

Project No: 6054-42000-025-00D

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

SY Team

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Collaborators

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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

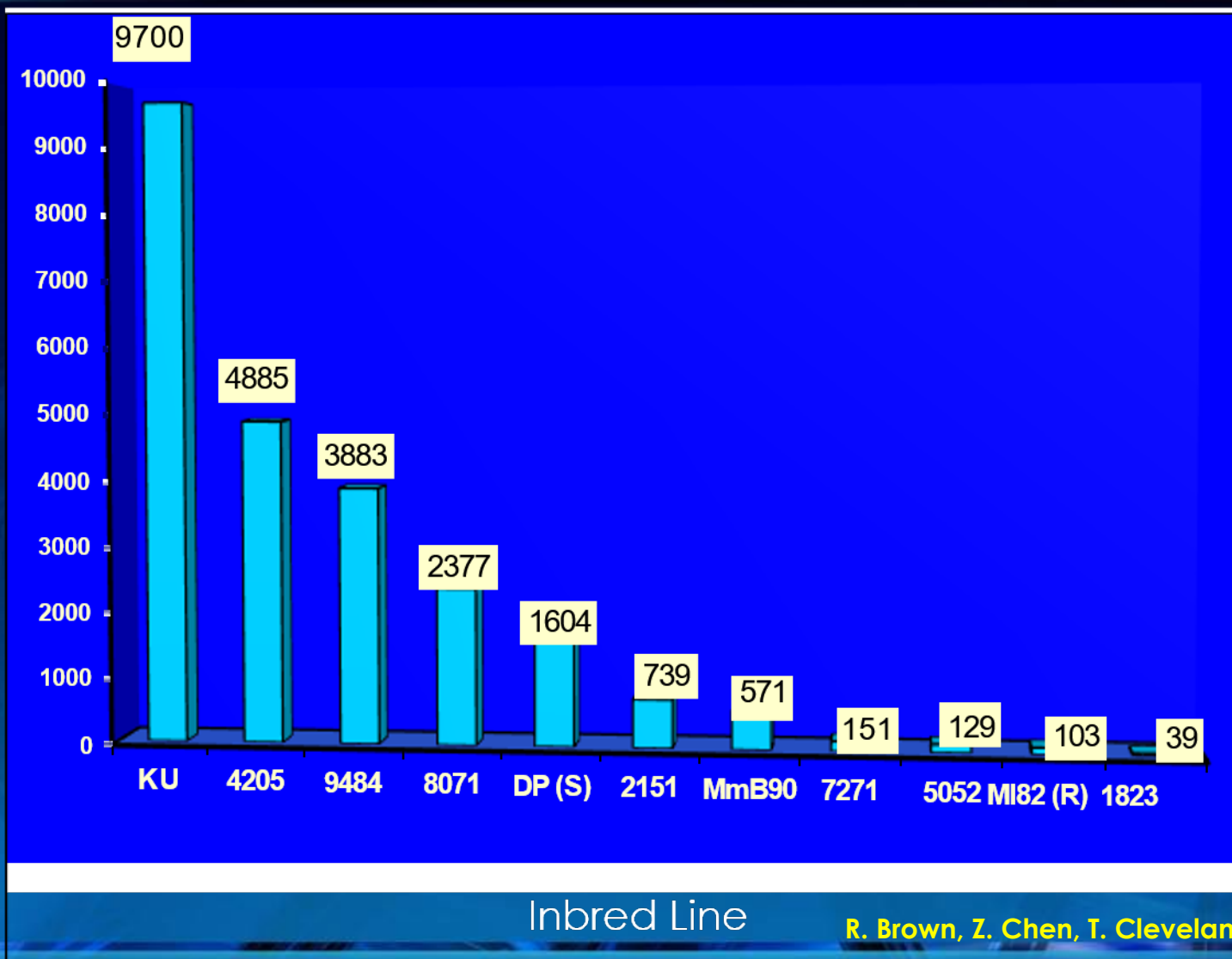
Breeding for Resistance in Corn



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 B₁ (ppb)



