INDUCIBLE RNA INTERFERENCE

IN *ASPERGILLUS NIDULANS*

By

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INDUCIBLE RNA INTERFERENCE

IN *ASPERGILLUS NIDULANS*

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CHAPTER I

INTRODUCTION

The Kingdom Fungi is an essential part of the ecosystem, playing a large role decomposing dead organic matter. Fungi are utilized in industry because they produce valuable enzymes, and they are used as food and in the production of food, such as soy sauce. Fungi can also cause infectious diseases in humans and animals, ranging from minor skin infections, such as ringworm, to serious and lethal infections, such as systemic mycosis. The number of people with compromised immune systems is rising due to advanced HIV infections, chemotherapy for cancer patients, and other predisposing factors that make one more susceptible to fungal infections. There is a need for new, more effective antifungal agents to treat and prevent fungal infections. Ambruticin has potential to be an antifungal agent for humans and animals. The molecular targets of ambruticin are still unknown, and here we use an RNA interference (RNAi) approach to discover those targets. The RNAi construct was first tested with a developmental gene, *brlA*. Then a genomic library was developed with the RNAi construct and screened for resistance against the fungicide fludioxonil. These resistant strains were tested for cross resistance to ambruticin VS3 and sensitivity to salt. Our results confirm that ambruticin targets the membane components of the osmotic stress-sensing pathway.

CHAPTER II

INDUCIBLE RNA INTERFERENCE OF *brlAβ* IN *Aspergillus nidulans*

Abstract

RNA Interference (RNAi) is a eukaryotic mechanism where small RNA molecules regulate gene expression, and RNAi is used as a tool to silence expression of targeted genes. We have used *Aspergillus nidulans*, a multicellular fungus, to test an alcohol-dependent inducible construct for RNAi. This RNAi construct consists of inverted repeats of an alcohol dehydrogenase promoter ($alcA_{(p)}$) with a gene of interest located in a unique restriction enzyme site (*Bam*HI) between the promoters. Our gene of interest for silencing was *brlA*β, the longer of a two-transcript, differentially expressed gene, encoding a transcription factor that regulates asexual development and sporulation. The RNAi mutants show normal phenotypes on standard media containing glucose, but a remarkable loss of sporulation on $alcA_{(p)}$ inducing media containing threonine, similar to that seen of *brlA* mutants. Expression and lack of expression of *brlA* in the two respective growth conditions was confirmed with Northern blotting, RT-PCR, and Real Time RT-PCR. Our results confirm the RNAi construct induces silencing of a targeted gene. Even though only the *brlA*β transcript was targeted for RNAi, both transcripts (*brlA*α and *brlA*β) were not present on RNAi inducing conditions. Anti-sense and siRNA Northerns confirmed that the lack of *brlA*α expression was not due to RNAi mechanisms, but rather a result of reduced *brlA*β expression.

Introduction

 RNA interference (RNAi) is a natural eukaryotic cellular mechanism that can be utilized to silence gene expression. Dicer degrades double-stranded RNA into 21 base pair siRNA duplexes that associate with the RISC complex to degrade complementary mRNA, thus silencing gene expression (Bartel, 2004). RNAi mechanisms have been shown to be functional in targeting specific genes for silencing in *Aspergillus nidulans* through inverted-repeat transgenes (Hammond et al, 2008; Hammond & Keller, 2005) and siRNAs (Khatri & Rajam, 2007). RNAi has been demonstrated in other *Aspergillus* species, including the pathogen *A. fumigatus* (Bromley et al, 2006; Henry et al, 2007; Khalaj et al, 2007; Mouyna et al, 2004) and industrially important *A. oryzae* (Yamada et al, 2007).

 A. nidulans reproduces asexually by extending its hyphae radially and developing aerial conidiophores (Clutterbuck, 1969). The conidiophores are differentiated at the tip into vesicles, primary and secondary sterigmata (metullae and phialides, respectively), and uninucleate conidia, which are the asexual spores (Adams et al, 1998; Boylan et al, 1987; Mims et al, 1988). *Bristle* (*brlA*) mutants have normal hyphal extension, but they have elongated conidiophores that do not develop viable conidia (Clutterbuck, 1969). *brlA*'s null mutations result in complete obliteration of conidiophores, but in leaky mutations, vesicles and sterigmata may form but do not develop viable conidia (Adams et al, 1998; Clutterbuck, 1969). Leaky mutants are thought to have partial *brlA* expression, and variability between phenotypes may be a result of a dose effect (Adams et al, 1998; Prade & Timberlake, 1993). The misscheduled expression of *brlA* leads to cessation of hyphal elongation and hyphal tip conidiation. *brlA* is expressed prior to and in an

epistatic relationship with other developmental genes, including *abaA* and *wetA* (Adams et al, 1988; Mirabito et al, 1989). *brlA* has two, overlapping transcripts, *brlA*α and *brlA*β. The *brlA*β transcript begins at 851 base pairs upstream of the *brlA*α transcript, and has an intron that circumvents the *brlA*α transcription initiation region (Prade & Timberlake, 1993). Previous models of development propose that *brlA*β initiates development, and *brlA*α continues development through a feedback pathway with *abaA* (Han et al, 1993). A strain that lacks *brlA*β (-1290 to -404), and has an intact *brlA*α, has early *brlA*α expression, but *brlA*α expression ceases after 12 hours of development (Prade & Timberlake, 1993). More recently it was found that overexpression of *brlA*β activates expression of *brlA*α, even when *abaA* is absent (Han & Adams, 2001). We chose to study silencing of this unique *brlA*β transcript, and to examine how silencing of *brlA*β affects *brlA*α.

In this paper, we demonstrate that a construct with inverted repeats of an inducible *alcohol dehydrogenase* promoter ($alcA_{(p)}$) flanking *brlA*β can silence its expression through RNAi in *A. nidulans*. We found that when *brlA*β was silenced, *brlA*α expression was also mute. The lack of anti-sense RNA and siRNAs specific for *brlA*α indicate this was not due to the RNAi inactivation, but more likely that *brlA*β controls the expression of *brlA*α.

The $alcA_{(p)}$ is repressed in glucose but strongly induced in threonine, and it has been used for misscheduled expression of *brlA* (Adams et al, 1988), overexpression of *brlA*α and *brlA*β, individually (Han & Adams, 2001), overexpression of a random genomic DNA library (Marhoul & Adams, 1995), and gene silencing in *Penicillium expansum* by inverting the downstream gene (Schumann & Hertweck, 2007). By using

inverted repeats of an $alcA_{(p)}$ flanking a gene, induced expression of double-stranded RNA triggers the RNAi mechanism to silence that gene. Our construct contains the *brlA*β (-2902 to -404) in a unique *Bam*HI site, so that *brlA*β may be replaced by a genomic DNA library or a specific gene of interest. This approach of using a single construct to silence many genes will increase knowledge of the growth and development mechanisms of a multicellular, sporulating model organism.

Materials and Methods

  Plasmid construction. $alcA_{(p)}$ was amplified with PCR from a previously constructed plasmid pRP68A, and the resulting 622 base pair PCR fragment was cloned into pGEM-T Easy Topoisomerase cloning vector (Promega). This vector, pSW2, allowed the $alcA_{(p)}$ to have flanking *Eco*RI and flanking *Not*I sites. Plasmid pRP07, containing the *brlA*β upstream sequence (AN0973.3, -2902 to -404, Figure 1A) cloned into the *Bam*HI restriction site on pBluescript ks(+), was used as a vector for the *Eco*RI isolated $alcA_{(p)}$ from pSW2. A plasmid with the $alcA_{(p)}$ oriented toward the *brlA*β fragment was selected, pSW5. Plasmid pSW5 was used as a vector for an *argB* nutritional selection gene (Johnstone et al, 1985). Plasmid pDC1, where the *Bam*HI site had been filled-in through a Klenow reaction, was used as a source for *argB*. It was isolated using *Sac*I partial digestion, and cloned into the *Sac*I site of pSW5. The resulting plasmid was named pSW6. Plasmid pSW6 was used as a vector for the *Not*I isolated $alcA_{(p)}$ from pSW2. A plasmid containing inverted repeats surrounding *brlA*β was selected, so that both promoters in the plasmid were oriented toward the *brlA*β. The final

RNAi construct plasmid was named pSW8 (Figure 1B).
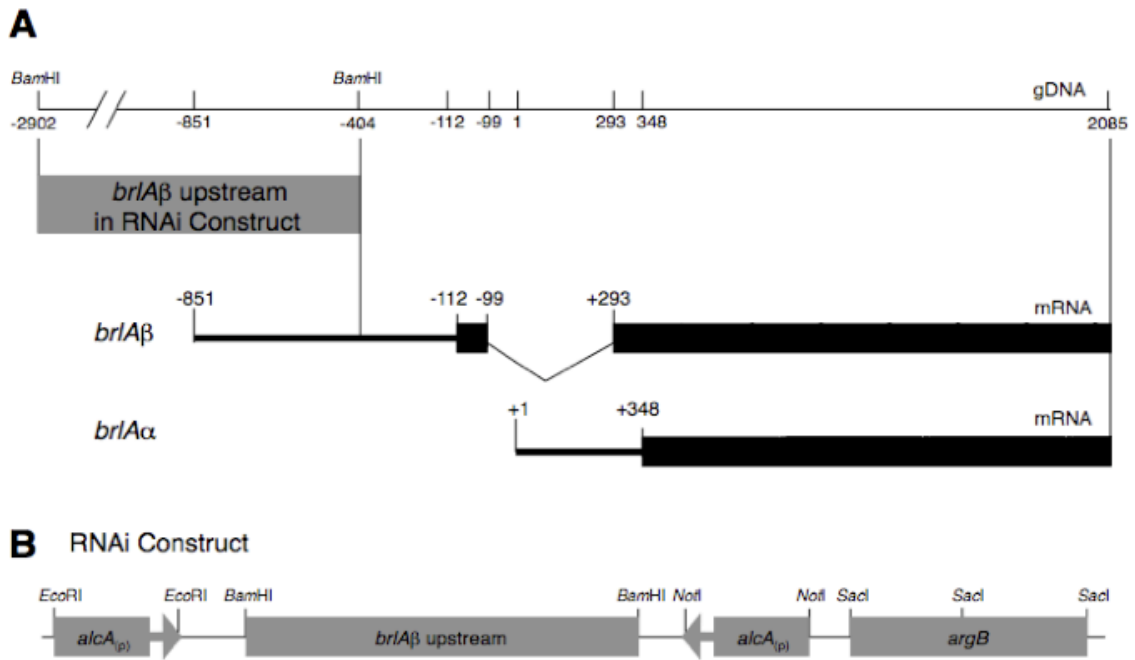
Figure 1. *brlA* Locus and RNAi Construct. **(A)** The *brlA* locus consists of two overlapping transcriptional units, *brlAα* and *brlAβ*. The top line represents the *brlA* genomic DNA (gDNA). The portion of the locus flanked by *Bam*HI sites (-2902 to -404) used in the RNAi construct included only *brlAβ*. The line of the transcripts (mRNA) represents untranslated RNA, and the box represents translated mRNA. **(B)** The RNAi construct consists of inverted repeats of inducible *alcA*(p)s flanking *brlAβ* in a unique *Bam*HI site and an *argB* marker.

Transformations were performed using *E. coli* SURE chemical competent cells (Stratagene). Additional competent cells were prepared (Inoue et al, 1990). Plasmid containing cells were recovered on LB Agar with 0.1mg/ml ampicillin. DNA was prepared according to Minipreparation protocol (Sambrook & Russell, 2001), and solutions were prepared with chemicals obtained from Sigma-Aldrich. Gene orientation

and sequences were verified using restriction enzyme mapping and sequencing. Sequencing was performed at Oklahoma State University's Core Facility on an ABI Model 3730 DNA Analyzer. All genes were isolated through gel electrophoresis in 1% agarose gels, and purified with a Gel Extraction Kit (Qiagen). Restriction enzymes, Ligases, and Polymerases were purchased from Invitrogen.

***Aspergillus nidulans* Transformation.** All strains were grown on *Aspergillus* Minimal Media (MM) (Pontecorvo et al, 1953). *A. nidulans* strains RMS11 (*pabaA1, yA2, argB::trpC B, veA1, trpC801*) and LR191 (*argB, pabaA1, pyrG, pyroA, yA2, veA1*) were used as parental strains. Spores were grown to germination, washed with 0.6 M $MgCl_2$, and digested with Drisilase (Sigma-Aldrich) at 30°C at 100 rpm for one hour. Protoplasts were washed twice with 0.6 M KCl and resuspended in STC50 (1.2 M sorbitol, 0.01 M $CaCl_2$, 0.05 M Tris-HCl pH 7.5). Linear DNA (10 μg) was added, and the mixture was incubated at room temperature for 5 minutes. 60% PEG4000 was added, mixed by rolling on the bench, and incubated at room temperature for 20 minutes. STC50 was added to dilute the mixture, mixed by inversion, and poured on three plates, with 1.2 M sorbitol and appropriate supplements, excluding arginine for nutritional selection of transformants. Plates were incubated three days to allow development of the transformants. Eight total pSw8 strains were obtained, and two pDC1 strains were obtained.

Analysis of Transformants. To extract genomic DNA, spores were grown on MM broth on Petri dishes for no more than 24 hours. The mycelia mats were harvested, washed with sterile water, and frozen with liquid nitrogen. Mycleia were lysed by adding 1 ml of genomic extraction solution (1% SDS, 50 mM EDTA) to 100 mg of tissue, heated

one hour at 68ºC, and separated by centrifugation. The supernatant was transferred, 45 µl of 5 M potassium acetate was added, and samples were incubated on ice 10-30 minutes and centrifuged. The supernatant was transferred, and the genomic DNA was precipitated with 95% ethanol. The genomic DNA was fished out with a glass hook, washed with 70% ethanol, and resuspended in TE with 10 mg/ml RNase.

PCR analysis of 10 ng genomic DNA was performed using Pfx (Invitrogen) according to manufacture's directions. Southern Blot analysis (Sambrook & Russell, 2001) of 5 µg genomic DNA, digested with *Eco*RI was performed using DIG High Prime DNA Labeling and Detection kit (Roche) or ^{32}P-dATP DNA labeled probe constructed with RadPrime DNA Labeling System (Invitrogen).

Phenotype analysis of transformants was observed by growing the strains on MM agar for 48 hours at 37°C. Spores were harvested from the plates by scraping with sterile water, and inoculated into MM broth. These cultures were incubated 24 hours, shaking 250 rpm at 37°C. Mycelia were harvested by filtration and washed thoroughly with sterile water. Each strain was plated on MM containing 100 mM glucose and 100 mM threonine as the sole carbon source. These plates were incubated for 24 hours for visible phenotypes. Images of plates were taken by scanning the plates using a Microtek ScanMaker 4700. Digital Photographs (Canon Powershot A620) were taken under a stereomicroscope (Nikon SMZ-U) at 60X magnification, and pictures of tape mounts were taken at 600X magnification under a light microscope (Nikon TMS).

RNA of each strain in each condition was harvested at 24 hours after developmental induction and purified with Tri Reagent (Sigma-Aldrich). RNA (10 µg) was separated on a formaldehyde agarose gel, blotted to a membrane, and fixed to the

membrane with UV light (Sambrook & Russell, 2001). DNA probes were constructed as previously mentioned, and ^{32}P-UTP RNA probes were constructed with Maxiscript T7 kit (Ambion). Hybridization and detection were carried out as previously described (Sambrook & Russell, 2001). For analysis of siRNAs, 5 μg of RNA of each sample was hybridized with a sodium carbonate treated probe (Catalanotto et al, 2002) with a mirVana miRNA detection kit (Ambion) and run on a 15% denaturing polyacrylamide gel.

Reverse transcriptase PCR (RT-PCR) analysis was begun by treating 2 μg of each RNA sample with DNase (Ambion) and converting it to cDNA with Reverse Transcriptase (Ambion). RT-PCR analysis was set up with Pfx (Invitrogen), 100 ng of cDNA template, and 300 μM of each primer into 50 μl total reactions. The reactions were carried out on MJ Research PC-200 Thermal Cycler for 22 cycles with the following conditions: 1. 94C for 2 minutes, 2. 94C for 30 seconds, 3. 50C for 30 seconds, 4. 68C for 5 minutes, 5. GoTo step 2. 21 times, 6. 68C for 5 minutes. Each sample had 8 μl run on a 1% agarose gel. The gels were stained with ethidium bromide and pictures were taken on a GelDoc-IT TS Imaging System (UVP).

Each Real-Time RT-PCR reaction was set up with SYBR Green PCR master mix (Applied Biosystems), 20 ng of cDNA, and 300 μM of each primer into 15 μl total reactions. Each reaction was performed in duplicate or triplicate on 96 well, optical plates on an Applied Biosystems 7500 Real-Time PCR System. Dissociation curves were performed for each reaction following the PCR and agarose gels were run on selected samples to ensure purity of products. For analysis, the threshold was set at 1.5 for the C_t value, and $\Delta\Delta C_t$ calculations were performed relative to Actin and the mean Δ

C_t. Gene expression fold change values were derived from $10^{(-\Delta\Delta Ct/\ln10/\ln2)}$, where the primer efficiency ($\ln10/\ln2$) was calculated experimentally for each primer set by PCR reactions of serial dilutions of genomic DNA. The average fold change and standard deviation were calculated for each sample and graphed on Microsoft Excel.

Table 1: Primers used in this study.

Primer	Sequence	Purpose
alcA(p) +	AAAAATCTTACTCCAGTGGTTCGG	clone alcA, DNA probe
alcA(p) -	GAGGCGAGGTGATAGGATTGGAAG	clone alcA, DNA probe
pSW8 brlA left	GCTGACGATCAGCTTTCTCC	sequencing, PCR
pSW8 argB right	GTTTCGCAATGGCTGTAGGT	sequencing, PCR
brlAβ left	GGATGCCACTTTCTCTCTGC	DNA probe, PCR
T7 and brlAβ left	ATATTAATACGACTCACTATAGGGGATGCCACTTTCTCTCTGC	RNA probe
brlAβ right	GTCTCAACCCGCACGTAGAT	DNA/RNA probe, PCR
brlAβ left 2	CGTTTAAGGGCGGGTCTATT	RT-PCR
brlAβ right 2	GAGAAGTGCCAGCCAGAGTC	RT-PCR
brlAα left	TCACCAACTCGCTCATTCAC	RT-PCR
brlAα right	ACGTTCCCTAAGCTTTGCAG	RT-PCR
brlAα and β left	ATATGGATCCTCACTCCCCAACAACACGTA	DNA probe, RT-PCR
T7, brlAα and β left	ATATTAATACGACTCACTATAGGTCACTCCCCAACAACACGTA	RNA probe
brlAα and β right	ATATGGATCCGAACTGCACCTGCTTGATGA	DNA/RNA probe, RT-PCR
creA left	CATGAGCCGTTCCCATTC	DNA probe, RT-PCR
creA right	AACGGAATTTGCGGTTGA	DNA probe, RT-PCR
brlAβ Forward	AAACAGCTAGTCCAGCCCTCTGTT	Real time RT-PCR
brlAβ Reverse	AGCAAGGCCAGGAATGTAGGCTAT	Real time RT-PCR
brlAα Forward	TTCATTCCTTCACTGGCCTCCACT	Real time RT-PCR
brlAα Reverse	AGCACCGTTCAGTTTACGTTCCCT	Real time RT-PCR
brlAα and β Forward	ACGAACTCCTGGTTCTGCTTCGAT	Real time RT-PCR
brlAα and β Reverse	ATTCGTTCCTGCCCTTCCATGCTA	Real time RT-PCR
actA Forward	TCAGGTTATCACCATCGGCAACGA	Real time RT-PCR
actA Reverse	TACCACCGCTTTCCAGACCAAGAA	Real time RT-PCR

Results

The RNAi construct integrates into the genome. In order to prove that the RNAi construct can integrate into the genome of *A. nidulans*, a sporulating, *argB* deficient strain was transformed. Eight resulting transformation strains were *argB* sufficient, and all strains sporulated on standard MM. Hyphal mats of these strains were grown and genomic DNA was extracted. Southern Blots were performed with *Eco*RI digested genomic DNA, using a DIG-labeled *alcA*(p) as a probe (data not shown). PCR reactions were performed on the genomic DNA with primers that were specific for the

