. *Pharmaceutical Biology.* 43(9): 790-796

Hudson, JB., Graham, EA., Lam, J. and Towers, GH. (1991). Ultraviolet-dependent biological activities of selected polyines of the asteraceae. *Planta Med.* 57(1):69-73

Hughes, TR. (2002). Yeast and drug discovery. *Funct Integr Genomics.* 2(4-5):199-211

Hughes, TR. *et al.* (2000). Functional discovery via a compendium of expression profiles. Cell. 102(1):109-126

Hui, KK., Yu, JL., Chan, WFA. (1989). Interaction of berberine with human platelet alpha-2 adrenoceptors. *Life Sci.* 49:315-324

Inbaraj, JJ., Kukielczak, BM., Bilski, P., Sandvik, SL. and Chignell, CF. (2001). Photochemistry and photocytotoxicity of alkaloids from Goldenseal (*Hydrastis canadensis* L.) 1. Berberine. *Chem Res Toxicol.* 14(11):1529-1534

Jamieson, DJ. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae.* Yeast. 14(16):1511-1527

Jensen-Pergakes, KL., Kennedy, MA., Lees, ND., Barbuch, R., Koegel, C. and Bard, M. (1998). Sequencing, disruption, and characterization of the *Candida albicans* sterol methyltransferase (*ERG6*) gene: drug susceptibility studies in erg6 mutants. Antimicrobiol agents and Chemotherapy. 42(5):1160-1167

Johnson, ES. and Blobel, G. (1999). Cell cycle-regulated attachment of the ubiquitin-related protein SUMO to the yeast septins. *J Cell Biol.* 147(5):981-994.

Johnston, SD., Enomoto, S., Schneper, L., McClellan, MC., Twu, F., Montgomery, ND., Haney, SA., Broach, JR. and Berman, J. (2001). *CAC3* (*MSI1*) Suppression of $RAS2^{G19V}$ is independent of chromatin assembly factor I and mediated by *NPR1*. Molecular and Cellular Biology. 21(5): 1784-1794

Jones, NP., Arnason, JT., Abou-Zaid, M., Akpagana, K., Sanchez-Vindas, P. and Smith, ML. (2000). Antifungal activity of extracts from medicinal plants used by First Nations Peoples of eastern Canada. *J. Ethnopharmacol.* 73(1-2):191-198.

Jungmann, J., Rayner, JC. and Munro, S. (1999). The *Saccharomyces cerevisiae* protein Mnn10p/Bed1p is a subunit of a Golgi mannosyltransferase complex. *J Biol Chem.* 274(10):6579-6585

Jutila, A., Rytömaa, M. and Kinnunen, PK. (1998). Detachment of cytochrome *c* by cationic drugs from membranes containing acidic phospholipids: Comparison of Lidocaine, Propranolol, and Gentamycin. Molecular Pharmacology. 54:722-732

Katzmann, DJ., Hallstrom, TC., Voet, M., Wysock, W., Golin, J., Volckaert G. and Moye-Rowley, WS. (1995). Expression of an ATP-binding cassette transporter-encoding gene (YOR1) is required for oligomycin resistance in *Saccharomyces cerevisiae.* Molecular and Cellular Biology. 15(12):6875-6883

Kaur, R., Castano, I. and Cormack, BP. (2004). Functional genomic analysis of fluconazole susceptibility in the pathogenic yeast *Candida glabrata*: roles of calcium signaling and mitochondria. *Antimicrob Agents Chemother.* 48(5):1600-1613

Kavanagh, K. Fungi: Biology and Applications. (2005). Wiley & Sons, Ltd. pp. 191-217

Kawakami T, Chiba T, Suzuki T, Iwai K, Yamanaka K, Minato N, Suzuki H, Shimbara N, Hidaka Y, Osaka F, Omata M, Tanaka K. (2001). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J.* 20(15):4003-4012

Kelly, SL., Lamb, DC., Baldwin, BC., Corran, AJ. and Kelly, DE. (1997b). Characterization of *Saccharomyces cerevisiae CYP61*, sterol delta22-desaturase, and inhibition by azole antifungal agents. *J Biol Chem.* 272(15):9986-9988

Klis, FM. (1994). Review: cell wall assembly in yeast. Yeast. 10(7):851-869.

Kerridge, D., Fasoli, M. and Wayman, FJ. (1988). Drug resistance in *Candida albicans* and *Candida glabrata. Ann N Y Acad Sci.* 544:245-259

Kluck, RM., Bossy-Wetzel, E., Green, DR., and Newmeyer, DD. (1997). The release of cytochrome *c* from mitochondria: a primary site for *Bcl-2* regulation of apoptosis. Science. 275:1132–1136

Ko, CH., Buckley, AM. and Gaber, RF. (1990). *TRK2* is required for low affinity K+ transport in *Saccharomyces cerevisiae*. Genetics. 125(2):305-312

Kowluru, R., Kern, TS. and Engerman, RL. (1994). Abnormalities of retinal metabolism in diabetes or galactosemia II. Comparison of gamma-glutamyl transpeptidase in retina and cerebral cortex, and effects of antioxidant therapy. *Curr Eye Res.* 13(12):891-896

Kutchan, TM. (1998). Molecular genetics of plant alkaloid biosynthesis. In: Cordell, G (ed) The alkaloids, Vol 50. Academic, San Diego, pp. 257-316

Leber, R., Fuchsbichler, S., Klobucnikova, V., Schweighofer, N., Pitters, E., Wohlfarter, K., Lederer, M., Landl, K., Ruckenstuhl, C., Hapala, I. and Turnowsky, F. (2003). Molecular mechanism of terbinafine resistance in *Saccharomyces cerevisiae. Antimicrob Agents Chemother.* 47(12):3890-3900

Lees, ND., Lofton, SL., Woods, RA. and Brad, M. (1980). The effects of varied energy and detergent on the growth of sterol mutants of *Saccharomyces cerevisiae. J Gen Microbiol.* 118:209-214

Lees, ND., Bard, M., Kemple, MD., Haak, RA. And Kleinhans, FW. (1979). ESR determination of membrane order parameter in yeast sterol mutants. *Biochim Biophys Acta.* 553(3):469-475

Lenschow, DJ., Walunas, TL. and Bluestone, JA. (1996). CD28/B7 system of T cell costimulation. *Annu Rev Immunol.* 14:233-258

Letchamo, W., Livesey, J., Arnason, JT., Bergeron, C. and Krutilina, VS. (1999). Cichoric acid and isobutylamide content in *Echinacea purpurea* as influenced by flower

developmental stages. Prespectives on new crops and new uses. J. Janick (ed.), ASHS Press, Alexandria, VA.