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

Development of Resistant Maize Lines

IITA Lines crossed with US Lines

↓
6 generations
(agronomic characteristics)

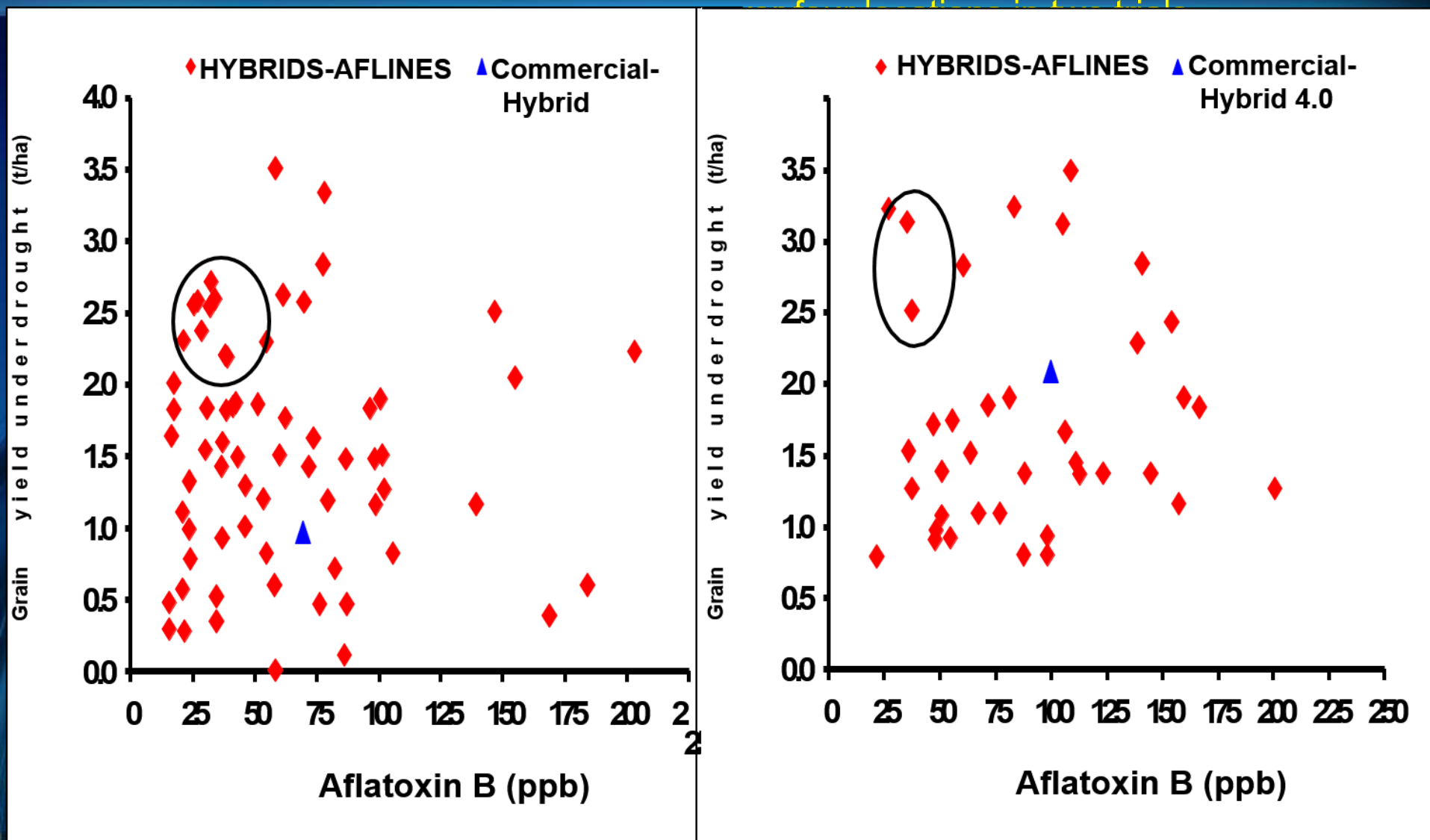
↓
10 generations

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

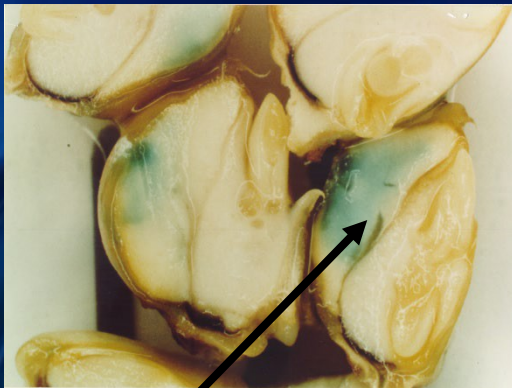
Lines	Fumonisin (ppm)	Aflatoxin-SRRC- USDA-KSA (ppb)		Aflatoxin-IITA- KSA (ppb)
	Average of three environments	First run	Second run	
