

# Project No: 6054-42000-025-00D

## Use of Classical and Molecular Technologies for Developing Aflatoxin Resistance in Crops

---

### SY Team

**K Rajasekaran**, Research Biologist, Lead Scientist

J Cary, Molecular Biologist

M Gilbert, Biochemist

vacant, Plant Pathologist (vice Brown)

MSU collaborators (Burger, Yao, Hruska)

Raj Majumdar, Postdoc

### Collaborators

Niranjan Baisakh, LSU

Zhi-Yuan Chen, LSU

Jesse Jaynes, Tuskegee U / Nexion LLC

Pooja Bhatnagar-Mathur, ICRISAT, India

Abebe Menkir, IITA, Nigeria

Ron Saylor, Uark (Retired)

Ahmad Fakhoury, SIU

Caryl Chlan, ULL (Retired)

# THREE LINES of DEFENSE APPROACH

1. Prevent the toxigenic fungus from reaching the crop (Conventional Farming Practices and Study of Fungal Ecology, Biological Control)
2. Understand the fungus if it does reach the crop (how and why the fungus makes the toxin and how to prevent it, regulation of fungal development and aflatoxin biosynthesis, -OMICs)
- 3. Prevent the fungus from invading the crop and producing aflatoxin (by altering physiology/genetics of susceptible crops, enhancing Host Plant Resistance)**

# Project Objectives

**Objective 1.** Develop aflatoxin-resistant corn with enhanced resistance traits against other mycotoxins and drought tolerance. Identify gene regulatory factors, networks and pathways related to resistance-associated proteins (RAPs). These data are then transferred to others to assist in selection by marker-assisted breeding.

**Objective 2.** Identify resistance associated protein (RAPs) genes from corn and cotton using transcriptomic analyses of the *Aspergillus flavus*-host plant interaction and evaluate for control of fungal growth and aflatoxin contamination.

**Objective 3.** Develop and evaluate transgenic corn and cotton containing over-expressed identified RAP genes (Objectives 1 and 2) or with RNA interference (RNAi)-based silencing of *Aspergillus flavus* genes critical to growth and aflatoxin production.

**Objective 4.** Advance and license the rapid, non-destructive hyperspectral imaging technology; develop and evaluate instruments suitable for different user platforms.

# ENHANCING HOST PLANT RESISTANCE

1. **Conventional Breeding** to improve resistance to aflatoxin contamination in cotton and corn
2. **Expression of antifungal/antimicrobial genes**, natural and synthetic, into cotton and corn for resistance to *Aspergillus flavus* and aflatoxin contamination
3. **Silencing of key *A. flavus* genes** vital for its growth, infection, and toxin production

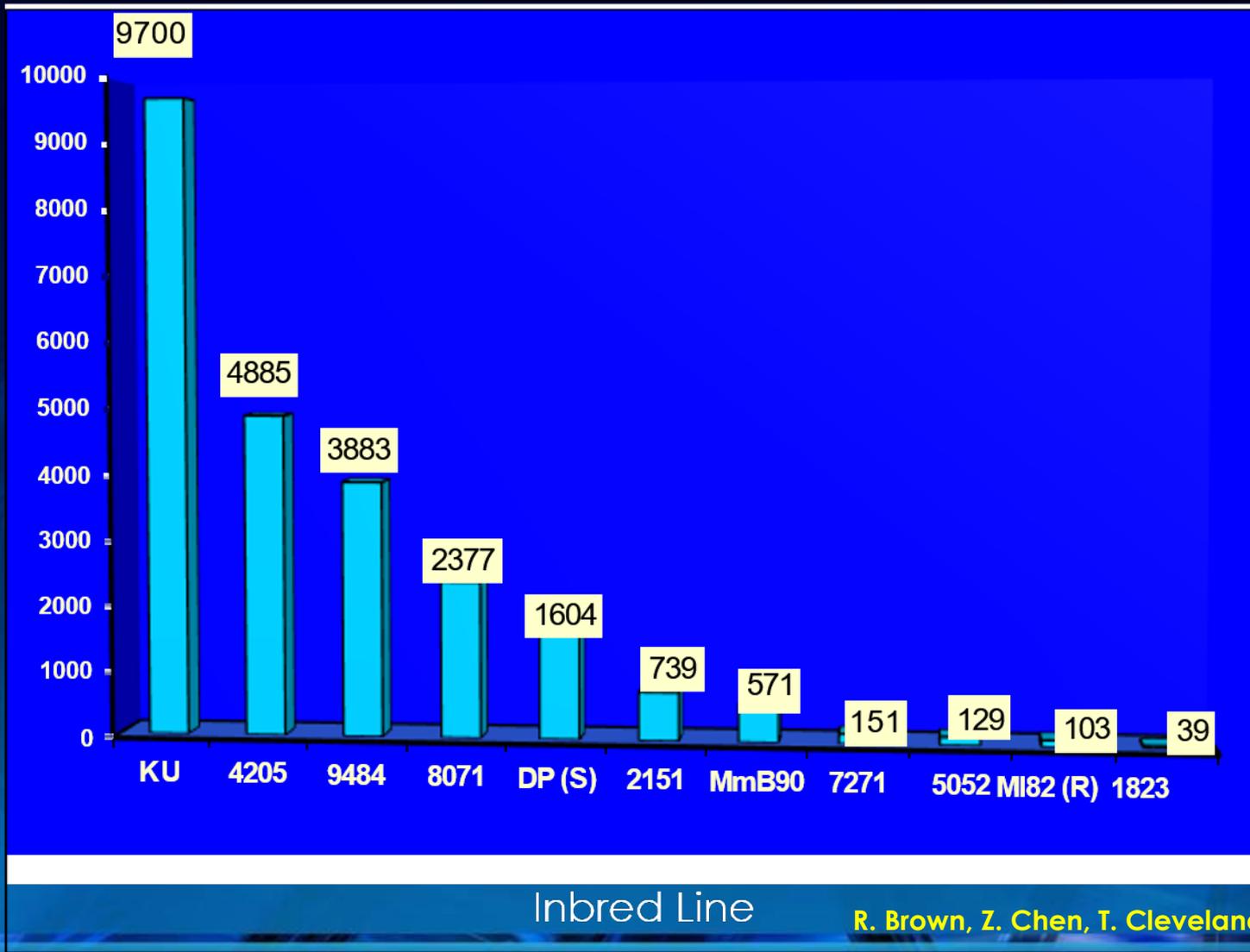
# Breeding for Resistance in Corn



*Aspergillus flavus* mycelial growth and sporulation on infected corn kernel

# Aflatoxin Levels in Representative Corn Inbred Lines from West Africa (IITA) Inoculated with *A. flavus*

Aflatoxin B<sub>1</sub> (ppb)



R. Brown, Z. Chen, T. Cleveland, A. Menkir

# Development of Resistant Maize Lines

IITA Lines crossed with US Lines

↓  
6 generations  
(agronomic characteristics)

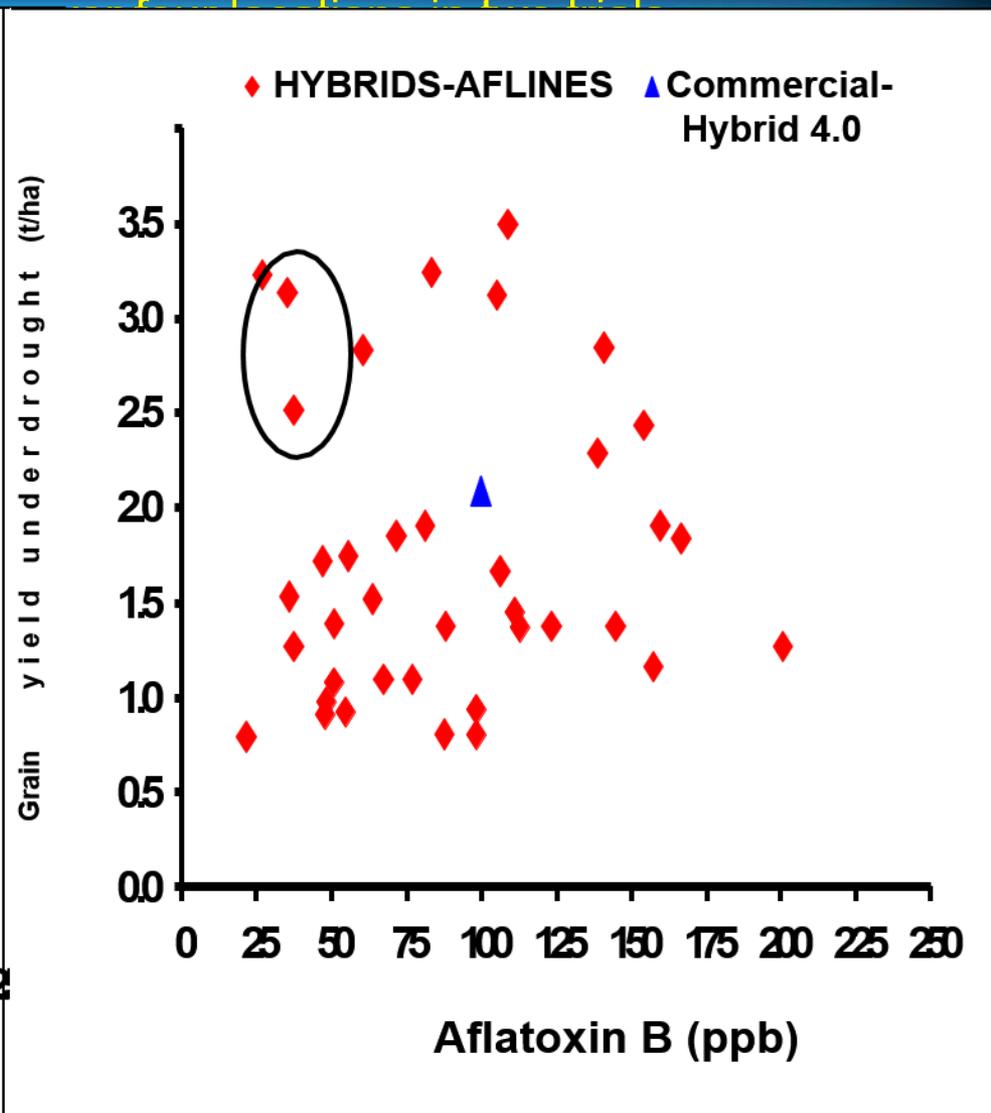
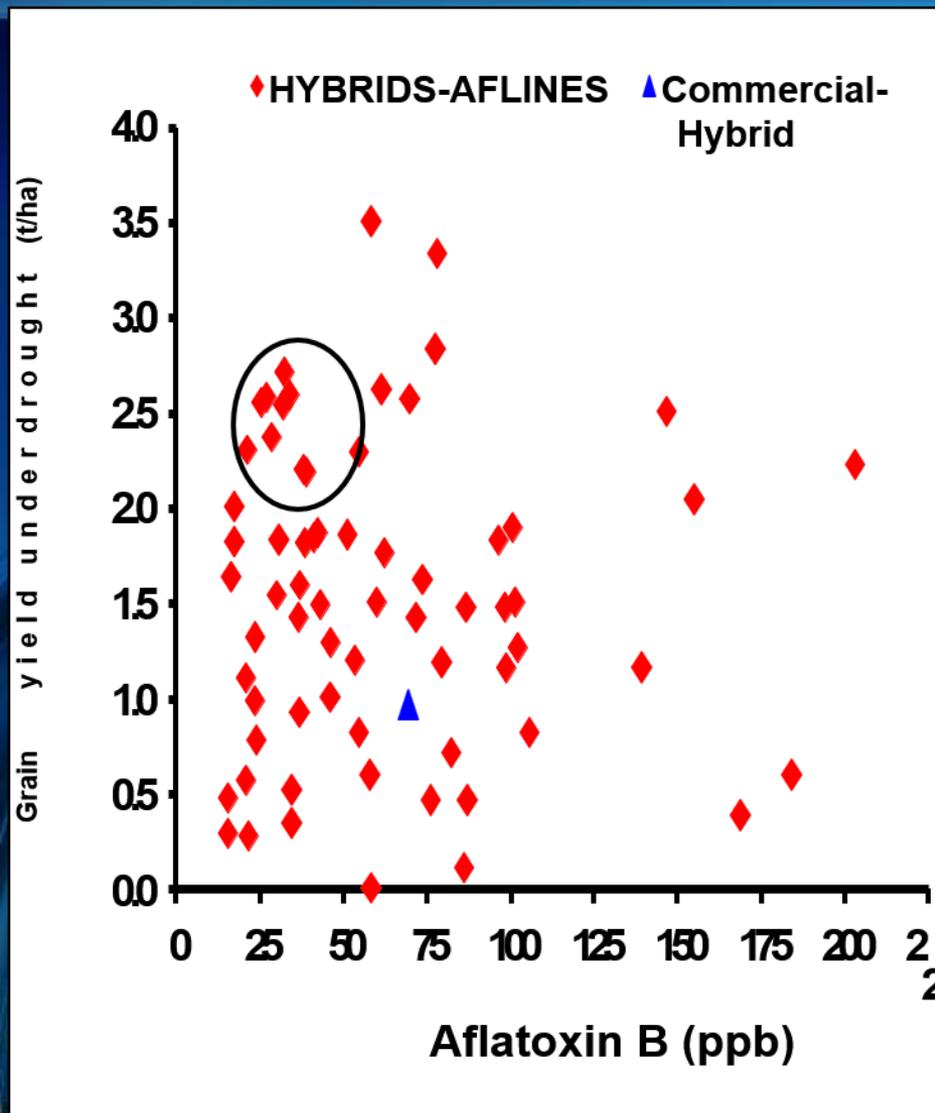
↓  
10 generations

