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Role of HogA and SskB Protein Kinases in Mycotoxin Production in the Filamentous Fungus *Aspergillus nidulans*

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Lauren Jurkowski

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Role of HogA and SskB Protein Kinases in Mycotoxin Production in the Filamentous Fungus *Aspergillus nidulans*

Student Name: Lauren Jurkowski

Faculty Supervisor: Dr. Ana Calvo

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ABSTRACT (100-200 WORDS): *Aspergillus nidulans* is a filamentous fungus that is often used as a model organism in genetics and molecular biology studies. The species has a well-characterized life-cycle and its genome has been sequenced. *Aspergillus* species are commonly found in the environment. Some species can cause health problems in humans. Some *Aspergillus* species produce aflatoxin, a toxic compound that causes acute necrosis, cirrhosis, and carcinoma of the liver in some animal species and can potentially cause similar symptoms in humans (Center for Integrated Fungal Research, 2005). *A. nidulans* produces the carcinogenic mycotoxin sterigmatocystin (ST), a precursor in the aflatoxin biosynthetic pathway (Adams, 1994). The focus of this project is to characterize the possible involvement of two *A. nidulans* stress-response protein kinases in the regulation of mycotoxin production. The two protein kinases

under study are HogA and SskB. They are part of a phosphorelay system that senses osmotic and oxidative stress and triggers an adaptive response to these stimuli (Miskei, 2009). Our results suggest that the HogA pathway negatively regulates ST biosynthesis. This is the first study of the connection between elements of the HogA response pathway and the regulation of ST production.

Abstract

Aspergillus nidulans is a filamentous fungus that is often used as a model organism in genetics and molecular biology studies. The species has a well-characterized life-cycle and its genome has been sequenced. *Aspergillus* species are commonly found in the environment. Some species can cause health problems in humans. Some *Aspergillus* species produce aflatoxin, a toxic compound that causes acute necrosis, cirrhosis, and carcinoma of the liver in some animal species and can potentially cause similar symptoms in humans (Center for Integrated Fungal Research, 2005). *A. nidulans* produces the carcinogenic mycotoxin sterigmatocystin (ST), a precursor in the aflatoxin biosynthetic pathway (Adams, 1994). The focus of this project is to characterize the possible involvement of two *A. nidulans* stress-response protein kinases in the regulation of mycotoxin production. The two protein kinases under study are HogA and SskB. They are part of a phosphorelay system that senses osmotic and oxidative stress and triggers an adaptive response to these stimuli (Miskei, 2009). Our results suggest that the HogA pathway negatively regulates ST biosynthesis. This is the first study of the connection between elements of the HogA response pathway and the regulation of ST production.

Introduction

The study of *Aspergillus* species is important because they are commonly found in the environment and some can cause severe health problems. They are often found in the soil, household dust, and food items (CDC). In humans, certain *Aspergillus* species can cause problems such as Aspergillosis and allergic respiratory symptoms (CDC). Importantly, some species, such as *A. flavus*, can infect the seeds of corn, cotton, and peanuts. The growth of some *Aspergillus* species on a food source can lead to the production of aflatoxin and contamination of the food source. Aflatoxin contamination compromises the safety of food supplies and causes significant economic losses every year (Dorner, 2005). *A. nidulans,* the organism under study in this project, produces the carcinogenic mycotoxin sterigmatocystin (ST) (Figure 1), a precursor in the production of aflatoxin (Adams, 1994).

In a preliminary analysis, seven *A. nidulans* kinase deletion mutants, received from the Fungal Genetics Stock Center (FGSC), were checked for production of ST. They were compared with a control strain, FGSC33. Two of those deletion mutants, HogA and SskB (Figure 2), showed higher levels of ST production than the control strain (Figure 3). The elevated toxin levels in these mutant strains provided preliminary evidence suggesting that the deleted genes, *hogA* and *sskB*, were repressors of toxin production.