TZIFRL01	1.4	345	440	4104
TZIFRL05	2.4	352	89	2917
TZIFRL04	2.6	864	29	8021
TZIFRL02	4.4	149	507	6514
TZIFRL03	4.5	202	51	5086
TZIFRL06	60.7	-	3228	10208
MI82 (Resistant)	-	325	404	-
P3142 or 9071 (Susc.)	-	2496	1531	19375
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

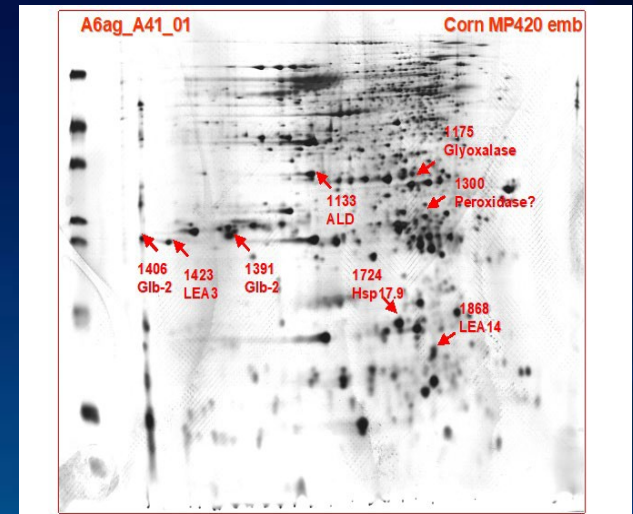


Blue color indicates presence of fungal (*A. flavus* GUS transformant) infection of the seed