RNAi construct, with the left primer specific for *brlA* and the right primer specific for *argB* (Table 1). These were also the primers used for sequencing the plasmid DNA before transforming it into *A. nidulans* (data not shown). The Southern and PCR results indicate that four of the transformation strains, named JB1, JB3, JB6 and JB8, contain the RNAi construct. Additional PCR reactions and Southern Blots were performed on transformation strains JB1 and JB3 (Figure 2). The first PCR primers used were *argB* and *brlA* specific as before (Figure 2A). This primer set gave strong products for the RNAi construct, JB1 and JB3, but no product for pBluescript or the wild type. Primers specific for the *brlA*β (Table 1) were also used (Figure 2B). Again strong products were present in the RNAi construct, JB1 and JB3, no product was seen in pBluescript, and a product was present for the native *brlA*β in the wild type. These results indicate the RNAi construct is integrated into the genome. In the Southern blots, genomic DNA was both not digested and digested with *Eco*RI and probed with an isotopically labeled PCR product specific for *brlA*β (Figure 2C). This blot shows that all the strains have a native *brlA* gene (4.6 kb), and JB1 and JB3 possess an additional *brlA* band that is a smaller size than the wild type (2.5 kb), due to the *Eco*RI digestion sites introduced to through the RNAi construct. The Southern blot results indicate again the RNAi construct is integrated into the genome and the native *brlA* gene is present in the strains as well without being disrupted by homologous recombination. The 2.5 kb band in the Southern blot is stoichoimetrically more dense than the 4.6 kb band, indicating that there could be more that one copy of the RNAi construct integrated into the genome. To further demonstrate that the RNAi construct is integrated into the genome of the RNAi strains, sexual crosses (Pontecorvo et al, 1953) between JB1 and JB3 with the wild-type *argB*

deficient strain were performed (data not shown). All *argB* sufficient progeny analyzed possessed the inducible silencing of the *brlA* phenotype.
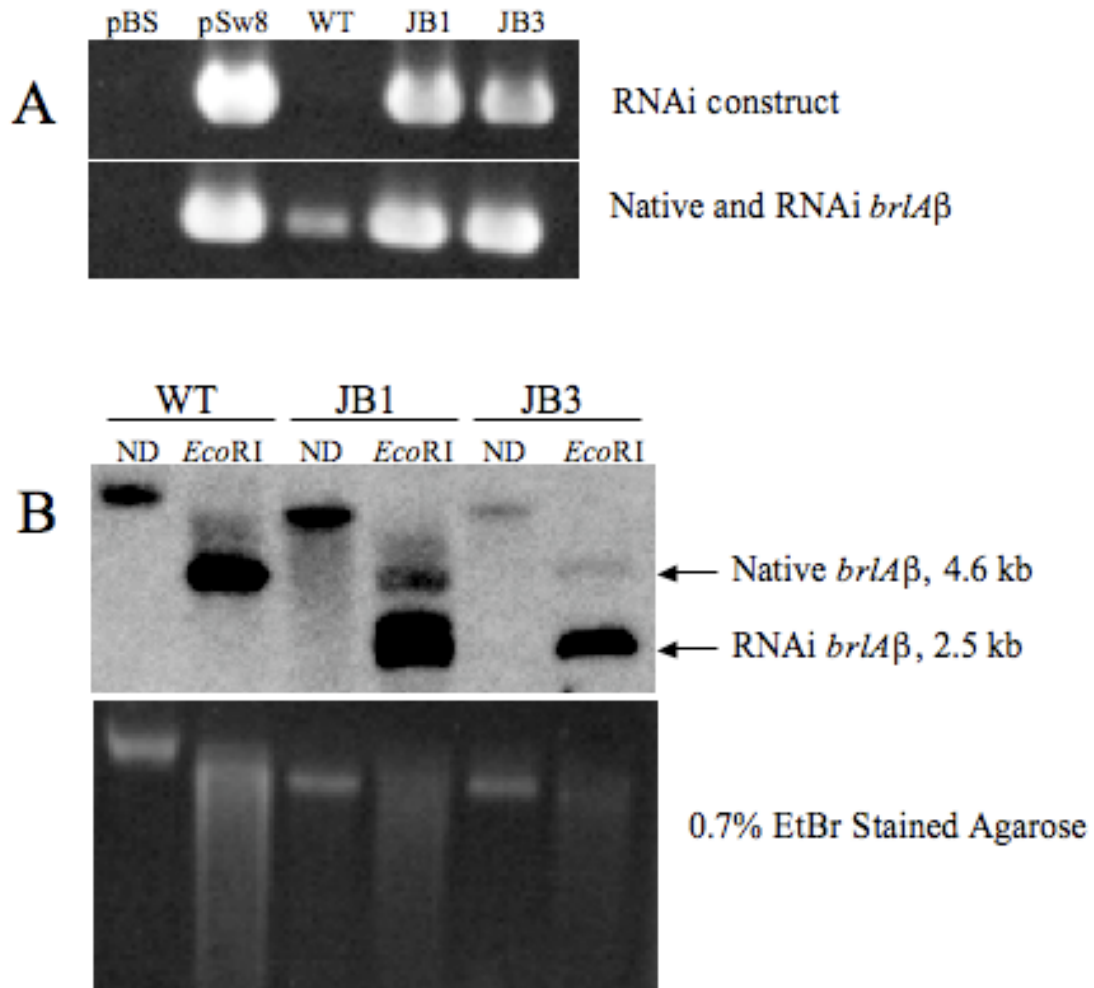
Figure 2. Integration of the RNAi construct into the genome. **(A)** Plasmid DNA from pBluescript ks(+) (pBS), the RNAi construct (pSw8) and genomic DNA isolated from the wild type (WT) and RNAi strains, JB1 and JB3, were all analyzed with PCR to verify presence of the RNAi construct (top band) and native and RNAi construct *brlAβ* (bottom band). **(B)** Southern blot analysis of wild type, JB1 and JB3 genomic DNA, both not digested (ND) and digested with *Eco*RI revealed presence of a 4.6 kb *brlAβ* band in all strains that is part of the native brlA locus and a 2.5 kb *brlAβ* band in JB1 and JB3 that is

part of the RNAi construct. Shown below is a photograph of the 0.7% agarose gel with ethidium bromide stained genomic DNA, from which the membrane was blotted.

The RNAi strains have inducible silencing of the *brlA* phenotype. *A. nidulans* conidiophore development is suppressed in submerged culture, but spores inoculated into submerged culture develop vegetatively to produce mature mycelia. Conidiophore development begins in a synchronized manner when the mycelia are plated and exposed to air (Axelrod, 1972; Boylan et al, 1987). The wild type, JB1 and JB3 were grown by inoculating spores in standard MM broth with appropriate supplements, and shaken 24 hours at 250 rpm. Mycelia was harvested by filtration, washed thoroughly with sterile water, and plated on either glucose or threonine MM (Figure 3). After 24 hours of developmental induction, it was clear that the wild type developed conidia on both glucose and threonine, but the RNAi strains developed conidia only on glucose. The lack of yellow-pigmented conidia makes the RNAi strains appear white on threonine (Figure 3A). The RNAi strains developed extended aerial conidiophores that did not continue development to produce viable conidia on threonine, just as the *brlA* phenotype exhibits (Figure 3B and C). The lack of full conidiophore and conidia development indicates that *brlA* is silenced when the RNAi strains develop in $alcA_{(p)}$ inducing conditions, but *brlA* is expressed normally without silencing in the presence of glucose that represses $alcA_{(p)}$.
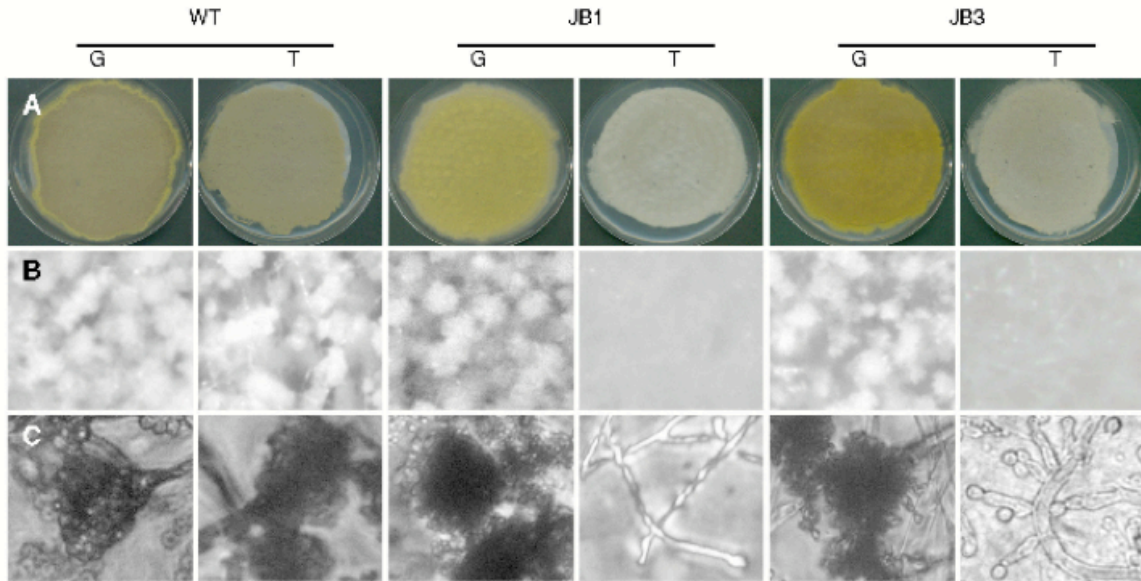
Figure 3. Phenotypes of RNAi Strains. **(A)** Plates that contained either glucose (G) or threonine (T) as the sole carbon source. **(B)** Plates under a stereomicroscope, 60X magnification. **(C)** Tape mounts under a light microscope, 1000X. These images show the wild type (WT) on both conditions, and the RNAi strains (JB1 and JB3) on glucose with normal development, but the RNAi strains on threonine show *brlA* phenotypes.

JB1 displays a complete *brlA* null phenotype with elongated, aerial conidiophores that do not develop vesicles, sterigmata or conidia. On the other hand, JB3 displays the *brlA* leaky phenotype that develops rudimentary vesicles and sterigmata in an abnormal manner, and it lacks viable conidia (Figure 4). The variability in the phenotypes of the two RNAi strains indicates that RNAi construct can be useful in constructing strains that have varied amounts of gene silencing.
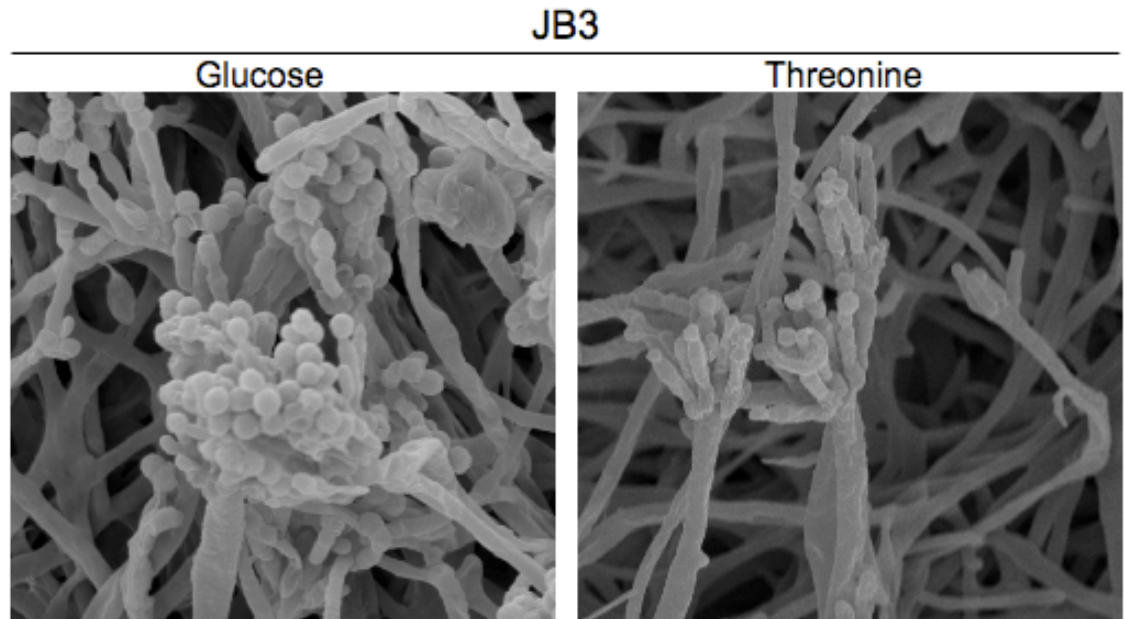
Figure 4. Scanning Electron Microscopy (SEM) Images of JB3. This RNAi strain produces chains of conidia in glucose, but it has a partial, or leaky, *brlA* phenotype in threonine, where conidiophores are partially formed. J.S. Barton fixed and coated the samples and ran the SEM at the OSU Microscopy Facility in Stillwater, OK.

Expression of *brlA* is reduced in silencing conditions. In order to prove that the expression of *brlA* is silenced on threonine, but not on glucose, gene expression analysis was performed with Northern blotting, RT-PCR, and Real Time RT-PCR (Figure 5). RNA used for these experiments was harvested at 24 hours after mycelia were plated. The Northern blots were probed with an isotopically labeled DNA probe specific to the downstream portion of *brlA*, where *brlA*α and *brlA*β transcripts overlap (Figure 5A). The Northern blot shows abundant *brlA* expression on glucose in all the strains. On threonine, the wild type had abundant *brlA* expression, but JB1 had a nearly absent signal, and JB3 had a drastically reduced signal for both *brlA*α and *brlA*β expression. As

a control *creA* was also probed to show similar expression in all strains and conditions, and rRNA is shown as a loading control. In the RT-PCR reactions (Figure 5B), the wild type had nearly equal PCR products in both glucose and threonine. Both JB1 and JB3 had abundant products from glucose, but on threonine JB1 had nearly no product and JB3 had much less product for both *brlA*α and *brlA*β. In order to quantify the fold change of the *brlA*α and *brlA*β transcripts, Real Time RT-PCR was employed (Figure 5C). In the wild type, *brlA*α and *brlA*β transcripts were nearly equal on both glucose and threonine. This indicates that no RNAi of *brlA* is occurring on threonine in the wild type. In JB1, *brlA* transcript level on glucose is approximately equal to that of wild type on glucose, but there is only slightly detectable abundance of *brlA*α and *brlA*β in JB1 on threonine. JB3 had a large amount of *brlA*α and *brlA*β on glucose, four to nearly five fold more transcripts than wild type on glucose, but JB3 *brlA*α and *brlA*β transcript level on threonine was three to four fold less abundant than on glucose. These experimental results indicate that the expression of *brlA* is indeed silenced in $alcA_{(p)}$ inducing conditions in the RNAi strains. The silencing in JB1 is more drastic than the silencing in JB3, which corresponds to the respective *brlA* null and leaky phenotypes. The silencing of both the *brlA*α and *brlA*β transcripts is clearly shown here, but this raised the question of whether the silencing of *brlA*α is due to RNAi mechanisms or lack of *brlA*β transcript.
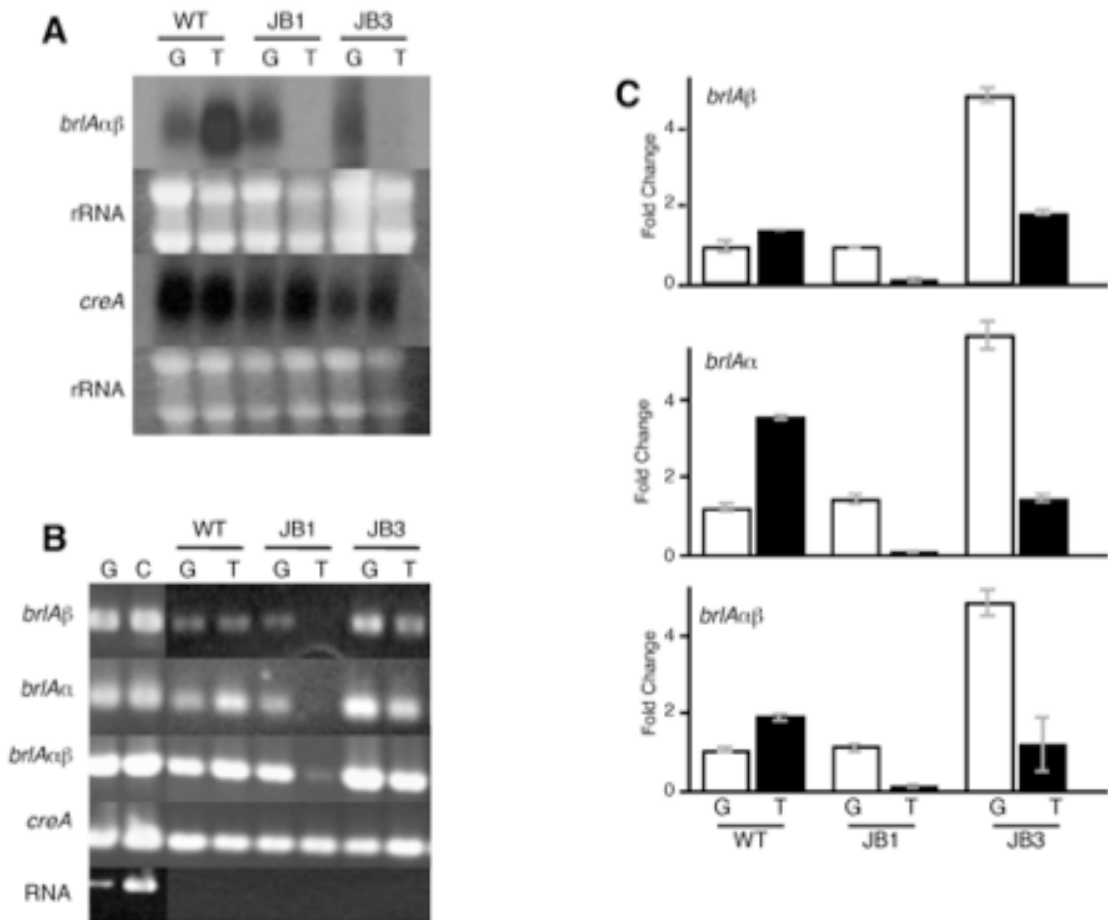
Figure 5. Expression of *brlA* in the RNAi strains. **(A)** Northern blot analysis of RNA indicates that *brlA* expression is present in the wild type (WT) in both conditions and the RNAi mutants (JB1 and JB3) in glucose (G), but not detectable on threonine (T). Expression of creA was consistent in all samples. **(B)** RT-PCR revealed the same pattern as the Northern: normal *brlA* expression in wild type in both conditions and RNAi mutants in glucose, insignificant amounts of *brlA* expression in JB1, and reduced but detectable amounts of *brlA* expression in JB3. *brlA*α and *brlA*β specific primers were used, as well as primers for the overlapping portion of *brlA*αβ. *creA* showed consistent expression in all strains and conditions. RNA samples revealed no products, to ensure that it was not contaminated with genomic DNA. Controls for each primer set was

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carried out with: genomic DNA (g), and a cDNA library (c). **(C)** Real Time RT-PCR allowed for the fold change quantification of the transcripts. Primers specific for *brlA*α, *brlA*β, and *brlA*αβ were used. The wild type had normal expression of brlA on both conditions. JB1 had normal brlA expression on glucose, but almost undetectable expression on threonine. JB3 had high levels of *brlA* expression on glucose, but drastically reduced expression on threonine.

Silencing of *brlA*β is due to RNAi. To verify that RNAi mechanisms are the direct cause of *brlA* silencing, Northern blots to detect anti-sense RNA and siRNAs were performed (Figure 6). These blots also verify that *brlA*β is the target of RNAi, not *brlA*α. Isotopically labeled RNA probes were constructed for the *brlA*β anti-sense Northern (Figure 6A), and a band approximately 400 bp, the predicted size of the *brlA*β transcript being targeted for silencing, was present in the RNAi mutant strains on threonine. Anti-sense RNA of *brlA*β was not detected in the wild type or in the RNAi strains on glucose. Anti-sense RNA of the overlapping portion of *brlA*α and *brlA*β (Figure 6B) was not detected in any of the strains or conditions. Just as in the anti-sense RNA Northern blots, siRNAs for *brlA*β (Figure 6C) were present in the RNAi strains on threonine, but not in the wild type or on glucose. No siRNAs were detected for the overlapping portion of *brlA*α and *brlA*β (Figure 6D). These Northerns also show that much more anti-sense RNA and siRNAs are present in the JB1 RNAi mutant than in the JB3 RNAi mutant. The variability in the RNAi corresponds to respective *brlA* null and leaky phenotypes of the RNAi strains.
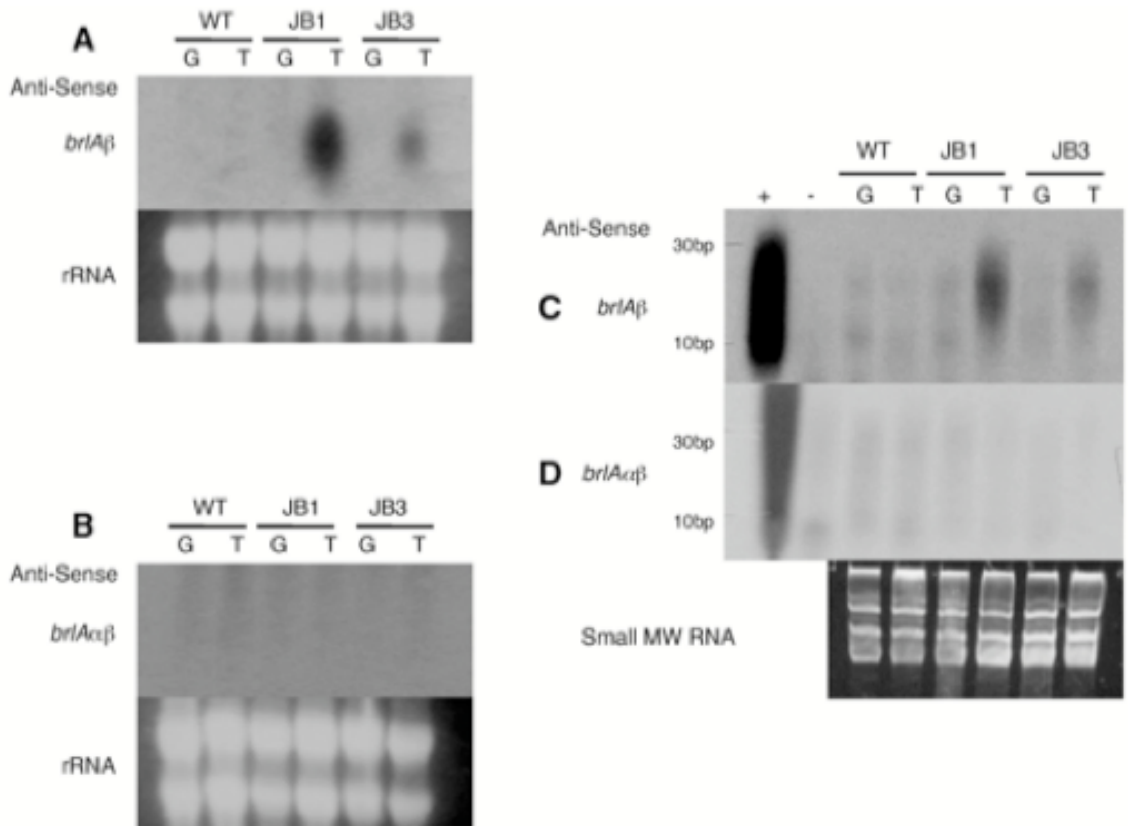
Figure 6. Blots showing RNAi of *brlA* in the RNAi strains. **(A)** Northern blot analysis of the wild type (WT), and RNAi strains (JB1 and JB3) in glucose (G) and threonine (T) using an RNA sense probe specific for *brlA*β detected complementary anti-sense RNA present in the JB1 and JB3 in threonine. **(B)** An RNA sense probe specific for *brlA*αβ did not detect any anti-sense RNA. **(C)** siRNAs specific for *brlA*β in the RNAi mutants on threonine were detected. No siRNAs were detected in the wild type or the RNAi mutants on glucose. **(D)** No siRNAs were detected using a probe specific for *brlA*αβ. This indicated that the RNAi mechanism is specifically targeting *brlA*β, not *brlA*α. Small molecular weight RNA is shown to verify the integrity of the RNA.