Release of six resistance lines  
**(TZAR 101-106) – GRIN**

SERAT2 Field trials at Starkville, College Station, & Lubbock

# Product Development: Breeding for resistance to aflatoxin accumulation

Mean aflatoxin values of hybrids formed from aflatoxin resistant lines with some levels



# Combined resistance to aflatoxin and fumonisin production

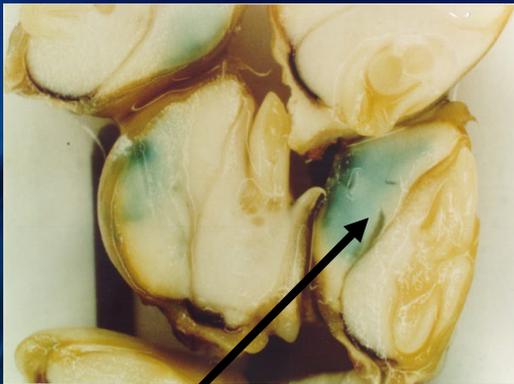
Screen maize inbred lines with low fumonisin values for resistance to aflatoxin relating new genetic variation

Lines	Fumonisin (ppm)	Aflatoxin-SRRC- USDA-KSA (ppb)		Aflatoxin-IITA- KSA (ppb)
	Average of three environments	First run	Second run	
TZIFRL01	1.4	345	440	4104
TZIFRL05	2.4	352	89	2917
TZIFRL04	2.6	864	29	8021
TZIFRL02	4.4	149	507	6514
TZIFRL03	4.5	202	51	5086
<b>TZIFRL06</b>	<b>60.7</b>	-	<b>3228</b>	<b>10208</b>
<b>MI82 (Resistant)</b>	-	<b>325</b>	<b>404</b>	-
<b>P3142 or 9071 (Susc.)</b>	-	<b>2496</b>	<b>1531</b>	<b>19375</b>
MEAN	19.2	2235	381	8520
LSD (0.05)	19.5	952	197	6371
CV (%)	126	141	164	53

# CORN PROTEOMICS (SRRC/LSU/Mississippi State)

- Discovery of corn lines “naturally” resistant to *Aspergillus flavus* invasion
- Identification of resistance factors/markers for breeding through natural product chemistry and proteomics
- Use of resistance factors/markers in breeding for enhanced resistance to aflatoxin contamination

Susceptible

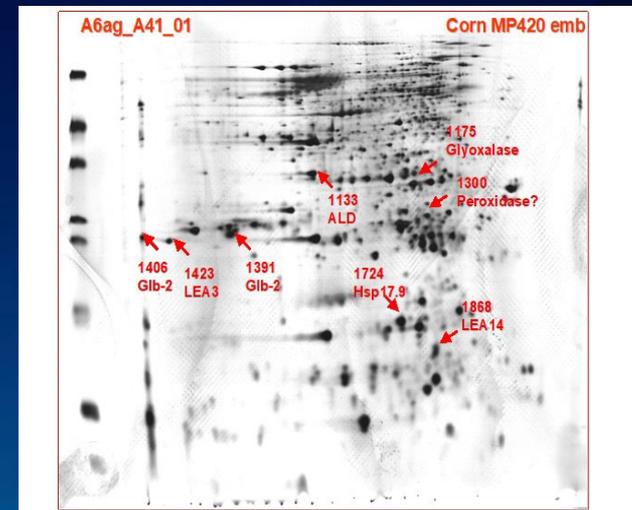


Blue color indicates presence of fungal (*A. flavus* GUS transformant) infection of the seed

Resistant



Blue color absent



The mechanism of this resistance is being studied through use of proteomic (and other biochemical) comparisons of “resistant” and susceptible” corn lines.

Proteomic analyses of new hybrids in progress

# Resistance Associated Proteins Identified

## Antifungal

Zeamatin

\**Trypsin/Amylase inhibitor 14 kDa (TI)*

\**PR-10*

\**PRms*

\**ZmWRKY* Transcription Factors

\* *$\beta$ -1,3-glucanase (PR-2)*

Ribosome inactivating protein (RIP)

TI-10 (10 kDa)

## Drought/Dessication-related

Water stress inducible (WSI)

Globulin I

Globulin II

Late embryogenesis abundant protein (LEA III)

LEA 14

## Oxidative Stress

Peroxiredoxin 1 (Per1)

Anionic peroxidase

## Heat Stress

Heat shock protein

## Osmostress Related

Glyoxylase (GLX I)

Aldose reductase (ALD)

## Regulatory (resistance)?

Serine Kinase

*\*Cloned, expressed, used in bioassays*

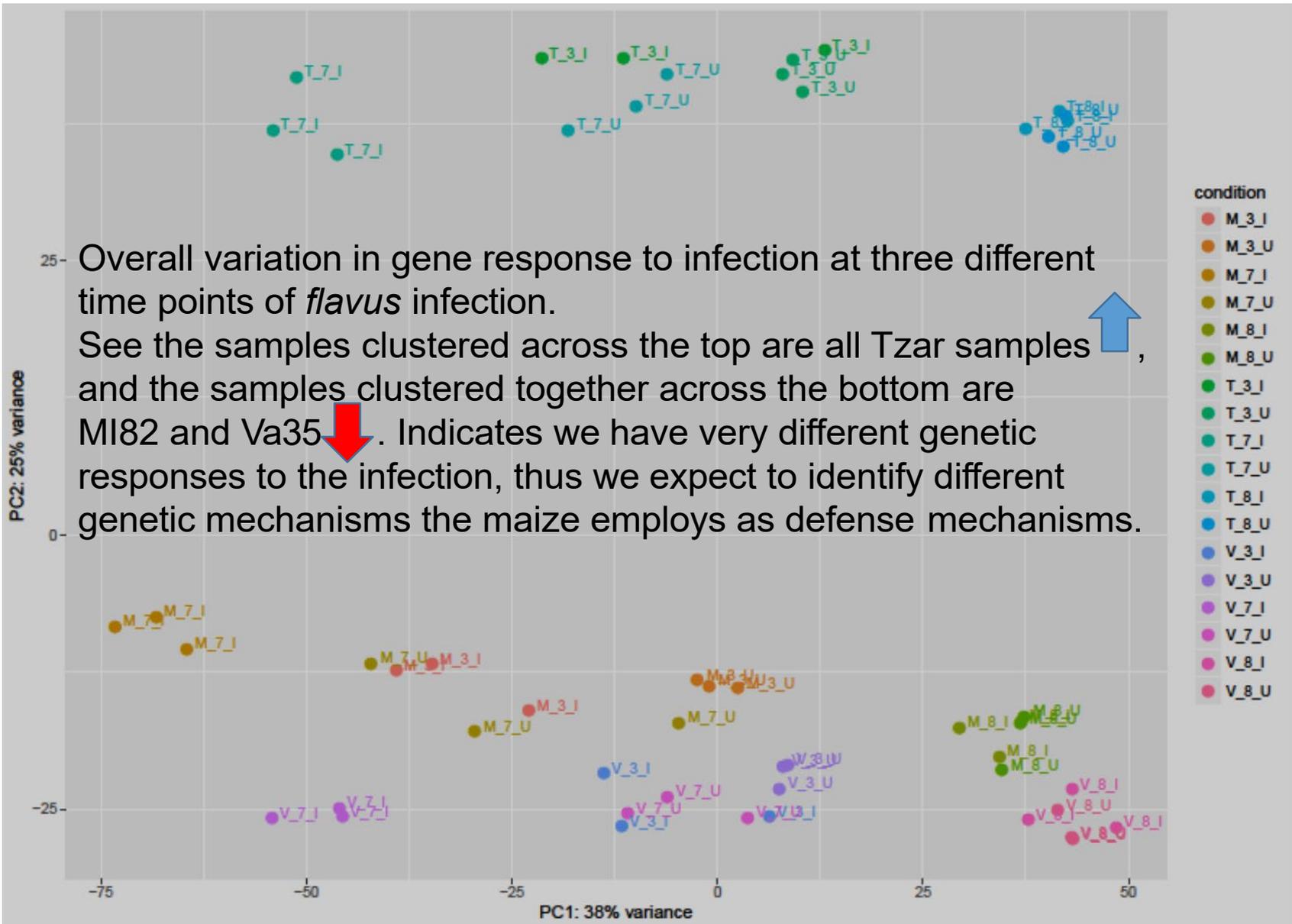
# Identification of *flavus*-resistance associated genes

