Abstract

Fungal infections are caused by a variety of fungi, and with a variety of clinical manifestations. Antifungal treatments are limited due to host toxicity and fungi gaining resistance. By utilizing the model organism *Saccharomyces cerevisiae*, we hope to elucidate the molecular mechanisms of fungal pathogenesis that we can then validate in the human pathogen *Candida albicans*, as well as explore options for novel therapies.

Small molecule signaling is a method by which single-cell organisms can communicate with one another, enabling them to coordinate gene expression. This is a useful tool because it allows microbes to turn on phenotypes that are only valuable when done in large numbers, such as bioluminescence, or virulence traits. We have previously shown that the yeast *Saccharomyces cerevisiae* synthesizes the secondary metabolite indole-3-acetic acid (IAA) from tryptophan. IAA is secreted into the environment, where it acts as a signal. At low concentrations, the IAA signals yeast to induce virulence traits, while at high concentrations, it is lethal.

The purpose of this thesis was to investigate the molecular mechanism of IAA (plant hormone auxin) regulation in fungi, specifically, *Saccharomyces cerevisiae* and the human pathogen *Candida albicans*. Towards this end, I first focused my efforts on evaluating the role of *S. cerevisiae* Grr1, as a putative IAA receptor. By evaluating the IAA response of several Grr1 mutants, I was able to show that the leucine-rich repeat region, while not required for function, likely plays a significant role in maintaining the structural integrity of the protein. Next, I evaluated IAA associated phenotypes, such as filamentation, surface adhesion and IAA uptake of *grr1Δ/Δ* mutant in the human pathogen *Candida albicans*. Together, these data support the hypothesis that GRR1 regulates IAA response, probably by regulating the IAA uptake carriers.

Table of Contents

List of Figures

List of Tables

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Introduction

Fungal Pathogenesis

Fungal diseases are a worldwide public health issue. The illnesses caused by fungi can range from superficial (athlete's foot), to deadly (blood stream infections). The three general classes of infections that the Mycotic Diseases branch of the Centers for Disease Control is concerned about are opportunistic infections, community acquired infections, and hospital acquired infections [1].

Opportunistic infections refer to infections that do not generally harm healthy individuals, but can cause massive damage to immunocompromised patients, including those with AIDS, individuals undergoing chemotherapy, or organ recipients. Some opportunistic infections are cryptococcal meningitis (caused by *C. neoformans*) and aspergilliosis (lung infection caused by *Aspergillus* species). Cryptococcal meningitis is one of the leading causes of death in HIV patients, and is a serious problem for other immunocompromised populations, while Aspergilliosis can be especially difficult in people with cystic fibrosis [1].

The second class is community acquired infections. These generally are caused by fungal spores that reside in soil. When the soil is disrupted, people inhale the spores, resulting in diseases such as blastomycosis, histoplasmosis, and coccidiomycosis (valley fever) [1]. Blastomycosis is caused by *Blastomyces dermatitidis* spores. Typically, the spores remain inactive in the host. Histoplasmosis is caused by *Histoplasma capsulatum* spores, and typically affects the lungs. This can be a severe problem for immunocompromised individuals, such as AIDS patients. Valley fever is caused by spores from *Coccidioides* species. The result is pneumonia, which is self-limiting. Overall, current antifungal drugs are capable of treating most infections in this class.

The last class is hospital acquired infections. The fourth most common cause of hospital-acquired infections is *Candida* species in the bloodstream (bloodstream infection) [2]. *Candida albicans* is the most prevalent fungal pathogen of humans. It is responsible for common clinical problems including oral thrush and vaginitis, but can also lead to life-threatening systemic infections in immunocompromised individuals, such as AIDS patients, resulting in 30-50% mortality rates. Overall, in the US alone, there are estimated to be about 10,000 deaths per year due to *Candida* infections. The estimated annual cost of treating nosocomial *Candida* infections exceeds $1 billion per year [3]. While several species of *Candida* are responsible for the deadly bloodstream infections, *C. albicans* is responsible for almost half (46%) [2]. For these reasons, we have chosen to focus our investigation of *C. albicans*.

Antifungal Agents

Treating fungal infection poses a unique challenge as fungi are eukaryotes. As such, they have much more in common with humans than the prokaryotic bacteria that colonize humans to cause infection, making finding drug targets substantially more difficult. Currently, anti-fungal drugs target fungus-specific pathways, in order to attack the fungi and not the human host. One of the major differences between fungal and human cells is the presence of a cell wall (Figure 1). The current drugs available to treat *Candida* infections in humans generally target the cell
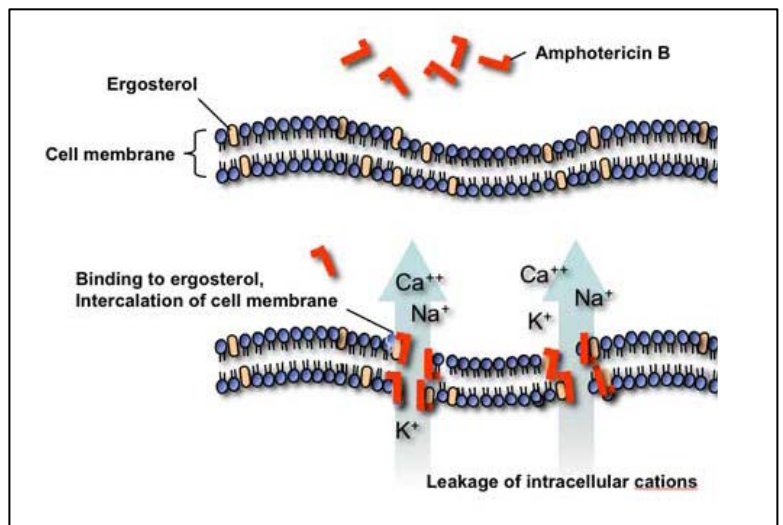

Figure 1: Fungal cell membrane and cell wall. Fungal-specific features include beta- (1,6) glucan, beta-(1,3)-glucan, chitin, ergosterol and squalene. (Image Courtesy of www.doctorfungus.org © 2005)

wall, or membrane, and can be classified into 4 main categories, based on their mechanism of action;

polyenes, azoles, echinocandins and allylamines.

(i) Polyenes

Polyenes, such as nystatin and amphotericin B, work by associating with ergosterol, and creating a porous channel through the cell wall (Figure 2). The result is that the membrane potential is lost, as monovalent cations leave the cells, resulting in yeast cell death [4]. Currently, very few *Candida* strains are resistant to polyenes. The few that are resistant have a defect in the ERG3 gene, responsible for ergosterol synthesis. These

Figure 2: Mechanism of polyene drug action. Polyenes associate with ergosterol to create pores in the cell membrane through which cations leave the cell, resulting in the loss of membrane potential. (Image Courtesy of www.doctorfungus.org © 2005)

strains have an increased proportion of other sterols in the cell wall [5].

Ergosterol is very similar in structure to cholesterol (Figure 3). This structural similarity means that at high doses, polyene drugs can be toxic to human cells containing cholesterol [6]. The result is that these drugs can only be used at low concentrations, limiting their efficacy as an antifungal agent.
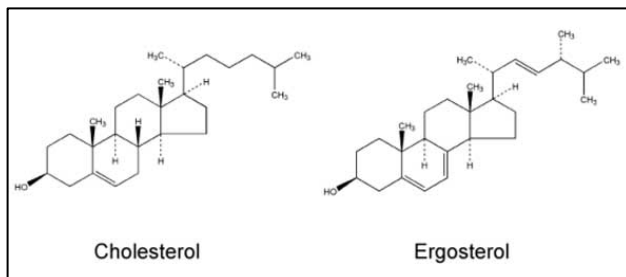
Figure 3: Structural similarity between cholesterol and ergosterol. Many antifungal drugs target ergosterol. Because ergosterol is extremely similar to cholesterol, host toxicity is observed at low concentrations. (Image Courtesy of www.doctorfungus.org © 2005)

(ii) Azoles

Azoles, such as fluconazol and itraconazol, work by interfering with the synthesis of ergosterol in the cell membrane, leading to the accumulation of toxic sterols in the membrane (Figure 4). Fluconazole resistant *Candida* strains are exceptionally prevalent; up to 1/3 of AIDS patients suffer oral thrush caused by azole resistant 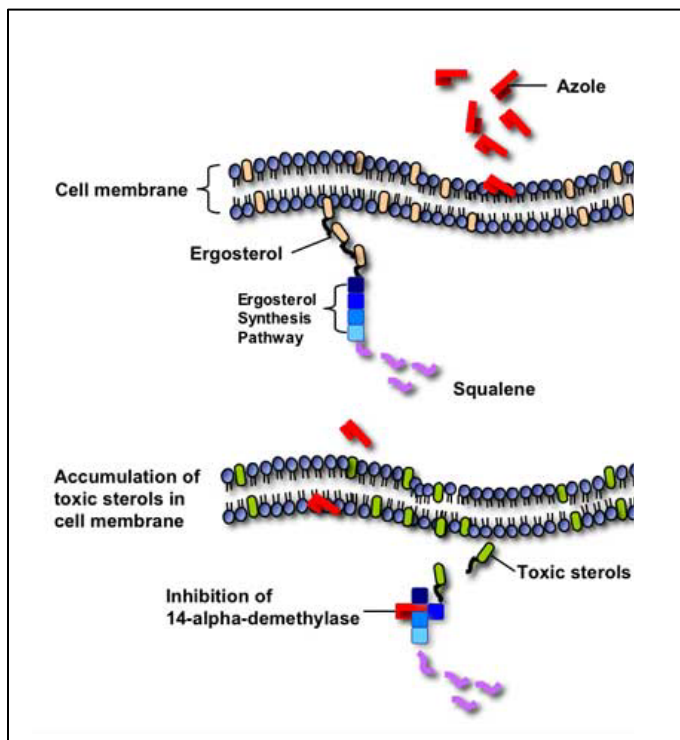*C. albicans* strains [5]. There are multiple mechanisms by which *Candida* gain resistance to azoles, outlined below. One species may use more than one mechanism, and they are thought to function in an additive manner [5].

One method by which *Candida* species evade the effects of azoles is by decreasing the intracellular drug concentration by the use of efflux pumps. Two of these are the Cdr pumps, and the Mdr pumps. It has been shown that *CDR1* and *CDR2* are upregulated in azole resistant strains of *C. albicans*, and *MDR1* is upregulated in (specifically) fluconazole resistant strains [7].

Figure 4: Mechanism of azole drug action. When azoles are present, 14-alpha-demethylase is inhibited, resulting in the accumulation of toxic sterols in the cell membrane, leading to cell death. (Image Courtesy of www.doctorfungus.org © 2005)

Another mechanism used by *Candida* to resist the effects of azoles is alteration of the enzyme that azoles target. Azoles function by binding to lanosterol C-14-alpha-demethylase, an enzyme critical to the synthesis of ergosterol in the cell membrane. It has been observed that some azole-resistant strains of *Candida* have a mutated *ERG11* gene, which is responsible for the synthesis of lanosterol C-

9

14-alpha-demethylase [5]. Additionally, the *ERG11* gene may simply be up-regulated, thereby diminishing the effects of the drug [8].

Lastly, as stated above, *Candida* with a defect in the *ERG3* gene, responsible for ergosterol synthesis, have been found to have membranes with very little ergosterol. This makes them resistant to polyenes, and also makes them resistant to azoles.

(iii) Echinocandins

Echinocandins, such as caspofungin, are less well understood than the aforementioned classes. They are believed to work by targeting the catalytic subunit of 1,3 beta glucansynthetase, which is involved in cell wall formation (Figure 5). When cell wall formation is disrupted in this manner, the cells lyse. Resistance to this class of drugs is rare, but it has been noted that resistant strains have amino acid substitutions between residues 641-649 and 1257-1364 of the catalytic subunit [7].