Lewis, K. and Klibanov, AM. (2005). Surpassing nature: rational design of sterile-surface materials. Trends in Biotechnology. 23(7):343-348

Lewis, K. (1999). Multidrug resistance: Versatile drug sensors of bacterial cells. *Curr Biol.* 9(11):R403-407

Lewis, WH. (1992). Plants used medically by indigenous people. In: Nigg, HN., Seigler D., eds. Phytochemical Resources for Medicine and Agriculture, Plenum Press, New York. pp. 33-74

Li, XK., Motwani, M., Tong, W., Bornmann, W. and Schwartz, GK. (2000). Huanglian, A Chinese herbal extract, inhibits cell growth by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells. *Mol Pharmacol.* 58(6):1287-1293

Liakopoulos, D., Doenges, G., Matuschewski, K. and Jentsch S. (1998). A novel protein modification pathway related to the ubiquitin system. *EMBO J.* 17(8):2208-2214

Lin, S., Tsai, SC., Lee, CC., Wang, BW., Liou, JY. and Shyu, KG. (2004). Berberine inhibits HIF-1alpha expression via enhanced proteolysis. *Mol Pharmacol.* 66(3):612-619

Lubelsky, Y., Reuven, N. and Shaul, Y. (2005). Autorepression of *Rfx1* gene expression: Functional conservation from yeast to humans in response to DNA replication arrest. *Mol Cell Biol.* 25(23):10665-10673

Lupetti, A., Danesi, R., van 't Wout, JW., van Dissel, JT., Senesi, S. and Nibbering, PH. (2002). Antimicrobial peptides: therapeutic potential for the treatment of Candida infections. 11(2):309-318

Lupetti, A., Danesi, R., Campa, M., Del Tacca, M. and Kelly, S. (2002). Molecular basis of resistance to azole antifungals. *Trends Mol Med.* 8(2):76-81

Maeng, HJ., Yoo, HJ., Kim, IW., Song, IS., Chung, SJ. and Shim, CK. (2002). P-glycoprotein-mediated transport of berberine across Caco-2 cell monolayers. Journal of Pharmaceutical Sciences. 91(12):2614-2621

Maesaki, S., Marichal, P., Hossain, MA., Sanglard, D., Vanden Bossche. H. and Kohno, S. (1998). Synergic effects of tactolimus and azole antifungal agents against azole-resistant *Candida albican* strains. *J Antimicrob Chemother.* 42(6):747-753

Mahady, GB. and Chadwick, LR. (2001). Goldenseal (*Hydrastis Canadensis*): Is there enough scientific evidence to support safety and efficacy? *Nutr Clin Care.* 4(5): 243-249

Manske, RHF. and Holmes, HL.(1953). The Alkaloids. volume III. New York: Academic Press. pp. 432

Marchetti, O., Moreillon, P., Entenza, JM., Vouillamoz, J., Glauser, MP., Bille, J. and Sanglard, D. (2003). Fungicidal synergism of fluconazole and cyclosporine in *Candida albicans* is not dependent on multidrug efflux transporters encoded by the *CDR1, CDR2, CaMDR1*, and *FLU1* genes. *Antimicrob Agents Chemother.* 47(5):1565-1570

Markovich, S., Yekutiel, A., Shalit, I., Shadkchan, Y. and Osherov, N. (2004). Genomic approach to identification of mutations affecting caspofungin susceptibility in *Saccharomyces cerevisiae. Antimicrob Agents Chemother.* 48(10):3871-3876

Martzen, MR., McCraith, SM., Spinelli, SL., Torres, FM., Fields, S., Grayhack, EJ. and Phizicky, EM. (1999). A biochemical genomics approach for identifying genes by the activity of their products. Science. 286(5442):1153-1155

Matsumori, N., Yamaji, N., Matsuoka, S., Oishi, T. and Murata, M.(2002). Amphotericin B covalent dimers forming sterol-dependent ion-permeable membrane channels. *J Am Chem Soc.* 124(16):4180-4181

Memarian, N., Alirezaie, J., Jessulat, M., Mir-Rashed, NH., Xu, J., Smith, ML. and Golshani, A. (2007). Colony size measurement of the yeast gene deletion strains for functional genomics. *BMC Bioinformatics.* (In press)

Merali S., Binns S., Paulin-Levasseur, M., Ficker C., Smith M.L., Baum B., Brovelli E. and Arnason J.T. (2003). Antifungal and anti-inflammatory activity of the genus *Echinacea.* Pharmaceutical Biology. Vol. 41. pp. 1-9

Mewes, HW., Albermann, K., Bahr, M., Frishman, D., Gleissner, A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, SG., Pfeiffer, F. and Zollner, A. (1997). Overview of the yeast genome. Nature. 387(6632 Suppl):7-65

Mikes, V. and Dadak, V. (1983). Berberine derivatives as cationic fluorescent probes for the investigation of the energized state of mitochondria. *Biochim Biophys Acta.* 723(2):231-239

Modak, S., Modak, MJ., and Venkataraman, A. (1970). Mechanism of action of berberine on *Vibrio cholerae* and *vibrio cholerae* biotype *eltor. Indian J Med Res.* 58 (11):1510-1522

Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, WA., Diaquin, M., Popolo, L., Hartland, RP. and Latge, JP. (2000). Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. *J Biol Chem.* 275(20):14882-14889

Murray, M, and Pizzorno, J. (1998). Encyclopaedia of Natural medicine. 2nd edition. Little, Brown and Company. pp.160