In order to study the regulatory role of *hogA* and *sskB* in toxin production in a wild-type genetic background, *veA+*, and the commonly used *veA1* background, fungal crosses were performed to attempt to change the genotype of each mutant. This eliminated any other possible mutation in their genetic background. The HogA strain *(wA3; argB2; ΔnkuAku70:: argB pyroA4; sE15 nirA14 chaA1 fwA1)* and SskB strain *(wA3; argB2; ΔnkuAku70:: argB pyroA4;*

sE15 nirA14 chaA1 fwA1) are each being crossed with another strain, WIM126 *(pabaA1, yA2, veA+)*. The genetic background was also changed to allow the strains to use nitrate as a nitrogen source. The strains previously required the use of urea and sodium thiosulfate as a nitrogen source, which is less conducive to studying toxin production. DNA analysis was performed on the resulting progeny of the crosses to determine their genotypes and after choosing strains to study, toxin analysis was performed.

Materials and Methods

Fungal Crosses

The parental strains, taken from glycerol stocks, were grown on glucose minimal medium (GMM) containing the supplements required for each strain to grow. HogA and SskB require pyridoxine, vitamin B6, to grow and use urea and sodium thiosulfate as nitrogen sources. WIM126 requires para-aminobenzoic acid (PABA) to grow. Liquid GMM containing all the required supplements was prepared to inoculate the freshly obtained spores. Two milliliters of liquid media was put in each of eight test tubes per cross. For each fungal cross, two test tubes were inoculated with each parental strain and four test tubes were inoculated with both parental strains using sterile toothpicks. The test tubes were incubated at 37°C in a dark incubator for two days.

After two days, the mycelia growing on the surface of the liquid media was collected and washed in distilled water to remove any media sticking to it. The mycelia was chopped into small pieces and four small pieces of mycelia were placed onto solid GMM (2% glucose) plates. The plates were incubated at 37°C in the dark for four days. After four days, plates that exhibited

heterokaryon formation were wrapped with masking tape to encourage the formation of cleistothecia. The plates were incubated at 37°C for five more days. Four cleistothecia from each plate were collected. The cleistothecia were rolled on solid 5% agar plates to remove the Hulle cells stuck to their surface. The cleistothecia were then broken open in distilled water to release the ascospores inside. The resulting ascospore suspension was diluted and varying amounts of the suspension (50 µl, 75 µl, 100 µl, and 150 µl) were spread onto solid GMM plates containing all of the necessary supplements. The plates were incubated at 37°C dark.

Individual colonies were picked from the spread plates to create a master plate on solid GMM plus all supplements. From the master plate, a recombination test was performed. A replicator was used to replicate the master plate and transfer the colonies to selective media. The selective media used was GMM with no supplements, GMM with pyridoxine and PABA, GMM with just pyridoxine, and GMM with just PABA. The colonies that grew on the selective media were compared and auxotrophs and prototrophs were identified.

The goal of the fungal crosses is to produce progeny strains of the same background (*veA+* or *veA1*) that either have the kinase gene *(K+)* or do not have the kinase gene *(K-)*. Four genotypes of progeny are desired: *(veA+, K+), (veA+, K-), (veA1, K+),* and *(veA1, K-).*

Genomic DNA Extraction

Spores from each cross progeny were used to inoculate liquid GMM with 0.5% yeast extract in petri dishes. The yeast extract allows fungus to grow quickly. The plates were incubated at 37°C dark for one day. The mycelia was collected from each plate and as much liquid as possible was squeezed out of the mycelia using paper towels. A small piece of each

mycelia sample was placed into an eppendorf tube and placed in liquid nitrogen to flash freeze the sample. Once all of the samples were flash frozen in liquid nitrogen, they were placed in a lyophilizer bottle and frozen at -80°C for 30 minutes. The samples were lyophilized for 2 hours.

Following lyophilization, the samples were crushed into a fine powder and 0.6 ml of LETS extraction buffer was added immediately to the powder. Following the LETS buffer addition, 0.6 ml of 25:24:1 phenol:chloroform:isoamyl alcohol was added to the solution. The solution was vortexed until it became a homogenous milky white mixture. The mixture was centrifuged for 10 minutes at 15,000 rpm.