Figure 5: Mechanism of echinocandin drug action. Echinocandins inhibit beta(1,3)-glucansynthetase action. This destabilizes the cell wall, leading to cell death. (Image Courtesy of www.doctorfungus.org © 2005)

(iv) Allylamines

The last major class of antifungal drugs is allylamines, including naftifine and terbinafine. These drugs work by inhibiting ergosterol synthesis[6]. Specifically, they inhibit squalene epoxidation, one of the

first steps in ergosterol synthesis. The result is two-fold; squaline builds up to toxic levels inside the

cells, and the cell walls are weakened due to lack of ergosterol. Squalene is an exclusively fungal

protein, which makes it an excellent drug target. Resistance, however, has been achieved by the use

of CDR efflux pumps, just as in azole resistant strains [9].

While all of these classes of drugs work in a slightly different manner, they all target the cell

membrane or cell wall. More importantly, yeast have developed methods to evade all of these. This

is, in part, because the drugs must enter the cells to work, and once the drug is in, there is selective

pressure to destroy it, or work around it. A novel, exclusively fungal target is needed, in order to

intelligently craft drugs that can fight yeast infections in humans. This thesis explores one such

avenue: targeting secondary metabolite signaling.

Disease Progression

Many fungi are normal, commensal human microflora found in the gastrointestinal and urogenital

tract, but frequently become problematic, particularly when the host is immunocompromised [3].

Candida species can somehow "sense" that the environment is optimal for invasion and then express

adhesins, which help them adhere to the epithelium of mucosal membranes, such as the oral cavity

and vaginal tract, as well as the plastics used for catheterization [10]. This is the first step of infection

[10]. One of the better understood classes of adhesins is the agglutinin-like sequence (Als)

glycoproteins.

There are 8 known Als proteins in *C. albicans*, and each is unique in its substrate affinity which is

determined by the N-terminal region. For instance, the ALS1 and ALS3 genes encode proteins that are

particularly efficient at binding endothelial cells, and epithelial cells, respectively [11]. Additionally, the Als proteins are very well conserved, which means that an antibody that targets one, may well target others, too. Vaccines of this variety would work by inhibiting the ability of the *Candida* to adhere to cells, which results in the halt of disease progression. The Edwards lab at UCLA has shown that vaccination of mice with recombinant N-terminal sequences of both Als1 and Als3 provides protection against bloodstream infections, as well as oropharyngeal and vaginal *Candida* infections [12]

Once the *Candida* have adhered, alternative proteins are expressed, and they begin to invade into the vascular space beneath the membrane. This is believed to be accomplished, in part, by the secretion of enzymes, such as secreted aspartyl proteases (SAPs), which are believed to help to break down the host cell membrane [13]. In further support of the role of SAPs in disease progression, researchers have found that vaccination targeting SAP2 has reduced the fungal load of mice infected with Candida bloodstream infections [14]. This is proposed to be due to the antibodies interfering with the enzymes' activity, as no signs of increased host immunity are present in these animals [14].

Another contributing factor to invasion of tissue is the dimorphic switch from yeast form to hyphal form. The hyphae, or filaments, are a foraging response. When the fungi are starved, or otherwise stressed, they respond by extending these protrusions in an attempt to secure nutrients. In the process of doing this, they penetrate the host membrane, gaining them access to the bloodstream [15]. In the lab, this phenotypic switch can be observed by microscopy. One challenge researchers face is controlling for the many known filamentation inducers, from pH, temperature and Ca^{2+} concentration, to carbon and nitrogen source, as well as many others [16]. For example, IAA has been

12

shown to induce filamentation in *S. cerevisiae*, but the concentration required to do so can vary immensely depending on the media. For instance, in rich media (such as YPD) up to 1.5 mM IAA can be required to induce filamentation, while as little as 20 µM IAA may be sufficient in minimal media with an alternative glucose source (such as SLAD plates).

Once the *Candida* have penetrated the membrane, they are exposed to the bloodstream where are disseminated throughout the body, leading to death in 30-50% of infected AIDS patients. When in the bloodstream, *Candida* have been shown to bind to plasmin (the active form of plasminogen), which grants them the ability to cross an *in vitro* blood-brain barrier. Additionally, when bound, the *Candida* are able to enhance fibrinolysis [17].

Small Molecule Signaling

A new approach to combating fungal infections is to manipulate their environment, in order to "trick" them into turning virulence traits off. If the small molecule used to communicate were somehow removed from the environment, or inactivated, the signal to activate virulence could potentially be disrupted. Microbes use small molecules to synthesize environmental information. The microbes are able to synthesize, secrete, recognize and respond to these small molecule signals, in a population-dependent manner [18]. This phenomenon has been very well studied in bacteria. The first described system is *Vibrio fischeri*. These bacteria naturally reside in the light organ of the Hawaiian squid *Euprymna scolopes*. Here, the bacteria multiply to exceptionally high numbers, resulting in the accumulation of high concentrations of the signaling molecules autoinducers 1 and 2 (AI1 and AI2). In turn, the AI molecules cause the expression of genes required for the production of bioluminescence. At regular intervals, the squid excretes almost all of the bacteria. The remaining bacteria lack

sufficient signaling molecule to continue to express the bioluminescence genes. The population increases again, until critical mass is reached, and the cycle continues (Figure 6) [19]. This is beneficial for both the squid, and the bacteria. The bacteria are housed in a nutrient rich, relatively safe environment, where they are able to multiply to levels much higher than achievable in the ocean. Meanwhile, the squid uses the light that is produced for counter-illumination which protects it from predators.

It is important to note that if only a single bacterium turned on these genes, it would not help the squid, and it would cost the bacterium a great deal of energy. Thus, this ability to regulate the expression in a population-density dependent manner is beneficial for all parties involved.
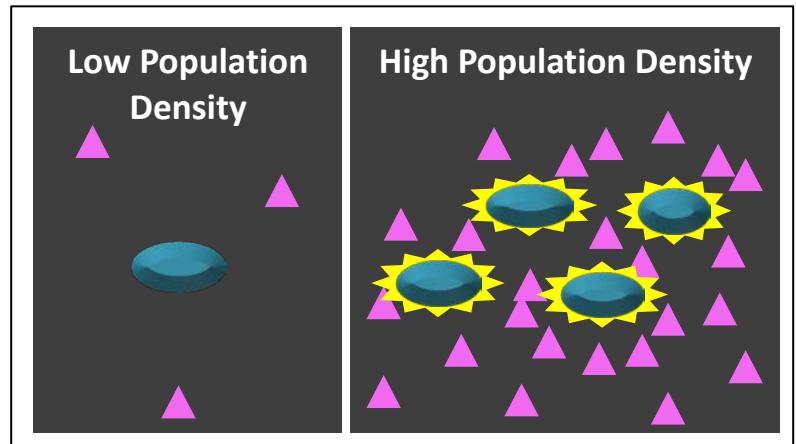
Figure 6: Small molecule signaling model. When microbes (blue ovals) reach a certain threshold density, enough of the small signaling molecule (purple triangles) accumulates, activating alternative gene expression. This results in such phenotypes as bioluminescence, or virulence traits.

While this example showcases the use of small molecule signaling as a symbiotic relationship, not all cases are beneficial for both parties. For example, the gram positive bacteria *Staphylococcus aureus* is part of normal human microflora. However, it is capable of using a small molecule, autoinducing peptide (AIP), to signal the secretions of deadly toxins [20]. Strains of *S. aureus* are classified based on the sequence of their AIP. Each strain's AIP is capable of eliciting response from its own species, while simultaneously inhibiting the pathways of the other species [20]. This ability to tell "kin" from "other" has very relevant clinical applications; it has been shown that *S. aureus* AIPII can be used to stop virulence progression of *S. aureus* I in mouse models [20].

While small molecule signaling has been well described in bacteria for more than 20 years, it has not been nearly as well studied in fungi. One well known cell density dependent phenotype in *C. albicans* is the long lag period before exponential growth observed when cells are diluted in fresh media. If the cells are diluted with conditioned media, this lag period is eliminated [21]. This observation suggests that there is some compound being synthesized by the yeast, and secreted into their environment that allows rapid exponential growth. It was determined that this compound is tyrosol [21]. Additionally, transcriptional profiling revealed that DNA-replication machinery and cell-cycle-control protein transcripts decline rapidly after dilution. Two major hypotheses to explain these phenomena have been proposed; tyrosol may help to control transcription of these genes, or, it could stabilize the transcripts after dilution.

Another phenotype observed in response to tyrosol is the phenotypic switch from cellular yeast form to invasive filamentous form, when cells are grown in permissive media (minimal, increased pH and increased temperature condition). This is believed to be as a result of the increased growth rate; tyrosol allows the cells to grow more quickly, thus synthesizing components more readily, resulting in the ability to form the filamentous protrusions.

Another compound that is related to this dimorphic phenotypic switch is farnesol. It has been shown that farnesol accumulates in the media at a rate that is roughly proportional to the number of cells present [15]. As is accumulates, it acts to suppress the switch to filamentous phenotype, even when grown in conditions favoring filamentation, such as the addition of serum [15]. In this way, it counter acts the effects of tyrosol.

15

Together, tyrosol and farnesol, and other secondary metabolites, such as IAA work to regulate the hyphal transition from yeast form to filamentous form in *C. albicans*. Tyrosol allows the cells to grow more rapidly, and allows the yeast cells to filament, while farnesol suppresses this effect, resulting in yeast cell morphology. This complex regulation of hyphal formation is only one way in which fungi use small molecule signaling to control gene expression.

The morphological transition, from the round yeast morphology, to the filamentous morphology is vital for disease progression. As noted earlier, hyphae formation is crucial for penetration through the host cellular membrane, a key step in disease progression. Fungi can exist in various forms (Figure 7), which can be induced by a variety of stimulators. *S. cerevisiae* and *C. albicans* can both exist in the yeast form, where mother and daughter cells fully separate during replication, resulting in two new, round yeast cells. Both fungi can also exist in a pseudohyphal form, where the daughter cells do not quite separate after cytokinesis, resulting in a chain of elongated cells*. C. albicans*, but not *S. cerevisiae* can also form true hyphae (also known as filaments or mycelia). In this form, single cells grow into elongated tubes, without constriction
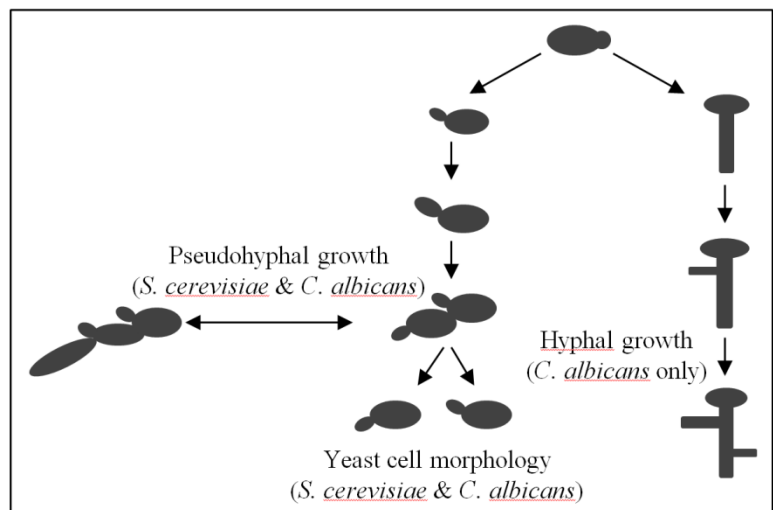
Figure 7: Fungal cell morphology. *S. cerevisiae and C. albicans* both exist in yeast and pseudohyphal form, but only *C. albicans* forms true hyphae.

indicating incomplete separation after cytokinesis [3] [22]. It is generally believed that the yeast form is most prevalent in bloodstream infections, where the yeast get carried throughout the body, while

the hyphal and pseudohyphal forms are most prevalent in superficial infections, and biofilm formation.

