

## FUNGI AND MYCOTOXINS

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### Overall Research Goals

The long-term goal of my research program is to find new ways to control fungal infestation and mycotoxin production by understanding toxigenic fungi. Two major fungal genera, *Aspergillus* and *Fusarium*, are targets of our research.

There are four main questions we ask and try to answer:

- 1) How do fungi grow? What genetic elements function in fungal growth?
- 2) What controls fungal growth? How is fungal growth controlled/modulated?
- 3) How do fungi sporulate? What are the components of the sporulation-specific signaling pathway?
- 4) How is biosynthesis of various mycotoxins controlled? Are there global controlling mechanisms for both sporulation and mycotoxin production?

Our previous studies in correlation with the pivotal roles of signal transduction in cellular regulatory, communicatory, and responsive processes lead us to hypothesize that heterotrimeric G-protein signaling components and RGS proteins are upstream determinants in controlling global processes of growth and development in *A. nidulans*. To test our hypothesis we have focused on uncovering the roles of heterotrimeric G protein components and RGS proteins in major *Aspergillus* and *Fusarium* species.

### Research Progress and Future Plans

#### Research on *Aspergillus*

We have been investigating signaling mechanisms controlling fungal growth, sporulation and mycotoxin production in a model filamentous fungus, *Aspergillus nidulans*, because this organism offers many of the experimental advantages of its unicellular relative, the yeast *Saccharomyces cerevisiae*. In addition, *A. nidulans* is a mycelial fungus and differentiates complex

tissues during its lifecycle, including multicellular reproductive organs termed conidiophores that each produce thousands of mitotically derived spores. Moreover, this fungus produces the mycotoxin (toxic secondary metabolite) called sterigmatocystin (ST), which is a penultimate precursor of the better-known aflatoxin.

We have made tremendous progress on identification and characterization of critical G protein signaling components during the past year. We have identified seven G-protein-coupled receptors, one G gamma subunit, another G protein signaling controller called phosducin, two additional protein kinase A catalytic subunits, one protein kinase C, and, more importantly, three additional RGS (Regulator of G protein Signaling) proteins. In order to efficiently characterize functions of these genes we have developed a new and innovative technology called Double Joint PCR. This new technology can be used for any selective markers and any fungi. This versatile DJ-PCR technology will allow us to quickly study multiple genes in a short time.

For understanding sporulation processes, we have identified an important and crucial gene that functions as a negative regulator for the asexual sporulation process. This is a new concept because only a linear activation of asexual sporulation has been considered previously.

Experimental plans for this year are as follows:

- 1) We will functionally characterize G protein component genes in controlling growth, sporulation and mycotoxin production. Genetic studies of downstream growth signaling components (GRCs) will also be carried out.
- 2) With the newly identified three additional RGS proteins, there are four RGS proteins and three G $\alpha$  subunits in *A. nidulans*. Not much study has been carried out for fungal RGS proteins. We will determine the mechanisms of each RGS protein's activities.
- 3) Genetic and biochemical studies of asexual sporulation will be carried out. We have identified a key genetic element that responds to the FluG-dependent development-specific signaling. We will further characterize the roles of this gene and the FluG factor (positive regulator).

### **Research on *Fusarium* species, Pathogenic Fungi**

We have already identified an important RGS protein from three *Fusarium* species, *F. graminearum*, *F. sporotrichioides* and *F. verticillioides*. In addition, we also found EST sequences that identified 9 of our 15 target signaling components in *F. sporotrichioides*. Some of these components are extremely conserved (~ 100% identity) among *Fusarium* species, allowing us to functionally characterize some of these genes in other *Fusarium* species without further identification of their own target genes.

As shown as bold and underlined in Table 1, we have identified (or found) two G alpha and one G beta subunits, two RGS proteins, one probable receptor and three protein kinase A catalytic subunits. Particularly, we expect that RGS1, a homolog of *A. nidulans* FlbA, will function as a global controller of growth, sporulation and mycotoxin production in three major *Fusarium* species.

**Table 1. Summary of identification of G-protein components and RGS proteins in *F. sporotrichioides*.**

| Components               | Target gene        | Homolog in <i>A. nidulans</i> |
|--------------------------|--------------------|-------------------------------|
| Heterotrimeric G-protein | <b><u>GPA1</u></b> | FadA                          |
|                          | <i>GPA2</i>        | GanA                          |
|                          | <b><u>GPA3</u></b> | GanB                          |
|                          | <b><u>GPB1</u></b> | SfaD (G $\beta$ )             |
|                          | <i>GPG1</i>        | GpgA (G $\gamma$ )            |
| RGS Proteins             | <b><u>RGS1</u></b> | FlbA                          |
|                          | <i>RGS2</i>        | RgsA                          |
|                          | <i>RGS3</i>        | RgsB                          |
|                          | <b><u>RGS4</u></b> | RgsC                          |
| Receptors                | <b><u>GPR1</u></b> | GprA                          |
|                          | <i>GPR2</i>        | GprB                          |
| Protein Kinase A         | <i>PKR1</i>        | PkaR                          |
|                          | <b><u>PKA1</u></b> | PkaA                          |
|                          | <b><u>PKA2</u></b> | PkaB                          |
|                          | <b><u>PKA3</u></b> | PkaC                          |

In this year we will further characterize newly identified genes. In addition, we will identify the rest of the target genes by various approaches, including the yeast two-hybrid system.