The resulting supernatant was transferred to a new eppendorf tube. A 1/10 volume of sodium acetate and 0.7 volumes of isopropanol was added to the supernatant. The solution was centrifuged at 15,000 rpm for 15 minutes resulting in the formation of a pellet. The supernatant was removed from the tube leaving the pellet behind, which was then washed with 500 µl of 70% ethanol. The mixture was centrifuged for 5 minutes at 15,000 rpm. The resulting supernatant was removed and the tubes were dried out. The pellet was re-suspended in 50 µl distilled water.

DNA Analysis

The DNA sample from each progeny strain was diluted (1:50) and the diluted samples were used to determine the genotype of each strain. Genotypes were determined by a series of polymerase chain reactions (PCR). PCR uses the enzyme DNA polymerase to direct DNA synthesis from deoxynucleotides on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3' end of a custom-designed oligonucleotide, a primer, which is annealed to a

longer DNA template. The primer, which is annealed to a single-stranded template that contains a region complementary to the primer, is used by DNA polymerase to elongate its 3' end to generate an extended region of double-stranded DNA (Watson, 2008).

Two primers, a forward and a reverse, are designed in order to amplify specific sequences of DNA. One is complementary to the 5' end of one strand of the DNA to be amplified and the other is complementary to the 3' end of the other strand. The DNA to be amplified is denatured and the primers are annealed to their target sequences. DNA polymerase and deoxynucleotide substrates are added to the reaction at this point and the enzyme extends the two primers. The reaction generates the double-stranded DNA over the region of interest on both of the strands of DNA from the template. The DNA is then subjected to repeated rounds of denaturation and DNA synthesis using the same primers producing a large quantity of double-stranded DNA (Watson, 2008).

Primers were designed for three different types of PCR reactions to determine the genotypes of the cross progeny (Figure 4). The primers for the first PCR reaction were designed to determine if the cross progeny were *veA+* or *veA1*. The strains that were *veA+* amplified and the strains that were *veA1* did not. WIM126 was used as a positive control. The primers for the second PCR reaction were designed separately for each kinase, although in each case, the strains that were Kinase+ *(K+)* amplified and the strains that were Kinase- *(K-)* did not. HogA was used as a positive control for the SskB progeny and SskB was used as a positive control for the HogA progeny. The primers for the third PCR reaction were designed to include a selectable marker, pyrG, from *Aspergillus fumigatus* (AfpyrG) if the kinase gene was deleted. In other words, if the strains are *K-*, they will amplify with the AfpyrG primers and be *AfpyrG+*. The kinases were used as positive controls for the AfpyrG PCR.

Following PCR, the samples are analyzed using gel electrophoresis. Electricity is used to separate DNA molecules by size as they migrate through a polyacrylamide gel matrix. The DNA migrates away from the negative end and toward the positive end because it is negatively charged. The heavier, larger DNA molecules do not migrate as far or as quickly as the smaller, lighter DNA molecules. The DNA bands are visualized by UV light. The UV light is absorbed by ethidium bromide, a compound used for detecting the presence of DNA because it intercalates between base pairs in the DNA double helix.

Toxin Analysis

Spores from cross strains (HogA x WIM126 #1 and #3/SskB x WIM126 #13 and #14) were inoculated on solid GMM with PABA and pyridoxine to obtain fresh spores. Spore suspensions were prepared and quantified. Top agar GMM (0.4% agar) with the correct supplements was prepared. The top agar was inoculated with spores from each strain and 5 ml of top agar containing spores was spread evenly onto 25 ml plates of GMM with supplements. The plates were incubated at 37°C under light and dark conditions for five days (Figures 5 and 6).

After five days, three 15mm cores were extracted from each plate. The cores were suspended in 5 ml of chloroform, vortexed, and allowed to sit for one hour. After one hour, the tubes containing the cores were vortexed again and 3 ml from the bottom of the tubes was extracted and moved to beakers. The chloroform was allowed to evaporate overnight and the toxin was concentrated in 500 µl of chloroform and moved to eppendorf tubes.

The concentrated toxin was analyzed for toxin production by thin layer chromatography (TLC). To run a TLC, the samples are loaded alongside a standard, in this case ST, on a silica gel

plate. The plate is placed into a solvent, benzene:glacial acetic acid in this experiment, in a closed chamber and the solvent is allowed to run up the plate by capillary action. The components of the sample separate on the plate and the plate is removed once the solvent nearly reaches the top of the plate. The plate is sprayed with aluminum chloride and put in an oven set at 80°C for ten minutes. The ST production can then be visualized under UV light.