In order to identify other signaling molecules responsible for the morphological transition, a chemical screen was performed. More than 50 chemicals were tested in order to find out which, if any, caused a phenotypic change [18]. Indole-3 acetic acid (IAA) was identified as one such compound.

Targeting the very molecule that fungi use to communicate may be an alternative way to treat fungal diseases. It is particularly appealing for resistant strains, because the resistance they have gained by substituting ergosterol in the membrane, or pumping drugs out of the cell would be useless against a drug that was active outside the cell. IAA may be one such molecule.

IAA: A Small Signaling Molecule

One of the oldest interests of plant biologists is the role of the auxin (IAA) *in planta*. As early as the late eighteen hundreds, Charles Darwin postulated that a substance which regulates growth must exist in plant cells [23]. About sixty years later, indole-3-acetic acid (IAA) was discovered to be the signaling molecule responsible for the growth inhibition of the primary root, the stimulation of lateral root initiation, and adventitious rooting. This directional growth is accomplished through the use of auxin concentration minima and maxima, and is important as it allows the plants to branch out their roots, increasing their ability to acquire nutrients and water [24].

Very shortly after IAA was isolated from plants, scientists began looking into whether IAA was synthesized in other organisms- namely, fungi. As early as 1959, Gruen reported that 67, out of 75

species of fungi that were screened, synthesized IAA. Since then, it has been determined that the

most preserved biosynthetic pathway for the synthesis of IAA uses tryptophan as a precursor, but

many species of fungi, including *Saccharomyces cerevisiae* have alternate, tryptophan-independent

biosynthetic pathways [25].

One possible explanation for the relationship between the production of a plant hormone in fungi is a

pathogen-host relationship [18]. In nature, yeast grow on, and eat from, the fruit of many plants. As

such, it is beneficial to the yeast to be able to sense when a plant is wounded, as it means the yeast

would benefit from growing directionally towards the wound-site, where the glucose is spilling from

the plant. However, this increased growth of yeast when a plant is wounded is detrimental to the

plant. A beneficial trait for the plant would be the ability to suppress the growth of yeast, particularly

when wounded.

It has been observed that when yeast are exposed to IAA at low levels, a phenotypic switch occurs and

pseudohyphal growth is observed, while at high levels of IAA growth is inhibited (Figure 8) [18]. These

observations support the hypothesis that yeast and plants use IAA to communicate, to benefit

themselves. What is yet to be understood is the molecular mechanism by which the IAA signal is

transduced into a pathogenic phenotype.
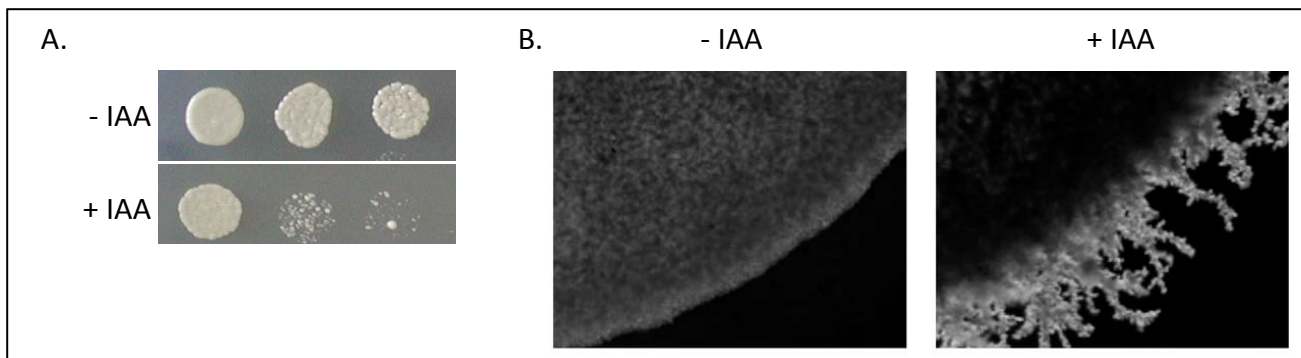
Figure 8: IAA inhibits growth, and induces filamentation in *S. cerevisiae*. Panel A: A dilution series of yeast was spotted onto plates with and without IAA. Plates containing high concentrations of IAA inhibit growth. Panel B: The edge of a yeast patch grown on filamentation-inducing media shows a change in phenotype due to exposure to IAA [18].

Molecular Mechanism of IAA Signaling

In order to better understand the role of IAA in pathogenesis, *Saccharomyces cerevisiae* was used as a model organism. *S. cerevisiae*, commonly known as baker's or brewer's yeast, is the simplest eukaryotic model organism, and is non-pathogenic, yet mimics the pathogenic traits of adhesion and (pseudo)hyphae formation, so it can be employed to study pathogenesis. Additionally, the genetic tools available for its manipulation are numerous, including ease of transformation with plasmids, and integration of DNA into the chromosomes via homologous recombination. The fact that the genome has been sequenced, and systematic genomic deletion libraries are available is particularly appealing. Additionally, yeast exist in both haploid and diploid forms, allowing the researcher the ability to study recessive traits in haploids, and use meiosis to genetically link genotype to phenotype. All of these aspects make yeast a desirable organism for study.

It has been proposed that when yeast is exposed to IAA, the transcription factor Yap1 translocates

from the cytoplasm to the nucleus, where it regulates transcription of amino acid/auxin permeases

(AAAPs, encoded by the AVTs in *S. cerevisiae*). These permeases allow greater uptake of IAA into the

cells. Additionally, an increased level of Flo11 (a surface glycoprotein) is observed, which causes

increased cell adhesion and filamentation (Figure 9) [26].
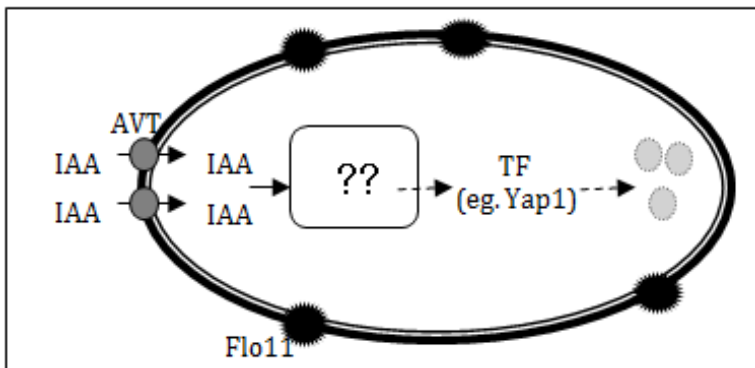
Figure 9: Maintenance of homeostasis: Yap1 acts to regulate turnover of IAA transport proteins (AVTs). Additionally, Flo11 is regulated, possibly by the same pathway. The molecular mechanism of the pathway(s) remain unknown.

What is not yet known is the molecular mechanism by which the signal is transmitted. In order to

begin to address this question, a whole genome screen for mutants sensitive to IAA was performed

(unpublished data). Cells deficient in Grr1, an F-box protein homologous to IAA receptor Tir1 in

plants, were identified as being hypersensitive to IAA.

Tir1 and the SCF Complex

Tir1 is an F-box protein that functions in the SCF complex which interacts with the plant hormone

auxin. This interaction mediates the proteosomal degradation of proteins that mediate the IAA

response (Figure 10).

The SCF complex is made up of 4 molecules, Skp1, Cdc53/cullin, Rbx1/Roc1 and an F-box protein, which interact with ubiquitination enzymes. The function of the SCF complex is to selectively ubiquitinate molecules for proteolytic degradation [27].
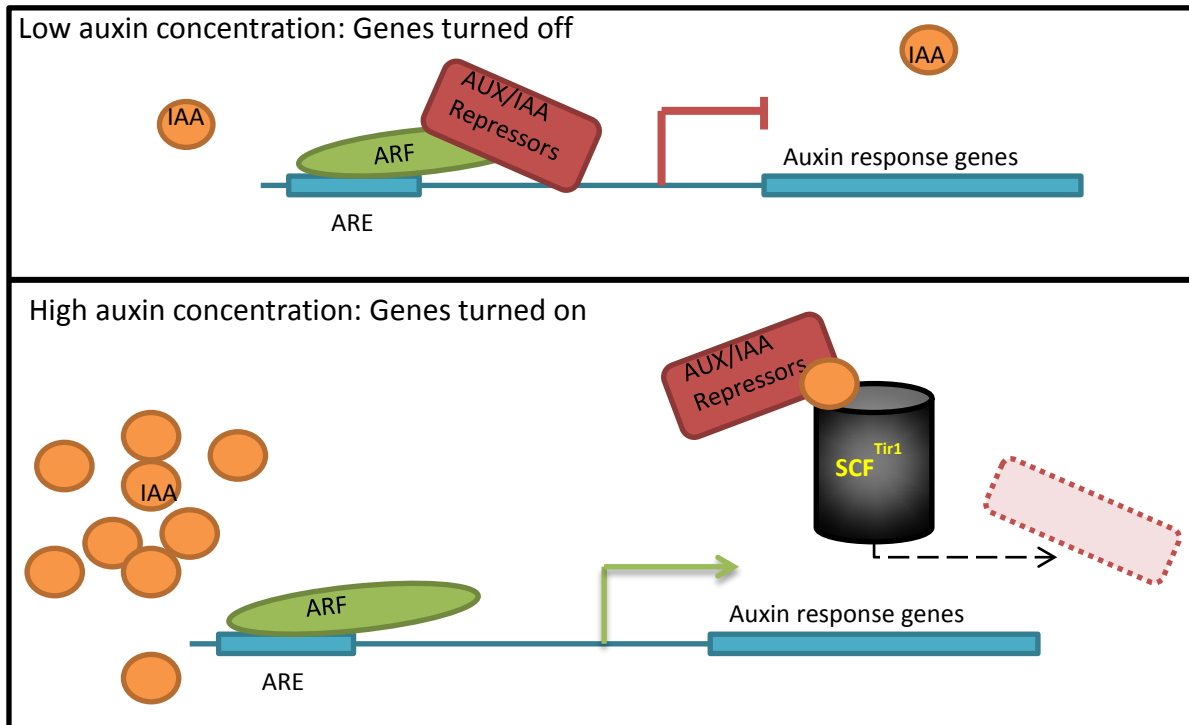
Figure 10: Tir1 model. When IAA is present at low concentrations, repressor proteins prevent the transcription of auxin response genes. When IAA is present, it interfaces with TIR1 in the SCF complex to facilitate the binding of the AUX/IAA repressor proteins, leading to ubiquitination and subsequent degradation of repressors.

This transcriptional regulation relies on auxin concentration to control the expression of genes. When there is very little auxin present, it is unlikely to bind many of the repressors to Tir1, therefore the repressors do not get tagged for degradation. When the auxin concentration reaches a particular threshold, the auxin, repressor and Tir1 are able to all bind, resulting in the tagging of the repressor for degradation. This mechanism is not unlike the small molecule signaling previously described in bacterial systems.