Nair, PN., Golden, T. and Melov, S. (2003). Microarray workshop on aging. *Mech Ageing Dev.* 124(1):133-138

Nakazawa, T., Horiuchi, H., Ohta, A. and Takagi, M. (1998). Isolation and characterization of *EPD1,* an essential gene for pseudohyphal growth of a dimorphic yeast, *Candida maltosa. J Bacteriol.* 180(8):2079-2086

Neiman, AM., Mhaiskar V., Manus, V., Galibert, F. and Dean, N. (1997). *Saccharomyces cerevisiae HOC1*, a suppressor of pkc1, encodes a putative glycosyltransferase. Genetics. 145(3):637-645

Nelissen, B., De Wachter R. and Goffeau, A. (1997). Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae. FEMS Microbiol. Rev.* 21(2):113-134

Nguyen-Nhu, NT. and Knoops, B. (2002). Alkyl hydroperoxide reductase 1 protects *Saccharomyces cerevisiae* against metal ion toxicity and glutathione depletion. *Toxicol Lett.* 135(3):219-228

Omar, S., Lemonnier, B., Jones, N., Ficker, C., Smith, ML., Neema, C., Towers, GH., Goel, K. and Arnason, JT. (2000). Antimicrobial activity of extracts of eastern North American hardwood trees and relation to traditional medicine. *J. Ethnopharmacol.* 73(1-2):161-170

Oliveira, EM., Martins, AS., Carvajal, E. and Bon, EP. (2003). The role of the GATA factors Gln3p, Nil1p, Dal80p and the Ure2p on *ASP3* regulation in *Saccharomyces cerevisiae.* Yeast. 20:31-37

Panwar, SL., Krishnamurthy, S., Gupta, V., Alarco, AM., Raymond, M., Sanglard, D. and Prasad, R. (2001). *CaALK8*, an alkane assimilating cytochrome P450, confers

multidrug resistance when expressed in a hypersensitive strain of *Candida albicans*. Yeast. 18(12):1117-1129

Park, KS., Kang, KC., Kim, JH., Adams, DJ., Johng, TN. And Paik YK. (1999). Differential inhibitory effects of protoberberines on sterol and chitin biosynthesis in *Candida albicans*. Journal of Antimicrobial Chemotherapy. 43:667-674

Parsons, AB., Brost, RL., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, GW., Kane, PM., Hughes, TR. and Boone, C. (2004). Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nat Biotechnol.* 22(1):62-69

Parsons, AB., Geyer, R., Hughes, TR., Boone, C. (2003). Yeast genomics and proteomics in drug discovery and target validation. *Prog Cell Cycle Res.* 5:159-166

Pasqual, MS., Lauer, CP., Moyna, P. and Henriques, AP. (1993). Genotoxicity of the isoquinoline alkaloid berberine in prokaryotic and eukaryotic organisms. Mutation Research. 286:243-252

Percival, SS. (2000). Use of Echinacea in medicine. *Biochem Pharmacol.* 15; 60(2):155-158

Piedras, P., Aguilar, M. and Pineda, M. (1998). Uptake and metabolism of allantoin and allantoate by cells of *Chlamydomonas reinhardtii* (Chlorophyceae). *Eur. J. Phycol.* 33(1):57-64

Pinner, RW., Teutsch, SM., Simonsen, L., Klug, LA., Graber, JM., Clarke, MJ., and Berkelman, RL. (1996). Trends in infectious diseases mortality in the United States. *JAMA.* 275(3):189-193

Pitt, JI. and Hocking, AD. (1997). Fungi and Food Spoliage. 2nd Edition. Clasgow: Blackie Academic and Professional. pp. 608

Popolo, L., Vai, M., Gatti, E., Porello, S., Bonfante, P., Balestrini, R. and Alberghina, L. (1993). Physiological analysis of mutants indicates involvement of the *Saccharomyces cerevisiae* GPI-anchored protein gp115 in morphogenesis and cell separation. *J. Bacteriol.* 175(7):1879-1885

Posteraro, B., Sanquinetti, M., Sanglard, D., La Sorda, M., Boccia, S., Romano, L., Morace, G. and Fadda, G. (2003). Identification and characterization of a *Cryptococcus neoformans* ATP binding cassette (ABC) transporter-encoding gene, *CnAFR1*, involved in the resistance to fluconazole. *Mol. Microbiol.* 47(2):357-371

Prasad, R. and Kapoor, K. (2005). Multidrug Resistance in Yeast Candida. *Int Rev Cytol.* 242:215-248

Rabbani, GH., Butler, T., Knight, J., Sanyal, SC., and Alam, K. (1987). Randomized controlled trial of berberine sulfate therapy for diarrhea due to enterotoxigenic *Escherichia coli* and *Vibrio cholerae*. *J Infect Dis.* 155(5):979-984

Radix Echinaceae from WHO Herbal Monograph, (1999) http://www.herbmed.org/Herbs/Herb6.htm#Category30Herb6

Ram, AF., Wolters, A., Ten Hoopen, R. and Klis, FM. (1994). A new approach for isolating cell wall mutants in Saccharomyces cerevisiae by screening for hypersensitivity to calcofluor white. Yeast. 10(8):1019-1030

Ram, AF., Kapteyn, JC., Montijn, RC., Caro, LH., Douwes, JE., Baginsky, W., Mazur, P., van den Ende, H. and Klis, FM. (1998). Loss of the plasma membrane-bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of beta1,3-glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. *J Bacteriol.* 180(6):1418-1424

Romani, L. (2000). Innate and adaptive immunity in *C. albicans* infections and saprophytism. *J Leukoc Biol.* 68(2):175-179. Review.

Romani, L. (1999). Cytokine modulation of specific and nonspecific immunity to *C. albicans*. Mycoses. 42(Suppl 2):45-48. Review.