## Completed transcriptomic analysis of cottonseed-*flavus* interaction

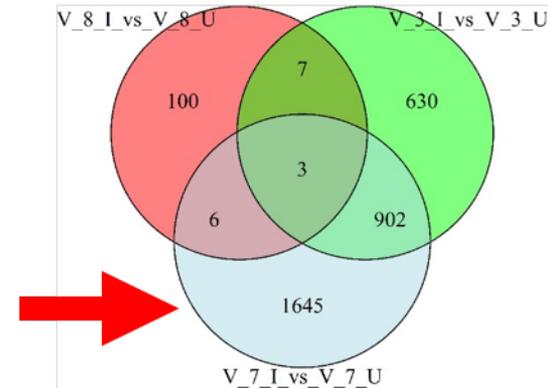
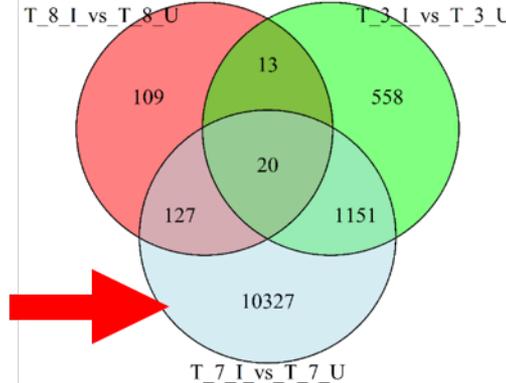
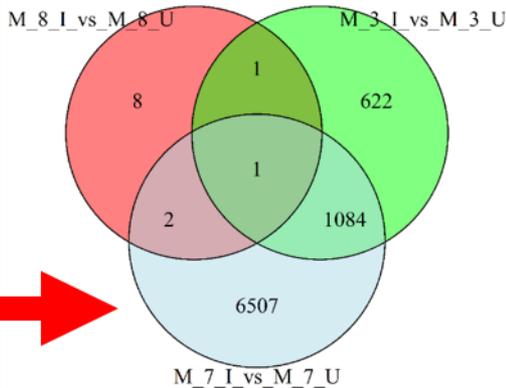
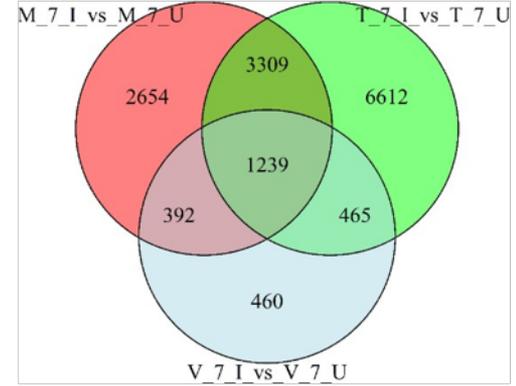
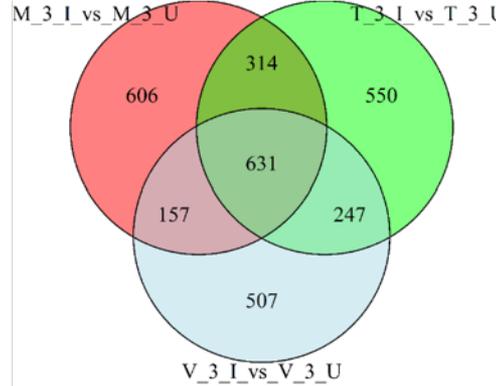
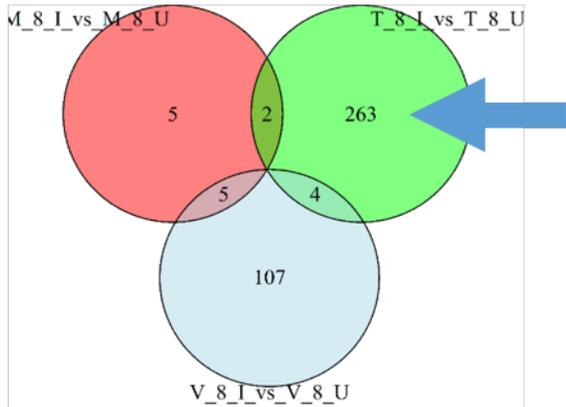
1. Identified candidate *Aspergillus flavus* resistance-associated genes from cotton transcriptome profiling and comparative transcriptome analysis with peanut and corn.
2. Transformation vectors were constructed with some genes that are being evaluated in transgenic cotton (spot11 catalase).
3. Isolated stress-inducible promoters and genes from an extremophile for their translational use in cotton or maize.

# Preliminary RNAseq analysis of *corn-flavus* interaction

## Principal Component Analysis of Gene Expression



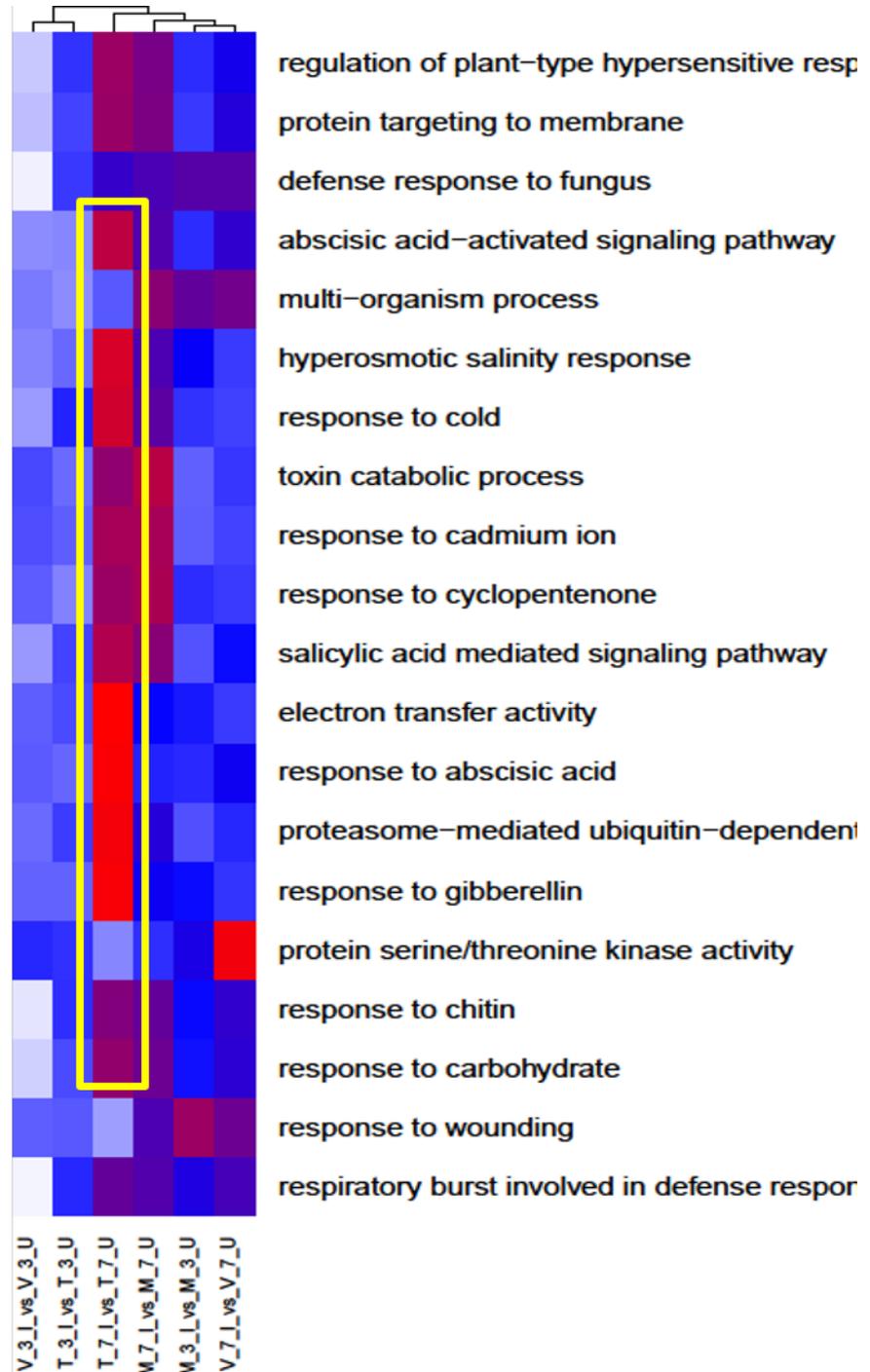
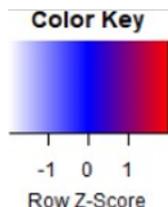
# Venn Diagrams of Differentially Expressed Genes



**M – MI82    T – TZAR102    V – Va35**

8h, 3d, and 7d samples

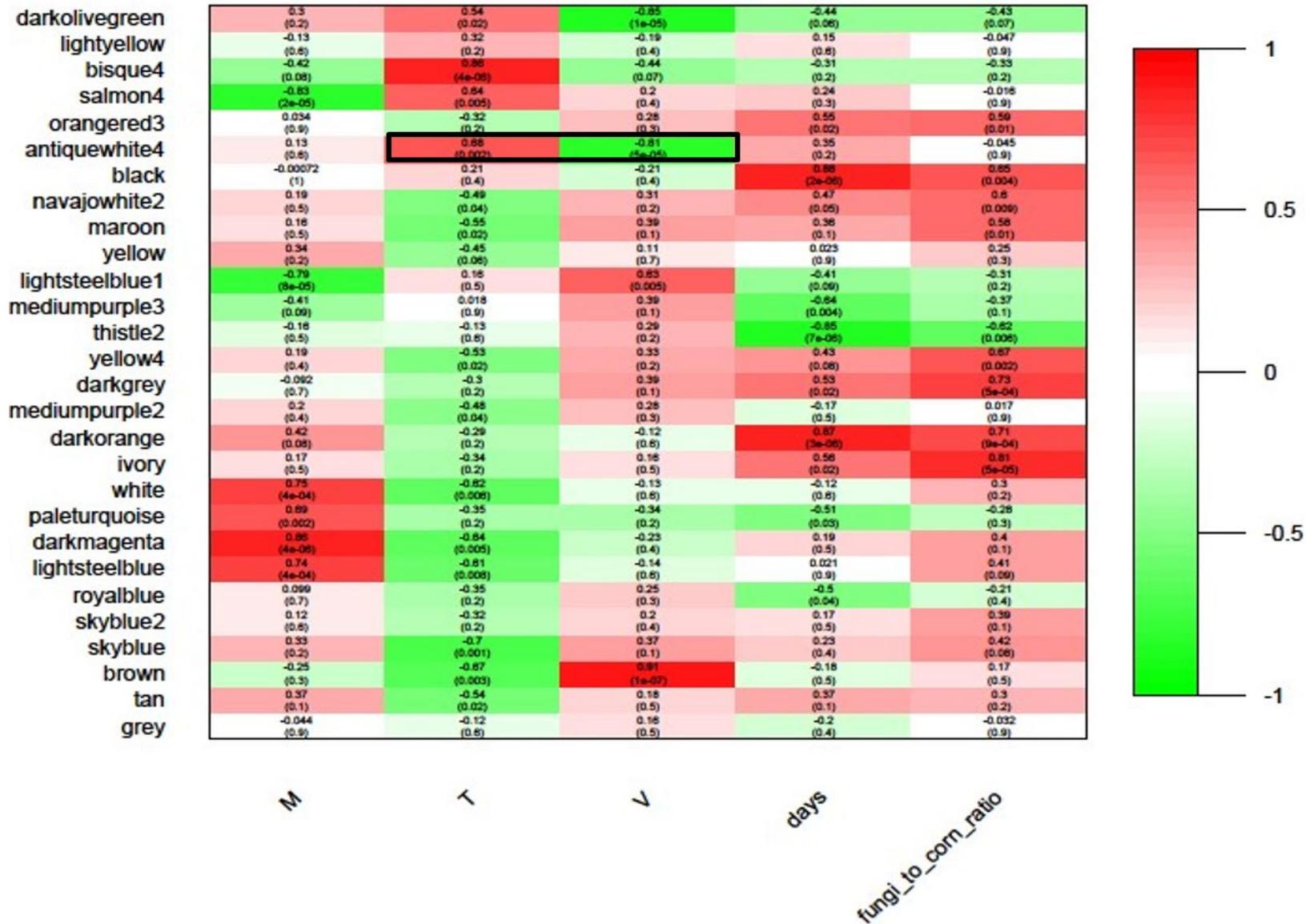
Heat map of overrepresented GO categories of upregulated genes showing  $-\log p$ -value