In order to better understand the mechanism by which proteins are ubiquitinated by the SCF[Tir1]

complex, Tir1 was crystallized with auxin bound. Two major hypotheses existed: auxin binds to an

allosteric site, thus changing the conformation of the protein to allow binding of repressors- or, auxin

enhances binding of the substrate to Tir1

by enhancing surface interactions [28].

The latter was determined to be true

(Figure 11). This model is the basis for

our model of Grr1-IAA interactions in

fungi.

Figure 11: Tir1, auxin, AUX/IAA protein interactions. Auxin binds in the concave surface formed by the leucine-rich repeat region. Here, it acts as a "molecular glue" enabling the substrate (AUX/IAA repressor proteins) to bind Tir [28]

Grr1: the Fungal Homologue of Tir1

There are 11 known F-box proteins in the yeast genome [29]. Grr1 is one of the very few of them to

have been shown to associate with the SCF complex, and have substrates identified as targets for

ubiquitination, such as cell division control protein Cdc6 [30]. As early as 1996, it was shown that the

SCF complex regulates transcription factors, such as Gcn4 [31]. Shortly thereafter it was shown that

Grr1 regulates the cell cycle by mediating the degradation of cyclins Cln1 and Cln2 [32]. Additionally,

it was observed that grr1Δ shows interesting phenotypes, such as decreased rate of growth and

elongated cell morphology. This led researchers to hypothesize that Grr1 mediates multiple pathways,

likely through association with the SCF complex [32]. One of these additional functions was found to

be in the glucose signal transduction pathway, where Grr1 has been shown to inactivate Rgt1, a

repressor of glucose-induced genes [31]. Another pathway may be regulating the IAA-mediated

virulence of fungi.

Aside from the F-box domain, which ubiquitinates proteins containing the degron sequence [29], Grr1 has another conserved domain; the LRR domain. This domain is frequently responsible for protein-protein interactions. In order to better understand the role of Grr1 in Cln1/2 regulation, the Wittenburg lab investigated the structure of Grr1. Structural analysis of Grr1 revealed that the LRR region formed a characteristic horseshoe shape with an exceptionally high number of cationic charges on the concave surface [33]. This led them to focus on this region for their study.

Mutational analysis of Grr1 included a carboxy-terminal deletion (CTΔ), a leucine-rich repeat deletion (LRΔ), and two more precisely targeted mutants, each with four basic residues on the aforementioned concave surface of the LRR mutated to either neutral glutamine (B4Q), or acidic glutamate (B4E). All constructs were confirmed via RT-PCR and shown to produce protein by western blotting. These studies revealed that the LRR and, to lesser extent, the C-terminus, were required for binding Cln1 and Cln2, and that this lack of binding was not due to the inability of the mutated protein to interact with the other SCF components [33]. When the LRR or C-terminus was deleted, the amount of Skp1 (an integral protein in the SCF complex) bound was decreased, leading them to conclude that while these mutants are still able to interact with the other SCF components, the nature of this interaction is likely modified [33].

Furthermore, the cationic concave surface of the LRR has a higher affinity for phosphorylated Cln2 protein, since the phosphate groups are negatively charged. The B4E mutant did not interact with Cln2, likely because of the dramatic change in charge. The B4Q mutant, however, interacted with Cln2 at roughly the same frequency as the wildtype, probably due to its slightly less positive charge in the LRR region. These data support the hypothesis that Cln2 interacts with positively charged concave surface of the LRR region of Grr1 [33].

Gic1 and Gic2 are phosphorylated proteins that interact with Grr1. In the B4E and LRΔ mutants, levels of Gic1/2 were stabilized, suggesting the mutants cannot bind them in the SCF to promote their degradation. This further supports the hypothesis that the cationic concave surface of the LRR is required for phosphorylated target identification/binding [33].

Grr1: A Potential IAA Regulator

Here we test the hypothesis that Grr1, a fungal F-box protein structurally similar to Tir1 (Figure 12) regulates IAA-mediated virulence traits, probably by facilitating the turnover of IAA transporters (Figure 9).
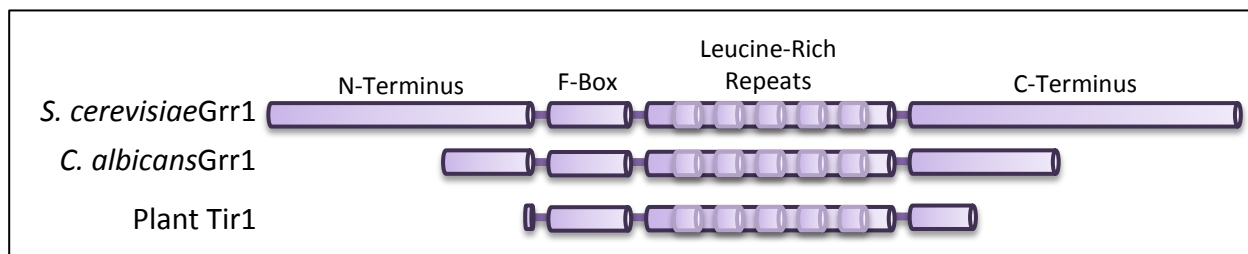
Figure 12: Comparison of Grr1 proteins and Tir1. F-box motif and leucine-rich repeat region are conserved in all three proteins, however length of both termini are highly variable. Figure is drawn to scale. *C. albicans* Grr1 shares 46% identity and 64% similarity to *S. cerevisiae* Grr1 [36].

The rationale for this hypothesis is based on the structural similarity of Grr1 and Tir1 (Figure 12), and the sensitivity profile of the *grr1Δ* mutant (Figure 7). The goal of this thesis was to better understand the role of Grr1 in the regulation of IAA, through mutational analysis, and in a human pathogen, *Candida albicans.*

Materials & Methods

Media Preparation: All media was prepared according to standard protocols [34]. Briefly, YPD Yeast Peptone Dextrose) consisted of 1% yeast extract, 2% peptone, 1% dextrose. SC (Synthetic Complete) media contains yeast nitrogenous base and dropout mix with 2% dextrose, and only those amino acids required for strain growth added. YNB (Yeast Nitrogenous Base) is the most limited media used, with only yeast nitrogenous base, 2% dextrose, and amino acids required for strain growth added. To make plates of these varieties, 2% final concentration of agar was added. SLAD plates consisted of 0.67% YNB, 0.05mM ammonium sulfate, 2% dextrose, and 2% washed agar.

All plates containing IAA were mixed as described above, and appropriate volume of 1M IAA in 70% ethanol was added to cooled media, just before pouring the plates. These plates were allowed to set for 7 days at room temperature in the dark before being used, and stored up to 7 additional days.

IAA Sensitivity Assay: All strains were grown in overnight cultures at 30°C in YPD media. Cell density was normalized by diluting cultures with fresh YPD to $OD_{600}=1$ (approximately 10^7 cfu/mL). Cells were then washed with water, and 10 μL spots of a 1:10 dilution series (in water) were spotted on solid agar SC supplemented with appropriate amino acids, containing IAA (20 μM *C. albicans*, or 30 μM *S. cerevisiae*). Control plates contained equal volume of the solvent (70% ethanol). Images were acquired after two days of growth at 30°C.

Adhesion Assay: All strains were grown in overnight cultures at 30°C in YPD media. Cell density was normalized by diluting cultures with fresh YPD to $OD_{600}=1$ (approximately 10^7 cfu/mL). Cells were then

washed with water. For *S. cerevisiae* strains, 20 µL were spotted onto solid agar YPD, with 0.5 mM

IAA. For *C. albicans* strains, 20 µL were spotted onto solid agar SC supplemented with appropriate

amino acids, with 100µM IAA. All control plates contained an equal volume of solvent (70% ethanol).

Spots were spread into triangular patches with a toothpick, taking care not to puncture the agar.

Samples were grown at 30°C for two days, and then stored at 4°C overnight. Plates were then washed

under a stream of running water, and the surface was gently rubbed with a gloved finger. This

treatment continued until the water ran clear. Images were acquired before and after washing.

Strains: Yeast strains in Table 1 were used throughout this study.

Lab Reference	Strain	Genotype	Source
Y769	sigma1278b	MATa; ura3/ura3	C. Wittenberg
Y770	grr1Δ	ura3-52/ura3-52 leu2::hisG/leu2::hisGhis3; grr1::LEU2/grr1::LEU2	C. Wittenberg
Y771	LRΔ	ura3-52/ura3-52 leu2::hisG/LEU2his3::hisG/HIS3; grr1L::KanMX2/grr1L::KanMX2	C. Wittenberg
Y772	CTΔ	ura3-52/ura3-52 leu2::hisG/LEU2his3::hisG/HIS3; grr1C::KanMX2/grr1C::KanMX2	C. Wittenberg
Y773	B4E	ura3-52/ura3-52 leu2::hisG/LEU2his3::hisG/HIS3; grr1B4E::KanMX2/grr1B4E::KanMX2	C. Wittenberg
SN250		his1Δ/his1Δ, leu2Δ::C.dubliniensis HIS1 /leu2Δ::C.maltosa LEU2, arg4Δ /arg4Δ, URA3/ura3Δ::imm , IRO1/iro1Δ::imm arg4Δ /arg4Δ, URA3/ura3Δ::imm , IRO1/iro1Δ::imm	S. Noble
grr1Δ	grr1Δ/Δ	grr1Δ/Δ in SN250	S. Noble
Y101	BY4741	MATα ura3-52 trp1 leu2-3,112 ade2 his3Δ pho3-1 pho5-1 ([HIS4-lacZ, ura3-52])	S. Harashima
Y681		MATα *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	E. Craig

Table 1: Strains used in this study

In an attempt to quantify these data, ImageJ software was used. First, the background intensity was found by acquiring three measurements of pixel intensity from the area immediately next to the triangle of growth and averaged. Next, three measurements of pixel intensity were acquired from the center of the triangle of growth and averaged. The average background was subtracted from the average growth to give one background adjusted value per triangle of growth. This was repeated for all triangles on all plates.

Next, to assess the adhesion of cells, the percentage of cells remaining post washing was calculated. In order to do this, the background adjusted values were compared for each plate, before and after washing, and represented as a percentage.

These percentages met the assumptions of normality and homogeneity of variance for a two-way analysis of variance reasonably well. The data were not transformed, as Zar [35] reports it is not warranted unless the largest sample size is greater than 5 times the smallest sample size. In this case, the smallest sample size was 2, and the largest 6.

Filamentation Assay: All strains were grown in overnight cultures at 30°C in YPD media. Cell density was normalized by diluting cultures with fresh YPD to $OD_{600}=1$ (approximately 10^7 cfu/mL). Cells were then washed with water, and 10 μL were spotted on SLAD plates with 20 μM IAA. Control plates contained an equal volume of solvent (70% ethanol). After 8 days of growth (*S. cerevisiae*), or 4 days of growth (*C. albicans*), at 30°C microscopic images of colony morphology were acquired.

Uptake Assay: This whole-cell assay used *C. albicans* strains grown at 30°C until the OD600 was 0.4 in YNB, supplemented with required amino acids. To measure IAA uptake, cells were harvested by centrifugation at 1,000 x g and were resuspended in MES (2-(N-morpholino) ethanesulfonic acid) buffer at pH 4.6 with 2% glucose. Duplicate samples were removed after the addition of approximately 5^7 cells to 4 mL volume medium containing 5.2 µCi ^{3}H-labeled IAA and 750 µM unlabeled IAA. Approximately 6.25^6 cells were collected on glass-fiber filters at various time points, and were washed extensively with buffer. The filters were placed in glass scintillation vials, were treated with 350 µl of 1 M NaOH for 10 minutes, and the solution was neutralized with 400 µl of 1 M acetic acid. Uptake of labeled IAA into cells was determined by using 5 ml of liquid scintillation fluid. Averages were taken for 4 independent samples, standard deviation was calculated, and a T-Test was performed.