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

**β-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*

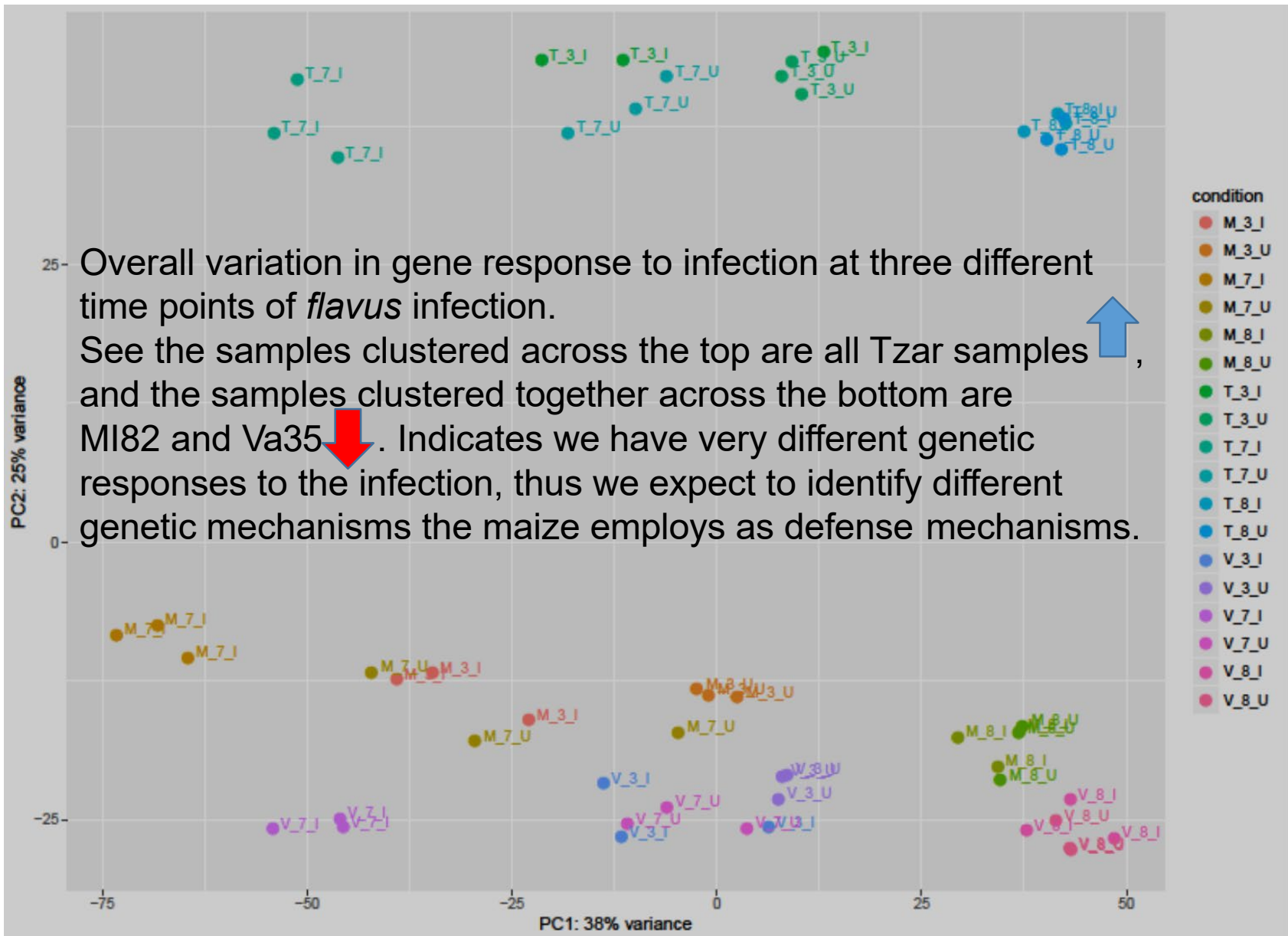
Identification of *flavus*-resistance associated genes