# Co-expression Network Analysis using WGCNA

## Module-trait relationships

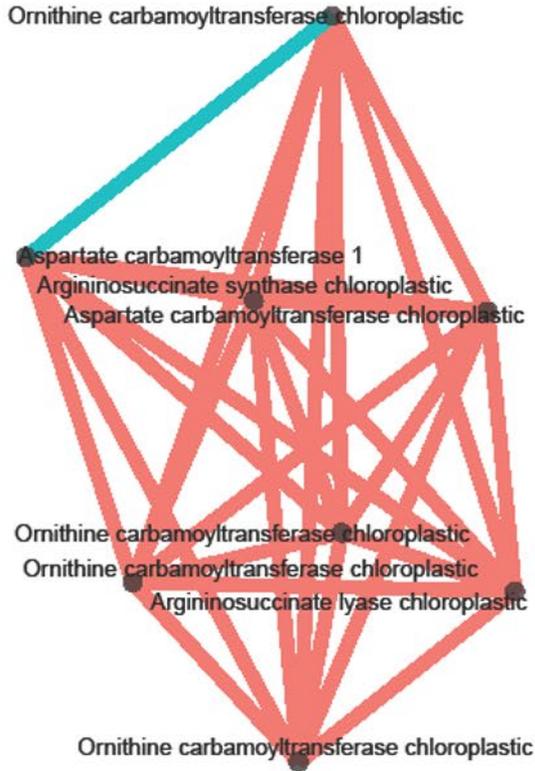
Gene Family Modules



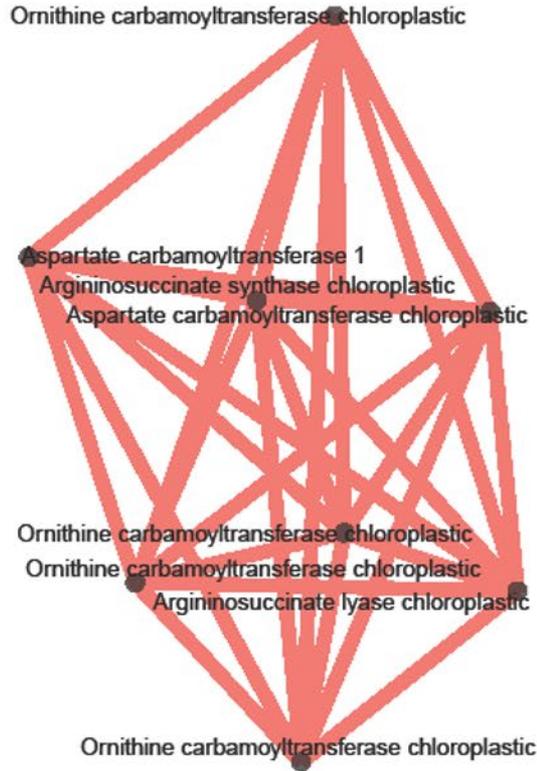
# Differential Correlation Analysis of Reactome Pathways

## Canavanine biosynthesis

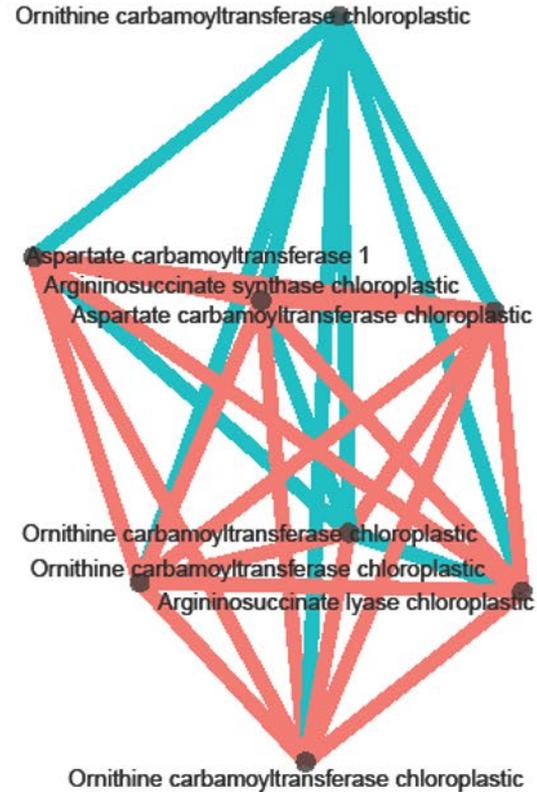
M



T



V



— weight > 0  
— weight < 0

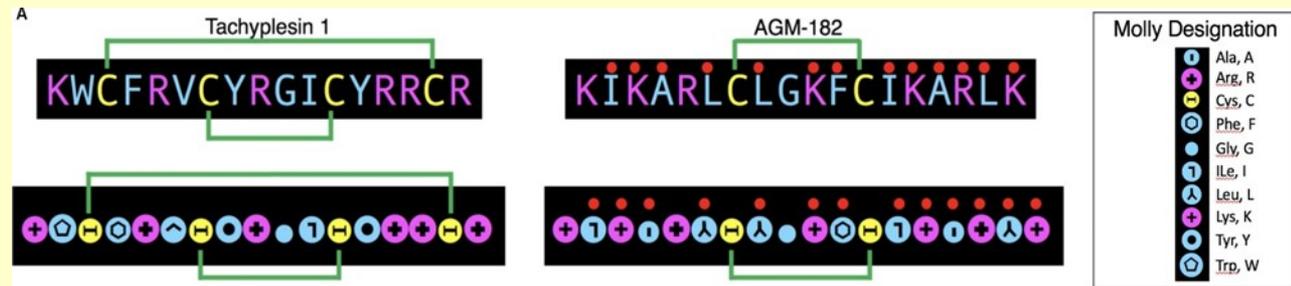
# Transformation of Maize

with the synthetic peptide gene  
AGM182

In-house Maize Transformation and Regeneration



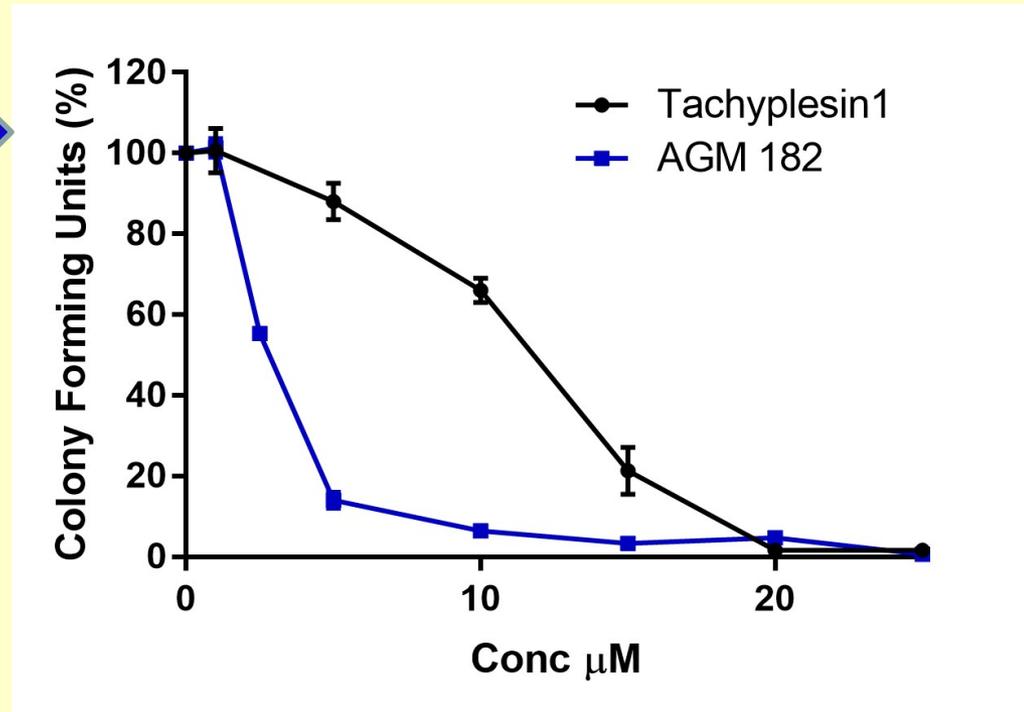
# AGM182



- Synthetic, linear, amphipathic,  $\beta$ -sheet; lytic
- ~90 bp product; 18 amino acid peptide
- Effective *in vitro* and *in planta* against several microbial pathogens including *A. flavus*
- Fairly resistant to protease activity
- non-toxic, non-hemolytic

# Designed Peptide AGM 182

- 5x Effective at lower concentrations than native tachyplesin on *A. flavus* →
- Killed germinated spores of *Fusarium verticillioides* and *Verticillium dahliae* at 5-10  $\mu\text{M}$  concentrations
- 18 amino acids – 5 lysine
- Potential to improve protein nutrition of kernel