Amplification of putative auxin response elements from yeast genome: Y101 was used as template in standard PCR with the following conditions: 1X standard Taq buffer, 200 µM dNTPs, 0.25 µM each primer (Table 2), 1 µg/ml yeast DNA template, and 0.02 units/µL standard Taq, in a final volume of 200 µL. Samples were initially denatured at 95°C for 5 minutes, then cycled 30 times through a 94°C denaturation for 1 minute, 50°C annealing for 2 minutes, and 72°C extention for 2.5 minutes, followed by a final 5 minute extension at 72°C. Samples were then purified via Qiaquick PCR spin columns, and eluted in 50 µL elution buffer.

Oligo Name	Sequence (5' to 3')	Distinguishing Features
YCR102C-5'	AGTCATGCGAGCTCAGTGGCGGCCGCACT TTGTATTTGTTTTGTTTGTTAG	PCR product will contain NotI compatible ends
YCR102C-3'	GATCATGCTCTAGACAGTGGCGGCCGCTTA CGGTTTCTGGAATTGCAACTTG	"
YLR460C-5'	AGTCATGCGAGCTCAGTGGCGGCCGCACT TTGTATTTGTTTTGTTTGTTAG	"
YLR460C-3'	GATCATGCTCTAGACAGTGGCGGCCGCTTA CGTTTGTAGGTTATCTGTAATT	"
YHL047C-5'	AGTCATGCGAGCTCAGTGGCGGCCGCACT TAGACAAACGCCTTTACGAATG	"
YHL047C-3'	GATCATGCTCTAGACAGTGGCGGCCGCTTA CATTTCCTTTGGCCCAAAGAGT	"
pYNL134C5'	AGTCATGCGAGCTCAGTGGCGGCCGCACT TTGTGATGGCCTACACACTTCG	"
pYNL1343'	GATCATGCTCTAGACAGTGGCGGCCGCTTA CGCTTGATATGCAGTATGATAT	"
Not1linker	GCGGCCGCAGCT	When annealed to self, contains one internal NotI site, and SacI compatible ends.

Table 2: Oligonucleotides used in this study.

Linker Insertion: In order to ensure (virtually) any PCR product could be cloned into this system, pAUR112 was modified to have a unique NotI digestion site, where it previously had a SacI site (Figure 13). NotI Linker was annealed by heating 100 μL of 100 μM NotI linker oligos in TE to 95°C in a heat block. When the block reached 95°C, it was turned off, and allowed to acclimate to room temperature (about 1 hour). This linker contained a central NotI site, and had SacI compatible ends, to facilitate ligation into SacI digested pAUR112 with T4
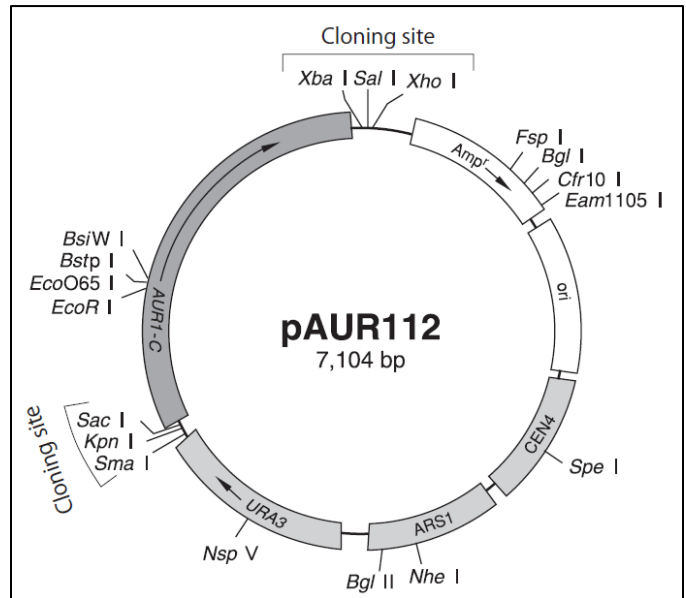
Figure 13 : pAUR112 cloning vector. Vector was digested with SacI and a linker with SacI compatible ends and internal NotI was ligated in. The NotI site was then used for ligation of PCR products with NotI compatible ends. [www.TaKaRa.com]

ligase at room temperature overnight. This construct was then transformed in DH5α cells according to manufacturer's instructions. Qiagen miniprep kit was used to purify samples, and insertion of NotI linker was confirmed via digest with NotI.

Cloning putative AREs: PCR amplified AREs with NotI compatible ends, were ligated to NotI digested plasmid pAURNotI, at a ratio of approximately 6:1 (insert:plasmid) with 200 units T4 ligase in a final volume of 10 μL at room temperature overnight. 1 μL of this reaction was transformed into DH5α cells according to manufacturer's instructions.   Qiagen miniprep kit was used to purify samples, and insertion of ARE was confirmed via various double digests, according to appropriate protocols from the manufacturer, and analyzed by gel electrophoresis.

Yeast transformation: Each ARE containing plasmid, as well as unmodified plasmid (control) was transformed Y681 via standard lithium acetate methods [34]. Transformation was plated on selective media (SC-Ura) in order to select for yeast containing plasmid.

Titration of Aureobasidin A: All plasmids contain the AUR1-C gene for aureobasidin A (AbA) resistance, so minimal inhibitory concentration of AbA had to be empirically derived. To do so, all strains were grown in overnight cultures at 30°C in YPD media. Cell density was normalized by diluting cultures with fresh YPD to OD_{600}=1 (approximately 10^7 cfu/mL). Cells were then washed with water, and 10 μL spots of a 1:10 dilution series (in water) were spotted on solid agar SC containing up to 3.3 μg/mL AbA. After 2 days of growth at 30°C, images were acquired.

Promoting growth with IAA: In order to evaluate the change in AbA growth inhibition in response to

IAA, cells were prepared as described above, then spotted onto solid agar selective media (SC-Ura)

containing 0.5µg/mL AbA and up to 50 µM IAA. Images were acquired after two days of growth at

30°C, and growth profiles were compared.

Results

Yeast can synthesize, secrete, detect and respond to IAA. When there is sufficient IAA in the

environment, the yeast switch from a non-invasive yeast form to an invasive pseudohyphal form [18].

In a whole genome screen, it was discovered that *grr1Δ* mutant is hypersensitive to IAA (unpublished).

Previous work in the Rao lab revealed that this hypersensitivity correlated with increased uptake of

IAA (Unpublished data).

The goal of this thesis was to investigate the role of Grr1 in regulating IAA-mediated virulence

phenotypes, by identifying the domain(s) of Grr1 required for IAA response in *Saccharomyces*

cerevisiae. Next, I tested the hypothesis that *Candida albicans* Grr1 is the functional homologue of *S.*

cerevisiae Grr1, as they share 46% identity and 64% similarity [36]. Finally, I wanted to identify cis-

regulatory elements that respond to IAA.

Phenotypic Characterization of *grr1* Mutants in *Saccharomyces cerevisiae*

Previously, the Rao lab had shown *grr1Δ* was sensitive to IAA, presumably because it takes up more

IAA. Therefore, I chose to investigate several Grr1 mutants (Table 1). These mutants were a gift from

Curt Wittenberg of the Scripps Institute [33].

The Grr1 protein is composed of several recognizable domains and regions (Figure 14). First, the F-box

motif is vital for the function of Grr1 in the SCF complex. Here, it is responsible for target

identification for the ubiquitin mediated degradation of proteins. The next known motif is the

leucine-rich repeat region. This region forms a characteristic horse-shoe shape, and is typically

involved with substrate binding in other proteins [28,33]. The F-box and LRR region are preceded by a large N-terminus and followed by a large C-terminus, the function of which is currently unknown. The homology of fungal Grr1 and plant Tir1 is limited to the F-box and LRR regions.

The first mutant used in this study was the leucine-rich repeat region deletion (LRΔ). This was of interest because the LRR region is a common site for protein-protein interactions. Next was the C-terminal deletion (CTΔ), of interest due to its large size, and unknown function. The last mutant of interest is the most subtle; four basic residues on the concave surface of the leucine-rich repeat region have been mutated to acidic glutamate (B4E). In molecular modeling, these four basic residues were predicted to protrude into the pocket made by the leucine-rich repeat region. By changing the charge so dramatically, this mutant was expected to show a distinct phenotype [33].
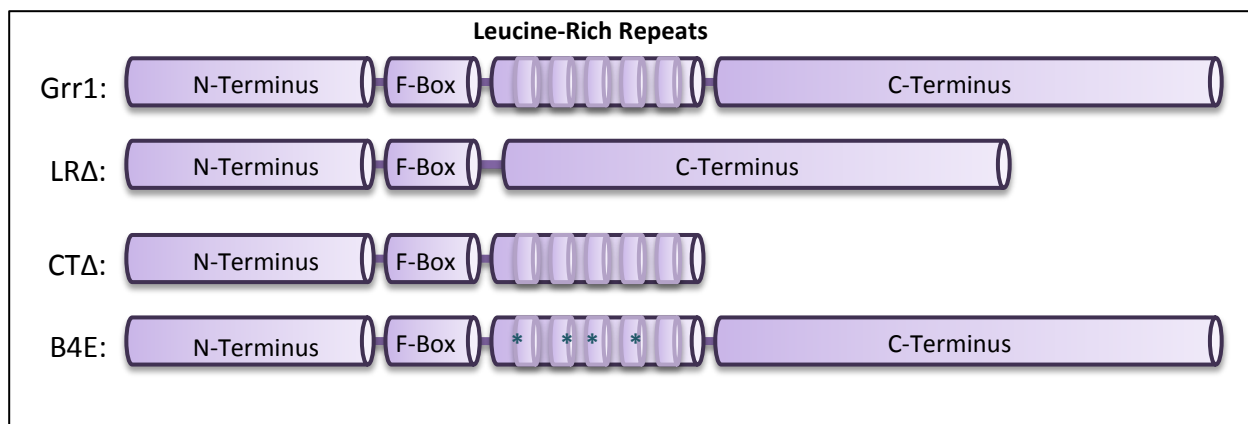
Figure 14: GRR1 gene structure. Mutants used for this study include leucine-rich repeat region deletion (LRΔ), carboxy terminus deletion (CTΔ), and a mutant with 4 point mutations, altering basic residues to acidic glutamate (B4E, indicated by *) [33]

B4E Mutant is Hypersensitive to IAA

grr1Δ has been previously shown to be more sensitive than wildtype to IAA-induced growth inhibition (Unpublished data). By testing various subtle mutations in the Grr1 protein, it could be determined which regions of the gene are responsible for this phenotypic change. 10-fold dilutions of overnight cultures were spotted on IAA-containing plates and incubated at 30°C for 2-3 days. Growth of all mutants (except for grr1Δ) was indistinguishable from wildtype in the absence of IAA. The B4E and *grr1Δ* mutants were severely inhibited in their ability to grow in the presence of IAA. In contrast, the LRΔ appeared to be slightly resistant to the effects of IAA, exhibiting more growth than the wildtype.