Results

DNA Analysis

The results of DNA analysis (Figure 7) confirmed that three of the four desired genotypes were obtained by the HogA x WIM126 fungal crosses: *(veA+, hogA-), (veA1, hogA-),* and *(veA+, hogA+)* (Table 1). If a band was present on the gel, then the strain was positive for whichever gene was being amplified. Strains #1 and #3 were chosen for further study because they are of the same VeA genetic background *(veA+).*

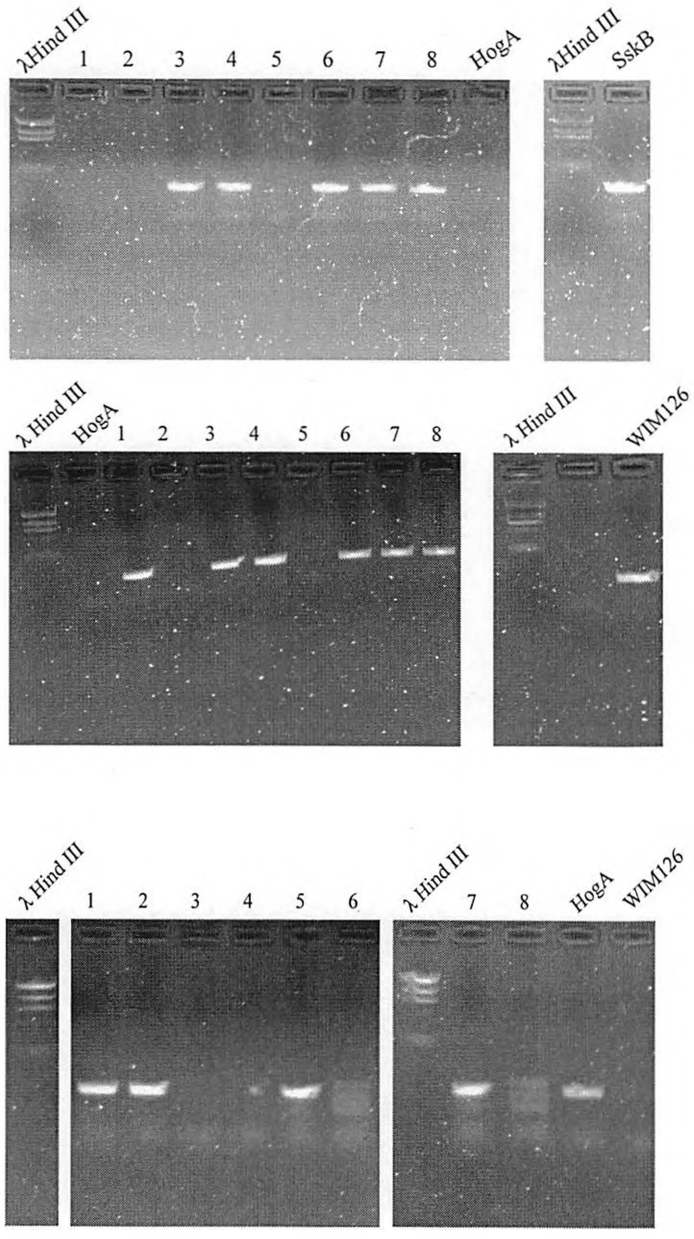
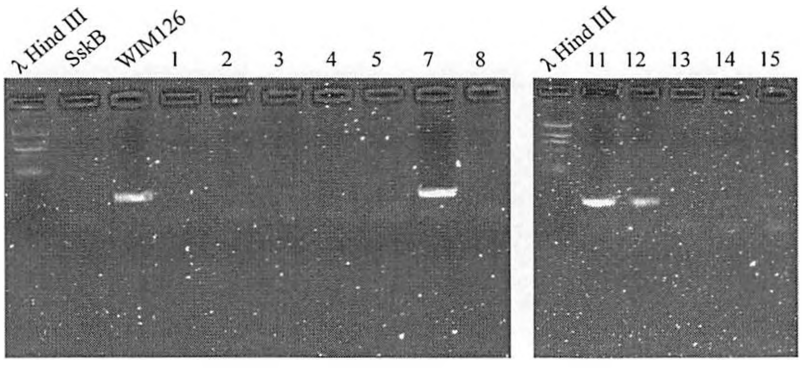


Figure 7. PCR results of HogA x WIM126 crosses: a) VeA+/VeA1, b) HogA+/HogA-, and c) AfpyrG+/AfpyrG-. Band presence indicates a positive result.