**AGM182 Transgenic maize**

# Reduced *flavus* growth in AGM182 transgenic kernels

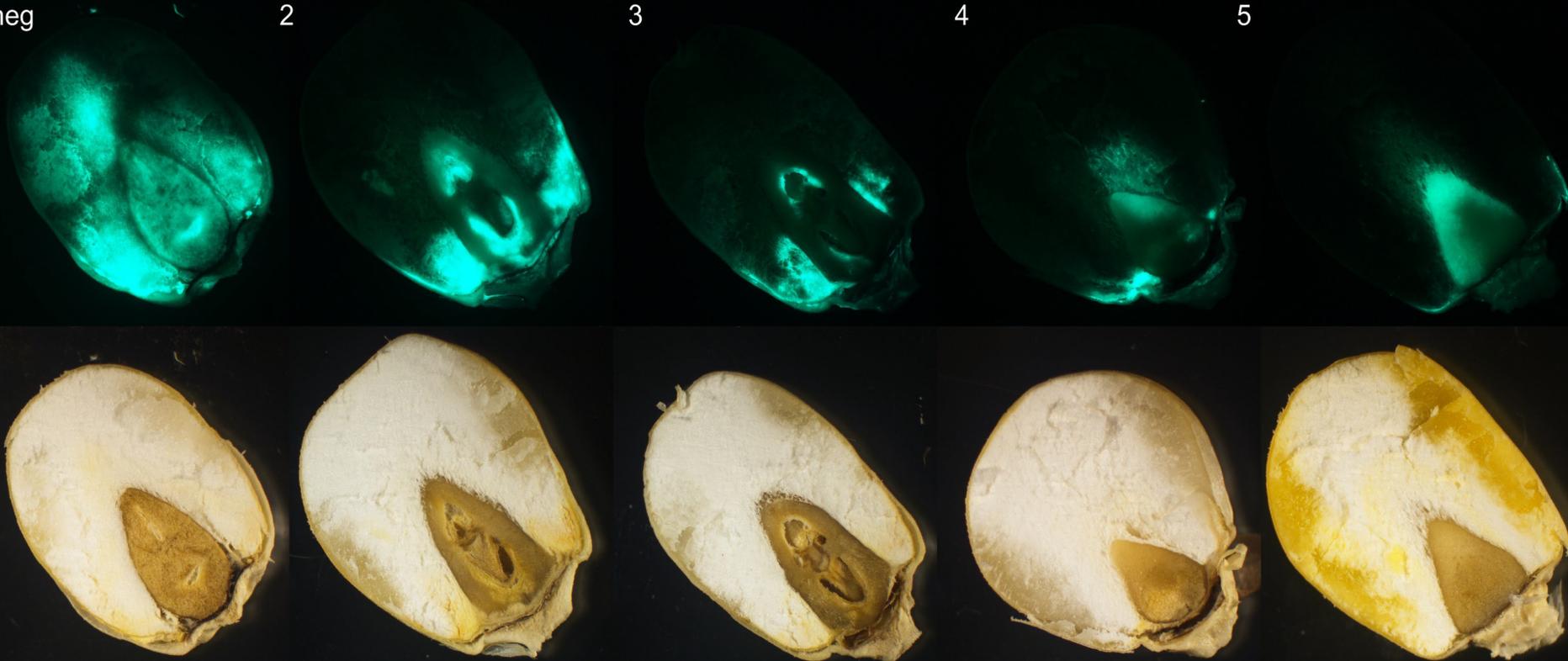
neg

2

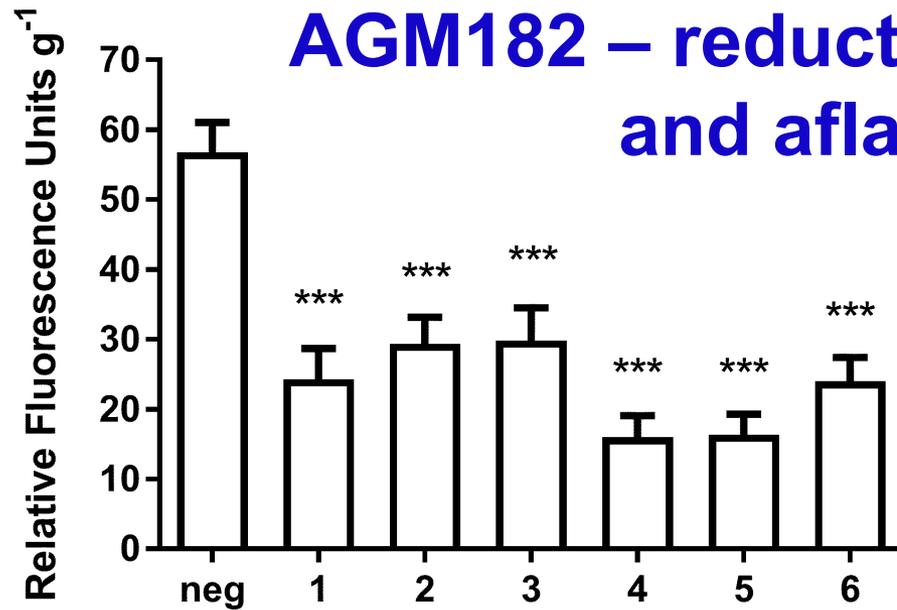
3

4

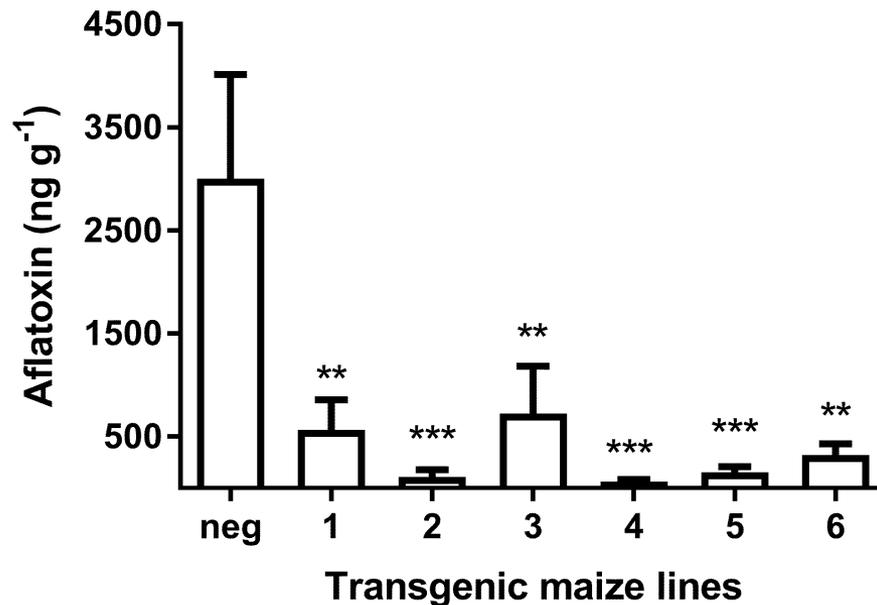
5



# AGM182 – reduction in fungal growth and aflatoxin levels



*A. flavus* growth



Aflatoxin production

# CRADA

**with Nexion LLC**

**To produce antifungal maize lines and to improve corn diet for poultry and swine**

**From FY19 for 3-4 years**

**A postdoc will be hired soon**

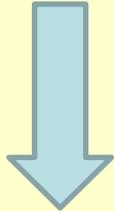
# Transformation of Maize

with an  $\alpha$ -amylase inhibitor from  
*Lablab purpureus* L.  
(hyacinth bean)

# *Aspergillus flavus* $\alpha$ -amylase

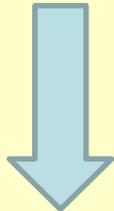


**Starch**



$\alpha$ -amylase  
*A. flavus*

**Glucose**



**Aflatoxin**

**Starch**



**Glucose**

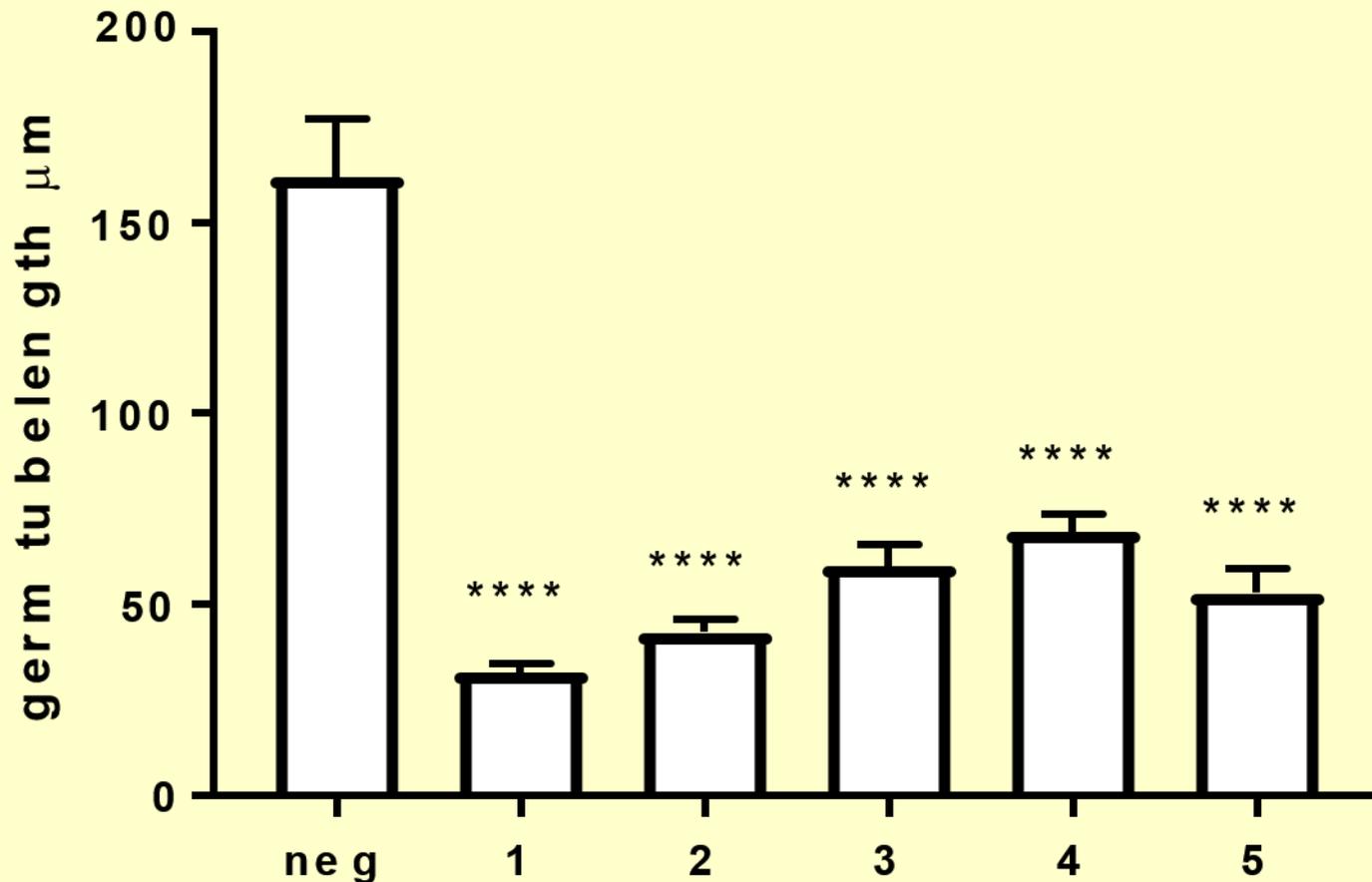


**No aflatoxin**

- $\alpha$ -amylase is an enzyme necessary for the breakdown of starch into glucose
- Mutant *A. flavus* strain lacking  $\alpha$ -amylase cannot infect maize kernels or produce aflatoxin