Romer, T., Delaney, S. and Bussey, H. (1993). *SKN1* and *KRE6* define a pair of functional homologs encoding putative membrane proteins involved in ß-Glucan synthesis. Molecular and Cellular Biology. 13(7):4039-4048

Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., and Borner, C. (1998). Bcl-2 prolongs cell survival after Bax-induced release of cytochrome *c*. Nature (Lond.). 391:496–499

Ruiz, C., Cid, VJ., Lussier, M., Molina, M. and Nombela, C. (1999). A large-scale sonication assay for cell wall mutant analysis in yeast. Yeast. 15(10B):1001-1008

Sabir, M., Akhter, MH. and Bhide, NK. (1978). Further studies on pharmacology of berberine. *Indian J Physiol Pharmacol.* 22:9-23

Saccharomyces Genome Deletion Project; *http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html*

Sambrook, J., Fritsch, EF., Maniatis, T. (1989). Molecular cloning, a laboratory Manual. Second edition. Cold Spring harbor Laboratory Press.

Sanglard, D. and Odds, FC. (2002). Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis.* 2(2):73-85

Sanglard, D. (2002). Resistance of human fungal pathogens to antifungal drugs. *Curr Opin Microbiol.* 5(4):379-385

Saporito-Irwin, SM., Birse, CE., Sypherd, PS. And Fonzi, WA. (1995). *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. *Mol Cell Biol.* 15(2):601-613

Saxena, D., Kannan, KB. and Brandriss, MC. (2003). Rapamycin treatment results in GATA factor-independent hyperphosphorylation of the proline utilization pathway activator in *Saccharomyces cerevisiae.* Eukaryotic Cell. 2(3):552-559

Schultes, RE. (1994). Amazonian ethnobotany and the search for new drugs. *Ciba Found Symp.* 185:106-12; discussion 112-115

Schumann, GL. (1991). Plant diseases: Their Biology and Social Impact. St paul, MN: APS press. pp. 397

Scott, JG. (1999). Cytochrome P450 and insecticide resistance. Insect Biochemistry and Molecular Biology. 29:757-777

Sheldon, WJ., Balick, MJ. and Laird, SA. (1997). Medicinal plants: can utilization and conservation coexist? The New York Botanical Garden, Bronx, NY. pp. 104

Semighini, CP., Marins, M., Goldman, MH. and Goldman, GH. (2002). Quantitative analysis of the relative transcript levels of ABC transporter *Atr* genes in *Aspergillus nidulans* by real-time reverse transcription-PCR assay. *Appl Environ Microbiol.* 68:1351-1375

Shemluck, M. (1982). Medicinal and other uses of the Compositae by Indians in the United States and Canada. *J Ethnopharmacol.* 5(3):303-358

Sherman, F. (1998). An introduction to the genetics and molecular biology of the yeast *S. cerevisiae.* Modified from Yeast genetics. The Encyclopedia of Molecular Biology and Molecular Medicine. Edited by Meyers, RA. VCH Pub., Weinheim, Germany. Vol. 6. pp.302-325

Simeon, S., Rios, JL. and Villar A. (1989). Pharmacologoical activities of protoberberine alkaloids plant. *Med. Phytother.*, XXIII, 202-250

Skinner, FA., Passmore, S. and Davenport, RR. (1980). Biology and activities of yeasts. The Society for Applied Bacteriology. Symposium series no. 9. Academic Press INC.

Small, E. and Catling, PM. (1999). Canadian Medicinal Crops. NRC Research Press. pp. 46

Smith, SJ. and Parks, LW . (1993). The *ERG3* gene in *Saccharomyces cerevisiae* is required for the utilization of respiratory substrates and in heme-deficient cells. Yeast. 9(11):1177-1187

Smyth, GK. and Speed, T. (2003). Normalization of cDNA microarray data. Methods. 31(4):265-273

Sofowora, A. (1982). Medicinal plants and traditional medicine in Africa. John Wiley & Sons,Ltd. pp. 88-107

Stanbrough, M., Rowen, DW. and Magasanik, B. (1995). Role of the GATA factors Gln3p and Nil1p of *Saccharomyces cerevisiae* in the expression of nitrogen-regulated genes. *Proc.natl.Acad. Sci. USA.* 92(21): 9450-9454

Stermitz, FR., Lorenz, P., Tawara, JN., Zenewicz, LA. and Lewis, K. (2000). Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. *Proc Natl Acad Sci USA.* 97(4):1433-1437

St-Germain, G., Laverdiere, M., Pelletier, R., Bourgault, AM., Libman, M., Lemieux, C. and Noel, G. (2001). Prevalence and antifungal susceptibility of 442 *Candida* isolates from blood and other normally sterile sites: results of a 2-year (1996 to 1998) multicenter surveillance study in Quebec, Canada. *J Clin Microbiol.* 39(3):949-953

Sullivan, D., Moran, G. and Coleman, D. (2005). Fungal diseases of human. John Wilely & Sons,Ltd. pp. 171-190

Taglicht, D. and Michaelis, S. (1998). *Saccharomyces cerevisiae* ABC proteins and their relevance to human health and disease. *Methods Enzymol.* 292:130-162

Taylor, N. (1965). Plant drugs that changed the world. George Allen & Unwin Ltd., London.

Terao, T., Hisanaga, E., Sai, Y., Tamai, I. and Tsuji, A. (1996). Active secretion of drugs from the small intestinal epithelium in rats by P-glycoprotein functioning as an absorption barrier. *J Pharm Pharmacol.* 48:1083–1089.

Timothy, C., Birdsall, ND., Gregory, S. and Kelly, ND. (1997). Berberine: Therapeutic Potential of an Alkaloid Found in Several Medicinal Plants. *Alt Med Rev.* 2(2):94-103

Tomishige, N., Noda, Y., Adachi, H., Shimoi, H., Takatsuki, A. and Yoda, K. (2003). Mutations that are synthetically lethal with a gas1Δ allele cause defects in the cell wall of *Saccharomyces cerevisiae. Mol Genet Genomics.* 269(4):562-573

Tong, AH. *et al.* (2004). Global Mapping of the Yeast Genetic Interaction Network. Science. 303(5659):808-813

Tong, AH. *et al.* (2001). Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science. 294(5550):2364-2368

Towers, GHN., Page, JE. and Hudson, JB. (1997). Light-mediated biological activities of natural products from plants and fungi. *Curr Org Chem.* 1:395-414

Trease, GE. and Evans, WC. (1978). Pharmacognosy, 11th edn. Bailliere Tindall, London.