Discussion

Inducing inverted repeats of *alcA* promoters that flank *brlA*β causes RNAi to nearly eliminate or severely reduce *brlA* expression. The unique RNAi construct integrated stably into the genome of the RNAi strains, and it did not disrupt the native *brlA* locus. This was verified through PCR and Southern analysis. The RNAi strains had normal conidiation and expression of *brlA* on $alcA_{(p)}$ suppressive media (glucose), but dramatic *brlA* phenotypes and loss of expression on $alcA_{(p)}$ inducing media (threonine). RNAi was the cause of the *brlA*β silencing, due to presence of anti-sense RNA and siRNAs on $alcA_{(p)}$ inducing media, but RNAi did not cause *brlA*α silencing. Lack of *brlA*α expression is most likely due to lack of *brlA*β expression and loss of feedback mechanisms to activate *brlA*α.

The RNAi construct used here serves as a powerful genetic tool for four reasons. First, $alcA_{(p)}$ is suppressed in glucose and strongly induced in threonine (Lockington et al, 1985). The $alcA_{(p)}$ can force expression of a downstream region of DNA (Adams et al, 1988; Han & Adams, 2001; Marhoul & Adams, 1995; Mirabito et al, 1989), and this is useful in isolating and examining essential genes. Mutants that die when an essential gene is knocked out by homologous recombination or silenced by a constitutively expressed promoter, may be isolated with our RNAi construct under $alcA_{(p)}$ suppressive conditions, and then examined under $alcA_{(p)}$ inducing conditions. Second, the orientation of the gene or gene fragment between the $alcA_{(p)}$s may be in either direction. Since they are transcribing RNA in both directions, double-stranded RNA will be produced regardless of the orientation of the DNA in the *Bam*HI site flanked by the $alcA_{(p)}$s.

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Orienting the gene to be silenced in either direction is an advantage over the currently popular use of inverted-repeat transgenes (ITRs) where the gene of interest must be cloned in specific orientations to produce double-stranded hairpin RNA (Hammond et al, 2008; Hammond & Keller, 2005; Nakayashiki et al, 2005; Yamada et al, 2007). The third reason our construct is a useful genetic tool is because the RNAi may nearly eliminate expression of a gene altogether or only downregulate gene expression. This variability in the silencing of the genes is probably due to where the RNAi construct has integrated into the genome. The RNAi construct can integrate into a typical wild type strain so that it may reside in a portion of the genome that has high or low amounts of transcription. Although variations between strains in the amount of gene silencing is sometimes considered a disadvantage (Nakayashiki, 2005), in our case the variability between strains allowed for a null and leaky *brlA* phenotype, and this difference between the amounts of gene silencing may prove useful in understanding the function of other genes that produce different phenotypes with partial expression. Finally, the fourth reason our RNAi construct is a powerful genetic tool is because any gene, gene fragment or a genomic library may be inserted in either orientation into the unique *Bam*HI site between the $alcA_{(p)}$s and examined with inducible gene silencing.

   A. nidulans serves as an asexual developmental genetic model due to its quick growth and development, ease of making developmental mutants, and ease of mapping the developmental loci through sexual crosses (Clutterbuck, 1969; Pontecorvo et al, 1953). Conidiophore development has been shown to involve expression of about 1,000 genes (Timberlake, 1980), and *brlA* is key in the initiation of proper conidiophore development due to its upstream regulation of other developmental specific genes, such

as *abaA* (Adams & Timberlake, 1990; Mirabito et al, 1989), *wetA* (Marshall & Timberlake, 1991), *rodA* (Chang & Timberlake, 1993; Stringer et al, 1991), and *stuA* (Miller et al, 1992). The regulation of *brlA* has been speculated and examined intensively, but there are still unanswered questions about it (Aguirre, 1993; Han & Adams, 2001; Han et al, 1993; Prade & Timberlake, 1993). The most current models suggest that *brlA*β initiates asexual development, since it is present in small quantities in vegetative cells, and *brlA*α continues the development through a feedback mechanism with *abaA* (Adams et al, 1998; Han & Adams, 2001; Han et al, 1993). *A. nidulans* contains two RNA-dependent RNA polymerases that could amplify the RNAi signal through transitive RNAi, shown to occur in *Caenorhabditis elegans* (Alder et al, 2003), but transitive RNAi has been absent in previous *A. nidulans* silencing experiments (Hammond & Keller, 2005). With a few rare exceptions, RNAi is sequence specific in fungi, not locus specific (Nakayashiki, 2005). If the RNAi signal were amplified, all RNAi strains would have null *brlA* phenotypes. One of our RNAi strains, JB3, has a leaky phenotype when induced, indicating lack of amplification of the RNAi signal and lack of transitive RNAi. Our results also show that RNAi was specific for *brlA*β, due to the presence of anti-sense RNA and siRNAs specific to *brlA*β, and lack of these specific for *brlA*α. The RNAi construct in this study only targeted *brlA*β for silencing. However, our results show that both *brlA*α and *brlA*β expression were not present or severely reduced when the RNAi strains were induced. Furthermore, in a *brlA*β knockout mutant, *brlA*α expression was not detected after 12 hours of developmental induction (Prade & Timberlake, 1993), and overexpression of *brlA*β induces expression of *brlA*α, even when

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abaA is not present (Han & Adams, 2001). This evidence strongly suggests that *brlAβ*

plays a key role in regulating expression of *brlAα*.

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CHAPTER III

FLUDIOXONIL AND AMBRUTICIN RESISTANCE IN *Aspergillus nidulans* USING

A NOVEL APPROACH WITH INDUCIBLE RNA INTERFERENCE

Abstract

Ambruticin is an antifungal agent with potential for use to treat fungal infections in humans and animals, and fludioxonil is a commonly used fungicide in agriculture. Evidence has pointed to the osmotic stress-signaling pathway to be the target of ambruticin and fludioxonil due to intracellular accumulation of glycerol. Further evidence of resistance conferred by mutations of upstream group III histidine kinases, an additive effect of resistance by response regulators, and improper activation of Hog1, in the HOG MAP kinase pathway, supports that ambruticin and fludioxonil mimic osmotic stress. Here we show that there are exceptions and inconsistencies in the proposed targets, and we use a novel method to identify resistance to ambruticin and fludioxonil with a genomic RNA interference library of *Aspergillus nidulans*. Nearly 300 strains in the library were found to have fludioxonil and ambruticin resistance, as well as sensitivity to osmotic stress. We further analyzed the 24 annotated multiple hits for the same ORFs and found that 19 are isolated with the membrane fraction and 21 have transmembrane domains. This indicates that downregulation of multiple genes may confer resistance to antifungals that affect the osmotic stress-signaling pathway.

Introduction

Ambruticin is an antifungal agent produced by the myxobacterium *Sorangium cellulosum*. Ambruticin S was first isolated and structure determined in the 1977 (Ringel et al, 1977). Ambruticin S was found to effectively kill a wide range of fungi with MICs lower than 1.5 μg/ml, including *Coccidiodes immitis, Trichophyton mentagrophytes, Histoplasma capsulatum, Botrytis cinerea, Aspergillus flavus* and *Candida parapsilosis*. Ambruticin was found ineffective in killing *Cryptococcus neoformans* and *Candida albicans*, (Ringel et al, 1977). Ambruticin was shown to be effective in mice against coccidioidomycosis (Levine et al, 1978) and histoplasmosis (Shadomy et al, 1978), and it was effective against *Trichophyton mentagrophytes* in guinea pigs (Ringel, 1978). Ambruticin was readily absorbed by the oral route in mice, guinea pigs and a single dog, and it prolonged the life of mice severely infected with *C. albicans* from 5 days to 15 days (Ringel, 1978). No noticeable side effects were observed in any of the above studies with reasonable doses, and no resistance to ambruticin was developed *in vitro* after 10 passages of *H. capsulatum* and *M. fulvum* (Ringel, 1978). In 1985, Simpkin suggested the mode of action for ambruticin in *Candida parapsilosis* was by inhibiting amino acid uptake into the cells, determined with experiments with ^{14}C-leucine (Simpkin, 1985).

Congeners of the ambruticins were separated by chromatography and identified in 1991 (Figure 1)(Hofle et al, 1991), and they were labeled as Ambruticin S, VS1 through VS5, and VS3 N-oxide. Ambruticin S has a hydroxyl group at the C5 position, and ambruticin VS compounds have quaternary ammonium groups at C5. The VS compounds were found to be more effective in antifungal activity and less sensitive to pH

changes than S (Hofle et al, 1991). A synthesized derivative of ambruticin VS4, called KOSN-2079 (MIC 1 µg/ml) was tested against a clinical strain of *Aspergillus fumigatus* in mice, reducing pulmonary fungal numbers and increase survival compared to the control group (Chiang et al, 2006). Two derivatives of Ambruticin, KOSN-2079 and KOSN-2089 (MICs 2.5 and 0.5 µg/ml) were used to treat mice infected with lethal doses of *Coccidioides*. 73-100% of the mice were effectively free of *Coccidioides* after treatment. The most promising of these results were of KOSN-2089 at 50 mg/kg/day, and the authors suggested that more mouse studies be done so that ambruticin can be used against *Coccidioides* infections in humans and animals (Shubitz et al, 2006).
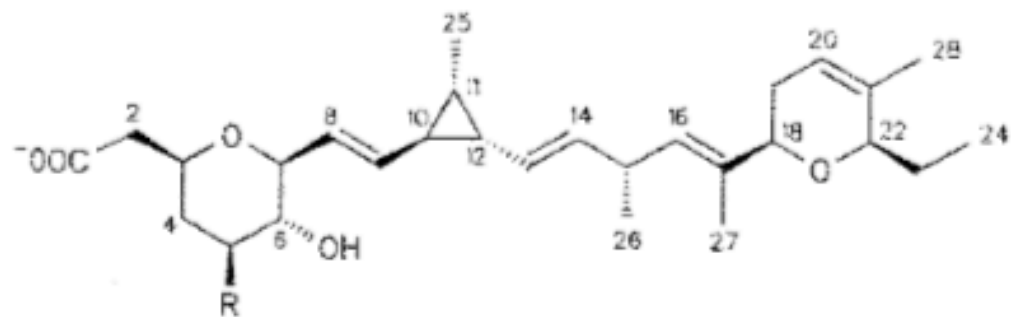
	R	Ambruticin
1a	OH	S
1b	NH_3^+	VS - 5
1c	$NH_2(CH_3)^+$	VS - 4
1d	$NH(CH_3)_2^+$	VS - 3
1e	$N(CH_3)_3^+$	VS - 1
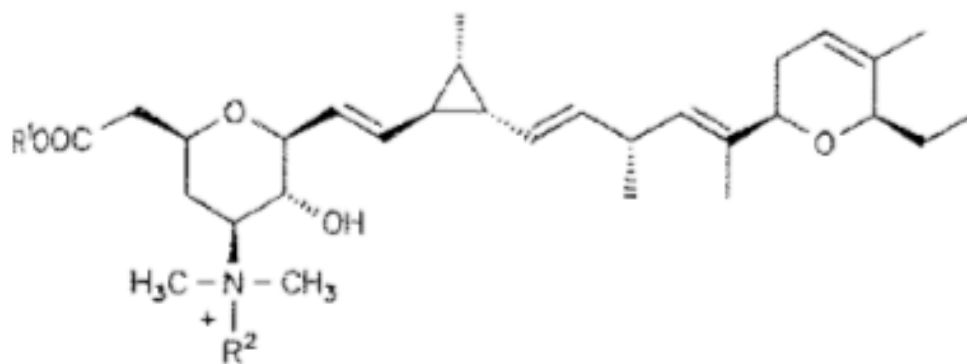
	R^1	R^2	Ambruticin
2a	CH_3	CH_3	VS - 2
2b	H	O^-	VS - 3 *N*-oxide

Figure 1. Structures of ambruticin congeners (Hofle et al, 1991).

An antifungal compound with similar structure was also isolated from *Sorangium cellulosum* called jerangolid A (Gerth et al, 1996). Strains of the yeasts *Hansenula anomala* and *Trichosporon terrestre*, that were resistant to jerangolid A were cross resistant to ambruticin VS3 and pyrrolnitrin, but the mechanisms of action was not known of any of these drugs at the time. It was found that *H. anomala* leaked [^{14}C]-2-aminoisobutyric acid into the supernatant, indicating an increase in membrane permeability (Gerth et al, 1996).

Pyrrolnitrin is a phenylpyrrole antifungal produced by several *Pseudomonas* species (Arima et al, 1965). Its derivatives, fludioxonil and fenpiclonil, are used as fungicides to control plant pathogenic fungi (Gehmann et al, 1990). Another group of fungicides, dicarboximides, showed cross resistance with the phenylpyrroles in *Botrytis cinerea* and *Fusarium vivale* mutants, and these resistant mutants showed sensitivity to high osmotic stress (Leroux et al, 1992). Fenpiclonil resistant strains of *Fusarium sulphureum* were also found to be osmotically sensitivity, and fenpiclonil caused accumulation of glycerol and mannitol in *F. sulphureum* mycelium (Jespers & Waard, 1995). Both fenpiclonil and fludioxonil were found to increase the accumulation of glycerol in the mycelium of *Neurospora crassa*, similar to an osmotic shock response, and inhibit a protein kinase (PK-III) (Pillonel & Meyer, 1997). Aromatic hydrocarbon fungicides, such as dicloran, also mimic the osmotic stress response, making it the target of three distinct classes of fungicidal agents, dicarboximides, phenylpyrroles, and aromatic hydrocarbons (Leroux et al, 2002; Ochiai et al, 2002).

The cross-resistance of pyrrolnitrin and ambruticin found by Gerth et al. above, led to the suggestion that the mechanism of action of ambruticin is interference with

osmoregulation, similar to phenylpyrroles. It was found that glycerol, triacylglycerols and free fatty acids accumulate in *H. anomala* cells treated with ambruticin VS3 and pyrrolnitrin. The cells swell and leak low molecular weight compounds, and the leakiness is assumed to kill the cells (Knauth & Reichenbach, 2000).

In *S. cerevisiae*, the HOG MAP kinase pathway regulates cellular turgor pressure and is well studied (Figure 2A) (Posas et al, 1996). It consists of a MAPKKK (Ssk2/Ssk22), a MAPKK (Pbs2) and a MAPK (Hog1). The cascade's upstream sensing and regulatory components consist of a hybrid histidine kinase signal transduction phosphorelay. The integral membrane histidine kinase (Sln1) consists of transmembrane sensing domain, a histidine kinase domain and a response regulator domain, and it is the only histidine kinase present in *S. cerevisiae*. The next component of the phosphorelay is a histidine phosphotransferase protein (Ypd1), and the final component is a response regulator protein (Ssk1) (Posas et al, 1996). Hog1 is activated, or phosphorylated, under high osmolarity conditions, activating transcription of up to 10% of yeast genes (Hohmann, 2002), including glycerol synthesis enzymes, such as glycerol-3-phosphate dehydrogenase (*gpd1*) and glycerol-3-phosphatase (*hor2*). Osmotic stress reduces the permeability of the plasma membrane to glycerol by inhibiting a glycerol transporter (Fps1). Glycerol accumulation in the cytoplasm restores the osmotic gradient between the cells and their environment. Deletions of *sln1* and *ypd1* are lethal, and deletion of *hog1*, *pbs2*, or *gpd1* are osmotically sensitive (Gustin et al, 1998). Although *S. cerevisiae* contains a single protein for each step of the histidine kinase phosphorelay, other fungi contain multiple histidine kinases (with varied sensory domains) and multiple response regulators, making the pathway more complex (Figure 2B, 2C, 2D) (Catlett et al, 2003).