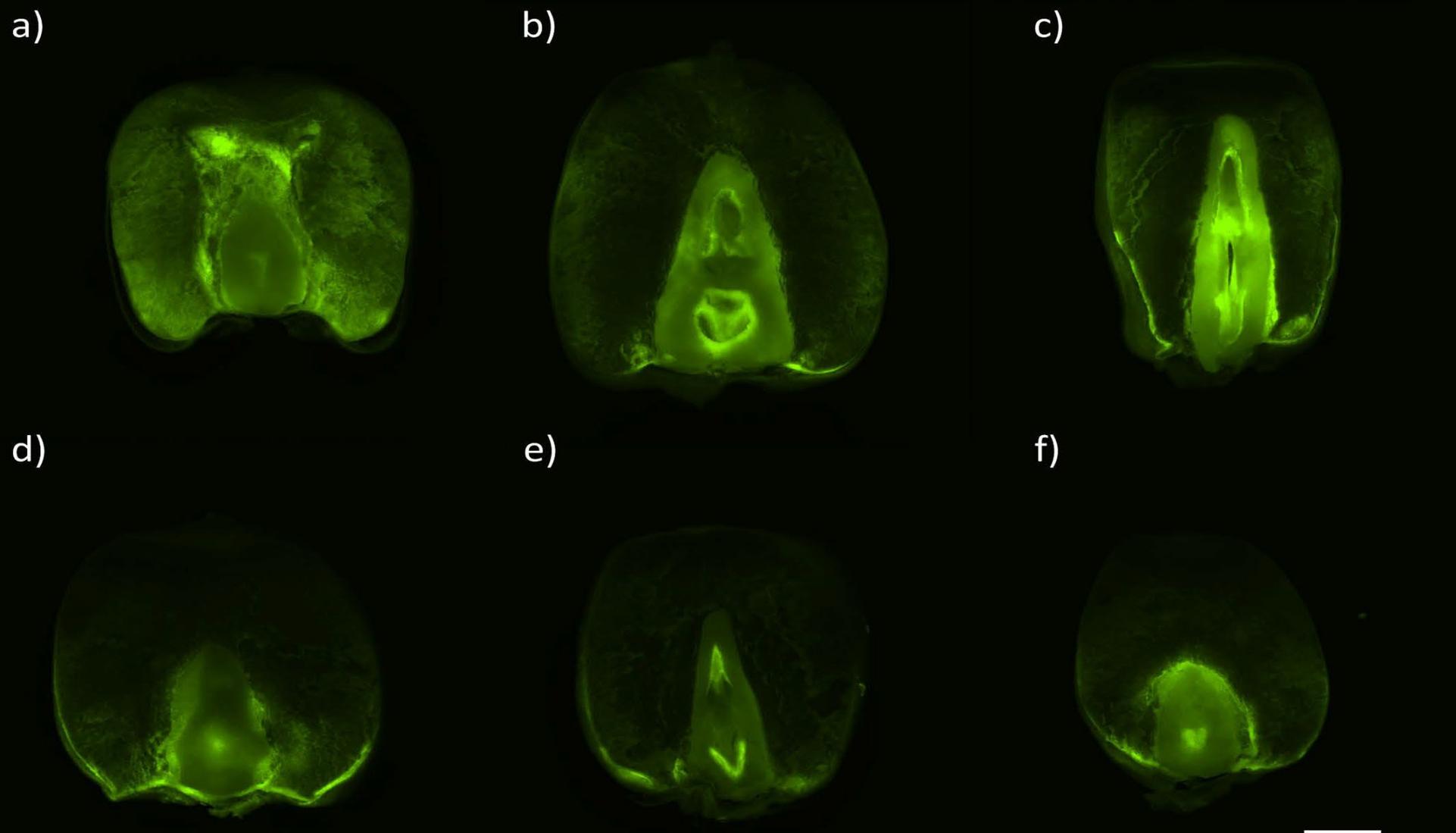
# Transgenic Corn Expressing the $\alpha$ -Amylase Inhibitor (AIP)

Effect of crude leaf extracts from transgenic AIP maize plants on *Aspergillus flavus* spore germination as compared with an isogenic negative control

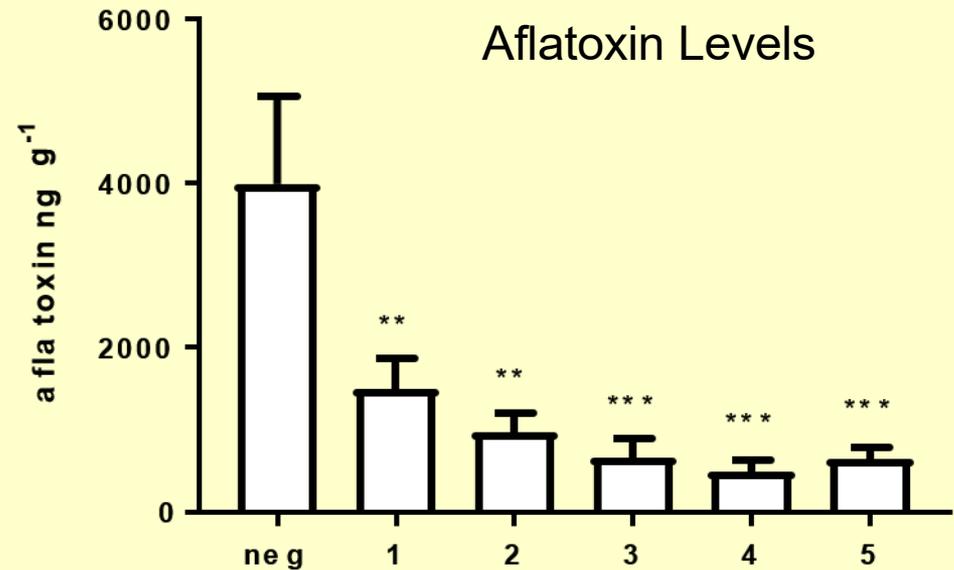
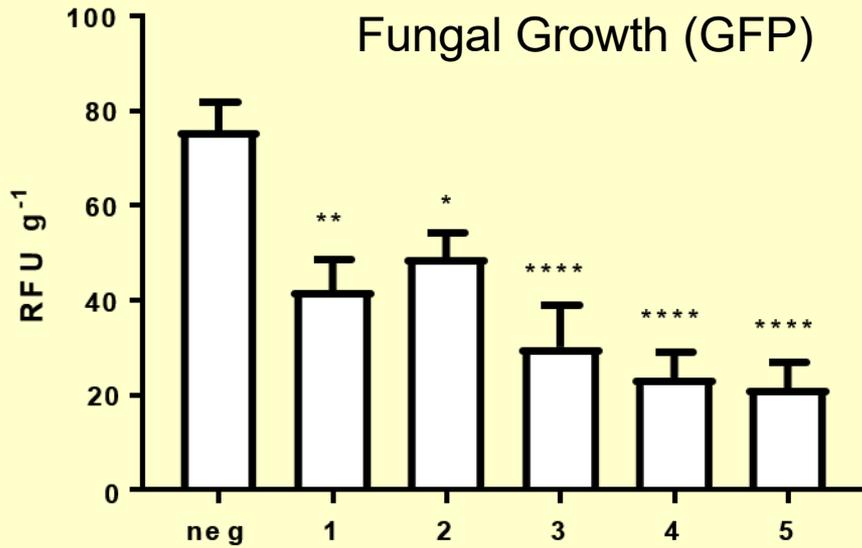


# Transgenic Corn Expressing the $\alpha$ -Amylase Inhibitor (AALP)

## Fungal Growth (=GFP)



# Transgenic Corn with Hyacinth Bean $\alpha$ -Amylase Inhibitor (AILP)



# Evaluation of Native Genes in Maize in Aflatoxin Resistance by RNAi-mediated Gene Silencing

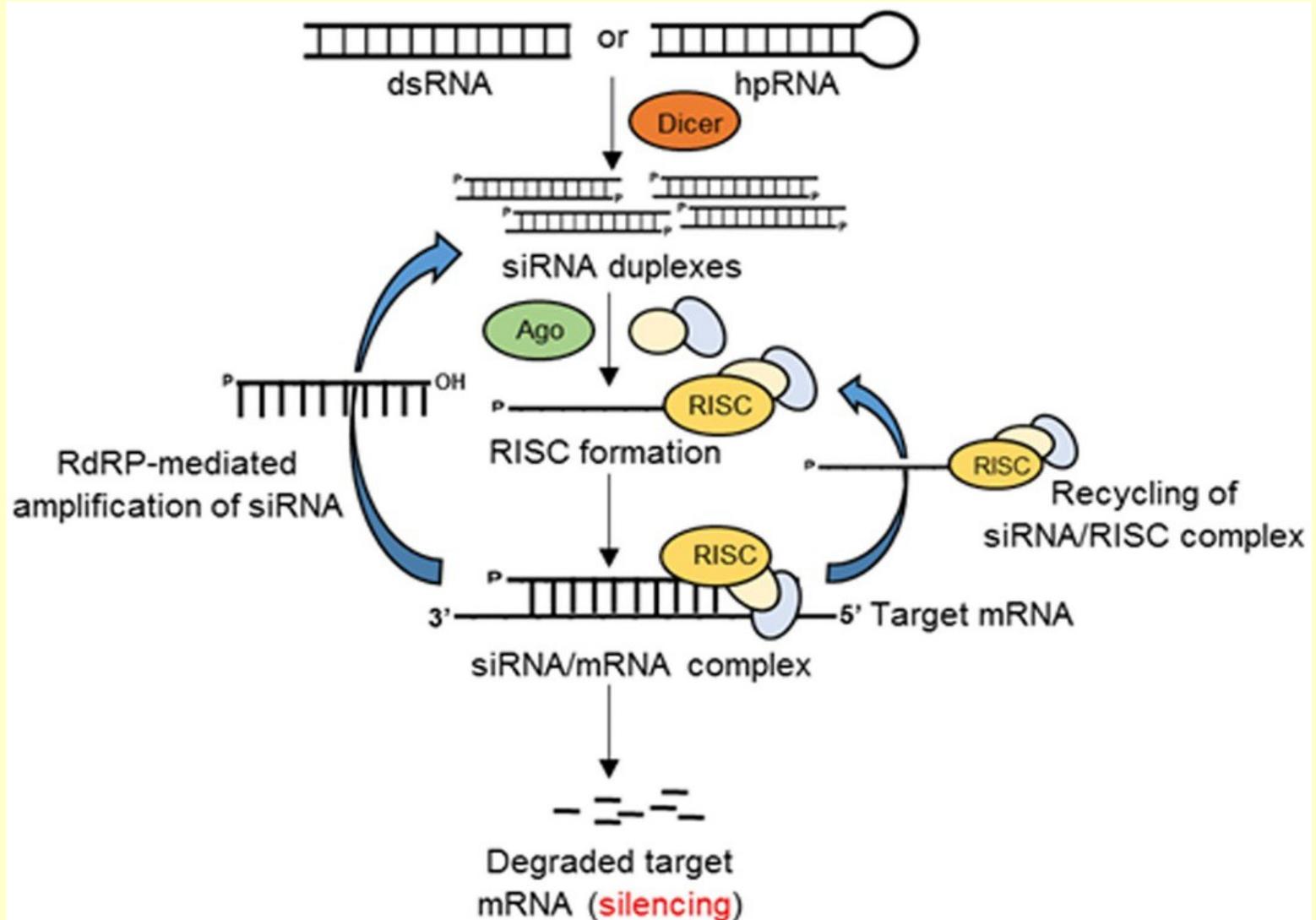
14 kDa TI

PR-10

$\beta$ -1,3-glucanase (PR-2)

PRms

# Development of transgenic corn for silencing of *A. flavus* genes critical to infection and aflatoxin production (Host-Induced Gene Silencing)