Tsai, CS. and Ochillo, RF. (1991). Pharmacological effects of berberine on the longitudinal muscle of the guinea-pig isolated ileum. *Arch Int Pharmacodyn Ther.* 310:116-131

Turner, WW. and Current, W. (1997). *In:*Srohl, WR. ed. Biotechnology of antibiotics. New York. Marcel Dekker Inc. pp. 315-334

Tyler, VE. And Brady, LR. (1988). Pharmacogonosy. Philadelphia. Lea & Febiger. pp. 475-476

Tyler, VE. (1993). Phytomedicines in Western Europe: Potential impact on herbal medicine in the United States, in AD. Klinghorn and MF. Balandrin, Eds., Human Medicinal Agents from Plants, Oxford University Press, Oxford, 25-37

Udagawa, S. and Yaguchi, T. (2005). Neosartorya (Eurotiales): taxonomy and significance in applied mycology. Biodiversity of fungi, their role in human life. Science Publishers, Inc. pp. 348

Van Berkel, MAA., Rieger, M., te Hessen, S., Ram, AF., van den Ende, H., Aebi, M. and Klis, FM. (1999). The *Saccharomyces cerevisiae CWH8* gene is required for full levels of dolichol-linked oligosaccharides in the endoplasmic reticulum and for efficient *N*-glycosylation. Glycobiology. 9(3):243-253

Vai, M., Gatti, E., Lacana, E., Popolo, L. and Alberghina, L. (1991). Isolation and deduced amino acid sequence of the gene encoding gp115, a yeast glycophospholipid-anchored protein containing a serine-rich region. *J Biol Chem.* 266(19):12242-12248

Vanden Bossche, H. and Koymans, L. (1998). Cytochromes P450 in fungi. Mycoses. 41 Suppl 1:32-38

Vanden Bossche, H., Willemsens, G. and Marichal, P. (1987). Anti-Candida drugs--the biochemical basis for their activity. *Crit Rev Microbiol.* 15(1):57-72

Vazquez, D. (1964). Uptake and binding of chloramphenicol by sensitive and resistant organisms. Nature. 203:257-258

Verpoorte, R., Van der Heijden, R. and Memelink, J. (2000). Engineering the plant cell factory for secondary metabolite production. Transgenic Research. 9:323-343

Vickers, MF., Yao, SYM., Baldwin, SA.,Young, JD. and Cass, CE. (2000). Nucleoside transporter proteins of *Saccharomyces cerevisiae*. Demonstration of a transporter (*FUI1*) with high uridine selectivity in plasma membranes and a transporter (*FUN26*) with broad nucleoside selectivity in intracellular membranes. *J Biol Chem.* 25;275(34):25931-25938.

Vimalanathan, S., Kang, L., Amiguet, V. T., Livesey, J. Arnason, J.T. and Hudson, J. (2005). *Echinacea purpurea* aerial parts contain multiple anti-viral compounds. *Pharmaceutical Biology.* 43(9):740-745

Wacker, A. and Hilbig, A. (1978). Virus inhibition by *Echinacea purpurea. Planta Medica.* 33(1):89-102

Wagner, R., de Montigny, J., de Wergifosse, P., Souciet, JL., and Potier, S. (1998). The ORF YBL042C of *S. cerevisiae* encodes a uridine permease. *FEMS Microbiol. Lett.* 159:69-75

Wang, YX. and Zheng, YM. (1997). Ionic mechanism responsible for prolongation of cardiac action-potential duration by berberine. *J Cardiovasc Pharmacol.* 30(2):214-222

Wang, T. and Bretscher, A. (1995). The rho-GAP encoded by *BEM2* regulates cytoskeletal structure in budding yeast. *Mol Biol Cell.* 6(8):1011-1024

Welihinda, AA., Beavis, AD. and Trumbly, RJ. (1994). Mutations in *LIS1* (*ERG6*) gene confer increased sodium and lithium uptake in *Saccharomyces cerevisiae*. *BBA.* 1198:107-117

White, TC., Marr, KA. and Bowden, RA. (1998). Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin Microbiol Rev.* 11(2):382-402

White, TC. (1997). The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14alpha demethylase in *Candida albicans*. *Antimicrob Agents Chemother.* 41(7):1488-1494

WHO Herbal Monograph. (1999). Radix Echinaceae from WHO Herbal Monograph, http://www.herbmed.org/Herbs/Herb6.htm#Category30Herb6

Winzeler, EA. *et al.* (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science. 285(5429):901-906

Wolfgar, H., Mamnun,YM. and Kuchler K. (2004). The yeast Pdr15p ATP-binding cassette (ABC) protein is a general stress response factor implicated in cellular detoxification. *J Biol Chem.* 279(12):11593-11599

Wolfger, H., Mamnun, YM. and Kuchler, K. (2001). Fungal ABC proteins: Pleiotropic drug resistance, stress response and cellular detoxification. *Res. Microbiol.* 152(3-4):375-389

Yamochi, W., Tanaka K., Nonaka, H., Maeda, A., Musha, T., Takai, Y. (1994). Growth site localization of *Rho1* small GTP-binding protein and its involvement in bud formation in *Saccharomyces cerevisiae. J Cell Biol.* 125(5):1077-1093

Yang,YL., Lin, YH., Tsao, MY., Chen, CG., Shih, HI., Fan, JC., Wang, JS. and Lo, HJ. (2006). Serum repressing efflux pump *CDR1* in *Candida albicans. BMC Molecular Biology.* 7:22

Yang, HT. and Wang, GJ. (2003). Transport and uptake characteristics of a new derivative of berberine (CPU-86017) by human intestinal epithelial cell line: Caco-2. *Acta Pharmacol Sin.* 24(12):1185-1191

Yang, YH., Dudoit, S., Luu, P., Lin, DM., Peng, V., Ngai, J. and Speed, TP. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res.* 30(4):e15