A *Saccharomyces cerevisiae*
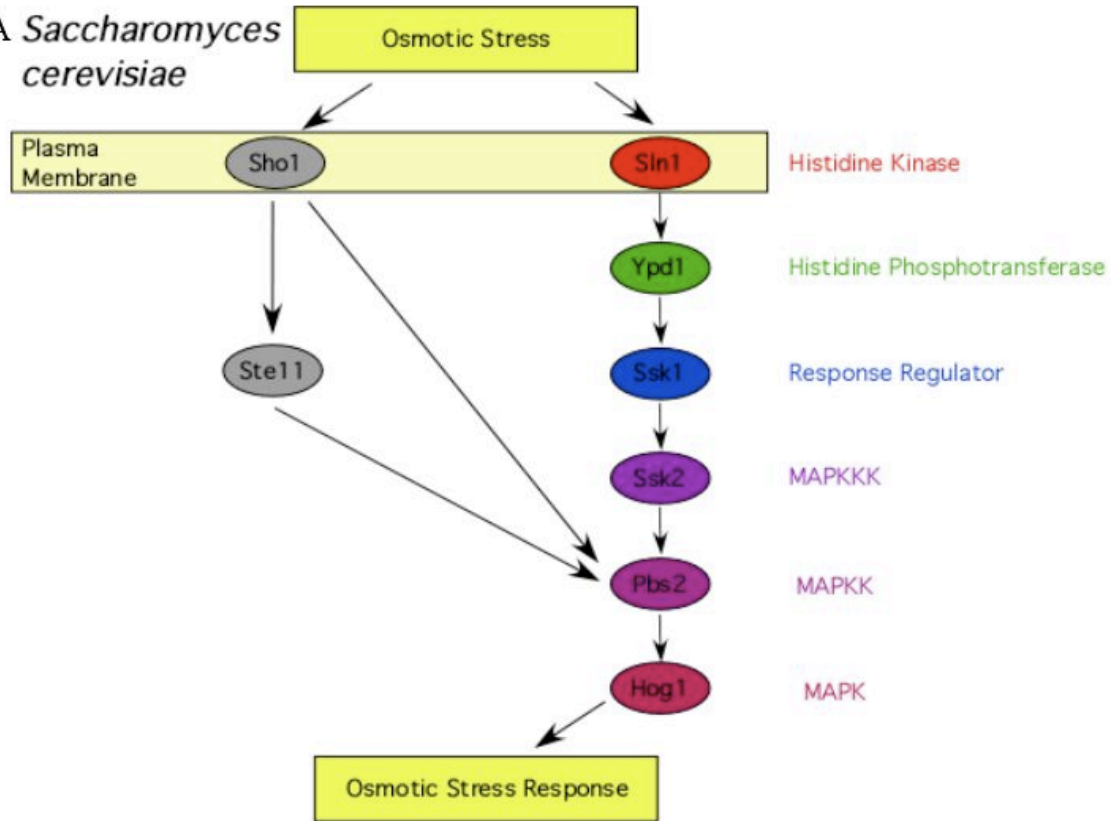
B *Candida albicans*
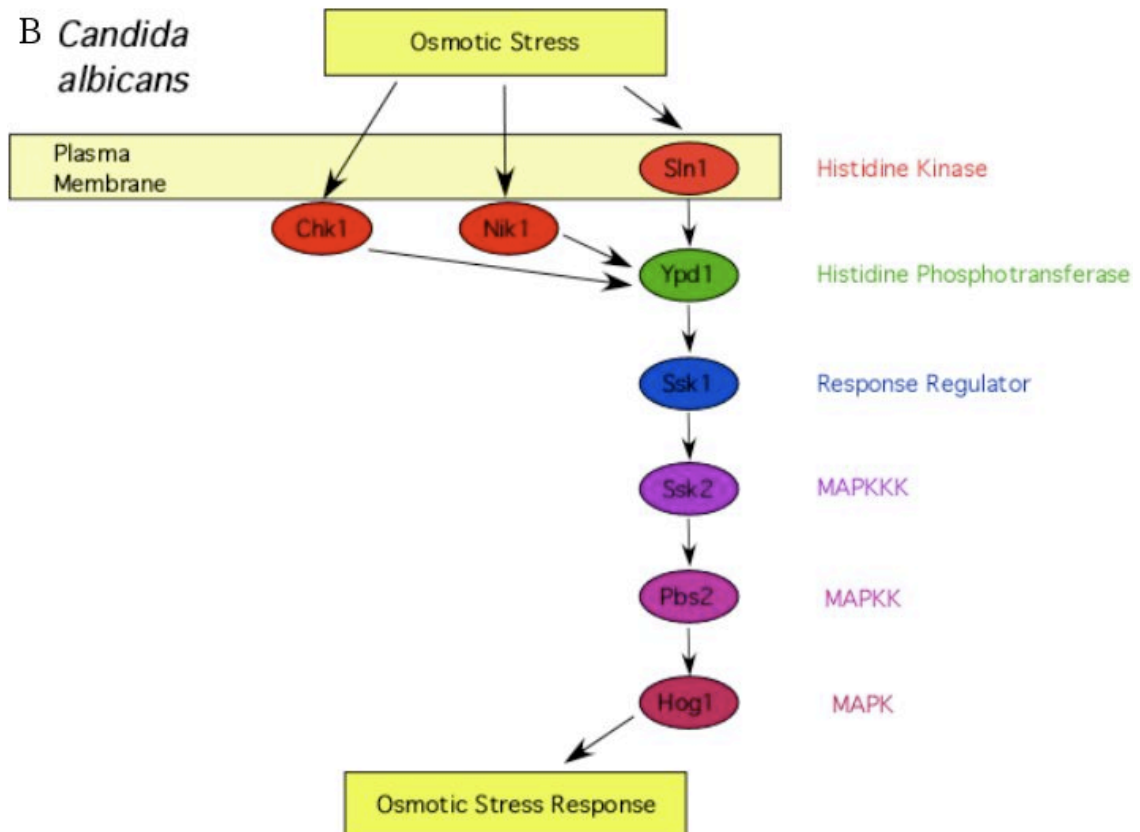
C *Neurospora crassa*
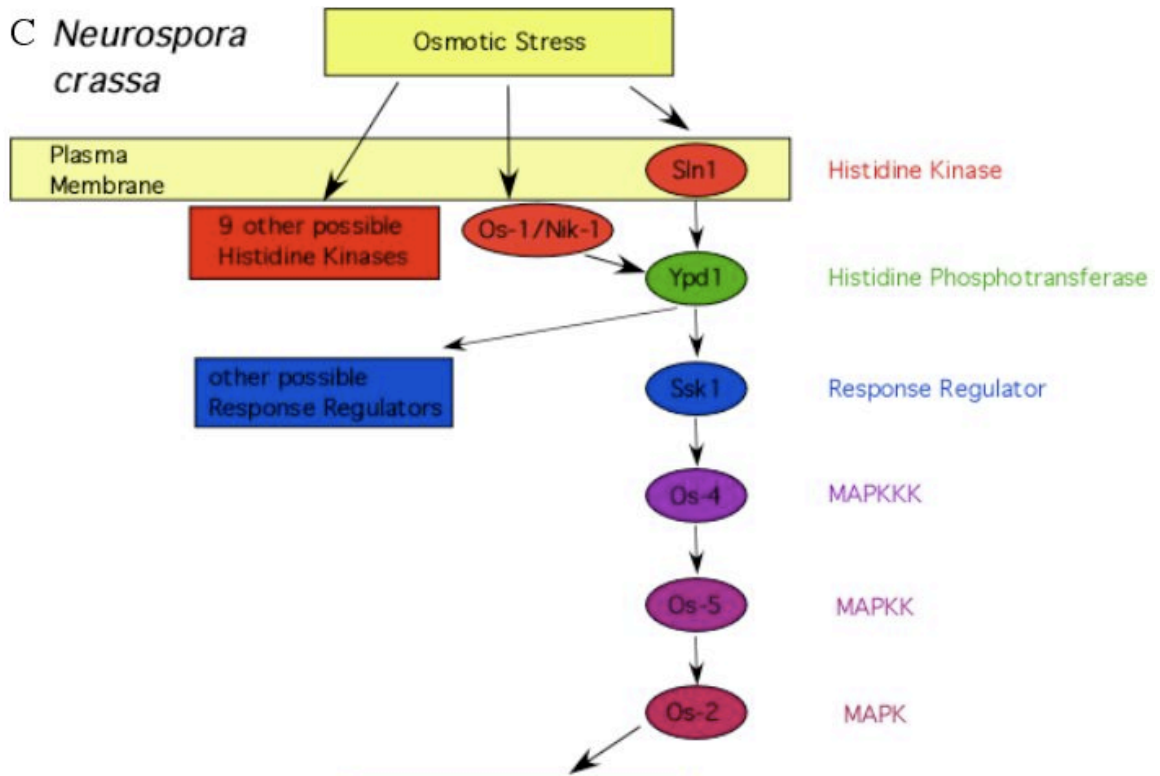
D *Aspergillus nidulans*
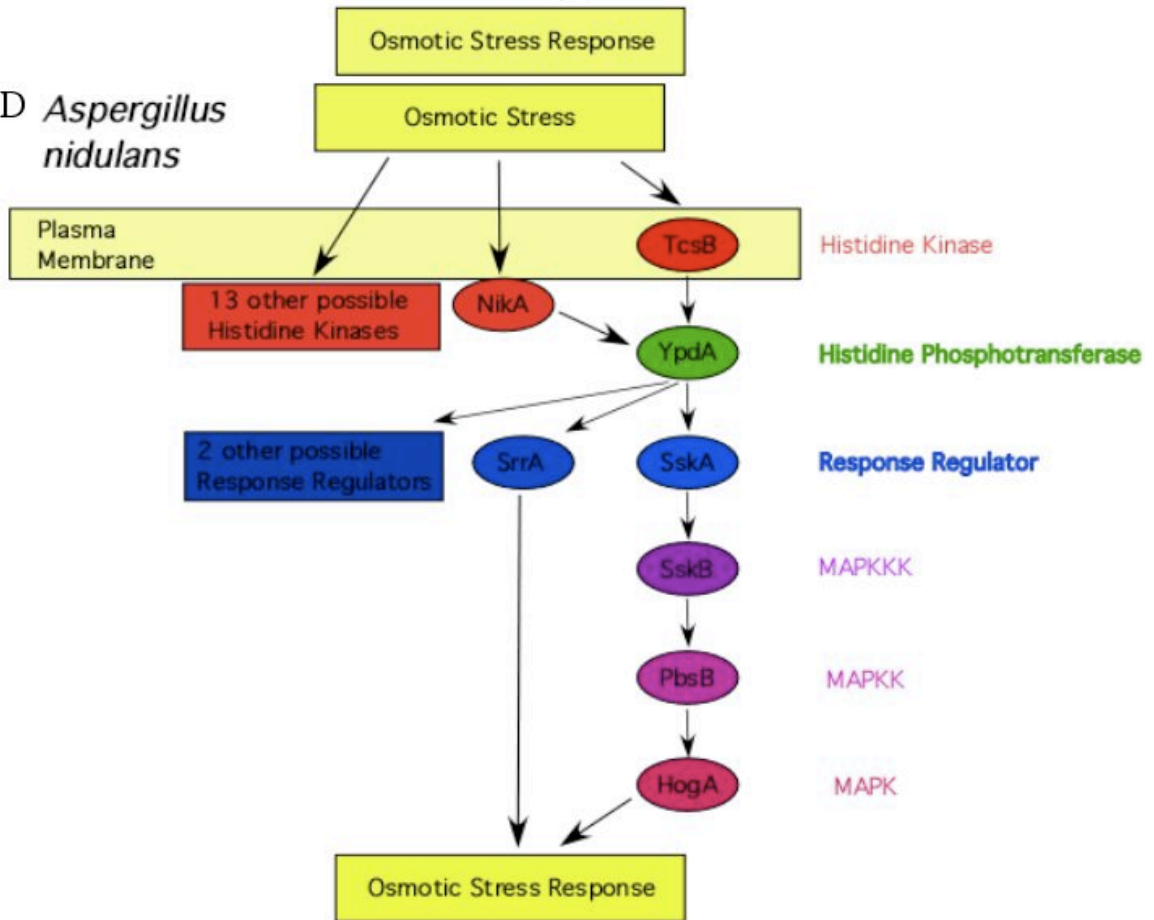
Figure 2. Comparing the Osmotic Stress-Signaling Pathways. (A) *Saccharomyces cerevisiae* only contains one histidine kinase, Sln1, and response regulator, but also uses an alternate Sho1 pathway to signal osmotic stress. (B) *Candida albicans* contains three histidine kinases, one of which is homologous to Sln1 and one categorized as a group III histidine kinase. (C) *Neurospora crassa* has 11 histidine kinases, but has not been well studied in other areas of the upstream signaling components. (D) *Aspergillus nidulans* contains 15 histidine kinases and four response regulators. All organisms have homologous MAPKKKs, MAPKKs, and MAPKs.

In *N. crassa*, osmotic sensitive mutants, *os-1*, *os-2*, *os-3* and *os-5* are resistant to dicarboximides and phenylpyrroles (Fujimura et al, 2000). The *os-1* gene is a hybrid histidine kinase predicted to be an osmosensor in *N. crassa*, containing six repeats of a 90 amino acid HAMP domain for sensing, a histidine kinase domain, and a response regulator domain (group III histidine kinase), and mutations in this gene resulted in resistance to phenylpyrroles (Ochiai et al, 2001). The group III histidine kinases, homologs of *os-1* containing the repeated HAMP domains, have also been found in other filamentous fungi to cause osmotic sensitivity and phenylpyrrole resistance, such as *Alernaria brassicicola* (Avenot et al, 2005), *Alternaria alternata* (Dry et al, 2004), *Botrytis cinerea* (Cui et al, 2002), *Cochilobolus heterostrophus* (Yoshimi et al, 2005; Yoshimi et al, 2004), *Magnaporthe oryzae* (Motoyama et al, 2008), and *Aspergillus nidulans* (Hagiwara et al, 2007).

Fludioxonil caused improper phosphorylation and activation of the homologous Hog1 protein in *C. lagenarium*, *C. heterostrophus*, *B. cinerea* (Kojima et al, 2004), *A. nidulans* (Furukawa et al, 2007), and *N. crassa* (Noguchi et al, 2007). HogA activation in *A. nidulans* activated the promoter of *gfdB* (glycerol-3-phosphate dehydrogenase) (Furukawa et al, 2007), and Hog1 activation in *N. crassa* activated three genes for glycerol synthesis, two for gluconeogenesis, and one for catalase (Noguchi et al, 2007).

Deletion of the group III histidine kinase in *C. heterostrophus* caused improper phophorylation of the Hog1 homolog (Yoshimi et al, 2005). Ambruticin VS4 and fludioxonil both caused improper phosphorylation of the *hog1* homologs in *A. brassiciola* and *N. crassa* (Dongo et al, 2009). *S. cerevisiae* does not contain a group III histidine kinase and is naturally resistant to fludioxonil, but in the presence of high salt conditions and ambruticin VS3, the group III histidine kinase of *M. oryzae* caused improper phosphorylation in *S. cerevisiae* Hog1 (Vetcher et al, 2007). It has been suggested that presence of group III histidine kinases are a prerequisite for ambruticin sensitivity (Dongo et al, 2009), but it is important to note here that *C. albicans* and *C. neoformans* both contain a group III histidine kinase (Bahn et al, 2006; Ochiai et al, 2002), but both organisms are naturally resistant to ambruticin (Ringel et al, 1977). We furthermore show in this work that *Aspergillus niger* contains a group III histidine kinase (An07g08100) and is naturally resistant to ambruticin VS3.

Several response regulators, the final protein in the histidine kinase signaling phosphorelay, have been studied in the fungi with focus on fludioxonil resistance. In *N. crassa* Rrg1 mutants were osmosensitive and fludioxonil resistant (Jones et al, 2007), but Rrg2 mutants have not been studied. In *Cryptococcus neoformans*, the response regulator

Ssk1 was found to modulate phosphorylation of Hog1, but Skn7 response regulator functions independently of Hog1 and mutants of Skn7 are resistant to sodium ion changes and fludioxonil (Bahn et al, 2006). In *C. heterostrophus*, both response regulators, Ssk1 and Skn7, acted additively to respond to fludioxonil, with the double mutant being most resistant (Izumitsu et al, 2007). In *A. nidulans*, deletion of SskA alone is not resistant to fludioxonil, and deletion of SrrA alone is only resistant to fludioxonil in liquid media, not solid media. A double deletion of SrrA and SskA was fully resistant on liquid and solid media, and suggests these response regulators also act additively to give fludioxonil resistance (Hagiwara et al, 2007).

Filamentous fungi with the homologous *hog1* deletions were resistant to fludioxonil and sensitive to high osmotic stress conditions in *N. crassa* (Zhang et al, 2002) and *Colletotrichum lagenarium* (Kojima et al, 2004). Deletion of *hog1* in the pathogenic basidiomycete, *C. neoformans*, was also resistant to fludioxonil and sensitive to a variety of stresses (Bahn et al, 2007), but it is important to note that even though the *hogA* deletion in *A. nidulans* is sensitive to osmotic stress (Han & Prade, 2002), it is not resistant to fludioxonil (Hagiwara et al, 2007). It is further shown in this work that *A. nidulans* with the *hogA* deletion is not resistant to ambruticin VS3.

Although the above work adds to the evidence of the mechanisms of ambruticin and fludioxonil resistance, the full picture is still incomplete. It is also not clear if the two drugs share the same target, because *A. niger, C. albicans* and *C. neoformans* possess natural sensitivity to fludioxonil, but also natural resistance to ambruticin, even though all contain a group III histidine kinase. It is also not clear why the *hogA* deletion in *A. nidulans* is not resistant to fludioxonil or ambruticin, when the HOG MAP kinase

pathway is presumed to be the drugs' primary target. Additionally, the finding that fludioxonil sensitivity is regulated by three different pathways in *C. neoformans* (Kojima et al, 2006) suggests that the HOG MAP kinase pathway is not the only target.

In this work, we will present a new approach to understanding the intricate changes that occur when the antifungals are applied and a new model for how these drugs target a complete pathway, rather than specific steps in the pathway. We used a unique RNA interference vector, that we previously used to silence *brlA*β (Barton & Prade, 2008), to construct a library with 10,000 RNAi strains. We screened the RNAi library for resistance to fludioxonil, then for cross resistance to ambruticin VS3 and sensitivity to osmotic stress. The silenced insert was rescued from the resistant strains, amplified by PCR, and sequenced. Genes with more than one hit were further analyzed *in silico*, and most contained transmembrane domains.

Materials and Methods

Strains and Media. *Aspergillus nidulans* RMS011 (*pabaA1, yA2, argB::trpC, veA1, trpC801*) was used as the parental strain in transformations and as a wild-type strain. *A. nidulans* A26 (*biA1*) genomic DNA was used for cloning the RNAi library, and it was obtained from the Fungal Genetics Stock Center (FGSC) in Kansas City, MO. Strains from the RNAi library are named after their plate and location. *Aspergillus* Minimal Media (MM) was used with appropriate supplementation (Pontecorvo et al, 1953).

DNA Cloning. *A. nidulans* A26 genomic DNA was prepared as follows: one full plate of spores were inoculated in liquid MM broth with biotin supplementation in a standard Petri dish and incubated overnight at 37°C. The hyphal mat was harvested and washed thoroughly with sterile water, frozen under liquid nitrogen, suspended in genomic extraction solution (1% SDS and 50mM EDTA), and heated at 68°C for 10 minutes. The hyphae was separated from the supernatant by centrifugation, and the supernatant was added to an equivalent volume of 5M potassium acetate. It was incubated on ice for 10 minutes, centrifuged, and the supernatant was added to 2.5 volumes of 95% ethanol and stored at -20°C overnight. The DNA was then collected with a sterile glass hook and suspended in TE containing 10 mg/ml RNaseA.

The genomic DNA was partially digested with Sau3AI, quality checked by agarose gel electrophoresis, and purified through a PCR purification kit (Qiagen). The RNAi construct, pSW8, from Barton and Prade 2008 was used as a vector for the RNAi library. It was digested with BamHI, dephosphorylated and also run though the PCR purification kit, due to the inability to heat inactivate BamHI. The vector and partially digested genomic DNA were ligated overnight at 4°C. The ligations were transformed into chemically competent *E. coli* SURE (Stop Unwanted REarrangements, Stratagene) by heat shock (Inoue et al, 1990).

Selected clones from the RNAi library were digested with EcoRI and run on agarose gel electrophoresis to check the integrity of the ligations. The RNAi library DNA was prepared with an endotoxin free plasmid preparation kit (Qiagen) in preparation to be transformed into *A. nidulans*.

***Aspergillus* Transformation.** This procedure was taken from Yelton et al. (Yelton et al, 1984), with the following modifications. One plate full of *A. nidulans* RMS011 spores was inoculated in liquid MM broth with arginine and para-amino benzoic acid supplements and shaken overnight at 37°C. The mycelium was harvested by vacuum filtration and washed with 0.6 M $MgSO_4$. A cell wall degrading enzyme cocktail was added to the washed mycelium, consisting of 500 mg Lysing Enzymzes from *Trichoderma harzianum* (Sigma L1412-10G), 250 mg Lysozyme (Sigma L7651-2), and 100 mg BSA (Sigma A-4503), all suspended in protoplasting solution (1.1M potassium chloride, 0.1M citric acid, adjusted to pH 5.8 with potassium hydroxide, and filter sterilized). The mycelium/enzyme solution was incubated at 30°C with shaking for 3-5 hours. Protoplasts were harvested by vacuum filtration through miracloth (Calbiochem 475855), and washed by centrifugation three times with STC50 (1.2 M sorbitol, 10 mM calcium chloride, 50 mM Tris-HCl pH 7.5). After the final was, protoplasts were suspended in 1 ml of STC50. 150 μl of protoplasts were mixed with 50 μl (10 μg) DNA, and incubated at room temperature for 10 minutes. 2 ml of 60% PEG 4000 was mixed and incubated at room temperature for 20 minutes. The mixture was diluted with 12 ml of STC50 and plated on selective (excluding arginine as a supplement) MM plates containing 12 M sorbitol as an osmotic stabilizer, and incubated at 37°C for 3 days. Transformants of the RNAi library were transferred to 96 well plates and stores in 20% glycerol, 10% lactose at -80°C in duplicate.

Analysis of RNAi Library Strains. Fludioxonil (Sigma-Aldrich Riedel-de Haën 46102) and Ambruticin VS3 (former Kosan Biosciences, Hayward, CA) were both suspended in

DMSO at the respective concentrations of 10 mg/ml and 5 mg/ml. The RNAi library was screened for fludioxonil resistance on $alcA_{(p)}$ inducing MM (100 mM threonine and 0.1% sucrose) at its MIC, 2.5 μg/ml. Resistant strains were transferred to new plates and tested for resistance on twice the MIC of both fludioxonil (5 μg/ml) and ambruticin VS3 (1 μg/ml). The selected strains were also screened for salt sensitivity on 1 M sodium chloride, using VM salts in the MM instead of nitrate salts and incubation at 30°C.

Strains were picked directly from the ambruticin VS3 selection plates and streaked for lawn growth on standard Petri dishes containing MM and para-amino benzoic acid (PABA) as a supplement. After 2-3 days of incubation at 37°C, spores were harvested with 1 ml of sterile water and stored at 4°C. 10 μl of the spore suspensions were mixed with 100 μl of alkaline PEG reagent (Chomczynski & Rymaszewski, 2006), and incubated at 68°C for 15 minutes. 50 μl PCR reactions were set up with 0.5 μl of the lysed spore solution. The PCR reactions were carried out with primers SK and T3, which were modified to be more specific than the standard primers. They are:

T3 5'-AGCGCGCAATTAACCCTCACTAAA-3'

and SK 5'-TCCCGCGGCCGCTCTAGAACTAGT-3',

ordered from Integrated DNA technologies (IDT). The PCR reaction was as follows: 1. 94°C for 2 minutes, 2. 94°C for 30 seconds, 3. 58°C for 30 seconds, 4. 72°C for 2 minutes, 5. Go To step 2 38 times, 6. 68°C for 5 minutes. 10 μl of the PCR reaction was run on a 1% agarose gel, bands were excised with a sterile blade, and run through a gel extraction kit (Sigma-Aldrich). This DNA was sent to Oklahoma State University's Recombinant DNA/Protein Core Facility to be sequenced by Lisa Whitworth on a ABI

model 3730 DNA Analyzer. The sequences were blasted against the *Aspergillus nidulans* genome at CADRE, NCBI and Broad.

Verification of Resistance. Subclones were made from either the rescued RNAi insert PCR product or by designing primers specifically for the ORF of the targeted genes. The PCR products were digested with BamHI, and ligating into the BamHI site of the RNAi construct, pSW8. The PCR products were run through the PCR purification kit before and after digestion, and pSW8 was run through the PCR purification kit after digestion and dephosphorylation. The ligations were run overnight at 4°C, and transformed into *E. coli* SURE cells as above. Clones were screened by colony PCR (Sambrook & Russell, 2001) and then further confirmed by restriction digestion analysis and sequencing. The plasmids were transformed into *A. nidulans* RMS011 as above. Twenty-three strains of each resulting transformation were plated on 2.5 µg/ml fludioxonil and incubated no more than seven days at 37°C.