Lastly, the CTΔ mutant was indistinguishable from the wildtype. Figure 15 shows a representative micrograph, and the trend was observed over several replications (over 10 times). These results suggest that the C-terminus is not required for IAA-induced growth inhib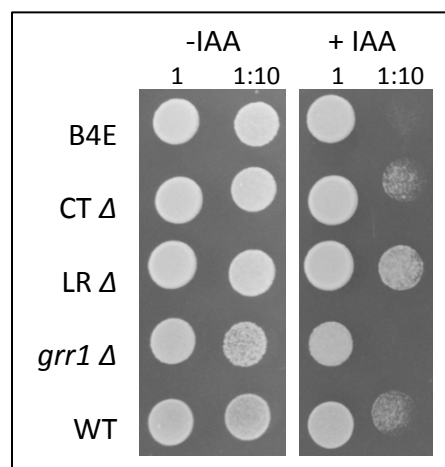ition. The apparent discrepancy between the IAA-resistant LRΔ and IAA-sensitive B4E mutants is noteworthy. The B4E mutant was indistinguishable from the *grr1Δ* mutant, while the LRΔ was not. This suggests that four point mutations in the LRR region render the protein inactive, while deleting the entire LRR region does not.

Figure 15: GRR1 mutants are more sensitive to IAA. Samples were spotted at OD600 = 1, and a 1:10 dilution on plates containing IAA.

B4E mutant shows pseudohyphal formation in response to IAA

Pseudohyphal growth is a foraging response associated with increased virulence. In order to better characterize IAA-mediated pseudohyphal growth, all mutants were grown in overnight cultures, then spotted on minimal media plates containing sub-inhibitory concentrations of IAA. After eight days of

growth in the dark (to prevent photodegradation of IAA), microscopic images were acquired to

analyze colony morphology and pseudohyphal growth. The experiment was repeated several times,

and a representative image is shown below (Figure 16). The CTΔ and B4E mutant were slightly

hyperfilamentous, as observed by the minimal filamentation present in the absence of IAA; however,

at a low concentration of IAA which does not enhance filamentation in the wildtype, the *grr*Δ and B4E

mutants filamented extensively. In contrast, the wildtype, LRΔ and CTΔ cells did not respond to IAA.

All strains were able to filament at higher concentrations of IAA, suggesting that these mutants are

capable of filamentous growth; they are simply not responding to the low level of IAA they are

exposed to. These data are congruent with the previous growth inhibition data; the B4E mutant

showed filamentation patterns similar to those of the *grr1*Δ mutant. Again of note, the LRΔ

phenotype was more similar to the wildtype than the B4E mutant, suggesting that mutating four

residues within the LRR region abolishes protein activity, but removing the entire LRR region does not.
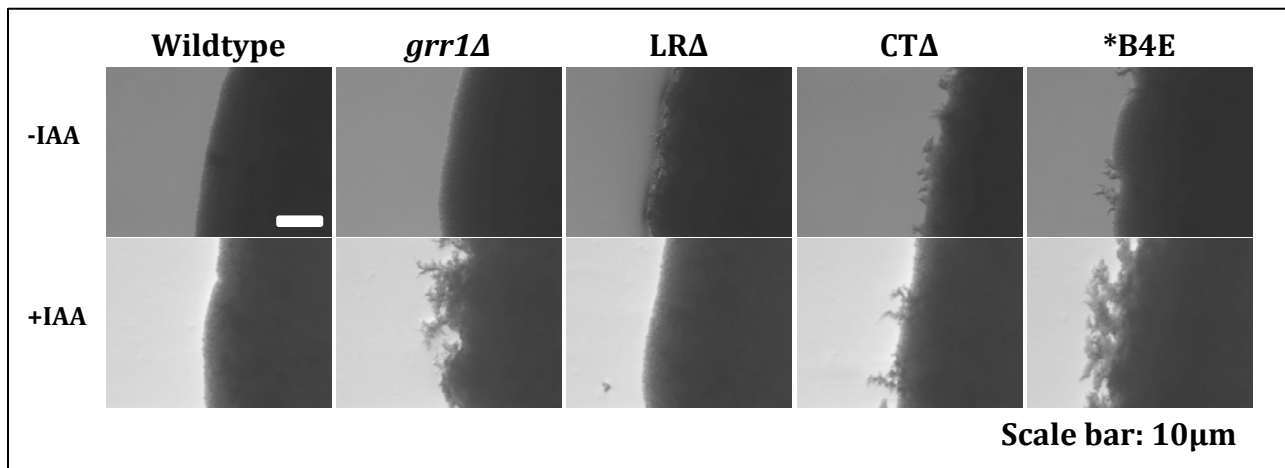
Figure 16: IAA induces filamentation in GRR1 mutants. Overnight cultures were spotted and grown on plates containing IAA, and then the edge of the colony was observed. The no IAA control shows that the CTΔ and B4E mutants are slightly hyperfilamentous. IAA increases in the amount of filamentation present in the *grr1*Δ and the B4E mutants.

B4E mutant grows invasively in response to IAA

Low levels of IAA enhance *grr1Δ* surface adhesion [18], an important virulence phenotype. To

determine which region(s) of the protein is required for this response, subtle mutations of *Grr1* were

grown in overnight cultures and patched onto rich media agar plates containing IAA. Next, the non-

adherent cells were washed away by gently rubbing the surface of the agar under running water. It

was observed that the agar surface beneath the *grr1Δ* and B4E mutant growth patches on IAA plates

were textured, where the other samples, and plates lacking IAA, felt smooth and undisturbed. The

micrographs are representative of several repetitions, which showed the same trend (Figure17A). All

mutants showed increased adhesion without IAA, but only the *grr1Δ* mutant showed quantifiable IAA

induced adhesion in this assay (Figure 17B). These data suggest that IAA induces surface adhesion in

the *grr1Δ* mutant, and possibly causes some change in B4E mutant adhesion behavior, as observed by

the change in agar surface texture.
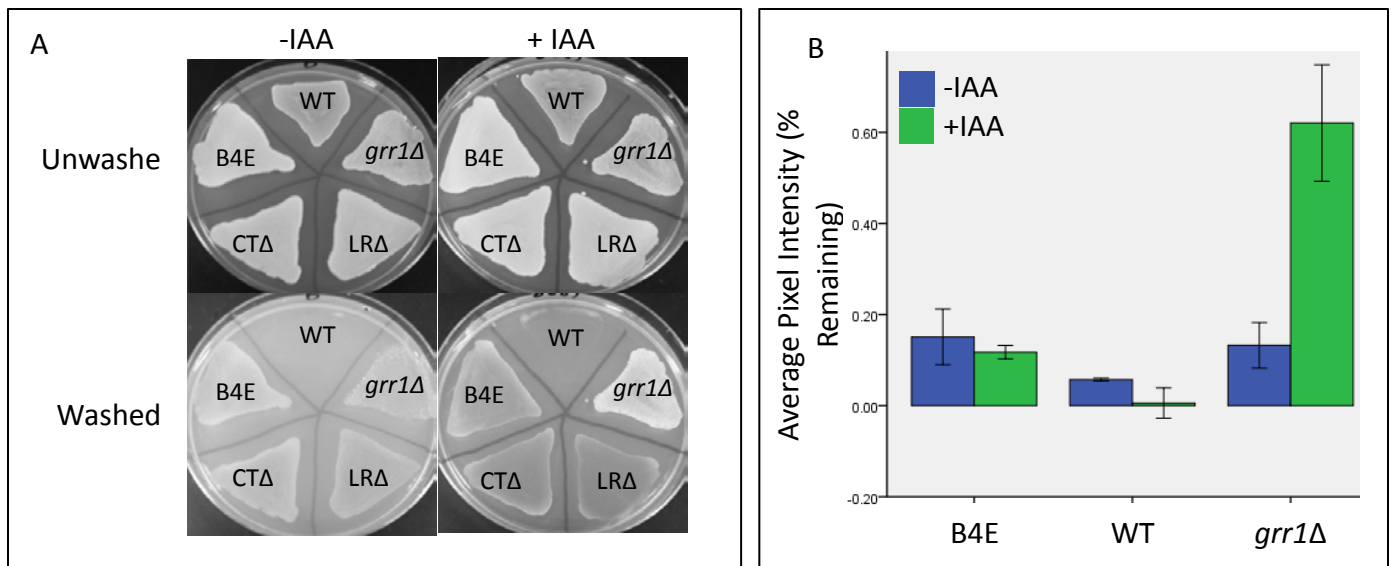
Figure 17: IAA induces adhesion of GRR1 mutants. Overnight samples were patched on plates containing IAA, and grown for 2 days. The plates were then washed under running water.   Panel A: The no IAA control shows that all mutants adhered slightly to the plates. When IAA was present in the plates, the amount of adhesion increased in the *grr1Δ*. Panel B: ANOVA followed by Tukey post-hoc tests revealed *grr1Δ* +IAA is significantly different from all other groups (p <0.05)

In summary, the B4E mutant phenocopied the *grr1Δ* mutant, with regard to IAA response. This is an important discovery, because it shows that the structural integrity of the protein is required for proper ability to respond to IAA. Since IAA mediates virulence responses, this knowledge could help with the development of a new drug target.

The Role of Grr1 in the Regulation of IAA in the Human Pathogen *Candida albicans*

To investigate whether the mechanism of IAA regulation was conserved in pathogenic fungi, I evaluated the IAA response of *C. alibicans grr1Δ/Δ* mutants. The *grr1Δ/Δ* mutant was obtained from the stock center (Table 1) [37].

IAA inhibits growth of *C. albicans grr1Δ/Δ* mutants

S. cerevisiae and *C. albicans* Grr1 are structurally similar (Figure 12). In order to determine whether they were functionally similar, the *Candida albicans grr1Δ/Δ* mutant was investigated for IAA sensitivity. *Candida albicans grr1Δ/Δ* mutant is sensitive to IAA (Figure 18), similar to *S. cerevisiae grr1Δ* (Figure 14). Overnight cultures of the strains were grown, and dilutions were spotted onto plates containing IAA. Growth patterns were observed after 2 and 3 days of growth. The *grr1Δ/Δ* mutant grows slightly less than the wildtype when no exogenous IAA is added, likely due to the effects of Grr1 on cell cycle regulation. A much greater difference, however, is noted when comparing the *grr1Δ/Δ* mutant to the isogenic wildtype strain in the presence of IAA. Here, IAA has severely suppressed growth in the mutant, but
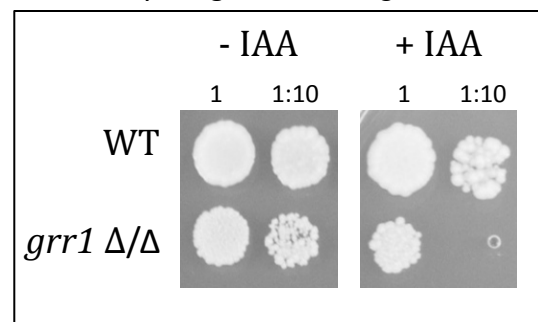
Figure 18: *grr1Δ/Δ* is sensitivite to IAA. Samples were spotted at OD600 = 1, and a 1:10 dilution on plates containing IAA.
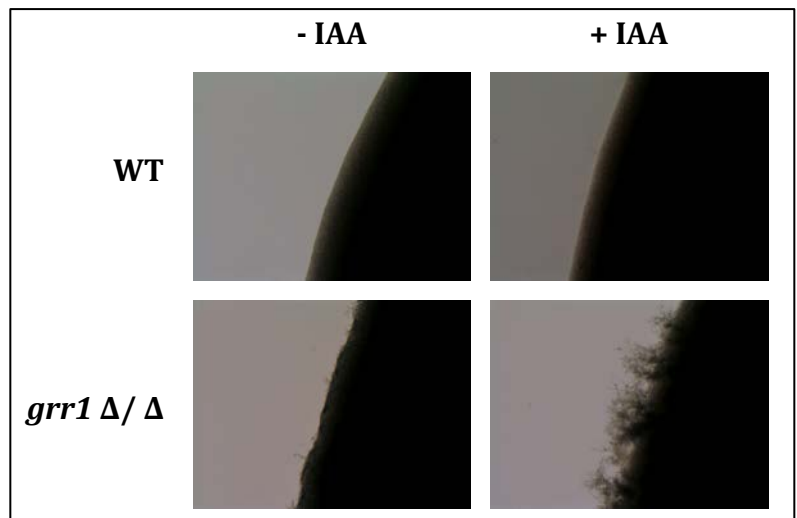
only had a very modest effect on the wildtype. Figure 18 shows a representative micrograph, and the trend was observed over several replications. These results suggest that Grr1 plays a vital role in IAA mediated inhibition of growth in *C. albicans*.