The outcome of this research will help us elucidate the signals controlling mycelial growth and provide an understanding of how this pathway impacts sporulation and mycotoxin production in the major toxigenic fungal genera *Aspergillus* and *Fusarium*. Understanding signaling mechanisms will provide us with new tools for manipulating fungal cellular response, which will eventually help us to find new approaches to prevent fungal infection and mycotoxin production in crops.

## TRANSGENIC APPROACHES TO REDUCE FUNGAL INFECTION AND MYCOTOXIN PRODUCTION IN WHEAT

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### Introduction

Fungal pathogens cause significant reductions in yield and grain quality in wheat. In addition, some fungi produce mycotoxins, which can make the grain unfit for consumption. Fusarium head blight (FHB), caused mainly by the fungal pathogen *Fusarium graminearum*, has become a widespread and devastating disease in wheat over the past decade. Infection of wheat with FHB leads to yield loss, reduced germination in subsequent generations, and losses in grain quality. Reduced grain quality results from thin kernels, contamination of kernels with hyphal mass and production of mycotoxins in the grain by the invading fungus. The main mycotoxin produced by the FHB pathogen is deoxynivalenol (DON), generally referred to as vomitoxin. Mycotoxins produced by FHB present a major health hazard to humans and animals consuming infected grain or grain products. Large-scale research efforts have been undertaken both in the US and worldwide to develop systems to control FHB in wheat. One of the most promising areas of research is utilization of genetic engineering to study resistance mechanisms, increase resistance to FHB and to reduce mycotoxin production. Our research focuses on genetic engineering of wheat with antifungal and resistance pathway regulatory genes to gain insights on resistance mechanisms to FHB and to enhance resistance to the disease. We are also conducting research aimed at cloning tissue-specific promoters to be used in later genetic engineering efforts to employ targeted expression of resistance transgenes.

### Materials and Methods

**Transgene constructs:** First generation plasmid constructs contained the antifungal gene, hordothionin, or resistance cascade regulatory gene, NH1 (rice homologue of *Arabidopsis* NPR1), under the control of the constitutive, maize ubiquitin promoter and ubiquitin intron 1. Second generation constructs are being developed containing a pericarp-specific promoter (cloned by our group) driving the antifungal and resistance cascade regulatory genes. The selectable marker gene in the constructs was *bar* (which imparts resistance to the herbicidal compound bialaphos) driven by the maize ubiquitin promoter and intron 1. The screenable marker genes *Uida* (*gus*) and *gfp* were included in some constructs to follow and optimize transformation efficiency.

**Target tissues:** Several regenerable tissues were tested as targets for transgene delivery in wheat. Those tissues included embryogenic callus

derived from mature wheat embryos, embryogenic callus derived from immature (10–14-day-old) embryos, and direct delivery of transgene DNA into scutellar tissue of immature embryos. Experiments were conducted to optimize initiation, maintenance, and regeneration of callus cultures from each of the tissues (Mendoza and Kaeppler, 2001). This was necessary to achieve acceptable transformation efficiencies.

**Transgene delivery:** Transgene constructs were coated onto gold microparticles, and the microparticles delivered into target tissues using the “gene gun” (PDS1000/He, Biorad Inc.). Standard bombardment parameters were used. Bombarded tissues were placed on selection medium (containing bialophos) following a one-week rest period on culture maintenance medium.

**Selection and regeneration of transgenic cell lines:** Bombarded tissues were grown on selection medium, with biweekly subculturing to fresh medium, for approximately 12 weeks. Putative transgenic sectors were then placed on plant regeneration medium to produce shoots. When shoots reached 1–2 cm in size, they were placed on rooting medium. Following root development, plantlets were transferred to soil in pots and grown in the greenhouse to maturity.

#### **Research Progress and Plans:**

Transgene constructs were assembled during the first year of funding, sequenced and tested in transient assays for expression. Constructs exhibiting high transient expression levels were used in later stable transformation experiments. Tissue culture optimization experiments were also carried out during the first and second year of funding and were described in previous progress reports. Optimized culture protocols were developed (Mendoza and Kaeppler, 2002) and used as part of the transformation procedure.

To date, plants were regenerated from 32 independent transgenic cell lines. Preliminary molecular genetic analysis indicates transgene integration into the wheat genome. Transgenic (T0) wheat plants from 10 events were fertile and produced seeds. Plants from 3 of the events contained the hordothionin gene. Plants from the remaining 7 fertile lines contained the NH1 gene. T1 seed arising from self-pollination of the 10 transgenic wheat lines was planted in the greenhouse recently to advance lines to the T2 and T3 generations.

Molecular genetic assays will be performed on T2 and T3 plants to demonstrate transmission of the transgenes to progeny for confirmation of stable integration. Advancement of transgenic lines to later generations is also necessary to produce plants homozygous for the transgenes. The homozygous lines and the related sister “null” lines (controls) will then be subjected to transgene expression and pathogen resistance assays.

*PUBLICATIONS:*

Mendoza, M.G., Kaepler, H.F. 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). *In Vitro Cell. Devel. Biol.* 38(1):39–45 (2002).

Skadsen, R.W., Puthigae, S., Federico, M.L., Kaepler, H.F. 2002. Cloning of the promoter for a novel barley gene, *LEM1*, and its organ-specific promotion of gfp expression in lemma and palea. *Plant Mol. Biol.* (in press).