Results

Testing *Aspergilli* against ambruticin VS3 and fludioxonil. Susceptibility assays using a 96-well format was designed to measure the effectiveness of ambruticin VS3 and fludioxonil at increasing concentrations. The drugs were tested in a 1% agar minimal nutrient media containing low osmotic (VM media) and high osmotic strength (1M NaCl). The maximum dose that supports growth (MDSG) was recorded for various

species of *Aspergilli* (Table 1). *A. nidulans* wild-type, *A. flavus*, *A. glacus*, and the pathogenic *A. fumigatus* all grew on ambruticin VS3 at or less than 0.5 μg/ml on both low and high osmotic stress, but *A. niger* was naturally resistant to ambruticin VS3, with 10 and 15 μg/ml supporting growth on low and high osmotic stress, respectively. The fludioxonil MDSG pattern was different from fludioxonil in that it did not support growth of *A. nidulans*, *A. niger*, and *A. fumigatus*, but it support growth of *A. glacus* on both low and high osmotic strength and *A. flavus* on low osmotic strength only. These results imply that ambruticin VS3 and fludioxonil have different targets within the osmotic stress signaling pathways, and these species of *Aspergilli* have differences in their osmotic sensing and signaling pathways. It was unexpected that *A. niger* was resistant to ambruticin VS3 because it does contain a group III histidine kinase, An07g08100, and also indicates that group III histidine kinases are not a direct interacting target of ambruticin, as suggested in other studies.

Table 1 *Susceptibility to ambruticin VS-3 and fludioxonil in Aspergillii*

| STRAIN | Maximum dose (ug/ml) | | | |
| | Ambruticin | | Fludioxonil | |
	LS	HS	LS	HS
A. nidulans	0.5	0.1	0.5	0.075
A. glaucus	0.5	0.5	15	150
A. niger	10	15	0.1	0.001
A. fumigatus	0.1	0.05	1	0
A. flavus	0.1	0	15	0

LS Low salt medium - VM medium

HS High salt medium - VM medium amended with 1M NaCl

SIK1 and suppressor mutants. Four *A. nidulans* suppressor strains of the *hogA* (SIK1) temperature dependent high osmotic strength sensitive phenotype were isolated and tested against a variety of antifungal drugs: terbinafine, amphotericin B, fludioxonil, fenpiclonil, and ambruticin VS3 (Table 2). The four *hogA* suppressor strains were completely susceptible to terbinafine and amphotericin B, which target ergosterol biosynthesis and cell membrane ergosterol, respectively (Richard D. Cannon & Mikhail V. Keniya, 2009). Surprisingly, both *A. nidulans* wild-type (WT) and SIK1 were resistant to amphotericin B, but only at low osmotic strength. On fludioxonil, fenpiclonil (both phenylpyrroles), and ambruticin VS3, WT, SIK1, and three of the *hogA* suppressor strains were susceptible, but one *hogA* suppressor strain (F14) was cross resistant to the drugs (Figure 3). These results indicate that *hogA* is not the only osmotic stress signaling pathway that phenylpyrroles and ambruticin VS3 affect, because SIK1 is not resistant. The *hogA* suppressor mutants were isolated by S. Bangaru, and the antifungal assays were performed by E. Lackey, J. Fought, R. Howard, D. Thomas, and R. Prade.

Table 2 *Aspergillus nidulans* susceptibility to various antifungal and resistance of osmotic mutants to ambruticin VS-3 and fludioxonil

A. nidulans Strain	Maximum dose that supports growth (ug/ml)								
	Ergosterol interference			Osmotic interference					
	Terb	Amphotericin B		Fludioxonil		Fenpiclonil		Ambruticin VS-3	
	LS/HS	LS	HS	LS	HS	LS	HS	LS	HS
WT	0.05	15.00	2.50	2.50	0.50	7.50	5.00	0.50	0.10
SIK1(*ΔhogA*)	0.05	15.00	5.00	5.00	1.50	5.00	5.00	0.50	0.50
F14 (S*hogA*)	0.05	2.50	1.50	15.00	15.00	15.00	10.00	15.00	15.00
F21(S*hogA*)	0.05	5.00	1.50	5.00	2.50	7.50	5.00	0.50	0.10
C18 (S*hogA*)	0.05	2.50	1.50	1.50	1.50	7.50	7.50	0.50	0.50
B20 (S*hogA*)	0.05	5.00	1.50	1.00	1.50	0.50	5.00	0.03	0.03

LS Low salt medium - VM medium
HS High salt medium - VM medium amended with 1M NaCl
Terb Terbinafine
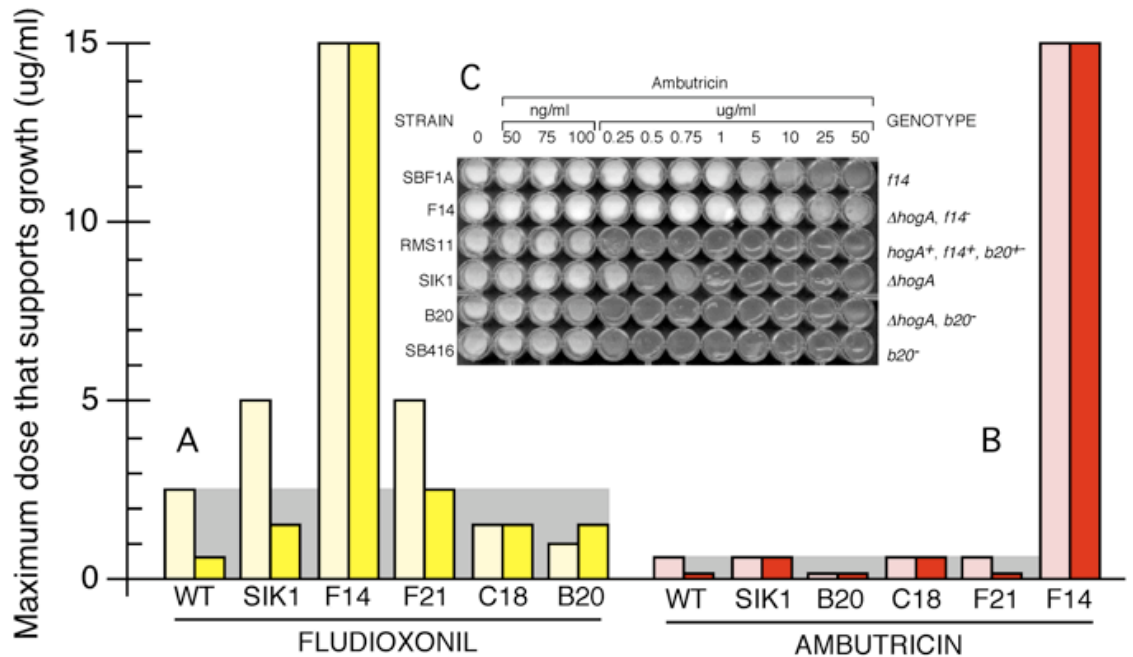
Figure 3. Aspergillus nidulans Δ*hogA* strain, SIK1, and Δ*hogA* suppressor strains on fludioxonil and ambruticin VS3. (A) Wild-type (WT), DhogA (SIK1), and four Δ*hogA* suppressor strains showing the maximum dose that supports growth (MDSG) in μg/ml concentrations. All strains are sensitive to fludioxonil except F14. The light yellow bar is low salt conditions and the dark yellow bar is high salt conditions. (B) The same strains on ambruticin VS3, again all strains are sensitive except F14. The light red bar is low salt conditions and the dark red bar is high salt conditions. (C) A 96-well based assay of the strains on ambruticin VS3 showing sensitivity of all strains except those with the F14 mutation.

Silencing of *ypdA*. The full open reading frame of *A. nidulans ypdA* gene, that encodes the histidine phosphotransferase, the intermediate between histidine kinases and response regulators in the histidine kinase phophorelay signaling pathway, was PCR amplified with primers that had engineered *Bam*HI sites on each 5' end and cloned into the RNAi construct. Because *ypdA* is a lethal knockout (Vargas-Perez et al, 2007), the RNAi approach was a suitable alternative. *A. nidulans* was transformed using the arginine selectable marker and two strains were recovered, LN421 and LN121 (Figure 4). Both strains were sensitive to osmotic stress with 1M NaCl, but neither strain was resistant to ambruticin VS3 nor fludioxonil. These results indicate that YpdA is not a direct target of ambruticin and fludioxonil, further complicating the molecular roles played by the antifungal drugs. If YpdA is truly the only intermediate to the histidine kinase phosphorelay signaling, and the osmotic stress signaling pathway is the only affected pathway by the drugs, one would expect the strains to grow on the drugs when *ypdA* is downregulated. The work with *ypdA* was performed by D. Qualls and R. Prade.
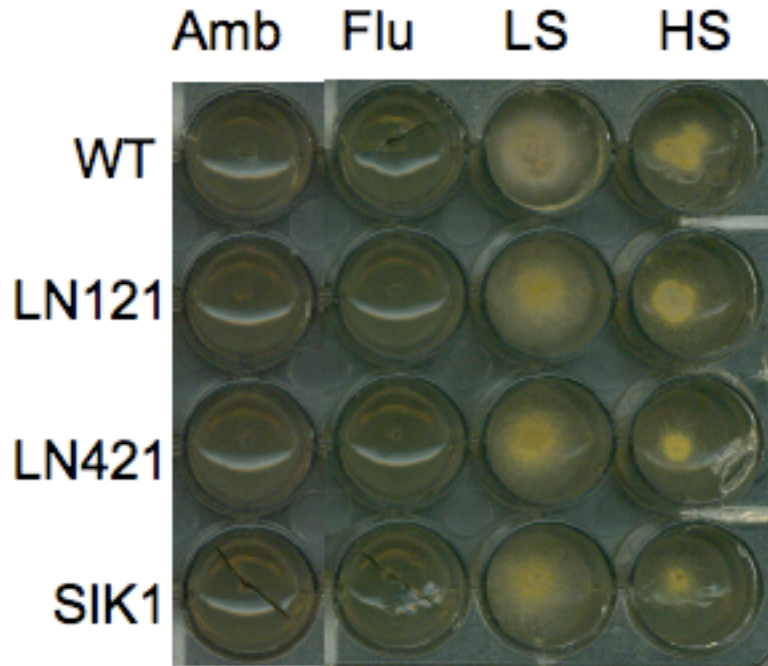
Figure 4. Silencing of *ypdA*. This assay shows four strains: wild-type (WT), *ypdA* silenced strains (LN121 and LN421), and Δ*hogA* (SIK1), growing on *alcA*(p) inducing media (100 mM threonine) containing 1 µg/ml ambruticin VS3 (Amb), 2.5 µg/ml fludioxonil (Flu), low salt VM *Aspergillus* media (LS) and high salt VM *Aspergillus* media with 1M NaCl (HS). None of the strains grew on the drugs, while all grew well on low salt media, and LN421 and SIK1 showed the most sensitivity to high salt.

Bioprocess Engineering of Ambruticins. *Sorangium cellulosum* ATCC 25532 was obtained from ATCC and cultured according to their instructions, using only cellulose filter paper as a carbon source (Figure 5A). Five one liter cultures were grown at 30°C in rich fermentation media, including additional carbon sources and XAD-1180N resin to absorb the ambruticins. The resin was isolated from the media, washed with water, and the ambruticins eluted with methanol. The eluted solution was rotary evaporated, and a separatory funnel was used to extract the ambruticins in ethyl acetate. The extracts were lyophilized, and the ambruticins were separated by column chromatography. The ambruticins were identified with Thin Layer Chromatography (TLC), Mass Spectrometry, High Pressure Liquid Chromatography (HPLC), and LC MS. The mass spec and LCMS were run at University of California at Irvine, and the HPLC was run with Dr. Andrew Mort at Oklahoma State University. See J.S. Barton's final report for details on fermentation, isolation and characterization of the ambruticins. Antifungal assays were performed against *A. nidulans* RMS011 and *Candida albicans* (sample #1) and killed with varying degrees (Figure 5B, 5C, and 5D). This work was performed by L.M. Barton, J.S. Barton, and R.A. Prade.
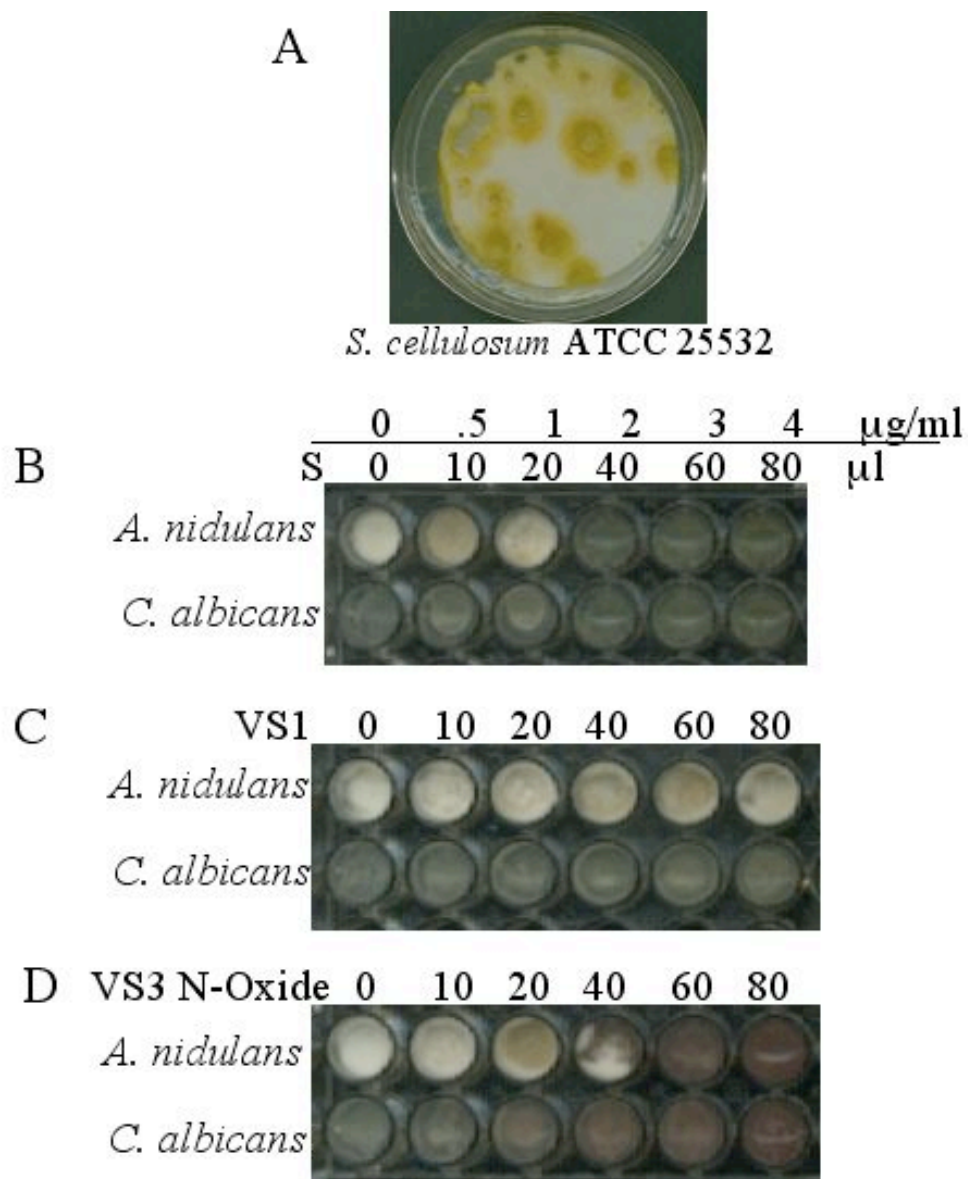
Figure 5. Bioprocess engineering of ambruticin. (A) *Sorangium cellulosum* ATCC 25532 cultured on cellulose filter paper as a carbon source. (B) Activity of ambruticin S, killing both *A. nidulans* and *C. albicans* at 2 μg/ml. (C) Activity of ambruticin VS1, reducing growth of both organisms at higher concentration, but not killing. (D) Activity of ambruticin VS3 N-oxide with a distinctively pink color, killing *A. nidulans* at 3 μg/ml, but only reducing growth of *C. albicans* at higher concentration. All antifungal photos were taken after 48 hours of growth at 37°C.

The RNAi Library. The RNAi construct previously used to demonstrate locus specific, inducible RNAi of the *brlA*β gene in *Aspergillus nidulans* (Barton & Prade, 2008) was used in this study as a vector to construct a genomic library (Figure 6). The genomic DNA was ligated into the unique *Bam*HI site of the RNAi construct (see Materials and Methods). The unique *Bam*HI site of the RNAi construct is located between inverted repeats of alcohol dehydrogenase promoters ($alcA_{(p)}$), which are inducible on threonine and repressible on glucose (Adams et al, 1988). When induced, the promoters initiate transcription of the downstream sequence, forming double stranded RNA that causes silencing of the homologous mRNA transcripts. The RNAi construct also contains a functional arginine biosynthesis (*argB*) gene to use as a selectable marker to transforming the parental strains (Johnstone et al, 1985).

A. nidulans genome is arranged into 8 chromosomes, totaling about 31 Mb in size, with approximately 11,000-12,000 genes (Galagan et al, 2005). The RNAi Library contains more than 50,000 individual clones with average insert size of 600 base pairs, which covers the genome one time. Nearly 10,000 *A. nidulans* strains were isolated and transferred to 96-well plates for storage and future use. In this format the strains can be stamped onto selective media to easily screen for phenotypes. This is a powerful tool that can be used to screen for many types of phenotypes, for example developmental mutants. The silenced insert of the selected strains can then be rescued by polymerase chain reaction, PCR, of genomic DNA, and identified by sequencing and blasting against the publicly available annotated genomic sequence.
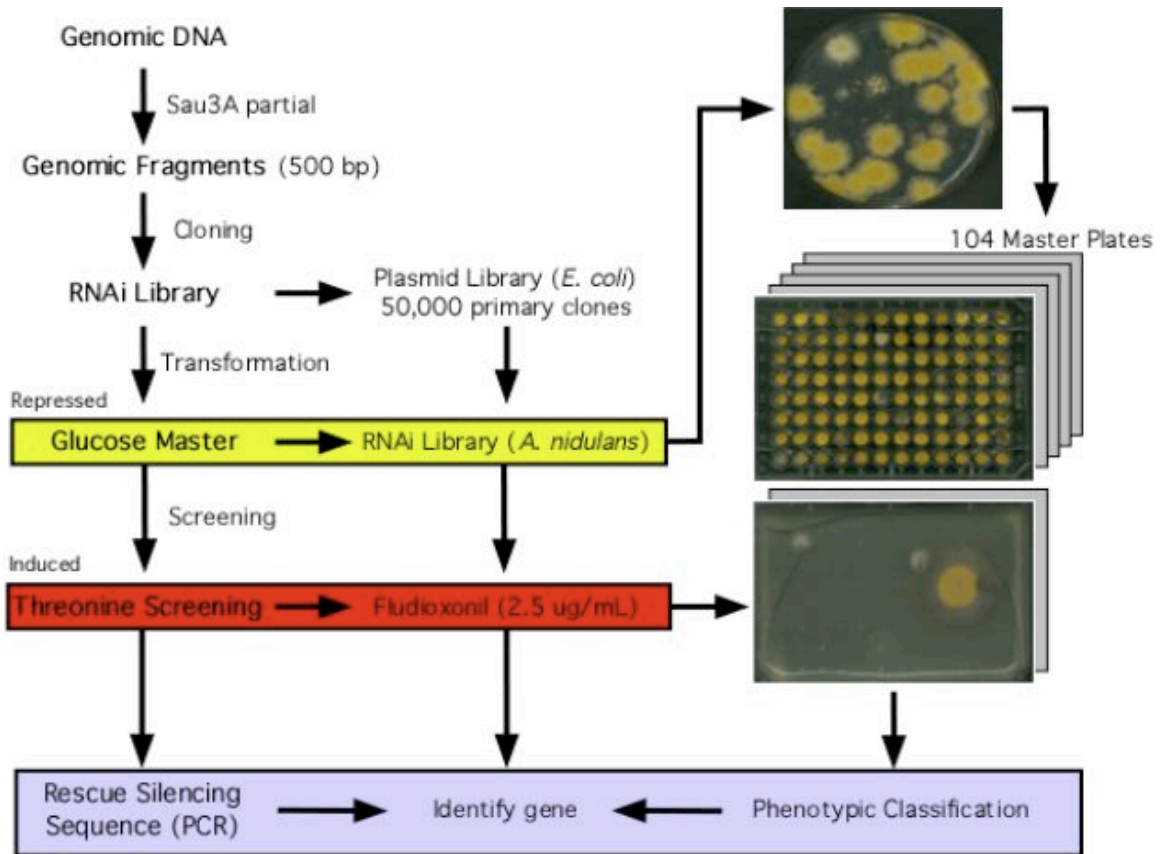
Figure 6. Construction and Analysis of the RNAi Library. The RNAi library was constructed by ligating genomic DNA fragments into an RNAi construct vector. Each strain in the library is preserved on a glucose (RNAi repressing) master plate. The library was screened by plating on threonine (RNAi inducing) selective plates, here fludioxonil was used to further screen the library. Phenotypes were classified according to cross resistance to ambruticin and osmotic stress sensitivity. The silenced insert was rescued by PCR amplification and identified by sequencing.