IAA induces hyphae formation in *C. albicans grr1Δ/Δ* mutants.

Filamentation is required for pathogenic fungi to penetrate host membranes, and thus is an important virulence phenotype. In *S. cerevisiae*, filamentation is induced by IAA. In order to determine whether the *grr1Δ/Δ* mutant is sensitized to IAA, filamentation of the mutant was observed in the presence and absence of IAA. Filamentation is induced in *C. albicans grr1Δ/Δ* at sub-inhibitory concentrations of IAA, similar to *S. cerevisiae grr1Δ* mutant

Figure 19: IAA induces filamentation in *grr1Δ/Δ*. Overnight cultures were spotted and grown on plates containing IAA, then the edge of the colony was observed. *grr1Δ/Δ* responds to low levels of IAA, while no change is observed in the wildtype.

(Figure 16). Filamentation of strains patched on minimal media were observed microscopically after 4 days of growth (Figure 19). The experiment was repeated several times, and a representative image is shown below. The micrograph in Figure 18 shows that at low concentrations of IAA that do not affect wildtype cells, the *grr1Δ/Δ* are affected, suggesting that the *grr1Δ/Δ* has been sensitized to IAA, and can detect low concentrations of IAA that the wildtype cells cannot.

IAA induces invasive growth in *C. albicans* Grr1Δ/Δ mutant.

Adhesion is the first step of infection, and the *S. cerevisiae grr1Δ* mutant showed increased surface adhesion in response to IAA (Figure 17). In order to test whether *C. albicans grr1Δ/Δ* mutant mimics this phenotype of their counterpart in *S. cerevisiae*, overnight cultures were grown, and then patched onto plates containing IAA and allowed to grow for two days. Next, the non-adherent cells were washed away by gently rubbing the agar under running water. At low concentrations of IAA, *grr1Δ/Δ* cells adhere to the agar surface, while wildtype wash off (Figure 20). Since this response to IAA is considered a foraging response, there is a known "edge-effect" where the cells around the edge are not nutrient deprived, while the center of the patch is starved and therefore responds to IAA. These data indicate that Grr1 regulates the surface adhesion of cells treated with IAA.
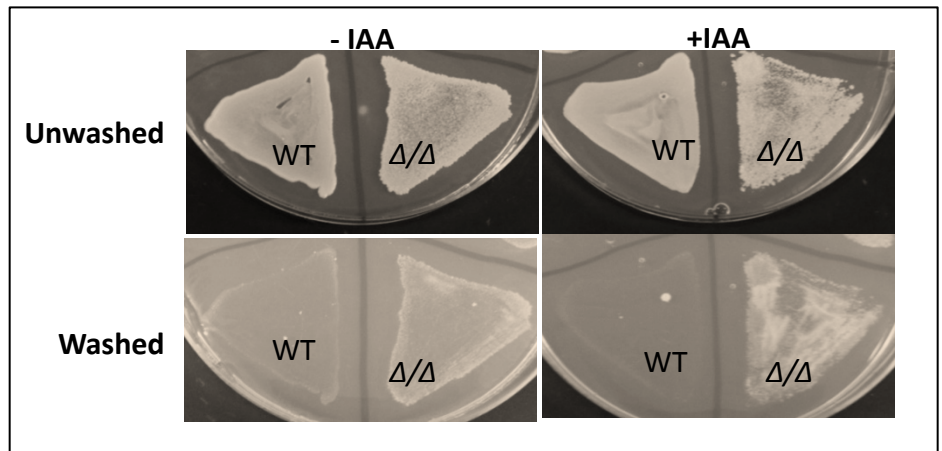
Figure 20: IAA induces surface adhesion of *grr1Δ/Δ*. Overnight cultures were patched on plates containing IAA, and then grown for 2 days. The plates were then washed under running water. The no IAA control shows that the mutant adhered slightly more to the plates. When IAA was present in the plates, the amount of adhesion increased in the *grr1Δ/Δ* mutant, while no change was seen in the wildtype.

IAA uptake is increased in *C. albicans grr1Δ/Δ* mutant.

Phenotypic characterization of *grr1Δ/Δ* mutant suggests it is more responsive to IAA than its wildtype counterpart (Figures 18-20). This result is consistent with the hypothesis that IAA can enter the mutant cells more easily than wildtype cells. To test this hypothesis, IAA uptake was measured by a

radioactivity filter binding assay [18]. The *grr1Δ/Δ* accumulates twice as much IAA as the wildtype

(Figure 21), presumably by up-regulating putative IAA transporters. The AAP (amino acid permease)

proteins in *C. albicans* are homologous to the AAAP (or AVT) transporters of *S. cerevisiae*. This

information, together with the mutant analysis of *C. albicans grr1Δ/Δ*, suggests that *C. albicans* Grr1 is

a functional homologue of Grr1 in *S. cerevisiae*. This information implies that by decreasing IAA

uptake in cells via the AAP transporters, virulence traits may be controlled.
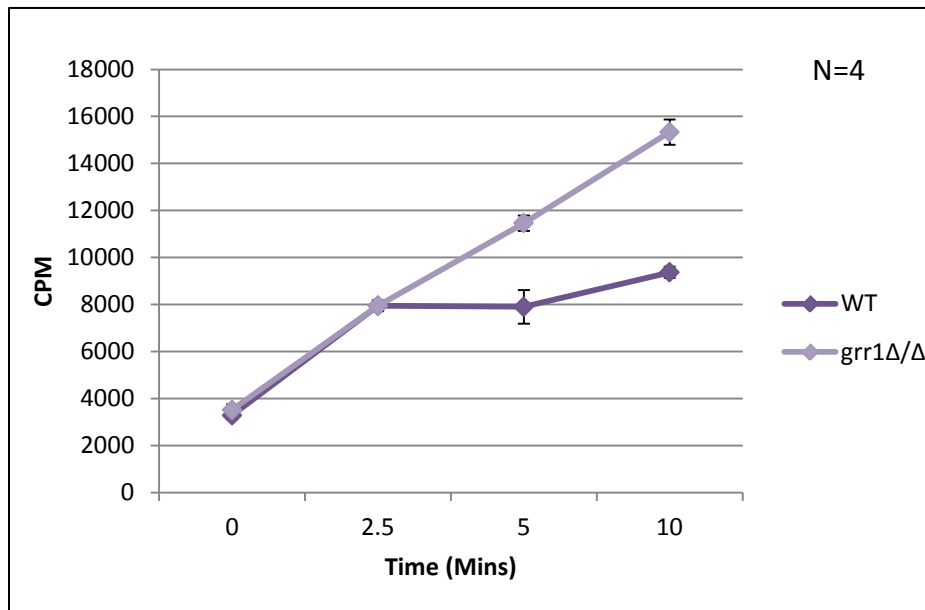
Figure 21: IAA uptake is increased in grr1 mutant.   Overnight cultures were freshly diluted and grown until log phase. Cells were harvested, and then incubated with radiolabeled IAA. Cells were then filtered, washed, and radioactivity was measured. Error bars indicate one standard deviation from the mean. P<0.001

In summary, the *Candida albicans grr1Δ/Δ* mutant mimics the IAA phenotypes of the *S. cerevisiae*

grr1Δ mutant with regards to the virulence traits of adhesion and filamentation, in response to IAA.

This information suggests that *S. cerevisiae* can be used as a model organism to study fungal

pathogenesis, and that Grr1 plays a vital role in IAA-induced virulence traits.

Identification of Cis-regulatory elements in *S. cerevisiae*

IAA regulates gene expression in plants via auxin response factors, ARFs, which are trans-acting factors, and auxin response elements, AREs, which are cis-acting sequences. We know that IAA gets into *S. cerevisiae* cells (unpublished data) and results in phenotypic changes that are indicative of increased virulence (Figures 14-16). Therefore, we believe that IAA probably regulates transcription factors (analogous to ARFs) that in turn regulate gene expression. These transcription factors likely bind to specific DNA sequences (analogous to AREs). To identify putative AREs, we analyzed the 5' intergenic regions of genes that were upregulated in response to IAA (Unpublished microarray data). Four genes (YCR102C, YLR460C, YHL047C, and YNL134C) (Table 3) that were expressed over two times above standard deviation were chosen for further analysis.

Gene Locus	5' Upstream Region (putative ARE) Size	Description of Upregulated Genes
YCR102C	2332 bp	Similar to *C. carbonum* toxD gene, member of the quinone oxido reductase family [38-40]
YLR460C	2030 bp	Member of the quinone oxido reductase family, up-regulated in response to the fungicide mancoze [39-41]
YHL047C	1708 bp	Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; responsible for uptake of iron bound to the siderophore triacetyl fusarinine C [42,43]
YNL134C	567 bp	Similarity to dehydrogenases [44-46]

Table 3: Putative auxin response elements

These intergenic regions (the sequence upstream of the target gene until the stop codon of the previous gene) were ligated into a reporter construct (modified pAUR112, see Materials and Methods for full description) upstream of the antibiotic (aureobasidin) resistance gene (AUR1-C) (Figure 13). If a transcription factor (such as an ARF) were to bind the putative ARE, the AUR1-C gene for antibiotic

resistance would be activated. Construct orientation was verified via endonuclease double digests.

These constructs were then transformed into *Saccharomyces cerevisiae*, along with stock pAUR112

plasmid (negative control). These samples were grown overnight, and spotted onto plates containing

up to 3.3μM antibiotic. Aureobasidin at 0.5μg/mL was sufficient to inhibit growth in all samples,

except YNL134C. This construct was not inhibited by up to 3.3 μg/mL concentration of aureobasidin

(Figure 22). It was concluded that the reporter construct containing the upstream sequence of YNL134C could not be used for this study because it likely contains the binding site for another transcription factor, which constitutively turned on the antibiotic resistance gene. For this reason, only three reporter constructs, with the upstream sequences of YCR102C, YLR460C, and YHL047C, were pursued.
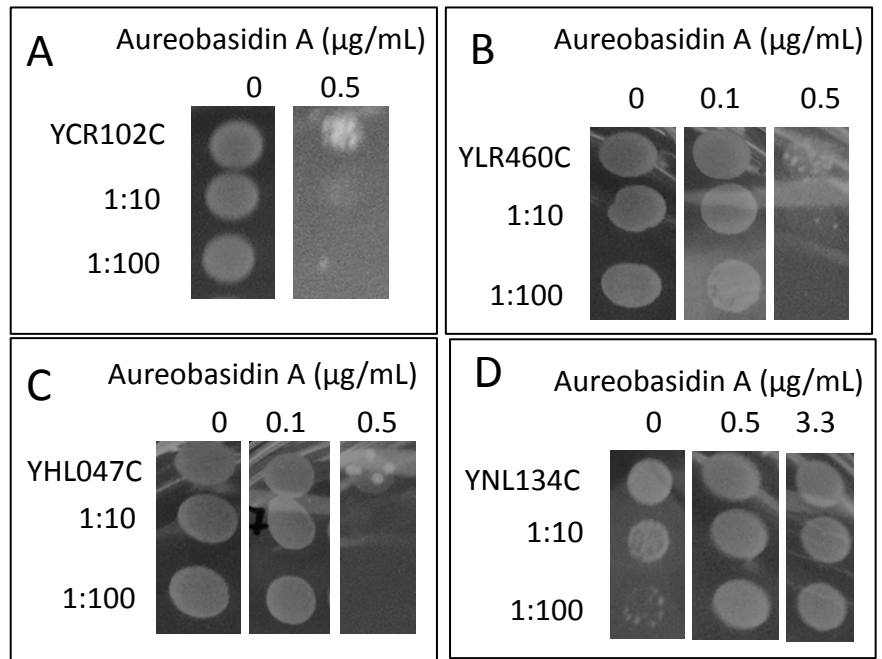
Figure 22: Concentration of aureobasidin A required to inhibit growth. Overnight cultures were spotted on plates containing antibiotic ranging from 0 to 3.3μg/mL. Panels A, B and C exhibit growth inhibition at 0.5μg/mL. Panel D exhibits no growth inhibition at the maximum tested concentration.