Yang, J., Liu, X., Bhalla, K., Kim, CN., Ibrado, AM., Cai, J., Peng, TI., Jones, DP. and Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. Science. 275:1129–1132

Yao, R., Zhang, Z., An, X., Bucci, B., Perlstein, DL., Stubbe, J. and Huang, M. (2003). Subcellular localization of yeast ribonucleotide reductase regulated by the DNA replication and damage checkpoint pathways. *PNAS.* 100(11):6628-6633

Zaman, GJ., Flens, MJ., van Leusden, MR., de Haas, M., Mulder, HS., Lankelma, J., Pinedo, HM., Scheper, RJ., Baas, F., Broxterman, HJ. and Borst, P. (1994). The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc Natl Acad Sci U S A.* 91(19):8822-8826

Zhang, K., and Zhao, H. (2000). Assessing reliability of gene clusters from gene expression data. *Funct Integr Genomics.* 1(3):156-173

APPENDIX: I

	ORF	Gene	Gene Regulation			
			2 h	4 h	5 h	6 h
1	YAL031C	*GIP4*	0.5	-0.338	1.707	-0.216
2	YAL032C	*PRP45*	0.774	1.236	1.947	0.879
3	YAL046C		0.739	0.494	1.067	2.604
4	YAL051W	*OAF1*	-0.657	-1.386	-1.516	-1.707
5	YAL060W	*BDH1*	0.031	0.746	0.99	2.618
6	YAL063C	*FL09*	0.725	1.443	1.574	1.788
7	YAL064W		0.074	0.843	0.252	2.195
8	YAR023C		0.997	1.799	-0.392	-0.066
9	YAR071W	*PHO11*	-1.371	-1.514	-1.797	-0.533
10	YBL001C	*ECM15*	0.526	0.883	1.166	2.413
11	YBL011W	*SCT1*	0.149	-0.001	0.132	2.436
12	YBL015W	*ACH1*	-0.256	0.26	0.661	2.981
13	YBL059W		-0.525	-0.197	0.122	2.093
14	YBL063W	*KIP1*	0.027	0.604	0.426	2.627
15	YBL066C	*SEF1*	-0.973	-0.993	-1.363	-1.656
16	YBR009C	*HHF1*	0.637	1.078	1.136	1.753
17	YBR010W	*HHT1*	0.692	1.802	1.403	1.766
18	YBR027C		-0.352	0.818	0.357	3.088
19	YBR071W		0.267	0.513	0.042	1.676
20	YBR090C		0.297	0.846	0.917	2.957
21	YBR133C	*HSL7*	0.338	0.744	0.468	1.724
22	YBR137W		0.415	0.949	1.191	1.833
23	YBR153W	*RIB7*	0.256	0.872	1.01	2.655
24	YBR193C	*MED8*	2.341	0.127	0.369	-0.15
25	YBR213W	*MET8*	-0.881	-1.075	-1.672	-1.396
26	YBR216C	*YBP1*	0	0.42	0.678	3.044
27	YBR229C	*ROT2*	-1.393	-1.8	-2.099	-2.15
28	YBR256C	*RIB5*	1.038	1.652	1.953	1.453
29	YBR264C	*YPT10*	0.114	0.287	0.756	2.64
30	YBR296C	*PHO89*	-2.028	-1.434	-1.478	-1.173
31	YCL023C		-0.134	-0.239	0.5	2.031
32	YCR020C-A	*MAK31*	0.919	0.567	1.421	1.902
33	YCR063W	*BUD31*	0.062	-0.307	0.19	2.038
34	YCRX20C	*Has been deleted from SGD*	0.117	-0.356	0.782	2.284
35	YDL015C	*TSC13*	0.145	0.104	0.432	1.884
36	YDL127W	*PCL2*	-0.118	-0.358	0.07	1.605
37	YDR010C		0.065	0.293	0.086	1.91
38	YDR022C	*CIS1*	0.167	-0.158	-0.885	2.8
39	YDR035W	*ARO3*	1.204	2.097	1.619	1.7

40	YDR036C	*EHD3*	-0.356	-1.471	-1.899	-1.595
41	YDR070C	*FMP16*	0.004	-0.181	0.425	2.542
42	YDR090C		0.311	0.344	0.141	1.839
43	YDR134C		0.483	1.001	1.449	1.814
44	YDR158W	*HOM2*	2.161	0.947	1.227	1.294
45	YDR197W	*CBS2*	0.33	-0.216	0.512	2.102
46	YDR227W	*SIR4*	-0.833	-2.34	-1.702	-1.707
47	YDR325W	*YCG1*	0.078	-0.043	0.306	2.579
48	YDR389W	*SAC7*	0.026	0.011	0.633	2.271
49	YDR444W		-1.047	-1.704	-1.84	-2.092
50	YDR486C	*VPS60*	0.418	1.202	1.638	1.017
51	YDR521W		-0.402	-0.337	-0.292	2.219
52	YEL009C	*GCN4*	0.445	1.252	1.635	1.575
53	YEL035C	*UTR5*	-0.213	-0.79	-0.24	2.748
54	YEL039C	*CYC7*	0.125	0.444	0.829	1.772
55	YER018C	*SPC25*	0.39	-0.366	0.129	2.551
56	YFL014W	*HSP12*	1.222	0.228	0.658	2.085
57	YFL021W	*GAT1*	-0.842	-1.408	-1.803	-2.112
58	YGL014W	*PUF4*	-1.726	-2.157	-2.736	-3.415
59	YGL088W		-0.134	-0.14	-1.327	-1.812
60	YGL102C		-0.002	0.871	0.958	1.848
61	YGL207W	*SPT16*	-0.809	-0.789	-1.347	-1.606
62	YGR027C	*RPS25A*	0.351	0.591	0.369	1.632
63	YGR199W	*PMT6*	-1.16	-1.91	-2.106	-2.419
64	YGR279C	*SCW4*	1.662	0.619	0.828	1.281
65	YHR003C		1.657	0.665	0.749	0.618
66	YHR016C	*YSC84*	1.116	1.384	0.916	1.797
67	YHR018C	*ARG4*	1.546	1.277	1.812	1.259
68	YHR065C	*RRP3*	1.736	0.645	0.859	0.63
69	YHR071W	*PCL5*	0.904	2.687	2.63	1.964
70	YHR215W	*PHO12*	-1.301	-2.204	-2.216	-2.181
71	YIL026C	*IRR1*	-1.384	-1.271	-2.028	-1.709
72	YIL066C	*RNR3*	-1.581	-2.011	-2.487	-2.782
73	YIL097W	*FYV10*	1.722	0.462	0.159	0.023
74	YIR034C	*LYS1*	1.345	1.64	1.657	1.437
75	YJL057C	*IKS1*	-0.065	0.708	0.265	1.675
76	YJL181W		-0.138	0.201	0.278	1.921
77	YJL185C		0.317	0.726	0.745	1.612
78	YJL196C	*ELO1*	1.919	0.649	0.855	0.842
79	YJL200C		1.634	0.706	0.693	0.678
80	YJR025C	*BNA1*	0.705	1.715	1.35	1.505
81	YJR109C	*CPA2*	2.597	2.173	2.17	1.324
82	YJR130C	*STR2*	0.793	0.634	1.884	1.24
83	YJR152W	*DAL5*	0.145	0.28	-0.566	1.727
84	YKL049C	*CSE4*	0.418	0.471	0.789	3.165