Completed transcriptomic analysis of cottonseed-*flavus* interaction

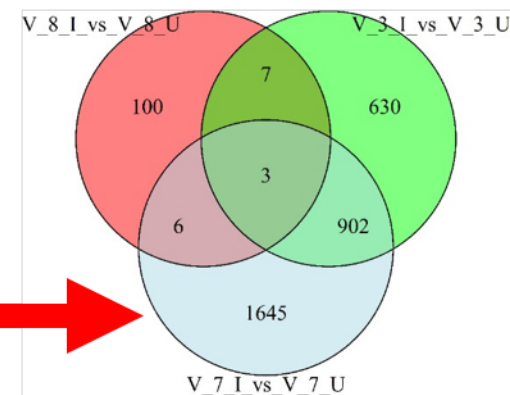
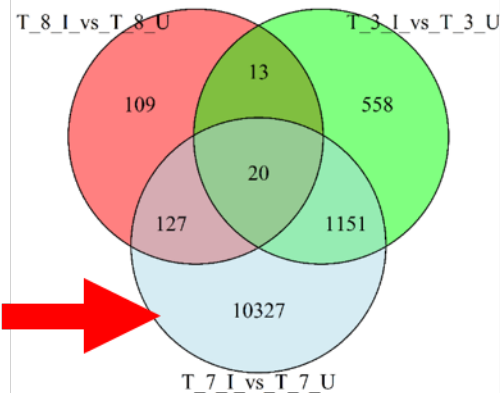
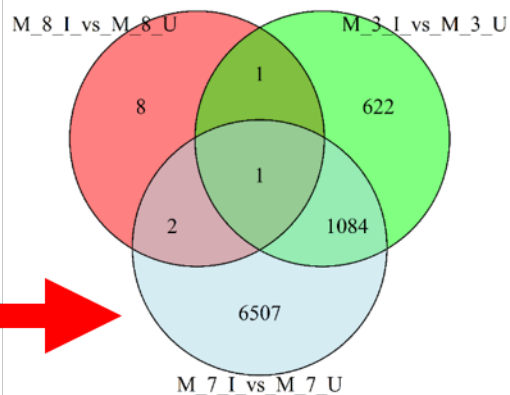
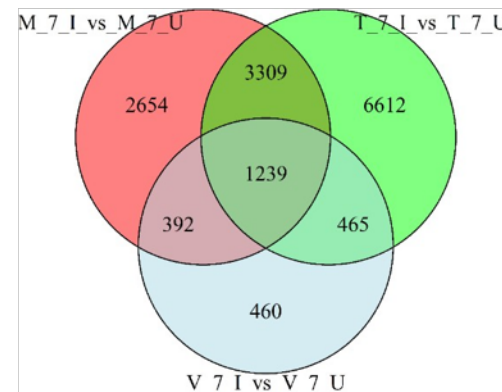
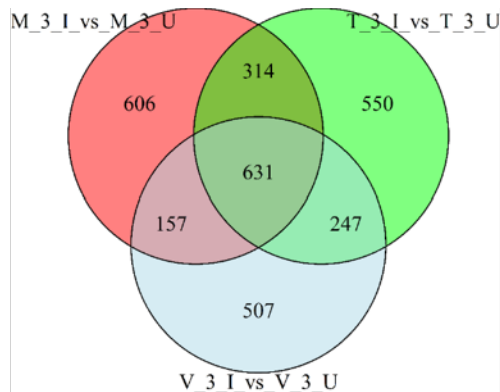
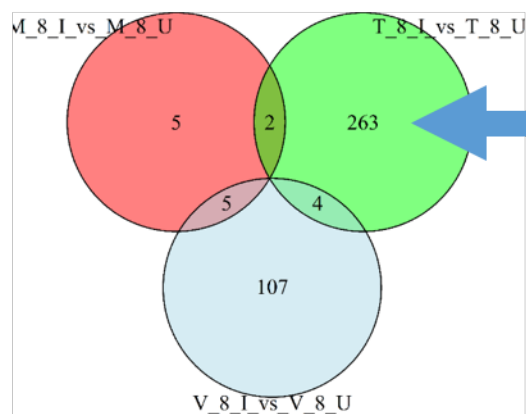
1. Identified candidate *Aspergillus flavus* resistance-associated genes from cotton transcriptome profiling and comparative transcriptome analysis with peanut and corn.
2. Transformation vectors were constructed with some genes that are being evaluated in transgenic cotton (spot11 catalase).
3. Isolated 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