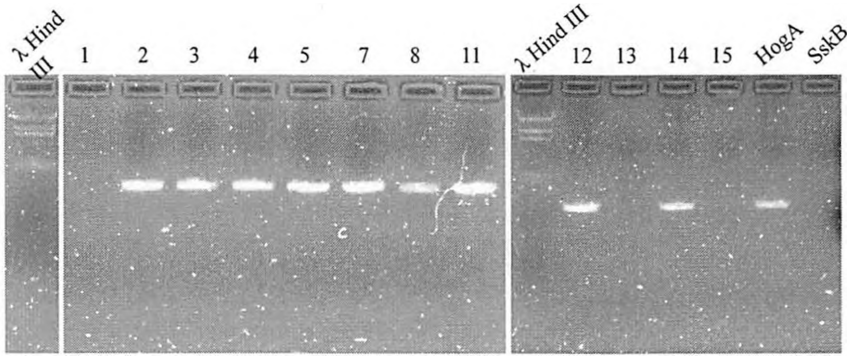
HogA x WIM126 Crosses	VeA+/VeA1	HogA+/HogA-	AfpyrG+/AfpyrG-	auxotrophy
1	VeA+	HogA-	AfpyrG+	pyro
2	VeA1	HogA-	AfpyrG+	pyro
3	VeA+	HogA+	AfpyrG-	pyro
4	VeA+	HogA+	AfpyrG-	pyro
5	VeA1	HogA-	AfpyrG+	paba/pyro
6	VeA+	HogA+	AfpyrG-	paba/pyro
8	VeA+	HogA+	AfpyrG-	paba/pyro

Table 1. Genotypes of HogA x WIM126 cross strains determined by PCR reactions.

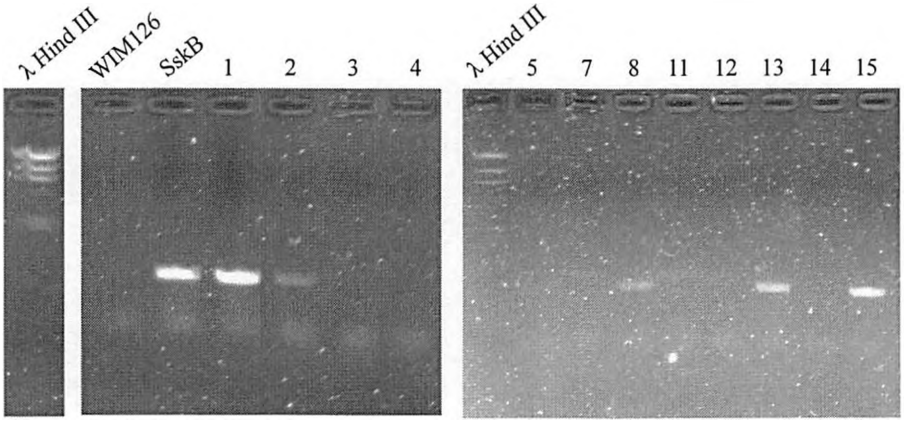
The results of DNA analysis for the SskB x WIM126 fungal crosses (Figure 8) showed that three of the four desired genotypes were obtained: *(veA1, sskB-), (veA1, sskB+),* and *(veA+, sskB+)* (Table 2). If a band was present on the gel, then the strain was positive for whichever gene was being amplified. Strains #13 and #14 were chosen for further study because they are of the same VeA genetic background *(veA1).*



(a)



(b)



(c)

Figure 8. PCR results of SskB x WIM126 crosses: a) VeA+/VeA1, b) SskB+/SskB-, and c) AfpyrG+/AfpyrG-. Band presence indicates a positive result.