85	YKR015C		-0.075	0.226	1.041	2.317
86	YKR019C	*IRS4*	0.1	0.543	0.589	1.64
87	YKR079C		0.037	0.446	0.858	1.886
88	YLL037W		0.249	0.733	0.312	2.068
89	YLR110C	*CCW12*	1.188	1.219	1.626	1.829
90	YLR162W		-2.121	-2.51	-2.831	-2.709
91	YLR249W	*YEF3*	0.666	-0.012	1.641	0.241
92	YLR259C	*HSP60*	-0.654	-1.265	-1.176	-1.949
93	YLR382C	*NAM2*	-1.527	-2.356	-2.665	-2.568
94	YLR398C	*SKI2*	-1.228	-1.157	-1.705	-1.637
95	YLR454W		-2.063	-2.475	-2.854	-3.406
96	YML045W		0.016	0.274	1.737	0.444
97	YMR181C		0.523	0.798	0.888	1.91
98	YMR185W		0.144	0.222	0.025	1.619
99	YNL298W	*CLA4*	0.023	0.11	-0.028	1.6
100	YNR038W	*DBP6*	1.662	0.612	0.994	0.639
101	YOR039W	*CKB2*	-0.08	0.648	0.541	1.646
102	YOR083W	*WHI5*	-0.651	-0.533	-0.655	-1.779
103	YOR290C	*SNF2*	-1.577	-2.262	-2.686	-2.855
104	YOR393W	*ERR1*	-1.005	-1.191	-1.795	-1.344
105	YPL188W	*POS5*	0.529	1.036	1.808	0.896
106	YPR080W	*TEF1*	-0.095	1.223	1.039	1.666
107	YPR149W	*NCE102*	1.803	0.689	0.515	1.678

APPENDIX: II

	ORF	Gene	GDA % Growth Reduction	Category
1	YAL014C	*SYN8*	11	T
2	YAL059W	*ECM1*	25	CW
3	YBL007C	*SLA1*	26	CW
4	YBL027W	*RPL19B*	18	GE
5	YBL042C	*FUI1*	55	T
6	YBL048W		12	UN
7	YBL055C		20	UN
8	YBL086C		13	UN
9	YBL106C	*SR077*	8	T
10	YBR025C		14	UN
11	YBR168W	*PEX32*	28	CELL.ORG
12	YBR195C	*MSI1*	14	CC
13	YBR199W	*KTR4*	14	GE
14	YBR218C	*PYC2*	17	M
15	YBR232C		33	UN
16	YBR244W	*GPX2*	9	STRESS
17	YBR245C	*ISW1*	14	GE
18	YBR298C	*MAL31*	15	T
19	YCL011C	*GBP2*	12	CELL.ORG
20	YCL012W	*BUD3*	16	MATING
21	YCL023C		14	UN
22	YCR030C	*SYP1*	14	UN
23	YCR033W	*SNT1*	14	GE
24	YDL037C	*BSC1*	8	UN
25	YDL075W	*RPL31A*	16	GE
26	YDL090C	*RAM1*	6	GE
27	YDL091C	*UBX3*	16	UN
28	YDL106C	*PHO2*	17	GE
29	YDL187C		13	UN
30	YDL214C	*PRR2*	22	OTHER
31	YDL234C	*GYP7*	16	T
32	YDR010C		23	UN

33	YDR024W	*FYV1*	27	UN
34	YDR125C	*ECM18*	16	CW
35	YDR197W	*CBS2*	25	GE
36	YDR231C	*COX20*	23	M
37	YDR253C	*MET32*	15	GE
38	YDR346C	*SVF1*	12	STRESS
39	YDR369C	*XRS2*	10	MATING
40	YDR387C		8	UN
41	YDR395W	*SXM1*	14	T
42	YDR501W	*PLM2*	24	CC
43	YDR504C	*SPG3*	16	UN
44	YDR513W	*GRX2*	46	STRESS
45	YDR524C	*AGE1*	18	T
46	YDR530C	*APA2*	20	M
47	YEL041W	*YEF1*	15	M
48	YEL067C		11	UN
49	YER020W	*GPA2*	10	OTHER
50	YER047C	*SAP1*	15	UN
51	YER068W	*MOT2*	36	MATING
52	YFL021W	*GAT1*	20	GE
53	YFL056C	*AAD6*	7	STRESS
54	YFR008W	*FAR7*	17	MATING
55	YGL013C	*PDR1*	48	GE
56	YGL034C		17	UN
57	YGL127C	*SOH1*	11	GE
58	YGL173C	*KEM1*	29	GE
59	YGL255W	*ZRT1*	18	T
60	YGR001C		11	UN
61	YGR008C	*STF2*	17	STRESS
62	YGR052W	*FMP48*	41	UN
63	YGR070W	*ROM1*	15	CW
64	YGR177C	*ATF2*	16	M
65	YGR178C	*PBP1*	24	GE
66	YGR230W	*BNS1*	11	MATING
67	YGR240C	*PFK1*	14	M
68	YGR249W	*MGA1*	15	CC