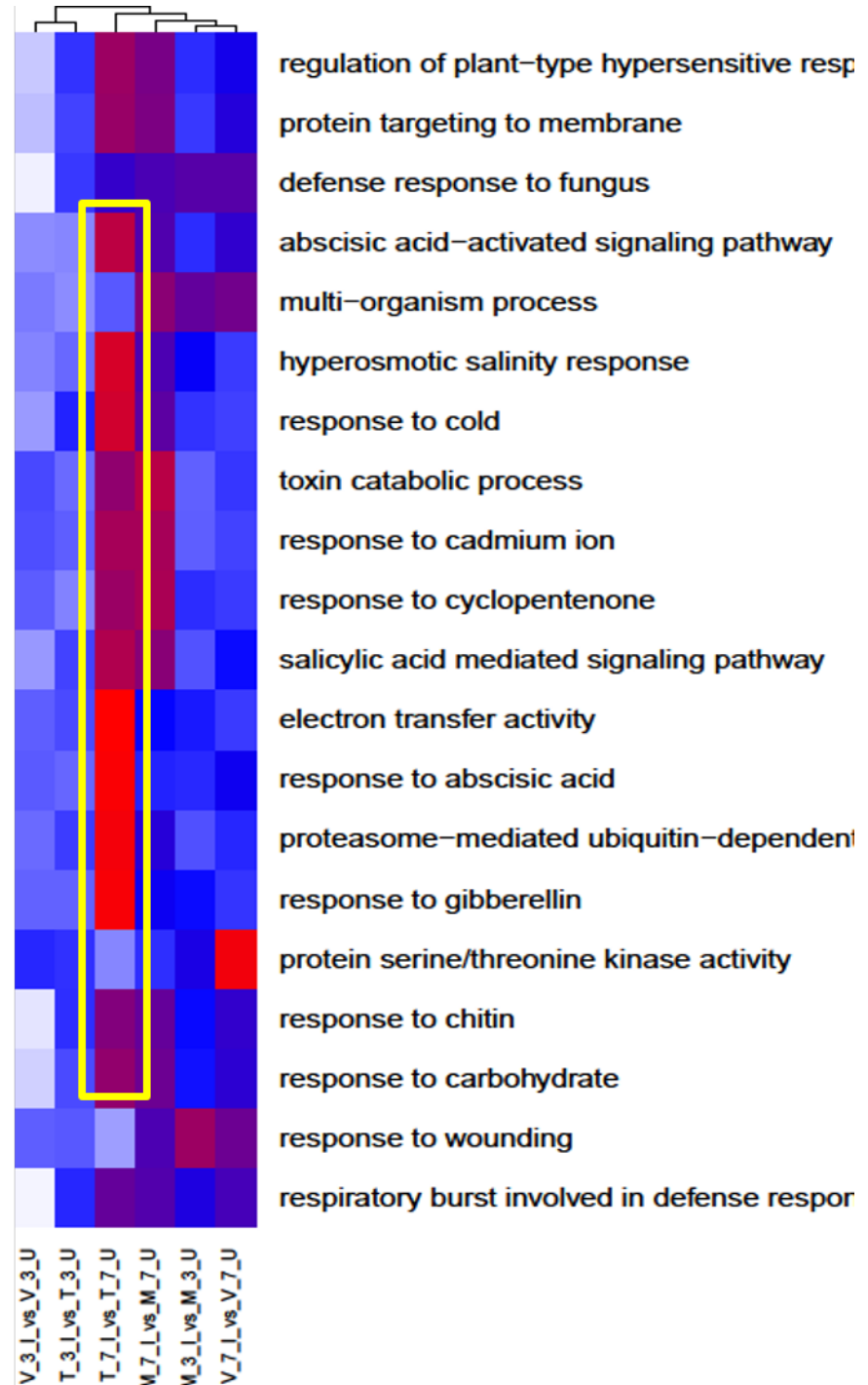
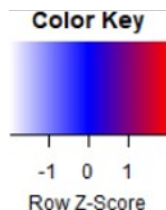
Venn Diagrams of Differentially Expressed Genes