SskB x WIM126 Crosses	VeA+/VeA1	SskB+/SskB-	AfpyrG+/AfpyrG-	auxotrophy
1	VeA1	SskB-	AfpyrG+	pyro
2	VeA1	SskB+	AfpyrG-	pyro
3	VeA1	SskB+	AfpyrG-	pyro
4	VeA1	SskB+	AfpyrG-	pyro
5	VeA1	SskB+	AfpyrG-	pyro
7	VeA+	SskB+	AfpyrG-	paba/pyro
11	VeA+	SskB+	AfpyrG-	paba/pyro
12	VeA+	SskB+	AfpyrG-	paba/pyro
13	VeA1	SskB-	AfpyrG+	paba/pyro
14	VeA1	SskB+	AfpyrG-	paba/pyro
15	VeA1	SskB-	AfpyrG+	paba/pyro

Table 2. Genotypes of SskB x WIM126 cross strains determined by PCR reactions.

Toxin Analysis

For the HogA x WIM126 crosses, strain #1 *(hogA-)* showed lower levels of ST production than strain #3 *(hogA+)* (Figure 8).

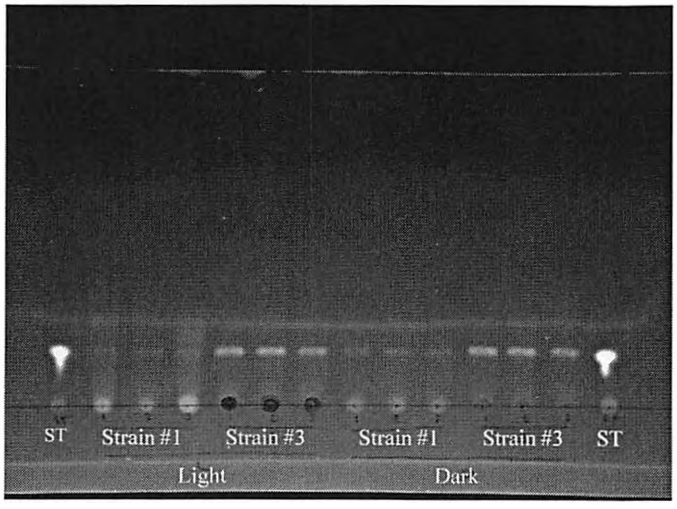


Figure 8. TLC plate showing ST production of HogA x WIM126 cross strains #1 and #3.

For the SskB x WIM126 crosses, strain #13 *(sskB-)* showed lower levels of ST production than strain #14 *(sskB+)* (Figure 9).

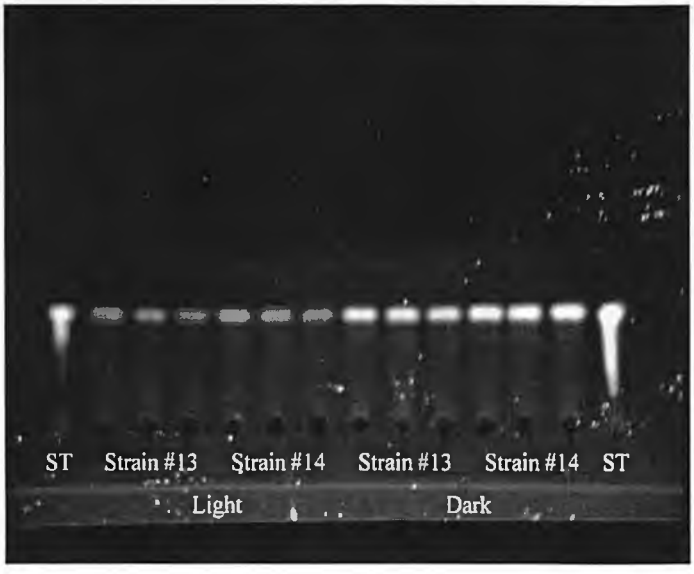


Figure 9. TLC plate showing ST production of SskB x WIM126 cross strains #13 and #14.