69	YGR275W	*RTT102*	24	GE
70	YGR281W	*YOR1*	45	T
71	YHR008C	*BUB2*	11	CC
72	YHR029C	*YHI9*	18	UN
73	YHR031C	*RRM3*	14	CC
74	YHR039C	*MSC7*	22	MATING
75	YHR077C	*NMD2*	24	GE
76	YHR138C		38	UN
77	YHR146W	*CRP1*	16	UN
78	YHR153C	*SPO16*	34	MATING
79	YIL001W		24	UN
80	YIL023C	*YKE4*	18	UN
81	YIL035C	*CKA1*	17	CC
82	YIL039W		33	UN
83	YIL097W	*FYV10*	27	M
84	YIL103W	*DPH1*	16	GE
85	YIL112W	*HOS4*	25	GE
86	YIL134W	*FLX1*	11	T
87	YIL141W		31	UN
88	YIL153W	*RRD1*	13	CC
89	YIR029W	*DAL2*	13	M
90	YIR036C		40	UN
91	YIR039C	*YPS6*	25	UN
92	YJL073W	*JEM1*	28	MATING
93	YJL080C	*SCP160*	12	MATING
94	YJL084C		21	UN
95	YJL089W	*SIP4*	14	GE
96	YJL095W	*BCK1*	23	OTHER
97	YJL126W	*NIT2*	49	UN
98	YJL129C	*TRK1*	40	CELL.ORG
99	YJL137C	*GLG2*	17	M
100	YJL145W	*SFH5*	13	T
101	YJL146W	*IDS2*	15	MATING
102	YJL181W		15	UN
103	YJL190C	*RPS22A*	15	GE
104	YJR021C	*REC107*	10	MATING

105	YJR039W		37	UN
106	YJR055W	*HIT1*	18	UN
107	YJR079W		16	UN
108	YJR082C	*EAF6*	45	UN
109	YJR149W		31	UN
110	YJR153W	*PGU1*	25	OTHER
111	YKL091C		22	UN
112	YKL124W	*SSH4*	11	UN
113	YKL150W	*MCR1*	16	M
114	YKL177W		41	UN
115	YKL178C	*STE3*	16	MATING
116	YKL187C		16	UN
117	YKL201C	*MMN4*	15	STRESS
118	YKL221W	*MCH2*	14	T
119	YKR030W	*GMH1*	11	T
120	YKR069W	*MET1*	42	GE
121	YKR080W	*MTD1*	16	M
122	YLL018C-A	*COX19*	27	T
123	YLR018C	*POM34*	22	T
124	YLR032W	*RAD5*	17	STREE
125	YLR044C	*PDC1*	18	M
126	YLR049C		16	UN
127	YLR110C	*CCW12*	16	CW
128	YLR165C	*PUS5*	27	GE
129	YLR176C	*RFX1*	17	STRESS
130	YLR287C		17	UN
131	YLR320W	*MMS22*	36	STRESS
132	YLR348C	*DIC1*	15	T
133	YLR373C	*VID22*	18	GE
134	YLR389C	*STE23*	36	M
135	YLR431C	*ATG23*	16	T
136	YLR450W	*HMG2*	55	M
137	YLR456W		27	UN
139	YML006C	*GIS4*	20	OTHER
140	YML008C	*ERG6*	52	M
141	YML010W	*SPT5*	17	GE

142	YML012W	*ERV25*	15	T
143	YML068W	*ITT1*	16	GE
144	YML078W	*CPR3*	27	OTHER
145	YMR021C	*MAC1*	30	GE
146	YMR029C	*FAR8*	32	MATING
147	YMR055C	*BUB2*	38	CC
148	YMR086C-A		13	UN
149	YMR127C	*SAS2*	11	GE
150	YMR138W	*CIN4*	18	CELL.ORG
151	YMR144W		12	UN
152	YMR157C	*FMP39*	16	UN
153	YMR166C		34	T
154	YMR189W	*GCV2*	25	GE
155	YMR224C	*MRE11*	26	GE
156	YMR295C		12	UN
157	YNL012W	*SPO1*	11	MATING
158	YNL052W	*COX5A*	49	M
159	YNL071W	*LAT1*	15	M
160	YNL108C		8	M
161	YNL123W	*NMA111*	18	M
162	YNL197C	*WHI3*	38	CC
163	YNL217W		34	UN
164	YNL228W		15	UN
165	YNL264C	*PDR17*	36	T
166	YNL283C	*WSC2*	18	CW
167	YNL311C	*SKP2*	16	GE
168	YNR008W	*LRO1*	18	M
169	YNR014W		35	UN
170	YNR036C		13	GE
171	YNR040W		12	UN
172	YNR064C		19	STRESS
173	YOL029C		19	UN
174	YOR039W	*CKB2*	19	CC
175	YOR061W	*CKA2*	20	CC
176	YOR073W	*SGO1*	17	MATING
177	YOR133W	*EFT1*	21	GE

178	YOR135C	*IRC14*	12	UN
179	YOR140W	*SFL1*	10	GE
180	YOR165W	*SEY1*	30	CELL.ORG
181	YOR265W	*RBL2*	16	CELL.ORG
182	YOR286W	*FMP31*	49	UN
183	YOR290C	*SNF2*	17	GE
184	YOR359W	*VTS1*	10	T
185	YPL042C	*SSN3*	32	MATING
186	YPL097W	*MSY1*	10	GE
187	YPL174C	*NIP100*	41	CC
188	YPL202C	*AFT2*	12	STRESS
189	YPL232W	*SSO1*	16	T
190	YPL264C		12	UN
191	YPR018W	*RLF2*	17	GE
192	YPR030W	*CSR2*	23	CW
193	YPR060C	*ARO7*	10	GE
194	YPR065W	*ROX1*	35	GE
195	YPR191W	*QCR2*	33	M