Screening for Drug Resistant Strains. The RNAi library was screened for resistance to the drug fludioxonil and cross-resistance to the drug ambruticin VS3 and salt sensitivity. The selected strains had the silenced insert rescued by PCR and sequenced to identify to gene target of interest (Figure 3, Table 3). The RNAi library strains were plated on $alcA_p$ inducing, selective media containing 2.5 µg/ml of the fungicide fludioxonil, which is available to purchase (Sigma-Aldrich Riedel-de Haën 46102). The resistant strains were transferred to new 96-well plates and screened again at twice the MIC of fludioxonil and ambruticin VS3 (limited availability), and salt sensitivity. The strains were then put into classes based on their phenotypes. The selected strains were then PCR amplified with the T3 and SK primers whose location on the RNAi construct is shown, and sequenced with the SK primer. The sequence was then blasted against the *Aspergillus nidulans* genome.

Table 3. Phenotype Classifications

Fludioxonil 5 ug/ml	Ambruticin VS3 1 ug/ml	Salt Stress 1M NaCl	# of Strains
R	R	S	287
R	R	R	74
R	S	S	12
S	R	S	1
S	R	R	1
S	S	R	50
S	S	S	65

Fludioxonil and Ambruticin Resistance Mechanisms. Thirty-five RNAi inserts were found multiple times in the resistant strains (Table 4). Twenty-four of the inserts had annotations and twelve were hypotheticals. The *argB* gene, AN4409 ornithine carbamoyl transferase, was found four times, implying an unexpected rearrangement occurred during integration into the fungal genome. It must be addressed that multiple transmembrane transporters were found among the hits. If the function of the transporter is to allow the drugs to enter the cell, then it is not clear why silencing of more than one transporter causes resistance because the drug should be able to enter through another transporter. Gene Ontology results showed that fourteen of the twenty-four annotated multiple hit inserts were isolated from the membrane fraction, seven were unknown, and the other three included ubiquitination, transport and nucleolus. Twenty-one of the twenty-four had at least one transmembrane domain, and they were varied in their known processes, ranging from amino acid metabolism, transport, lipid binding and metabolism, and chitin synthesis.

Table 4. Multiple Hits of Resistant RNAi Strains with Annotated and Hypothetical Function

	HITS	GENE	TMD	FUNCTION	PROCESS	COMPONENT
KNOWN FUNCTION						
1	5	AN7165	9	plasma membrane stress response protein Ist2	unknown	membrane fraction
2	4	AN5134	0	glutamate Synthase	amino acid metabolism	membrane fraction
3	2	AN3729	17	1,3 beta-D-glucan synthase	glucan biosynthesis	membrane fraction
4	2	AN1276	12	MFS sugar transporter	transport (sugar)	membrane fraction
5	2	AN0890	12	MFS Multidrug Transporter	transport (multidrug)	membrane fraction
6	2	AN1189	12	calcium ATPase, response to cation stress, osmotic stress	calcium transport	membrane fraction
7	2	AN4477	11	L-glutamine transmembrane transporter activity	amino acid export from vacuole	membrane fraction
8	2	AN4367	9	chitin synthase class III	chitin synthesis	membrane fraction
9	2	AN8660	5	flavin binding monooxygenase	transport (K+)	membrane fraction
10	2	AN7923	4	GAL4-like Zn2Cys6 binuclear cluster - not much homology	transcription factor	transmembane
11	2	AN2403	4	Acyl-CoA synthetases (NRPS-like)	lipid metabolism	transmembane
12	2	AN6093	3	esterase lipase	esterification lipid	membrane fraction
13	2	AN3927	3	Inositol phospholipase (response to osmotic stress)	phospholipase C activity	membrane fraction
14	2	AN6785	3	phosphatidylinositol transfer protein (CRAL/TRIO Sec14)	lipid binding protein	membrane fraction
15	2	AN4962	2	UPF0132 domain	unknown	membrane fraction
16	2	AN6138	2	bimA, sep1 DNA damage checkpoint	anaphase promoting complex	transmembane
17	2	AN5387	2	proline racemase - DUF453 domain	amino acid metabolism	transmembane
18	2	AN2866	2	NAD binding oxidoreductase	unknown	transmembane
19	2	AN3827	1	ribosome biogenesis	ribosome large subunit	nucelolus
20	2	AN3006	1	esterase lipase	esterification lipid	membrane fraction
21	2	AN2610	1	cytochrome P450	secondary metabolism	transport
22	2	AN2144	1	urease accessory portein UreD	urease accessory protein	unknown
23	2	AN5003	0	C2H2 possibly not a TF	transcription factor	unknown
24	2	AN5906	0	ring finger protein U-box domain	protein transport	unknown
HYPOTHETICALS						
25	3	AN3532	0	hypothetical	unknown	unknown
26	3	AN6634	1	hypothetical	unknown	transmembane
29	2	AN1738	7	hypothetical	unknown	transmembane
36	2	AN8933	3	hypothetical	acyl-CoA thioesterase	transmembane
34	2	AN8310	3	hypothetical	esterase lipase	transmembane
27	2	AN0308	3	hypothetical	Serine/threonine phosphatase 2C	transmembane
31	2	AN5663	2	hypothetical	unknown	transmembane
32	2	AN7362	1	hypothetical	serine/threonine phosphatase 2A	transmembane
28	2	AN1605	0	hypothetical	unknown	unknown
30	2	AN2292	0	hypothetical	maturation of SSU-rRNA	membrane fraction
33	2	AN8200	0	hypothetical	unknown	unknown
35	2	AN8492	0	hypothetical	methyltransferase (LaeA-like)	unknown

Confirming Resistance by RNAi. To verify that the RNAi construct is conferring resistance to the strains, rather than random integration, subclones were constructed and tested for resistance. The first set of transformants were originated from the rescued PCR products, then religated into the RNAi construct. The PCR products were chosen due to a single, clean band given by the rescuing PCR strain. The resistance rate of the substrains on fludioxonil was as high as 17%, much higher than the 3% resistance rate given by the RNAi Library, suggesting a correlation between the silenced insert and resistance. The second set of transformants were originated from the RNAi construct with inserts cloned from specific ORF hits with PCR primers. The cloning with the specific PCR products was much cleaner than those from the rescued PCR products. Ten specific hits were selected for subcloning. Those with multiple hits subcloned are AN7165 (plasma membrane stress response protein Ist2), AN0890 (MFS multidrug transporter), AN1189 (calcium ATPase), AN2144 (urease accessory protein UreD), AN3927 (inositol phosphosphingolipid phospholipase C), and AN4477 (L-glutamine transmembrane transporter). Those with single hits subcloned are AN4447 (group VII histidine kinase), AN4479 (group III histidine kinase NikA), and AN9168 (glycerol/H+ symporter Stl1). Additionally, two more genes were subcloned into the RNAi construct in separate experiments, AN2005 (histidine phosphotransferase YpdA) and AN5134 (glutamate synthase). The YpdA subclone showed sensitivity to salt, but was not resistant to fludioxonil or ambruticin VS3, indicating that it is not the target of the drugs. YpdA is a lethal knockout (Vargas-Perez et al, 2007), making it a good choice for the RNAi construct because viable mutants can be recovered, and YpdA can be partially downregulated.

Discussion

  In this study we show that the group III histidine kinase, histidine phosphotransferase, and MAPK HogA are not the direct target of ambruticin VS3. We used an inducible RNAi vector that we previously showed effectively silences *brlA*β in *Aspergillus nidulans*. A genomic RNAi library was constructed and stocked for future use, and a high-throughput screen was done with the library to test for fludioxonil resistance. Fludioxonil is a phenylpyrrole fungicide commonly used in agriculture, known to mimic osmotic stress response in fungi. It is known that deletions and mutations of certain intermediates in the osmotic stress-signaling pathway, including the histidine kinase phosphorelay and the HOG MAP kinase pathway, can cause resistance to fludioxonil. It is also known that the antifungal, ambruticin, acts in a similar manner as fludioxonil, and the same osmotic stress-signaling pathway is affected (see introduction). Therefore, we screened the fludioxonil resistant RNAi strains isolated from the library for cross-resistance to ambruticin VS3, and found that the majority of strains do show cross-resistance to both drugs. We also found that most of the resistant strains showed sensitivity to osmotic stress, as expected. After rescuing and sequencing the silenced DNA fragments from the RNAi strains, we found that inserts that were found in multiple strains were associated with the membrane and had transmembrane domains.

  The RNAi library is a powerful genetic tool to understand gene function in *A. nidulans* and serves as a model for other filamentous fungi. There are nearly 10,000 individual strains in the RNAi library, which represents approximately one strain for each gene in the genome. There are advantages of the RNAi library, including the ability to screen for many phenotypes easily by stamping the master plates onto any type of selective media. A genomic library rather than a cDNA library allows regulatory regions

of the genome to be examined, rather than limiting research to transcripts. Because the RNAi library is an inductive system, genes that are lethal when deleted may be examined because they the stains are first isolated, then gene silencing is induced. Silencing of the gene may also be partial to understand phenotypes of downregulation, rather than just deletion, as in the case of the leaky *brlA* phenotype.

As with any genetic library, there are disadvantages of the system, including that the RNAi constructs are not targeted to a specific location in the genome for integration. Therefore it is possible that interruption of genes could cause a non-specific phenotype. This issue may be addressed by rescuing the RNAi construct from the genome to map its location of integration. Another inadvertent possibility is integration of multiple RNAi constructs into a single strain, so that multiple PCR products are rescued from a single strain. This may be addressed by making specific silencing constructs for each rescued sequence to determine the gene-silencing event causing the phenotype. During the cloning of the RNAi library, the possibility of cloning of concatamers into the RNAi construct is possible, and this issue must be addressed in a similar manner a multiple integration events, by silencing each sequence individually. Finally, because the RNAi library DNA was introduced to the cell as a circular plasmid, there is a possibility that the plasmid will linearize in the *alcA*$_{(p)}$ or the genomic insert, and gene silencing cannot occur despite its integration into the genome. Because the RNAi construct plasmid is approximately 5 kb and the size of the *alcA*$_{(p)}$s and genomic insert is approximately 1.8 kb, there is an approximately 75% chance that the RNAi construct can cause gene silencing in a strain in which it is integrated, but the non-silencing strains will be eliminated through the phenotype selection process.

Genes found through the RNAi library are primarily transmembrane domain proteins. They can be further categorized into osmotic regulatory function or amino acid synthesis/transport function related to osmotic regulation. The genes that we chose to specifically subclone have previously been associated with sensitivity to osmotic stress. The group III histidine kinase deletion of *Aspergillus nidulans* (NikA) confers partial sensitivity to osmotic stress (Vargas-Perez et al, 2007), and they have are implicated in osmotic stress response in other fungi (see introduction). Calcium ATPase in *C. neoformans*, Eca1, found in the sarcoplasmic and endoplasmic reticulum is involved in various stress tolerances, including osmotic stress, and virulence (Fan et al, 2007). The calcium ATPase of *N. crassa*, NCA-2, is also involved in osmotic stress response (BegoÒa Benito, 2000). The NRPS, Non-Ribosomal Peptide Synthetase, produces bacterial and fungal cyclic peptides. Four NRPS enzymes are conserved among ascomycetes. In *C. heterostrophus* two NRPS enzymes, NPS6 and NPS10, are hypersensitive to various stresses, including osmotic stress (Gillian Turgeon et al, 2008). Inositol phosphosphingolipid phospholipase C in *S. cerevisiae*, Isc1, is involved in sodium and lithium ion tolerance and is an early step in salt induced signaling pathways (Christian Betz, 2002). The glycerol/proton symporter of *S. cerevisiae*, Stl1, is osmoresponsive and induced by Hog1 (Nadal et al, 2003), and it is required for glycerol uptake in *C. albicans* (Kayingo et al, 2009). The plasma membrane stress response protein in *S. cerevisiae*, Ist2, is involved in response to salt stress, and the deletion strain is salt sensitive (Entian et al, 1999). Ist2 has also been implicated in maintenance of amino acid levels and ion homeostasis (Kim et al, 2005).

Two of the specific subclones are involved in amino acid synthesis and transport related to osmotic stress response. In bacteria and plants, it is known that glutamate serves as a precursor to proline, and proline synthesis is a response to salt stress (Francisco Berteli, 1995). Glutamate synthase, GltS, has been shown to increase expression during osmotic stress in *Bacillus subtilis* (Dirk Hˆper, 2006), tomatoes (Francisco Berteli, 1995), and barley and rice (Ueda et al, 2006). The amino acid transporter, whose gene ontology was L-glutamine transmembrane transporter activity, has not been well studied in fungi; although, glutamine synthetase has been shown to be induced by osmotic stress in sunflowers (Santos et al, 2004), indicating the importance of glutamine in osmotic stress response.

Other proteins that are specific subclones and have not been well studied with special regard to osmotic stress are the urease accessory protein, UreD and the group VII histidine kinase. An MFS (Major Facilitator Superfamily) multidrug transporter is not involved in the osmotic stress response, but they are involved in transporting molecules across the membrane (Higgins, 2007). In *B. cinerea* an ABC transporter, similar to MFS transporters, were found to carry fludioxonil across the membrane (Vermeulen et al, 2001).

We have shown that the *A. niger* group III histidine kinase, and the *A. nidulans* histidine phosphotransferase YpdA, and MAP kinase HogA are not the targets of ambruticin VS3, but the RNAi library confirms that ambruticin does target the osmotic pathway in many different steps. This work demonstrates that ambruticin and fludioxonil may not have one specific target, but affect the entire osmotic stress-signaling pathway and represents a new model for antifungal molecular mechanisms. This work also

demonstrates the robustness of the osmotic stress-signaling pathway; that is, it has evolved to function although one or more of its components have been downregulated in expression. In an effort to explain why downregulation of many genes may cause resistance to the drugs is that they are important in the secondary response to osmotic stress, rather than the primary response of the histidine kinase and HOG MAP kinase pathways. The secondary response caused by the downregulated genes may be essential in glycerol production and balancing of the internal osmolyte concentrations so that the cell may be stabilized during osmotic stress. This is to say that genes important in secondary responses may not be directly causing resistance, but they may be "suppressors of sensitivity."

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APPENDICE

Appendix 1. Key to Reordered Plates: Strains Taken Directly From FluR Master Plates.

Plate3	1	2	3	4	5	6	7	8	9	10	11	12
A	P1C9	P1E1	P1E4	P2A3	P2D5	P3D6	P3G10	P4D1	P5E3	P5E8	P5F5	P7B3
B	P10B2	P10D10	P2E4	P5E3	P5E8	P7B3	P11C11	P11C5	P11D6	P11E11	P12A3	P12A5
C	P12B5	P12C10	P12F6	P12G6	P12H7	P13A1	P13A2	P13B5	P13F6	P14A8	P14A9	P14B6
D	P14G4	P14G10	P14H5	P15A1	P15A3	P15A11	P15C9	P15E6	P15E7	P15F11	P16B2	P16C3
E	P15G2	P16A2	P16D5	P16D8	P16E3	P16E5	P16E7	P17A3	P17A6	P17A10	P17G7	P17H6
F	P17H9	P18B9	P18D7	P18E10	P18H1	P18H7	P19A6	P19B3	P19E11	P19F3	P19G4	P20C3
G	P20C8	P20C10	P20F1	P20E5	P20G6	P20G12	P20H8	P21A11	P21C4	P21D5	P21D10	P21G8
H	P21G8	P21H8	P22A11	P22B7	P22C10	P22D5	P22D11	P22D12	P22E12	P23A10	P23B5	P23B12

Plate4	1	2	3	4	5	6	7	8	9	10	11	12
A	P23C2	P23C9	P23D12	P23F6	P23G1	P24C8	P24E1	P24G2	P25B7	P28C12	P29D5	P29F9
B	P30A10	P30C11	P31E9	P31A3	P31D1	P31A9	P31A2	P28F1	P30D7	P31C5	P31E5	P31E9
C	P32B4	P32D12	P32F9	P32G3	P33G2	P33G3	P33G7	P33G9	P34F3	P34G5	P37F5	P37G8
D	P37G9	P38H5	P39C5	P40F2	P42E10	P46H5	P48H9	P53D10	P53E6	P53E7	P53E8	P55B6
E	P55C2	P60B8	P61E7	P80E6	P83C10	P83D9	P85C3	P86D4	P94B8	P64D3	P91G7	P62C4
F	P62D10	P81C3	P71H8	P58F9	P100B9	P27D2	P28A1	P28B11	P28C12	P28D9	P29B1	P29D5
G	P30A10	P30C11	P30G11	P31E9	P31G5	P31H12	P31A3	P31D8	P31D1	P31A9	P31A2	P3G10
H	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011

Plate5	1	2	3	4	5	6	7	8	9	10	11	12
A	p1C1	P1C4	P1C9	P1D7	P2D2	P2C6	P2G6	P3A2	P3C4	P3C3	P3C11	P3H4
B	P5E1	P6C1	P6B7	P6F2	P6G1	P7A4	P8B8	PD11	P8E3	P8G5	P8G6	P8G9
C	P8H8	P9C3	P9B7	P9C9	P9D9	P9D12	P9E6	P9E7	P9E12	P9G1	P9G5	P9g10
D	P9G11	P9H8	P9H10	P9H11	P10A8	P10A9	P10D2	P10D10	P11B9	P11C6	P11F5	P11A8
E	P12A9	P12C10	P13A3	P13C10	P13F5	P13G6	P13H1	P13H2	P14A5	P14A5	P14C6	P14G8
F	P14H3	P15B10	P15C11	P15D9	P15F1	P15F8	P15F9	P16B4	P16h10	P17C7	P17D3	P17D6
G	P17E4	P17F1	P17F6	P18E3	P18F5	P18F9	P18F11	P18F11	P18G5	P18G6	P18H4	P19C8
H	P19F8	P19H4	P20A7	P20B8	P20D4	P20G3	P20H6	P21B2	P21C7	P21C10	P21D3	P21E3

Plate6	1	2	3	4	5	6	7	8	9	10	11	12
A	P21F3	P21F6	P21G10	P21H10	P22A5	P22C2	P22C4	P22E4	P22G4	P22H6	P23A7	P23A9
B	P23B1	P23B12	P23D3	P23G7	P23G12	P23H9	P24C1	21H8	P24D9	P24E11	P24G3	P25A3
C	P25B6	P25D12	P25E12	P25G5	P25H7	P25H10	P26A8	P26D2	P27A11	P27B5	P27B11	P27C1
D	P27C8	P27C12	P27E7	P27E12	P27F1	P27F8	P27G6	P27H11	P27H12	P28A5	P28A8	P28C3
E	P28D1	P28D11	P29A7	P29B1	P29B5	P29C12	P29D2	P29D6	P29E2	P29G8	P29G10	P29H1
F	P29H3	P29H5	P29H9	P30B7	P30C3	P30D1	P30D3	P30E7	P30G7	P30G8	P30G11	P30H5
G	P31A2	P31A11	P31A12	P31C3	P31D2	P31E1	P31E9	P31E6	P31F5	P31G1	P31G2	P31G4
H	P31G10	P31H1	P31H4	P31H6	P31H7	P32A8	P32B2	P32B11	P32C7	P32D9	P32E2	P32F1

Plate7	1	2	3	4	5	6	7	8	9	10	11	12
A	P32G2	P32G9	P32G12	P32H2	P33A8	P33C5	P33E4	P33H7	P34A9	P34B7	P34B8	P34B9
B	P34E4	P34E11	P34F2	P34F5	P34F11	P34G1	P34G5	P34G11	P34H2	P34H8	P35A3	P35B9
C	P35C6	P35C11	P35E9	P35F1	P35F5	P36B3	P36C12	P36G2	P36G12	P37B6	P37D11	P37F1
D	P37F2	P37F9	P37F12	P37G1	P37G2	P37G4	P37H6	P38B7	P38D1	P38D5	P38D9	P38E2
E	P38E5	P38E10	P38E11	P38E12	P38F4	P38F11	P38F12	P38G1	P38G5	P38G8	P38G10	P38H3
F	P38H11	P39A4	P39B8	P39C3	P39C10	P39C12	P39D2	P39D12	P39E2	P39E3	P39E6	P39E8
G	P39F4	P39F5	P39G9	P39H4	P39H6	P39H10	P40A9	P40C1	P40D11	P40E10	P40F4	P40G11
H	P101B4	P101B5	P101B11	P101C8	P101C10	P101D1	P101D2	P101D8	P101D11	P101E8	P101E12	P32G2

Plate8	1	2	3	4	5	6	7	8	9	10	11	12
A	P101F3	P101G8	P101H4	P101H8	P102A8	P102B2	P102C11	P102E7	P102F10	P102G2	P102G4	P102G12
B	P102H6	P103C5	P103C12	P103E12	P103H12	P104C6	P104E5	P104G5				
C												
D												
E												
F												
G												
H	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011	RMS011

Appendix 2. Reordered Plate 3 Phenotypes (1 is growth, 0 is no growth).