Conclusions and Future Work

The results of the toxin analysis are contradictory to the results that were expected. Initial toxin analysis indicated that the HogA and SskB kinases were possible repressors of ST production. The deletion mutants had elevated levels of ST production. Once crossed and in the same genetic background, it was expected that the kinase+ strains would have lower levels of ST production and the kinase- strains would have higher levels of ST production, but the opposite is seen on the TLC plates. In order to determine if this result is correct, we will focus on HogA and complementation will be carried out through transformation. The *hogA*- strain will be complemented to regain the *hogA* gene and toxin analysis will be repeated to observe ST production.

Appendix

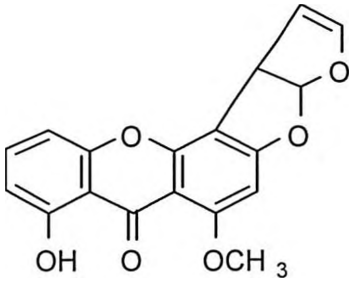
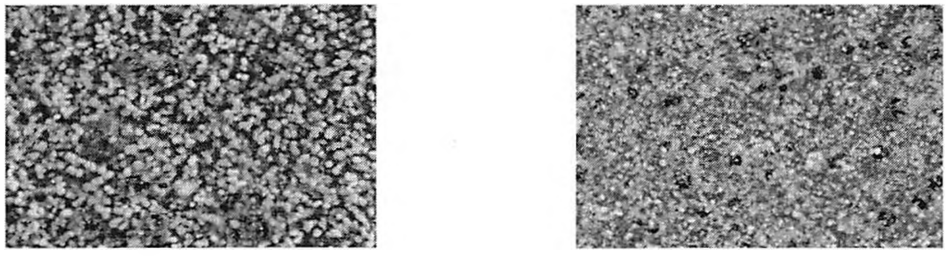


Figure 1. Chemical structure of the mycotoxin sterigmatocystin produced by *Aspergillus nidulans.*



(a) (b)

Figure 2. *Aspergillus nidulans* deletion mutant strains a) HogA and b) SskB.

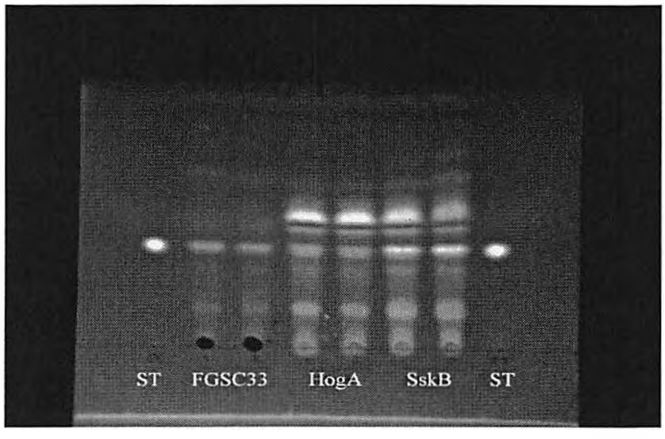


Figure 3. Thin Layer Chromatography plate showing elevated sterigmatocystin production by HogA and SskB deletion mutants.

Forward	5′- GACCCTCTTTTCTATCCT - 3′
Reverse	5′- AAGCTCTCGCGCGCTTG - 3′

(a)

Forward	5′- GGCGCGCCTATGGCGGAATTTGTACGTGCCC - 3′
Reverse	5′- GCGACTTGACGAACCGCAGAGTCT - 3′

(b)

Forward	5′- GGCGCGCCTATGGACCCTCAAGCGGTCCGC - 3′
Reverse	5′- GGATGCGCTGTTTCAAGCTCTTTATGG - 3′

(c)

Forward	5′- GATGTGACGACAACCCGAGAACTCC - 3′
Reverse	5′- GAGCAGCGTAGATGCCTCGAC - 3′

(d)

Figure 4. Primers designed for genotype determination of cross progeny: a) VeA+/VeA1, b) HogA+/HogA-, c) SskB+/SskB-, and d) AfpyrG+/AfpyrG-.