We anticipated that these sequences should respond to IAA. To test this hypothesis, samples were

spotted onto plates containing increasing concentrations of IAA, and a constant concentration of

antibiotic. The construct containing the putative upstream regulatory region of gene YLR406C showed

increased growth in response to increased IAA. The empty vector control, and the other three regions

were not able to express enough of the antibiotic resistance gene to overcome the antibiotic in the plates (Figure 22). These data indicate that there may be a transcription factor that is regulated by IAA, and binds upstream of gene YLR406C in order to regulate expression. Future directions for this work would be to use this construct in a yeast 1 hybrid screen, in order to identify this putative transcription factor.
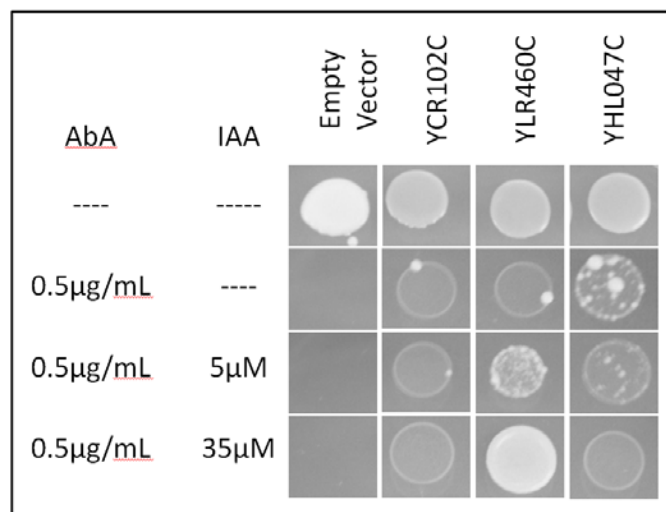
Figure 23: Upstream sequence YLR460C activates alternative gene expression in response to IAA. Overnight cultures were spotted on plates containing no antibiotic and no IAA (positive control), antibiotic and no IAA (negative control), and plates with antibiotic, and increasing concentrations of IAA. YLR460C showed a dose-dependent response to IAA.

Discussion

In summary, Grr1 is critical for fungal response to IAA, potentially as a soluble receptor. Changing four basic residues to acidic glutamate in the LRR region abolishes Grr1 activity. I propose that this is due to a conformational change in the protein structure which renders the active site inaccessible. Additionally, the similar IAA induced phenotypic changes observed in both fungi provide strong evidence that *Candida albicans*Grr1 is the functional homologue of *Saccharomyces cerevisiae* Grr1.

The purpose of this thesis was to investigate the molecular mechanism of IAA (the plant hormone auxin) regulation in fungi, specifically, *Saccharomyces cerevisiae* and the human pathogen *Candida albicans*. It has been established that *S. cerevisiae* synthesize IAA in both a tryptophan dependent and tryptophan independent manner [25], secrete IAA into the environment, transport IAA into the cells, and respond to IAA in several ways, including filamentation, surface adhesion, and growth inhibition (Figures 15-17). I focused my effort on evaluating the contribution of Grr1 to IAA regulation. Grr1 is an F-box protein homologous to the IAA receptor Tir1 in *Arabadopsis thaliana*. This homology suggests that Grr1 may play a similar role in fungi. Deleting *grr1* in *S. cerevisiae* has been shown to sensitize cells to IAA, but what remained to be shown was the specific domain(s) involved in the IAA response.

Tir1 is an F-box protein in the SCF complex. This complex is responsible for the degradation of AUX/IAA proteins (transcriptional repressors). The interaction between Tir1 and the AUX/IAA proteins is only made possible by the presence of IAA, which acts as a "molecular glue," filling a pocket in the Tir1 leucine-rich repeat region (Figure 11). By filling this cationic gap, the surface area for binding is increased, and degradation of the AUX/IAA proteins is possible [33].

Saccharomyces cerevisiae GRR1 is quite similar to Tir1, and previous work in the Rao lab indicates that the *grr1Δ* mutant is hypersensitive to IAA. It was hypothesized that Grr1 may be the functional homologue to Tir1. Several mutants were surveyed to determine the regions required for IAA response. Given that the leucine-rich repeat region of Tir1 forms a pocket where IAA binds, the leucine rich repeat region was a target of interest in this study.

Surprisingly, deleting the leucine-rich repeat region resulted in a phenotype virtually indistinguishable from the isogenic wildtype. Meanwhile, four point mutations in the LRR region (B4E protein) resulted in a phenotype virtually indistinguishable from the *grr1Δ* mutant in all phenotypic tests. Combined with the (previously known) fact that B4E protein is produced just fine [33], this suggests that the conformational change has affected the availability of the active site of the protein.

One of the main differences between plant Tir1 and yeast Grr1 is the carboxy terminal region (Figure 12). The C-terminus of Grr1 is substantially longer than that of Tir1, and its function is unknown. The C-terminal deletion mutant was slightly hyperfilamentous in the absence of IAA, however, when exposed to IAA, it showed no phenotypic change (akin to the wildtype response to IAA), suggesting that the carboxy terminal region of Grr1 is not involved in IAA response.

Candida albicans Grr1 is a functional homologue of *Saccharomyces cerevisiae* Grr1 with regard to IAA regulation. All of the assays performed on the *S. cerevisiae* mutants were performed on the *C. albicans grr1Δ/Δ* mutant, and all IAA-related phenotypes were recapitulated in the *Candida albicans grr1Δ/Δ* mutant: increased IAA sensitivity; filamentation and surface adhesion were increased in response to IAA; IAA uptake was increased in the *grr1Δ/Δ* mutant when compared to wildtype.

While this thesis presents clear indication that Grr1 regulates IAA response in both *Saccharomyces cerevisiae*, and *Candida albicans*, there is still much work to be done in order to better understand the molecular mechanism by which Grr1 regulates fungal response to IAA. The most parsimonious model that explains the phenotypic consequences of *grr1Δ* is that the F-box ubiquitin ligase protein Grr1 indirectly regulates the turnover of IAA transporters (AVT genes in *S. cerevisiae*) [18]. As cell surface proteins in *S. cerevisiae* are turned over by endocytosis, not by the proteasome, Grr1 probably does not recruit these AVTs to the proteasome. Like the Tir1 model (Figure 10), Grr1 likely functions through an intermediary, which in turn regulates levels of the transporters (Figure 24). For instance, Grr1 may tag a repressor protein that inhibits the transcription of the transcription factor Yap1 for degradation in the presence of IAA. Yap1 has been shown to negatively regulate the AVT transporters [18]. Thus, IAA-mediated activation of Grr1 releases the repression of a transcription factor, such as Yap1, resulting in the increase of IAA uptake carriers. Using this model, it follows that when Grr1 is not active, the repressor of the transcription factor will not be degraded, and no transporters will be degraded. This will result in an accumulation of transporters on the membrane, and cells that are hypersensitive to IAA. This is congruent with the data that has been presented here.
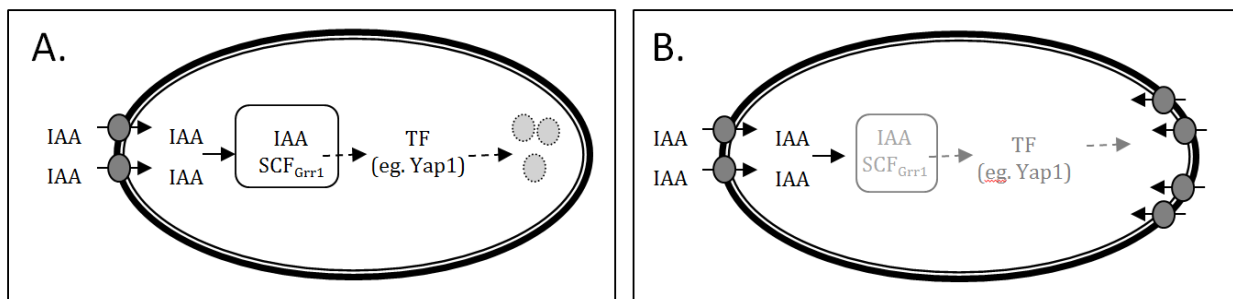
Figure 24: Proposed molecular model of Grr1 mediated IAA response.
Panel A: In wildtype cells, IAA homeostasis maintained by proper regulation of IAA uptake carriers.
Panel B: In *grr1Δ* cells, the SCF complex cannot function to relieve transcriptional inhibition of transcription factors that are responsible for the downstream degradation of uptake carriers.

Future directions for this work are numerous. First, in order for Grr1 to be a receptor for IAA, it must

bind IAA. In order to confirm the hypothesis that Grr1 is the receptor for IAA in yeast, a pull down

assay followed by a binding assay should be performed. Briefly, recombinant GST-IAA will be

expressed in *E. coli*, and purified using glutathione beads. Meanwhile, Grr1 will be cloned into yeast,

and expressed in the presence of ^{35}S translabel, resulting in radiolabeled Grr1. If Grr1 binds IAA,

incubating the beads together with the labeled Grr1 should result in labeled beads [47].

Next, the working model is that Grr1 regulates the turnover of IAA uptake carriers. In order to confirm

this hypothesis, GFP tagged Avt proteins can be tracked in the *grr1Δ* mutant by fluorescent

microscopy. Increased accumulation of Avt proteins in the *grr1Δ* compared to isogenic wildtype

would support our hypothesis (Figure 24).

Lastly, I have shown that the active site for IAA is not likely to be located in the carboxy terminal region

or the LRR region of Grr1 (Figures 15-17). As the F-box motif is required for association with the SCF

complex, it is unlikely that the active site for IAA is located within it. If an N-terminal deletion strain

were constructed, it could be determined whether this is where the active site for IAA is located.

Once this is determined in *Saccharomyces cerevisiae*, it would be interesting to see whether the active

site is located in the same place in *Candida albicans*, as their N-terminal regions are so different in

length (Figure 12).

These results are consistent with the hypothesis that IAA is a small molecule signal that regulates

virulence in the human pathogen *C. albicans*. This is an important finding, as is provides us an avenue

to understanding how fungal pathogens are communicating to turn on virulence traits. Developing a

strategy to disrupt this communication may be a method of treating the ever growing public health problem of drug resistant fungal infections.

One particularly appealing aspect of this new potential antifungal drug target is that it does not necessarily require the entry of the drug into the yeast cells. One main mechanism by which yeast gain resistance to drugs is by developing pumps to excrete the drugs. If the drugs cannot build up in the pathogen, the pathogen cannot be affected. As IAA is secreted from the cells, the drug target could be extracellular. This would mean the mechanism of developing efflux pumps would have no effect on the drugs ability to work.

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