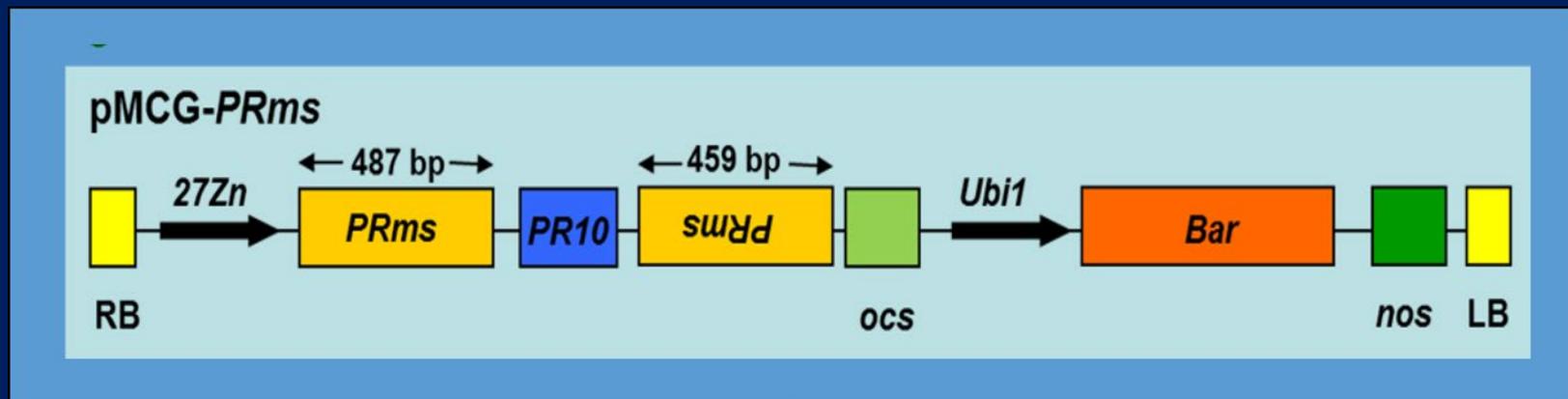


# RNAi-based Silencing of Maize PRms

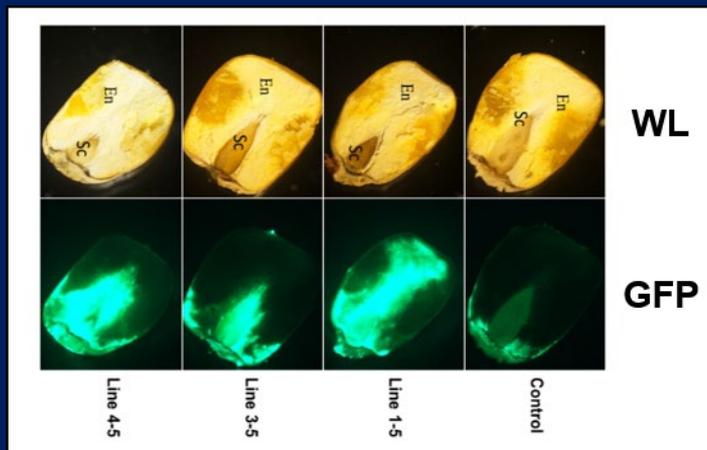
## Why PRms?

- PRms transcripts accumulate at the aleurone layer and scutellum of germinating maize seeds and their production is increased upon fungal infection- Casacuberta et al., 1991, 1992
- Progression of *A. flavus* infection in resistant maize lines was slowed at the scutellar-embryo interface- Dolezal et al., 2014
- RNA-Seq analysis of the *A. flavus*-maize interaction led to development of a gene expression network that identified PRms as a major network hub that may be involved in resistance to *A. flavus* infection- Musungu et al., 2016, Majumdar et al. 2017

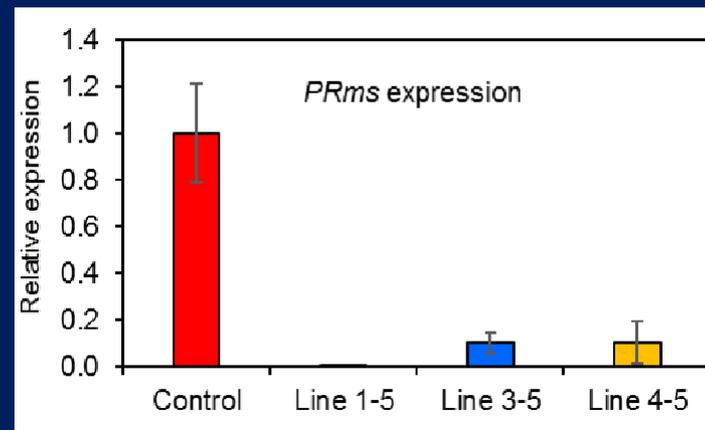
## Construction of PRms Silencing Binary Vector



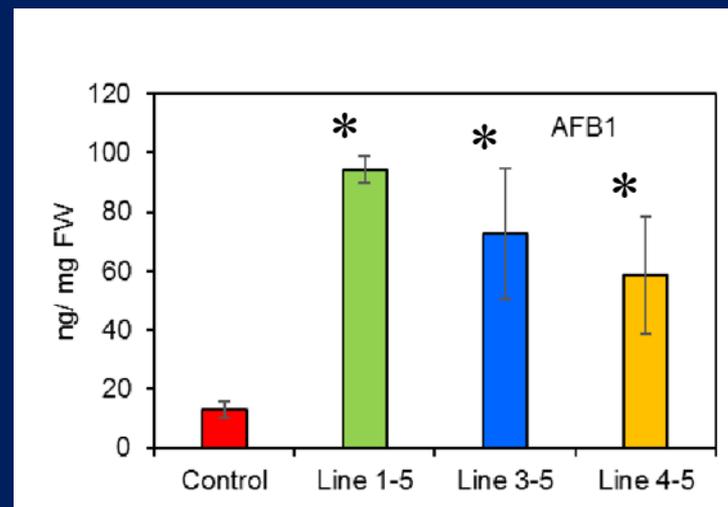
# Analysis of PRms-RNAi Lines



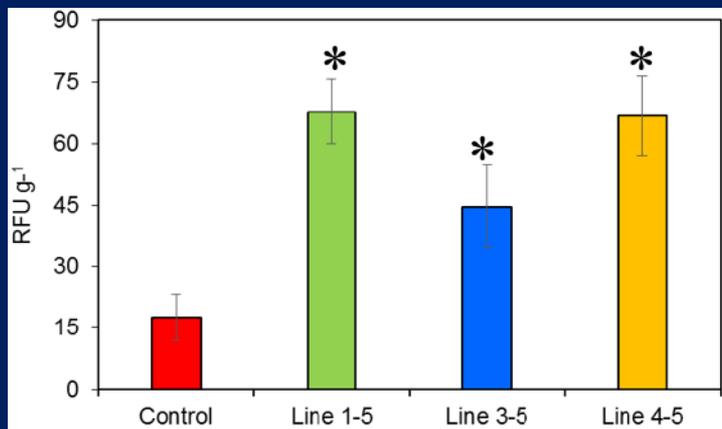
Kernels infected with AF70-GFP strain



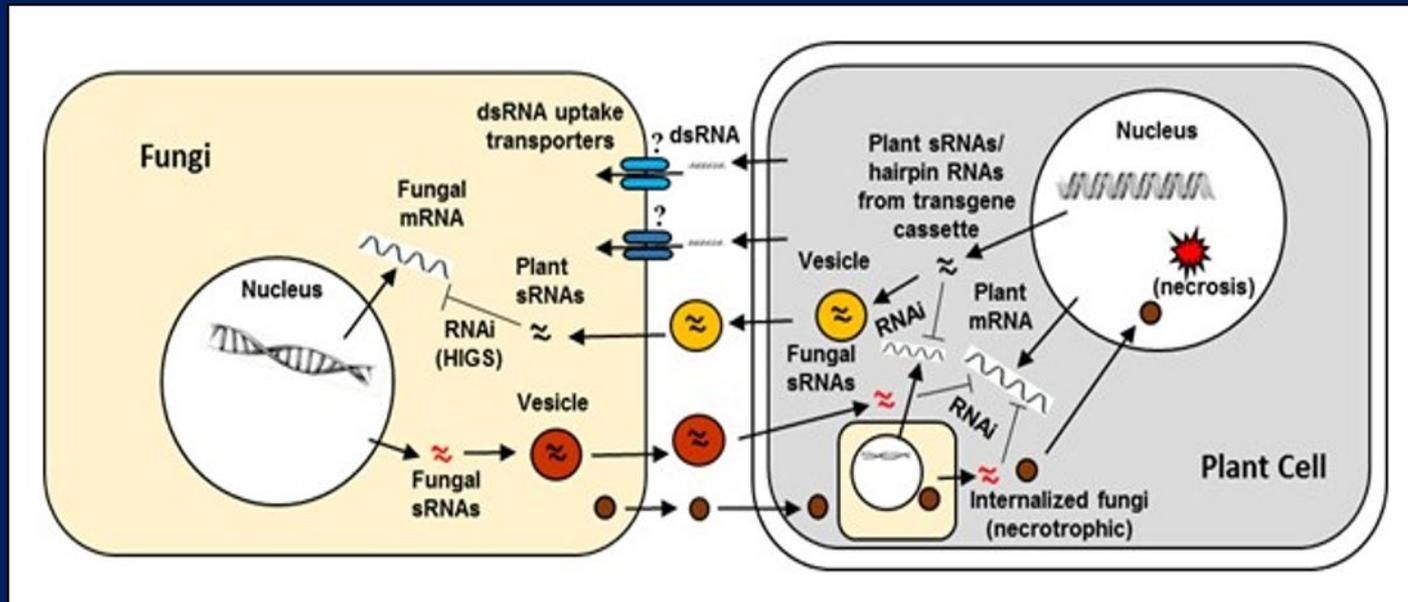
## AFB1 Production



## Fungal Growth



# Development of transgenic corn for silencing of *A. flavus* genes critical to infection and aflatoxin production (Host-Induced Gene Silencing)



# Host Induced Gene Silencing (HIGS)

*Does not require expression of foreign proteins in plants = consumer acceptance*

# RNAi in Maize to Target *A. flavus* Genes that are Critical for Growth and Toxin Production

## Growth

$\alpha$ -amylase- starch degradation

Alkaline protease- protein degradation

CYP51- ergosterol biosynthesis

## Toxin Production

VeA- global regulator of aflatoxin production

NsdC- global regulator of aflatoxin production

aflR- aflatoxin pathway- specific regulator

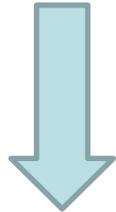
aflC (pksA)- aflatoxin biosynthetic gene



# *Aspergillus flavus* $\alpha$ -amylase



**Starch**



$\alpha$ -amylase  
*A. flavus*

**Glucose**



**Aflatoxin**

**Starch**



**Glucose**

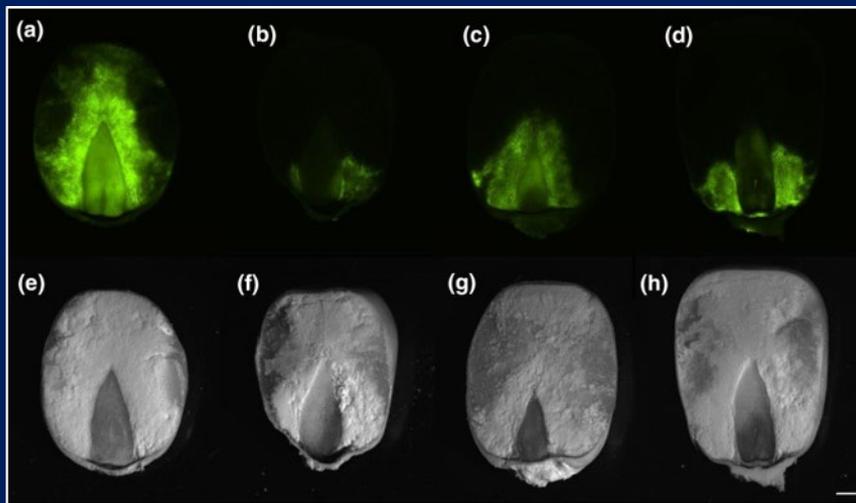


**No aflatoxin**

- $\alpha$ -amylase is an enzyme necessary for the breakdown of starch into glucose
- Mutant *A. flavus* strain lacking  $\alpha$ -amylase cannot infect maize kernels or produce aflatoxin