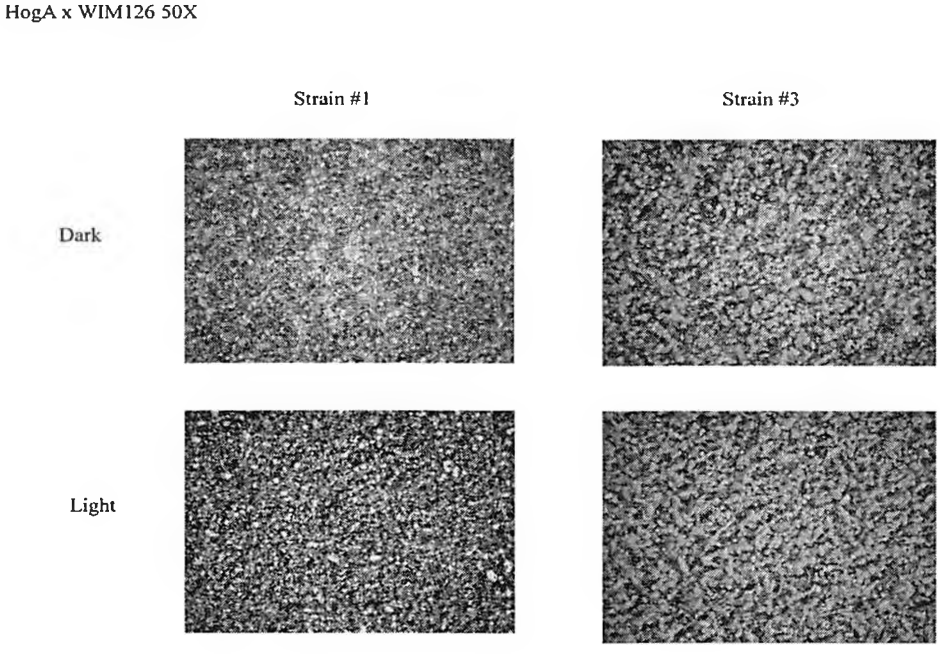


Figure 5. Top agar inoculated plates of HogA x WIM126 cross strains #1 and #3 grown under light and dark conditions at 37°C.

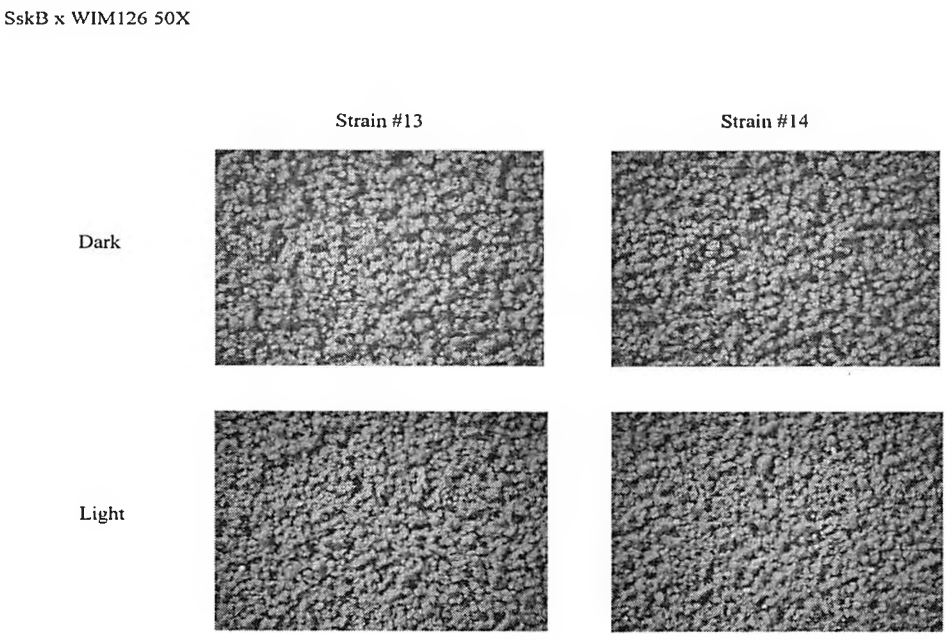


Figure 6. Top agar inoculated plates of SskB x WIM126 cross strains #13 and #14 grown under light and dark conditions at 37°C.

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