M – MI82 T – TZAR102 V – Va35

8h, 3d, and 7d samples

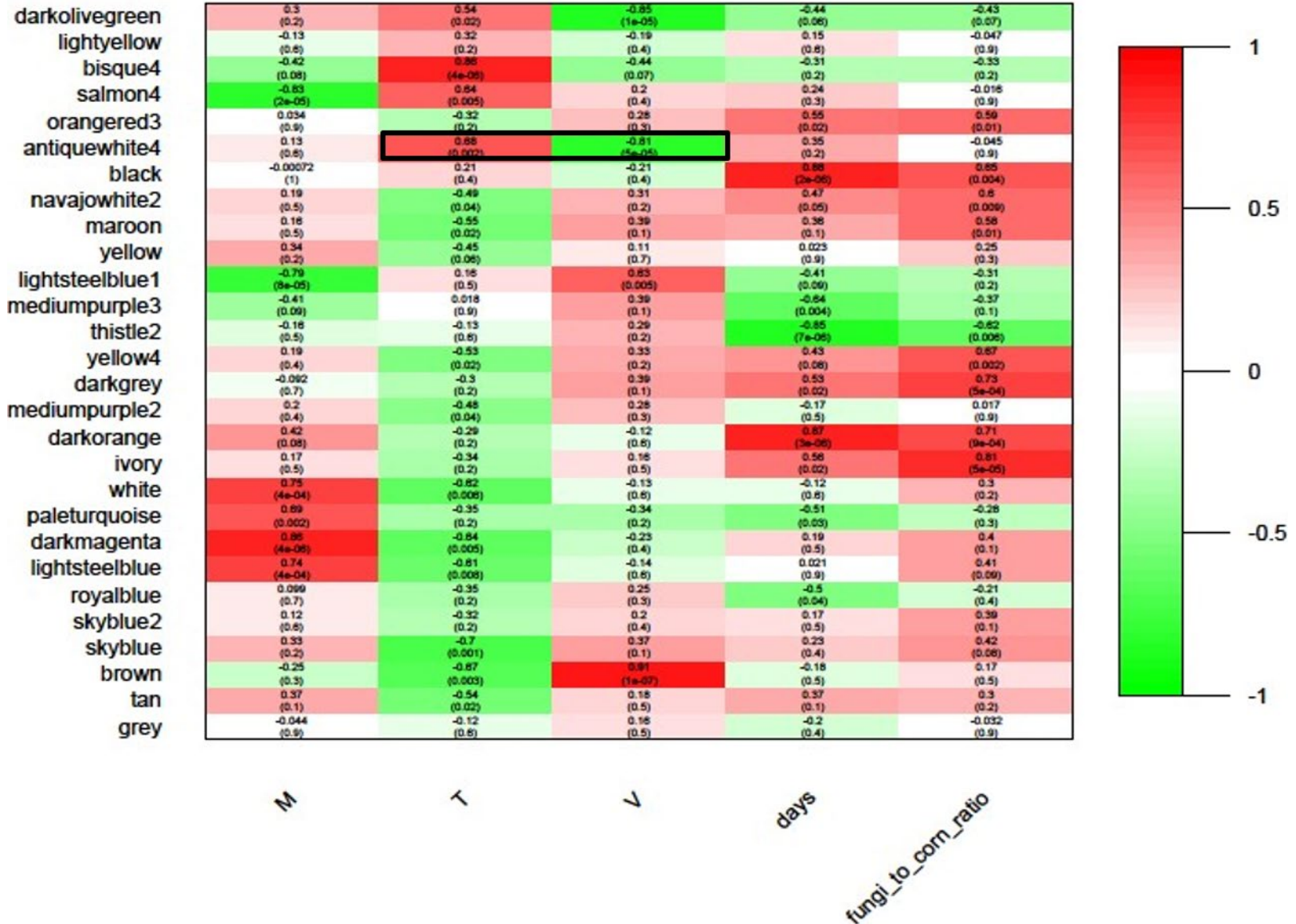
Heat map of overrepresented GO categories of upregulated genes showing -log p-value