# Fungal Growth on *amy*-RNAi Transgenic Maize Seed

## GFP Microscopy



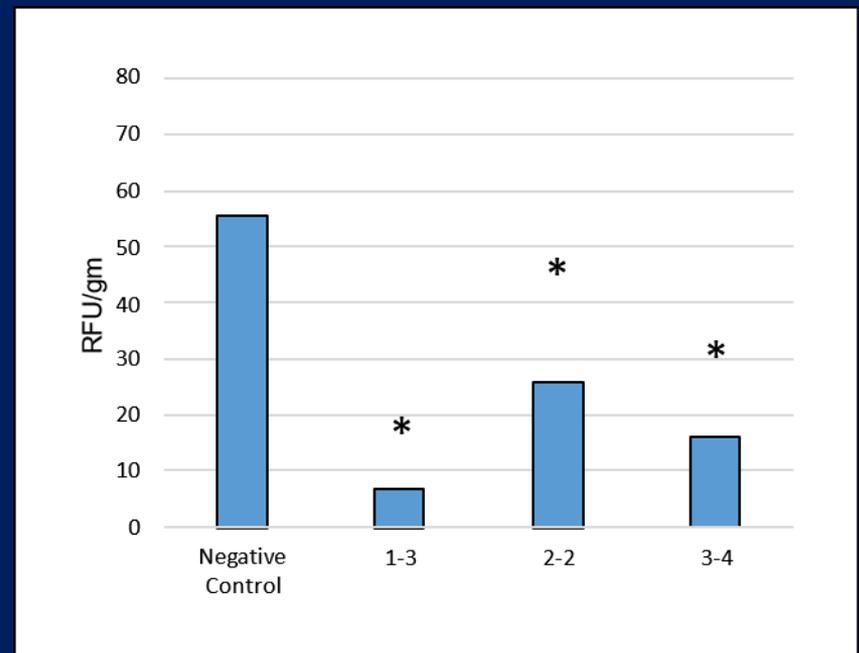
Control

1-3

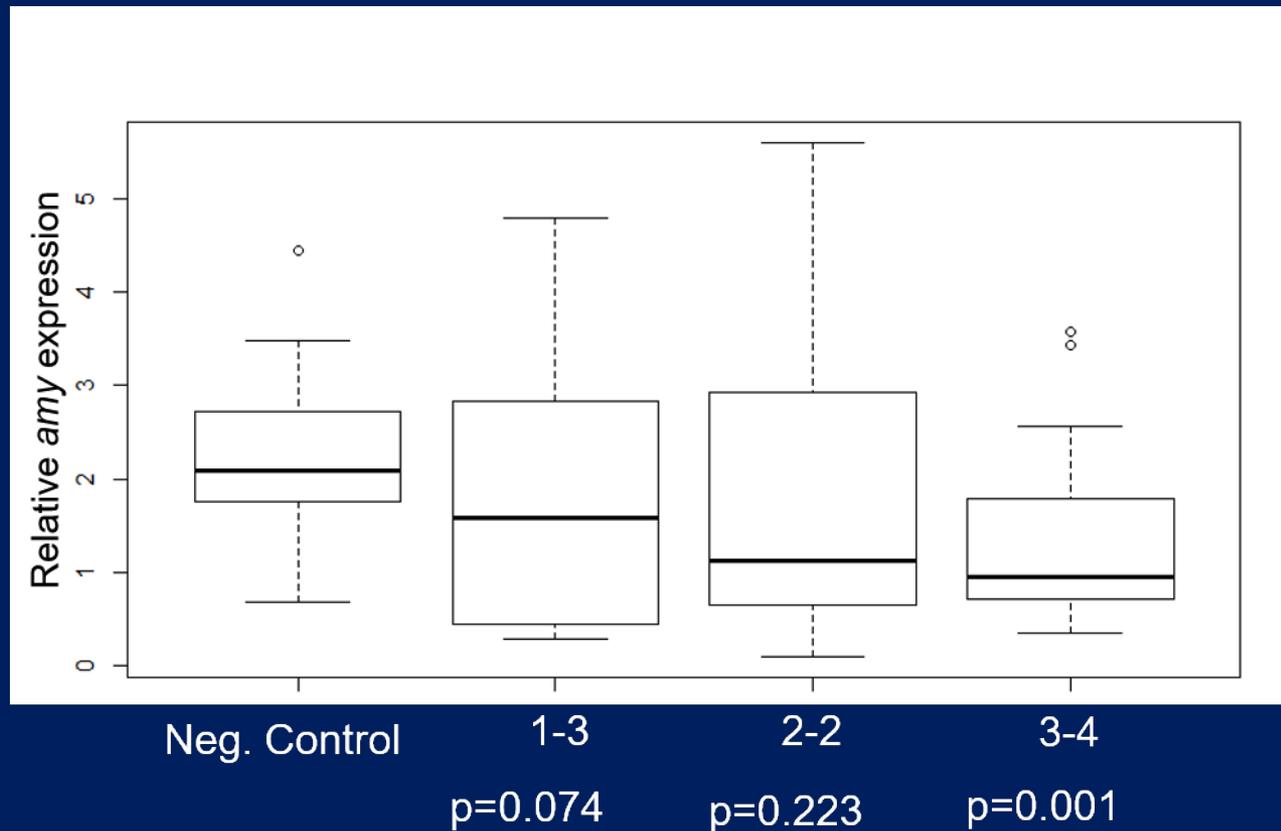
2-2

3-4

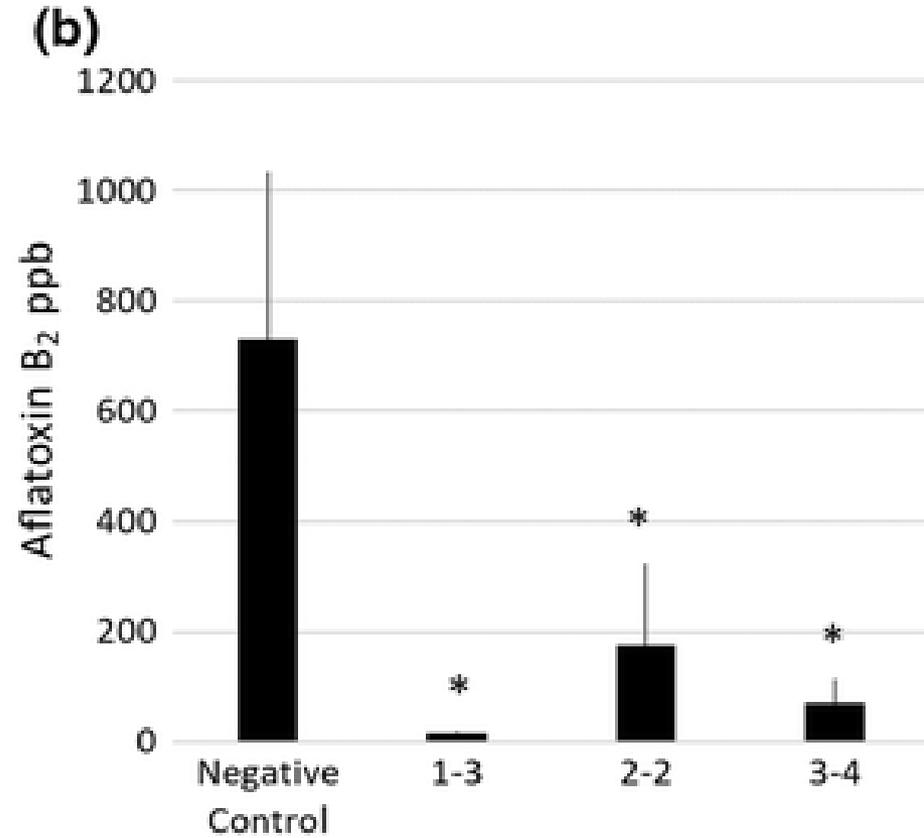
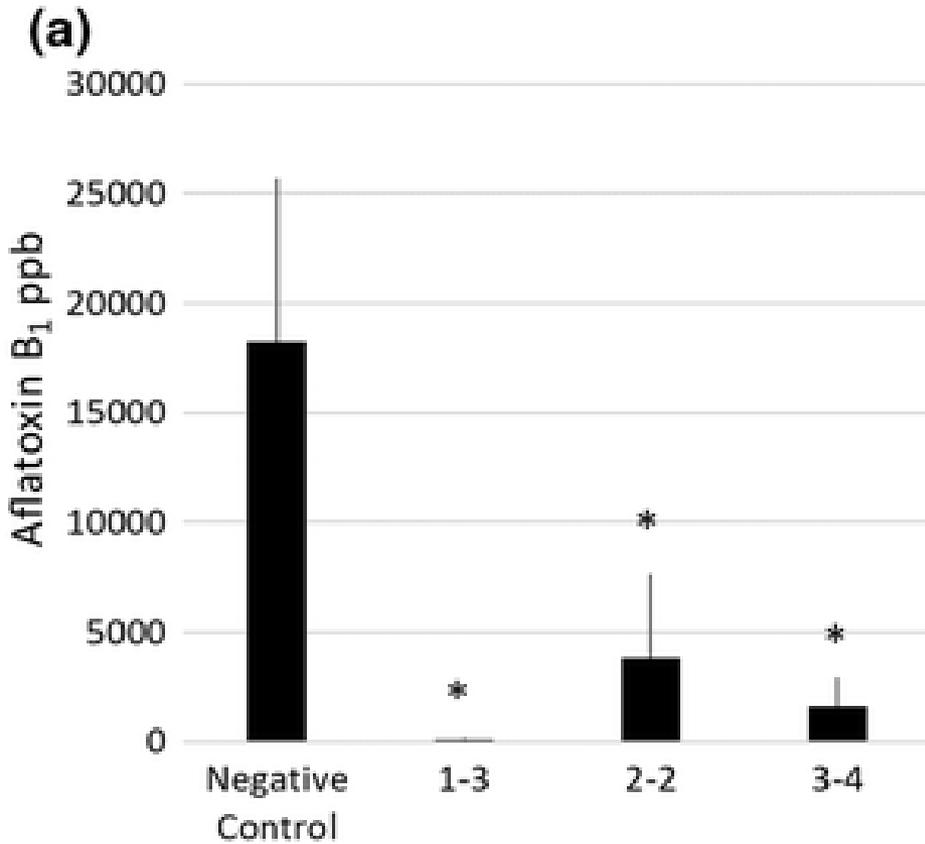
## GFP Fluorometric Analysis



# Amy Gene Expression in *amy*-RNAi Transgenic Maize Seed



# Reduced Aflatoxin levels in *amy*-RNAi transgenic kernels



# ENHANCING HOST PLANT RESISTANCE

## Summary

### Classical breeding

Aflatoxin resistant corn lines released; New hybrids being developed

Key antifungal proteins in corn identified by proteomics

### Transcriptomic analysis of host-*flavus* interaction

Antifungal genes, up or down regulated, identified in cotton and corn

### Transgenic expression of antifungal genes

Transgenic corn/cotton lines expressing natural or synthetic antifungal proteins or peptides demonstrated resistance to aflatoxin contamination

### Host-Induced Gene Silencing (HIGS)

Silencing of *flavus* genes critical for its growth and/or toxin production provided significant resistance to aflatoxin contamination in corn