Fludioxonil 3	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1	1	1	1	0	1	1	1	1	1	1
B	0	1	1	1	1	1	1	1	1	1	1	1
C	1	1	1	1	0	1	1	0	0	1	1	0
D	0	1	1	1	1	1	1	1	1	1	1	1
E	1	1	1	1	1	1	1	1	1	1	1	1
F	1	1	1	1	1	1	1	1	1	1	1	1
G	1	1	1	1	1	1	1	1	1	1	1	1
H	1	1	1	1	0	1	0	1	1	1	0	1

Ambruticin 3	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1	1	1	1	0	1	1	1	1	0	1
B	0	1	1	1	1	1	1	1	1	0	1	1
C	1	1	0	1	0	1	1	0	0	1	1	0
D	0	0	1	1	1	1	0	1	1	1	1	1
E	1	1	0	1	1	0	1	1	1	1	1	0
F	0	1	1	1	1	1	1	1	0	1	1	1
G	1	1	1	0	1	1	1	1	1	1	1	1
H	1	0	0	1	0	1	0	1	1	1	0	1

based on hyphal extension

VM 1M NaCl 3	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	0	0	1	1	0	0	0	0	0	0
B	0	0	0	0	0	0	1	1	1	1	1	0
C	1	0	1	1	1	1	0	1	1	0	0	0
D	1	0	0	1	0	1	0	0	0	0	0	0
E	0	0	0	0	1	0	1	0	0	0	0	0
F	0	0	0	1	0	1	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	1	0	1	0	0	0	1	0

Appendix 3. Reordered Plate 4 Phenotypes (1 is growth, 0 is no growth).

Fludioxonil 4	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1	1	1	1	1
C	1	1	1	0	1	1	0	0	1	1	0	1
D	1	1	1	1	1	1	0	1	1	0	1	1
E	1	1	1	1	1	1	1	1	0	1	0	1
F	0	1	1	1	1	1	1	1	1	1	1	1
G	1	1	0	1	1	0	0	1	1	1	1	1
H RMS011	0	0	0	0	0	0	0	0	0	0	0	0

Ambruticin 4	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1	1	1	1	1
C	1	1	1	0	1	1	0	0	1	1	0	1
D	1	1	1	1	1	0	1	1	1	0	1	1
E	1	1	1	1	1	1	1	1	0	1	0	1
F	0	0	1	1	1	1	1	1	1	1	1	1
G	1	1	0	1	1	0	0	1	1	1	1	1
H RMS011	0	0	0	0	0	0	0	0	0	0	0	0

VM 1M NaCl 4	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	1	0	0	0	0	1	0	0	1
B	0	0	0	0	0	0	0	0	0	1	1	0
C	0	0	1	1	0	0	0	0	0	0	1	0
D	1	0	0	0	0	0	0	0	0	1	1	0
E	0	0	1	0	0	1	1	0	0	0	1	0
F	1	0	0	1	1	0	0	0	1	1	0	0
G	0	0	1	0	0	1	1	1	0	0	0	0
H	1	1	1	1	1	1	1	1	1	1	1	1

Appendix 4. Reordered Plate 5 Phenotypes (1 is growth, 0 is no growth).

Fludioxonil 5	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1	1	0	1	1
C	1	0	1	1	0	1	1	1	1	1	1	0
D	1	1	1	1	1	1	1	1	1	0	1	1
E	0	0	1	1	1	1	0	0	1	1	1	1
F	1	0	1	1	1	1	1	0	0	0	1	1
G	1	0	0	1	1	0	1	1	1	0	1	0
H	0	1	1	1	1	0	0	1	1	1	1	1

Ambruticin 5	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1	1	0	1	1
C	1	0	1	1	0	1	1	1	1	1	1	0
D	1	1	1	1	1	1	1	0	1	0	1	1
E	0	0	1	1	1	1	0	0	1	1	1	1
F	1	0	1	1	1	1	1	0	0	1	1	1
G	1	0	0	1	1	0	1	1	1	0	1	0
H	0	1	1	1	1	0	0	1	1	1	1	1

VM 1M NaCl 5	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1	0	1	0	0	0	0	1	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	1	0	0
D	0	0	0	0	0	0	1	0	0	1	1	0
E	1	0	1	1	0	0	0	1	0	0	1	0
F	0	0	0	0	0	0	1	0	1	1	1	0
G	0	1	0	0	0	0	0	1	0	0	0	0
H	0	0	0	0	0	0	1	1	1	0	1	0

Appendix 5. Reordered Plate 6 Phenotypes (1 is growth, 0 is no growth).

Fludioxonil 6	1	2	3	4	5	6	7	8	9	10	11	12
A	1	0	1	1	1	1	1	1	1	1	0	1
B	0	1	1	1	1	1	0	1	1	0	1	1
C	1	0	1	1	1	1	0	1	1	0	1	1
D	1	1	1	1	0	1	0	1	1	0	0	1
E	1	0	0	0	1	1	0	1	1	1	1	0
F	1	0	1	0	1	1	0	1	1	0	0	0
G	1	1	1	1	1	1	0	0	1	1	1	0
H	1	1	1	1	1	0	1	1	0	1	1	1

Ambruticin 6	1	2	3	4	5	6	7	8	9	10	11	12
A	1	0	1	1	1	1	1	1	1	1	0	1
B	0	1	1	1	1	1	0	0	1	0	1	1
C	1	0	1	1	1	1	0	1	1	0	1	1
D	1	1	1	1	0	1	0	1	1	0	0	1
E	1	0	0	1	1	1	0	1	1	1	1	0
F	1	1	1	0	1	1	0	1	1	0	0	0
G	1	1	1	1	1	1	0	0	1	1	1	0
H	1	1	1	1	1	0	1	1	0	1	1	1

VM1M NaCl 6	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	0	0	1	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	1	1	0	0	0	0	0	0	0	0	0
D	0	0	0	0	1	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	1	0	0	0	0	1	1	1
G	1	0	0	0	0	0	0	0	0	0	1	1
H	0	0	0	0	0	0	0	0	1	0	0	0

Appendix 6. Reordered Plate 7 Phenotypes (1 is growth, 0 is no growth).

Fludioxonil 7	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	0	1	1	1	1
B	1	0	0	1	0	1	1	1	1	1	0	1
C	1	0	1	0	0	1	1	1	1	1	1	1
D	1	1	0	1	0	1	1	0	1	0	1	1
E	1	0	1	1	0	0	1	1	0	1	1	1
F	1	1	1	1	1	1	0	1	1	1	0	1
G	1	0	0	1	1	0	0	0	1	1	1	0
H	1	1	1	1	1	1	1	1	0	0	0	1

Ambruticin 7	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	0	1	1	1	1
B	1	0	0	1	1	1	1	1	1	1	0	1
C	1	0	1	0	0	0	1	1	1	1	1	1
D	1	1	0	1	0	1	1	0	1	0	1	1
E	1	0	1	1	0	0	1	0	0	0	1	1
F	1	1	1	1	1	1	0	1	1	1	0	1
G	1	0	0	1	1	0	0	0	1	1	1	0
H	1	1	1	1	1	1	1	1	0	0	0	1

VM1M NaCl 7	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1	0	0	1	1	0	0	0	1	1	1
B	0	1	0	0	0	1	1	1	0	1	1	1
C	1	0	1	0	0	1	1	0	0	0	0	1
D	0	0	1	0	0	0	0	0	1	0	0	0
E	0	1	0	1	1	0	0	1	0	0	0	0
F	1	0	0	0	1	0	1	0	0	0	0	0
G	0	0	0	0	1	1	0	0	0	0	0	0
H	0	0	0	0	1	1	1	1	0	1	1	0

Appendix 7. Reordered Plate 8 Phenotypes (1 is growth, 0 is no growth).

Fludioxonil 8	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	0	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1				

Ambruticin 8	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	0	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1				

VM1M NaCl 8	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1	0	0	1	0	0	0	0	1	0	0
B	0	0	1	0	0	1	0	0				

Appendix 8. Annotated Open Reading Frame Hits

RNAi Strain	Gene	Annotation
P20F1	AN3729	1,3 beta-D-glucan synthase
P11E11a	AN3729	1,3-Beta-D-glucan synthase
P103G5 4-64	AN1700	26S Proteasome Regulatory Subunit Rpn2
P12A3a 38-318	AN0840	2-isopropylmalate synthase, 1st leucine biosynthesis
P23A9 137-174	AN5331	3-hydroxylacyl-CoA dehydrogenase
P38B7	AN10661	5' nucleotidase
P6F2 4-91	AN9465	60S Ribosomal protein L9
P24C8 6-256	AN6581	ABC multidrug transporer, S. cerevisiae SNQ2-response to siglet Oxygen
P7A4 176-246	AN1103	acetoacetyl-CoA synthase
P27F8 206-280	AN6093	acetyl xylan esterase CAZY family
P29C12 206-331	AN6093	acetyl xylan esterase CAZY family
P38G5	AN3894	aconitate hydratase mitochondrial TCA cycle
P16B2a 1-109	AN0609	acyl CoA synthetase, N-acyl-N-hydroxy-L-ornithine synthesis
39H6	AN3006	alpha/beta hydrolase
P40D11	AN3006	alpha/beta hydrolase
P102H6 1-135	AN5725	alpha-1,2-mannosyltransferase
P24D9 7-211	AN8392	alpha-1,4-galactosidase CAZY family gh36
P4D1b	AN2200	alpha-ketoglutarate dependent taurine dioxygenase
P18E10	AN4477	amino acid transporter
P18E8 7-42	AN4477	amino acid transporter
P23B12 59-347	AN3287	amino acid transporter, C. albicans AGP2-response to osmotic stress
P18D7a 5-110	AN0842	AMP binding enzyme
P19F3	AN6138	Anaphase promoting, DNA damage check
P1C4b	AN5822	AnkA KinaseA DNA damage checkpoint
P18H4 450-607	AN8562	ankyrin repeat
P35B9	AN2364	Ankyrin Repeat protein
P29B5 8-184	AN1067	antibiotic biosynthesis monooxygenase
P8E3 410194	AN7016	AP-2 adaptor complex subunit alpha
P20G12a 199-229	AN0162	APSES TF
P30C3 53-242	AN4501	artA DNA damage checkpoint
P36G12	AN8329	aryl-alcohol dehydrogenase
P9D12	AN10125	ATP-dependent RNA helicase Drs1
P28A5 2-110	AN5491	Autophagy related protein-peripheral membrane protein Atg2
P32B4	AN7734	bHLH TF penR
P18H7 1-250	AN6138	bimA, sep1 anaphase promoting complex DNA damage checkpoint
P38E12 8-89	AN4323	branched-chain aminotransferase
P101D8-2	AN3216	B-Zip TF
P22C4	AN5634	C2 domain protein
P12F6 7-142	AN8741	C2H2 finger domain protein
P5E8	AN5003	C2H2 TF
P28A5 166-274	AN3391	C2H2 Zn(2)-Cys(6) DNA binding TF
P17A10b	AN1189	Calcium Ion P-type ATPase, N. crassa NCA-2-response to cation stress, osmotic stress
P30A10b	AN1189	Calcium Ion P-type ATPase, N. crassa NCA-2-response to cation stress, osmotic stress
P28C3 1-402	AN5708	CBS domain protein
P28C12c	AN0594	cell morphogenesis protein
P28B1b	AN1064	cell morphogenesis protein PAG1
P8D11a 220-422	AN4566	chitin synthase 1
P27F8 2-232	AN4367	chitin synthase class III
P29C12 4-388	AN4367	chitin synthase class III
P23B12 344-389	AN6317	Chitin synthase myosinfusion, C. albicans CHS3-response to osmotic stress
P3H4b 268-429	AN3851	choline dehydrogenase
P16B2a	AN5731	Chorismate Synthase, aromatic aminoacid biosynthesis
P24C8	AN7937	Concanamycin response protein
P33G3	AN2676	Conserved hypothetical protein
P31A1	AN6785	CRAL/TRIO domain
P31A2	AN6785	CRAL/TRIO domain
P12A3b 69-275	AN11114	Cyanide Hydratase/Nitrilase
P4D1a	AN3394	Cytochrome P450
P14A9b	AN2610	Cytochrome P450
P15A3a	AN2610	Cytochrome P450
I46-223	AN10613 & 10776	Cytochrome P450
P4D1a	AN3394	cytochrome P450

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P14G10 82-237	AN6523	cytokinesis protein sepA, C. albicans and S. cerevisiae BNI1-response to osmotic stress
P8D11b 92-215	AN0596	damaged DNA binding protein Ddb1
P28D11 8-178	AN7014	DEAD helicases superfamily
P6B7	AN1634	DEAD/DEAH box helicase
P28B11a	AN9014	Dehydrogenase FMN
34H8 100-211	AN4272	DNA excision repair Rad14
P17H9a 2-62	AN3620	DNA repair protein Rad1
P31E9	AN3890	DNA repair Rad4
P38G10	AN4206	DnaJ domain protein
P27C12 119-164	AN2238	DNAJ domain Psi
P3C3a-1 7-78	AN4758	dsDNA binding protein PDCD5
P12A5	AN3479	DUF1212 domain, membrane protein
P9H10	AN5387	DUF453 domain
P17E4	AN5387	DUF453 domain
P18H7 67-120	AN10769	DUF814 domain
P12A3a 2-88	AN1322	Dynamin family GTPase
P64D3	AN8877	ELMO/CED-12 family protein
P31A9	AN6563	Elongation factor 1-gamma (Cam1p)
P38E5	AN2690	endo-1,3(4) beta-glucanase
P27D2b 50-160	AN1908	essential cytoplasmic protein Ctr86
P29B1 77-200	AN0602	extracellular protein
P35B9	AN0353	F-box domain protein
P7A4 52-205	AN1758	FF domain protein
P10A9 7-99	AN8660	flavin binding monooxygenase
P6G1 2-95	AN8660	flavin binding monooxygenase
P28C12a	AN9017	FMN dependent dehydrogenase
P13A1 104-192	AN1249	Formin binding protein (FNB3)
P37H6b	AN3840	FPGS folylpolyglutamate synthase
P18F11-1	AN1055	G2/M phase checkpoint control protein Sum2
P29D5 13-69	AN6828	GATA TF Ams2
P18F11-2	AN1792	GDSL Lipase/Acylhydrolase family protein
P37H6a	AN8212	GIY-YIG catalytic domain
P5E8	AN4925	Glutamate Carboxypeptidase, Tre2
P28B11b	AN5134	Glutamate Synthase
P28C12	AN5134	Glutamate Synthase
P28C12b 109-344	AN5134	glutamate synthase
P28B11a 114-282	AN5134	glutamate synthase
P27C12 3-116	AN2096	glycerol transporter
P31D1	AN9074	Glycine Rich Conserved
P11C5b	AN2955	glycosyl transferase
P21G8a	AN4461	G-patch domain protein
P12C10 72-354	AN3141	GPI-anchored protein
P27H11b	AN8188	GTP cyclohydrolase
P32G12b	AN10853	HbrB -required for hyphal growth
P20G6B	AN6585	helicase DEAH-box RNA helicase (Dhr1)
P11F5a 256-379	AN4479	Histidine Kinase (with HAMP domains)
P29E2	AN4447	Histidine Kinase hk5 (no 5' domain)
P3G10	AN2005	Histidine Phosphotransferase (YpdA)
P9C3 75-151	AN5640	histone acetyltransferase
P14A9a	AN3096	Histone arginine methyltransferase
P18H4 550-718	AN6533	hypercellular protein A HypA, intragolgi transport polarity defects
P53E8	AN5003	hypothetical C2H2 zinc finger
P40A9	AN8173	Impact family protein
P17D6 174-272	AN0906	Importin beta Kap95
P32D12B	AN3927	Inositol phospholipase;salt stress response
P32D2b	AN3927	Inositol phosphosphingolipid phospholipase C S. cerevisiae ISC1-response to salt stress

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P4D1a	AN5595	Involucrin Repeat
P34G5 4-210	AN2823	Ion Channel
P103E12	AN8152	Isoamylalcohol oxidase
P17H9a 48-210	AN7547	Kinesin family
P11E11b 4-177	AN2459	Leucine rich repeat
P2G6	AN5714	leucine rich repeat domain protein
P23G12	AN5568	leucine rich repeat domain protein
P27H12 6-122	AN7005	leucine-rich repeat protein
P21D10a 72-243	AN2890	Lipase/Esterase
P32D2a	AN6230	Medusa
34H8 2-100	AN4445	methyltransferase DMAP1
P1C9	AN3869	mevalonate kinase
P39C5	AN11092	MFS monocaroxylate transporter
P35C9-2	AN0890	MFS Multidrug Transporter
P37H6c	AN0970	MFS Multidrug Transporter
P18H4 8-338	AN0890	MFS multidrug transporter
P31A9	AN1276	MFS sugar transporter
P22A5	AN5067	MFS Sugar Transporter
P19H4 198-315	AN0233	MFS sugar transporter
P31A9 6-51	AN1276	MFS sugar transporter
P33G2	AN9460	Microsomal signal peptidase subunit
P23G1	AN1986	mitochondrial chaperone Frataxin
P3A2	AN5724	mitochondrial export translocase Oxa2
P103G5 61-151	AN6193	Mitochondrial Serine Protease Pim1 heat response ATP dependent
P40E10 10-177	AN3942	MOSC domain
P8D11b 1-172	AN4618	Myb-like TF
P18E5a 223-397	AN10611	N-acetylglucosaminyl transferase component Gpi1
P31A9	AN2866	NAD binding oxidoreductase
P31A9 47-251	AN2866	NAD binding Rossmann fold oxidoreductase
P17G7b	AN2682	NADH flavin oxidoreductase
P29H9 178-227	AN1007	nitrate reductase
P18H7 10-225	AN9244	Non-ribosomal peptide synthase
P24C1	AN8504	nonribosomal peptide synthase GliP-like 5' overlap with copia-2
P17A6a	AN10297	NRPS-like enzyme
P29D6 18-117	AN10297	NRPS-like enzyme
P23B12 4-72	AN6444	NRPS-like enzyme
P40E10 11-146	AN8056	Nuclear Condensin complex 3
P16E3	AN1115	Nucleoside hydrolase
P37F2 2-189	AN2207	O-methyltransferase
P1E4	AN4409	Ornithine Carbamoyl transferase
P18E10	AN4409	Ornithine carbamoyl transferase
P18E8174-665	AN4409	ornithine carbamoyltransferase
P17D6 266-432	AN4409	ornithine carbamoyl-transferase, mitochondrial precursor
P15F11	AN6031	Oxidoreductase, 2-nitropropane dioxygenase
P8D11a 1-255	AN2497	palmitoyl-rotein thioesterase
P29D6 1-219	AN1671	PAP2 domain
P18H4 400-452	AN7626	paxillin Pxl1, Cdc42 and Rho1 GTPases
P38E2	AN2331	pectin lyase
P16B2b 6-173	AN8061	peptidyl-prolyl cis-trans isomerase
P35E9	AN8211	PHD TF Rum1
P18B9a 1-114	AN7418	Phenol 2-monooxygenase promoter

P46H5 6-283	AN4992	Phopholipid: diacylglycerol acyl transferase
P21H10	AN10791	phosphatidylinositol 4-kinase type II subunit alpha
P5E3	AN4234	Phosphoacetylglucosamine mutase
P5E3	AN4234	phosphoacetylglucosamine mutase, chitin synthesis
P40D11a 12-123	AN4992	Phospholipid diacylglycerol acyltransferase
P29G8	AN2614	plasma membrane hexose transporter
P1D7	AN7165	plasma membrane stress response protein Ist2
P2D6	AN7165	plasma membrane stress response protein Ist2
P11F5c	AN7165	plasma membrane stress response protein Ist2
P18F5	AN7165	plasma membrane stress response protein Ist2
P20D4	AN7165	plasma membrane stress response protein Ist2
P25H10	AN11191	polyketide synthase
P37G2 169-340	AN1036	polyketide synthase
P11E11b 149-233	AN6200	pre-rRNA processing protein Rrp12
P39E8	AN1731	proline oxidase
P39H6 23-101	AN1732	Proline specific permease, proline utilization transporter
P27D2c	AN7986	protein kinase
P15A11 12-100	AN4896	Protein Tyrosine Phosphatase 2
P24D9 196-298	AN8396	pyruvate decarboxylase
P20G6A 162-301	AN3063	regulatory factor Sgt1
P22D5 11-225	AN5787	Rho GTPase activator (Bem3)
P101D8-1	AN7783	Rho guanyl nucleotide exchange factor
P20C10	AN4522	Ribosomal protein L34
P23C9	AN3827	Ribosome biogenesis
P24C8 8-318	AN4065	ribosome biogenesis protein Kri1
P27D2b 135-191	AN3827	ribosome biogenesis protein Pescadillo
P28D11 184-230	AN9421	RING finger
P23D3b	AN6136	RING finger membrane protein
P3D6	AN5711	RLI DUF367 domains
P1C1	AN8276	RNA binding protein Nrd1
P18B9b	AN6178	RNA PolII TF SIII Subunit A promoter
P31C5	AN4570	RNA polmeraseII transcription factor Rtf1p
P39H10 2-142	AN0809	RNA Polymerase II largest subunit
P23F6A	AN2759	RNA processing protein
P34G11	AN2593	salicylate hydrolase
P40G11	AN1019	SCF Ubiquitin ligase complex subunit CulA
P20H8	AN10596	septin aspE
P6F2 88-254	AN10529	serine palmitoyl transferase, sphigolipid synthesis
P23F6B	AN0698	SH3 domain protein
P39G9	AN8210	SH3 domain protein
P25A3b	AN8499	siderochrome iron transporter
P28A5 106-171	AN5627	SonB nuclear pore complex-DNA damage-lethal deletion
P38E12 86-193	AN0640	sphinganine hydrolase BasA
P21D10b 11-72	AN2289	sucrose transporter
P3H4a 8-469	AN9168	sugar transporter, glycerol H+ symporter Stl1
P29C12 326-439	AN0573	TBC domain protein
P3H4b 425-543	AN4639	TORC1 growh control complex subunit Kog1, response to starvation
P15A3b	AN4670	Transcription factor SPT8
P8E3 1-76	AN3014	translation repressor/antiviral protein Ski3, TPR repeats
P38G1	AN0108	Tubulin Specific chaperone D
P9C3 155-399	AN6176	tubulin specific chaperone Rbl2
P27F8 109-255	AN5906	U-box domain
P29C12 109-255	AN5906	U-box domain
P7B3	AN4754	UBX domain protein
32G2	AN4962	UPF0132 domain
P101B4	AN4962	UPF0132 domain
P37D11	AN2144	Urease accessory portein UreD
P37B6b	AN2144	UreD Urease Accessory protein
P14G10 12-89	AN3720	vesicle coat complex COPII, subunit SEC24
P71H8	AN7526	vesicle mediated transport protein Bfr2/Che-1
P20C3	AN1168	VIC calcium ion channel, S. cerevisiae Cch1
P4D1a	AN6199	Zinc Carboxypeptidase

P27E12	AN0568	Zn(II)2Cys6 TF
P35C11	AN9236	Zn(II)2Cys6 TF
P40D11b	AN7923	Zn(II)2Cys6 TF
P39H6 22-130	AN7923	Zn(II)2Cys6 TF
P3C11	AN0863	Zn(II)2Cys6 TF
P9C9	AN10262	Zn(II)2Cys6 TF
P28D1 110-260	AN1927	Zn(II)2Cys6 TF
P21D10b 5-43	AN7189	ZN(II)2Cys6 TF
P25B7 150-196	AN3280	ZN(II)2Cys6 TF

Appendix 9. Hypothetical Open Reading Frame Hits.

P23H9a 3-121	AN0024	hypothetical
P83D9	AN0659	Hypothetical
P37F1	AN0845	hypothetical
P11D6a	AN10065	Hypothetical
P34G5 140-285	AN10065	Hypothetical
P26D2	AN10180	hypothetical
P17H9b	AN10372	Hypothetical
P26G10	AN10766	Hypothetical
P13A2	AN10923	Hypothetical
P3C3-2	AN10995	hypothetical
P32F9B	AN11011	Hypothetical
P29B5 121-269	AN1118	hypothetical
P25G5	AN11349	hypothetical
P3C3a-1 272-350	AN1507	hypothetical
P22D5 222-276	AN1605	hypothetical
P39H10 73-136	AN1605	hypothetical
P17A3	AN1738	hypothetical
P21C7	AN1738	hypothetical
P27A11 1-293	AN1823	hypothetical
P25A3a	AN1942	hypothetical
P15C9 13-82	AN2169	Hypothetical
P29H9 8-190	AN2197	hypothetical
P28B11b	AN2292	Hypothetical
P28C12b 257-354	AN2292	hypothetical
P11C5a	AN2376	Hypothetical
P32D12	AN2391	Hypothetical
P34A9	AN2467	hypothetical
P5F5	AN2504	Hypothetical
P2D2 130-186	AN2586	hypothetical
P101H8 2-83	AN2620	hypothetical
P20G12a 140-199	AN2643	Hypothetical
P16B2a 70-403	AN2660	Hypothetical

P16B2b 133-214	AN2680	Hypothetical
P9B7 6-149	AN2874	hypothetical
P17A10a	AN3021	Hypothetical
P21D10a 1-85	AN3512	Hypothetical
P1H10	AN3532	Hypothetical
P2A3	AN3532	Hypothetical
P2E4	AN3532	Hypothetical
P23D4a 106-291	AN3726	hypothetical
P28D16-138	AN3802	hypothetical
P9B7 141-230	AN3930	hypothetical
P2D2 3-136	AN4089	hypothetical
P8B8	AN4100	hypothetical
P3H4a 550-757	AN4108	hypothetical
P11C11	AN4868	Hypothetical
P3C3a-1 75-302	AN5042	hypothetical
P39H4	AN5245	hypothetical
P21F3	AN5326	hypothetical
P27H11a	AN5507	hypothetical
P14A9a	AN5663	Hypothetical
P38H11	AN5663	hypothetical
P19A6	AN5664	Hypothetical
P18E5a 1-237	AN5739	hypothetical
P14A9a	AN5807	Hypothetical
P30C3 7-67	AN6196	hypothetical
P29H9168-303	AN6538	hypothetical
P28B11b	AN6634	Hypothetical
P28C12b 8-120	AN6634	hypothetical
P28B11a 9-125	AN6634	Hypothetical
P23D12	AN6868	Hypothetical
P20G6A 16-134	AN6877	hypothetical
P20G6A 16-134	AN6877	hypothetical
P15E7	AN7362	Hypothetical

Appendix 9. Hypothetical ORF Hits, continued.

P19H4 12-155	AN7362	hypothetical
P25B7 92-168	AN7383	Hypothetical
P3H4b 197-340	AN7601	hypothetical
P21D10b 27-167	AN7789	Hypothetical
P18B9a 101-255	AN7883	Hypothetical
P29H1	AN8034	hypothetical
P100B9	AN8151	Hypothetical
P37G2 3-209	AN8175	hypothetical
P3H4b 10-207	AN8200	hypothetical
P37B6a	AN8200	hypothetical
P12C10	AN8310	Hypothetical
P11F5b 1-143	AN8310	hypothetical
P3C3b	AN8492	hypothetical
P21D3 270-374	AN8492	hypothetical
P23D4a 6-109	AN8493	hypothetical
P34E11a	AN8665	hypothetical
P6G1 75-253	AN8933	hypothetical
P10A9 81-257	AN8933	hypothetical
P14H5	AN8985	Hypothetical
P2D2 104-369	AN9036	hypothetical
P17G7a 169-361	AN9444	Hypothetical

Appendix 10. Annotated Non-ORF Hits.

RNAi Strain	Gene	Annotation
P5F5	AN0930/AN0929	Efflux Transporter/Kinase
P11C5a	AN0176/AN10033	GATA TF/COP1 coated vesicle
P11D6b	AN2020/AN2021	AhpA(hoxA)/beta transducin HET-E2C NACHT,WD40
P12A3b 1-70	AN4256/AN4255	Lipase/Hexokinase
P13A1 2-112	AN7682/AN7683	AP-1 clathrin assembly/Export protein Nce102
P15A11 111-269	AN4325/AN4324	Hypothetical/B-zip TF
P16A2	AN3739/AN3738	RNP domain/hypothetical
P16E7a	AN10770/AN6101	RTA1 Domain/Cytochrome P450
P16E7b	AN9467/AN8001	Protein Phosphatase Par1 stress/mRNA splicing Yju2
P17G7a 7-170	AN1963/AN1962	Hypothetical/HMG box transcriptional regulator
P18D7a 156-266	AN2769/AN2768	Transcription Initiation factor TFIID/Hypothetical
P20G12a 29-140	AN6672/AN6673	Hypothetical/alpha-fucosidase CAZY family GH95
P24G2	AN8231/AN8232	S-adensyl methionine decarboxylase/TFIID complex
P26G10	AN6888/AN6889	Endopeptidase/Zinc Finger
P26G10	AN7579/AN7580	Proteosome Regulator/Zinc Finger
P29D5	AN6785/AN11527	Oxidoreductase/Hypothetical
P33G2	AN1888/AN9459	fructose biphosphate aldolase/Disease resistance protein
P37G9	AN4356/AN7726	Hypothetical/Nuclear Pore Complex Sac3
P58F9	AN4547/AN4546	Coatomer subunit/KH domain RNA-binding protein
P62E4	AN8644/AN8643	Hypothetical/bZip TF Atf21
37D11	AN2505/AN2504	F-box domain/hypothetical
P38F12	AN6565/AN6564	hypothetical/37S Ribosomal Protein S9
P32G12a 1-190	AN2487/AN2488	Chromatin Remodeling Complex Subunit Arp5/hypothetical
P32G12a 137-237	AN10059/AN0372	C6 Finger domain protein/Integral membrane protein
P23A9 38-172	AN1356/AN1357	hypothetical/choline phosphatase cytidylyltransferase
P37G1 4-124	AN3113/AN3112	DMT family organic anion transporter/GlfA UDP-galactopyranose mutase
P37G1 74-179	AN0432/AN10103	mitochondrial NADH cytochrome b5 reductase MCR1-oxidative stress response/ HMG box protein
P22H6	AN8528/AN8529	beta-lactamase/Zn(II)2Cys6 TF
P37B7	AN2505/AN2504	F-box domain/hypothetical
P102C11	AN7483/AN7484	hypothetical/hypothetical
P101H8 80-153	AN10651/AN5210	hypothetical/pyruvate kinase pki
P34F5	AN1551/AN1552	beta-transglucosylase GH17 family/hypothetical
P40D11a 92-387	AN11520/AN6509	hypothetical/hypothetical
P27H12 118-229	AN6947/AN6948	hypothetical/transglycosidase GH16 family cell wall biosynthesis Type-1 TM
P22C2	AN2601/AN2602	MFS maltose transporter/lipase, esterase
P9B7 224-315	AN1425/AN1426	FarB, Zn(II)2Cys6 TF/serine carboxypeptidase
P9G11	AN8906/AN8907	hypothetical/C-4 methyl sterol oxidase
P9H8	AN1151/AN1150	carboxy-cis,cis-muconate cyclase/ acetylornithine aminotransferase, ornithine transaminase
P9H11	AN2476/AN2477	phosphoric ester hydrolase/plasma membrane channel protein Aqy1 (aquaporin)
P11F5a 11-470	AN10770/AN3101	RTA1 domain protein/cytochrome P450
P12C10 300-509	AN10791/AN6221	Phosphatidylinositol 4-kinase type II subunit alpha/GATA factor AREB
P14C6	AN6673/AN6674	alpha-fucosidase/hypothetical
P17D6 9-169	AN1705/AN1706	hypothetical/glutaminyl cyclase
P18E5b	AN10494/AN10502	hypothetical/ class V chitinase
P23H9a 117-273	AN2623/AN2622	acyl-coenzymeA: 6-aminopenicillanic acid acyltransferase/isopenicillin N synthetase ipnA (IPNS)
P27A11 205-318	AN2554/AN2555	lanthionine synthetase C/serine carboxypeptidase
P28C3 19-109	AN3422/AN3421	MAP kinase kinase/nucleoside diphosphate sugar epimerases
P29B1 7-193	AN0179/AN11262	oxidoreductase, short chain dehydrogenase, reductase family/hypothetical
P29H3	AN3304/AN3303	GABA transporter/hypothetical
P12F6 1-309	AN5156/AN5157	Pho80-like cyclin(phosphate regulator)/armadillo repeat protein
P15C9 77-292	AN0063/AN0062	Integral membrane protein (Ptm1)/hypothetal
P16D5a	AN2266/AN2265	DigA, S. cerevisiae Vps18(Pep3) hyphal growth, nuclear distribution, actin organization/serine, threonin
P16D5b 2-89	AN0508/AN0507	hypothetical/ZN(II)2Cys6 TF
P25B7 3-103	AN0288/AN0287	MYND domain/WD repeat containing protein
P27D2a 2-281	AN7463/AN7464	ammonium transporter C. albicans MEP1 (N2 starvation)/high affinity Ca2+,MN2+ P-type ATPase PMF
P29D5 64-140	AN7539/AN7540	hydrophobin/Eukaryotic translation initiation factor 3 Subunit EifCd
P30A10a 3-57	AN8907/AN8906	C-4 methyl sterol oxidase/hypothetical
P30A10a 49-258	AN9014/AN9013	FMN dehydrogenase family protein/Zn(II)2Cys6 TF
P80C6	AN8644/AN8643	hypothetical/B-Zip TF Atf21

Appendix 11. Hypothetical Non-Open Reading Frame Hits

P5F5	AN2383/AN2384	Hypothetical/Hypothetical
P12B5	AN1645/AN1646	Hypothetical/Hypothetical
P24C8	AN11652/AN9267	Hypothetical/Hypothetical
P16D5b 81-250	AN8086/AN8087	hypothetical/hypothetical
P19E11	AN5094/AN5095	hypothetical/hypothetical
P17C7	AN9054/AN9053	hypothetical/hypothetical
P35C9-1	AN1645/AN1646	hypothetical/hypothetical
P9C3 1-94	AN10339/AN2757	hypothetical/hypothetical
P21H8	AN9054/AN9053	hypothetical/hypothetical
P13C10	AN2465/AN11342	hypothetical/hypothetical
P21D5	AN2535/AN10318	hypothetical/hypothetical

Appendix 12. Repeating Element Hits.

P28F1	repeat, esp telomeres
P53E6	repeat, esp telomeres
P11F5b 117-209	non-ORF repeat, many hits
P28F1	repeat non-ORF
P53E6	repeat ORF and non-ORF
P15E7	Gypsy1 domain
P37G2 301-364	l-1_ANelement line
P32G2	Gypsy-1
P28F1	repeat non-ORF
P53E6	repeat ORF and non-ORF
1C4a	mariner-6_Anelement, many hits repeat
P11F5b 117-209	non-ORF repeat, many hits

Appendix 13. *Aspergillus nidulans* Strains.

Strain	Description
A26	green wild-type, requires biotin
RMS011	yellow wild-type, requires arginine and paba
LR121	yellow wild-type, requires arg, paba, pyro, ur/ue
SIK1	*hogA* deletion, requires paba
F14	*hogA* phenotype suppressor
F21	*hogA* phenotype suppressor
C18	*hogA* phenotype suppressor
B20	*hogA* phenotype suppressor
LN121	*ypdA* silenced, requires paba
LN421	*ypdA* silenced, requires paba
JB1	*brlA* silenced, requires paba, pyro, ur/ue
JB3	*brlA* silenced, requires paba, pyro, ur/ue

Appendix 14. *A. nidulans* RMS011 PEG-Mediated DNA Transformation

Introduction
The purpose of this procedure is to perpetuate the uptake of plasmid DNA into the *A. nidulans* genome. Protoplasts, which are small spherical cells that lack complete cell walls, must be prepared and harvested to perform the transformation.

Materials and Supplies
Media
-MM Agar plate containing Arginine and Paba (growing RMS011 spores)
-MM broth containing Arginine and Paba (growing hyphae)
-MM Agar plates containing Paba and 1.2M Sorbitol (recovery and selection)

Solutions
  1) Mycelium Wash Solution (250mL)
     0.6M MgSO4   36.972g
     -autoclave and store at RT

  2) Double Strength Protoplasting Solution (DSPS) (100mL)
     1.1M KCl   8.2016g
     0.1M Citric Acid   1.921g
     1M KOH   dropwise until pH 5.8
     -filter sterilize and store at RT

  3) STC 50 Solution (250mL)- Make fresh every 15 days
     1.2M Sorbitol   54.65g
     10mM CaCl2   0.37g
     50mMTris HCl (pH 7.5)   12.5mL
     -autoclave and store at RT

  4) 60% PEG Solution (10mL)- Make fresh daily
     PEG 4000   6g
     STC 50   5mL
     -microwave for 15-30 seconds
     -adjust final volume to 10mL
     -store at RT until use

Enzymes
     500 mg Lysing Enzymes from *Trichoderma harzianum* (Sigma L1412-10G)
     250 mg beta-D Glucanase G (InterSpex Products 0439-2)
     250 mg Lysozyme, from Chicken Egg White (Sigma L7651-1G)
     100 mg BSA (Sigma A-4503)

Protoplasts preparation
-Streak entire plate of RMS011 on MM+Arg+Paba plate and grow for 2 days at 37*C
-Spores MUST be fresh
-Harvest spores with sterile water and flamed spatula under hood
-Inoculate MM+Arg+Paba broth with spores and grow O/N at 30*C shaking at 250 RPM
-Harvest mycelium with suction flask, funnel, and filter paper (get about 2 grams)
-Wash with Mycelium Wash Solution (0.6M MgSO4)
-Wash with DSPS (Double Strength Protoplasting Solution)
-Transfer mycelium to 50mL plastic centrifuge tube

-Add enzymes and BSA to 5 mL of DSPS, vortex to dissolve

-Add all the enzymes to the mycelium solution, votex to put mycelium in solution
-Incubate at 30*C, shaking at 250 RPM for 3-5 hours (the thick solution should become more liquid with more time), do not exceed 5 hours
-You can observe the protos under the microscope at this time (they will remain stable in a suspension of STC 50, but will burst in a suspension of water), it is advisable to get an approximate concentration of protos using a hemocytometer

Harvesting and Washing Protoplasts
-Using a suction flask with a sterile glass tube placed inside and a sterile funnel with miracloth (Calbiochem Cat# 475855), transfer your protos to the funnel (the protos will go through the miracloth, but the mycelium will stick to the cloth), collect the supernatant in the sterile glass tube
-Transfer to an appropriate (50 mL) centrifuge tube
-Spin at 7500 RPM for 10 minutes
-Dump the supernatant, and resuspend the pellet in 45 mL STC50
-Repeat the previous 3 times to remove protoplasting enzymes
-Resuspend your final pellet in 1 mL of STC50
-Protos should be milky white in color
-Your Protos are now ready!
-Store extras in -80C by bringing protos to 20% glycerol

Transformation
-Add 10 ug DNA (linear preferred, but not necessary) to a 15 mL falcon tube
-DNA should be CsCl2 prep or Quiagen Endotoxin Free prepared (Cat. No. 12381)
-Add STC 50 to make volume of DNA 50 uL
-Add 150 uL protos, mix well by swirling, incubate at RT 10 minutes
-Add 2 mL PEG, mix well by gently rolling on bench, incubate at RT 20 minutes
-Add 12 mL STC 50, mix well by inverting tube
-Transfer 2 mL to each MM+Paba+1.2M Sorbitol plate and spread with a sterile glass hockey stick
-Leave plates with agar side down, and incubate at 37*C for 1 day
-Invert plates and continue to incubate at 37*C for 2 more days to see transformants

VITA

Lisa Michelle Barton

Candidate for the Degree of

Doctorate of Philosophy

Dissertation: INDUCIBLE RNA INTERFERENCE IN *Aspergillus nidulans*

Major Field: Microbiology, Cell and Molecular Biology

Biographical:

      Education:
      Bachelor of Science in Cell and Molecular Biology
      Oklahoma State University, Stillwater, Oklahoma in July, 2005.

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      Experience:
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      Qualifying Exam: Revealing Histidine Kinase Function in *Magnaporthe oryzae*
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      Publication: Inducible RNA Interference of *brlA_* in *Aspergillus nidulans*
            Eukaryotic Cell (7)11: 2004-2007
            November 1, 2008

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Name: Lisa M. Barton   Date of Degree: July, 2009

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Title of Study: INDUCIBLE RNA INTERFERENCE IN *Aspergillus nidulans*

Pages in Study: 87   Candidate for the Degree of Doctorate of Philosophy

Major Field: Microbiology, Cell and Molecular Biology

Scope and Method of Study:

A unique RNAi construct was tested with the developmental gene, *brlAβ*, in the fungal genetic model, *Aspergillus nidulans*. The construct contains inverted repeats of the inducible alcohol dehydrogenase (*alcA*) promoter surrounding a unique BamHI restriction enzyme site containing any gene of interest. The RNAi construct was utilized to generate a genome-wide RNAi library. The RNAi library was used here to screen for fludioxonil resistance. There is evidence that fludioxonil, a commonly used fungicide, activates an osmotic stress-signaling pathway. When it is aberrantly activated, the cells swell beyond capacity, leak their contents, and die. Ambruticin is an antifungal that could potentially be used in humans and animals to treat fungal infections, and it is also thought to aberrantly activate the osmotic stress-signaling pathway. The RNAi library strains resistant to fludioxonil were tested against ambruticin VS3 and sensitivity to osmotic stress. The silenced insert from selected strains was rescued from the genome by PCR of the integrated RNAi construct and sequenced to identify the silenced gene. Those genes that were identified more than one time were further analyzed *in silico*, and selected genes were chosen to independently confirm resistance conferred by silencing.

Findings and Conclusions:

The RNAi construct was found to integrate into the genome of the fungus and cause locus specific silencing of *brlAβ* in *Aspergillus nidulans*. The RNAi strains had inducible *brlA* phenotypes, and downregulation of *brlA* was confirmed by Northern blotting and Real-Time Reverse-Transcriptase PCR. The downregulation of *brlA* was found to be induced by RNAi with anti-sense Northern blots and siRNA Northern blots, and the silencing was specific for *brlAβ*, not *brlAα*, a downstream alternate transcript of the *brlA* locus.

Thirty-six genes were found more than one time to cause resistance to fludioxonil and ambruticin when silenced. Twenty-four of these had annotations, while twelve were hypothetical. Twenty-one out of the twenty-four annotated genes contain transmembrane domains, indicating that fludioxonil and ambruticin alter the plasma membrane in order to aberrantly activate the osmotic stress-signaling pathway. It also suggests there are multiple ways the drugs act upon the cell, rather that having a single target.

ADVISER'S APPROVAL: Rolf A. Prade, Ph.D.