Co-expression Network Analysis using WGCNA

Module-trait relationships

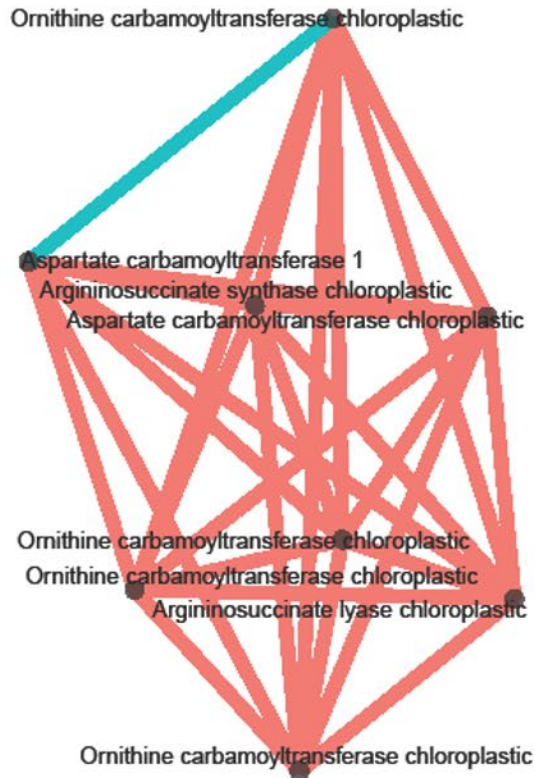
Gene Family Modules



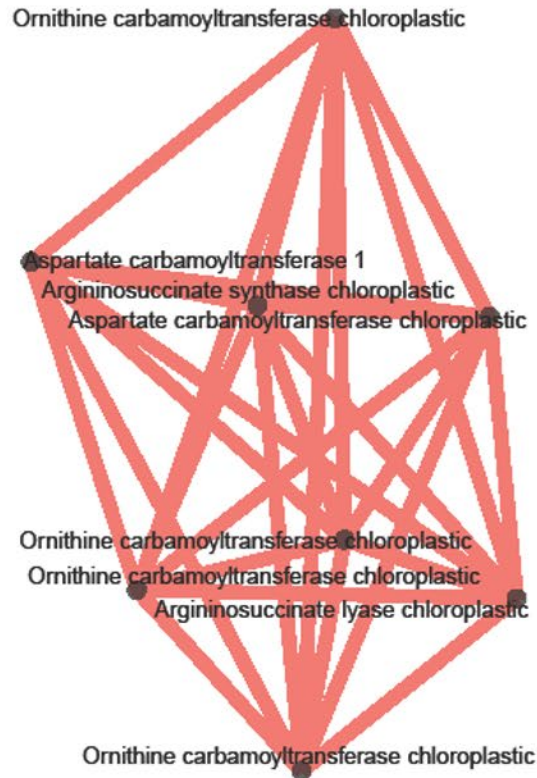
Differential Correlation Analysis of Reactome Pathways

Canavanine biosynthesis

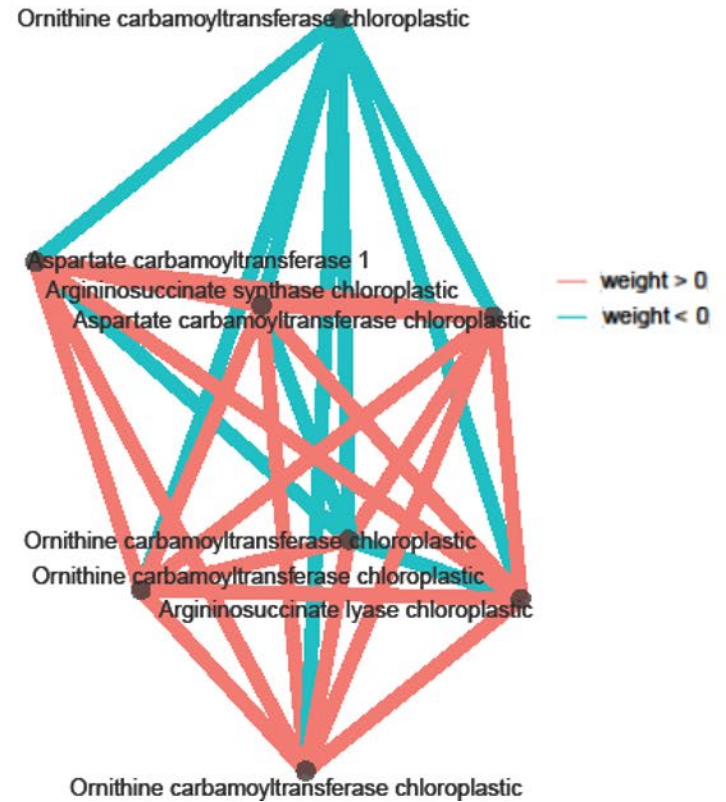
M



T



V



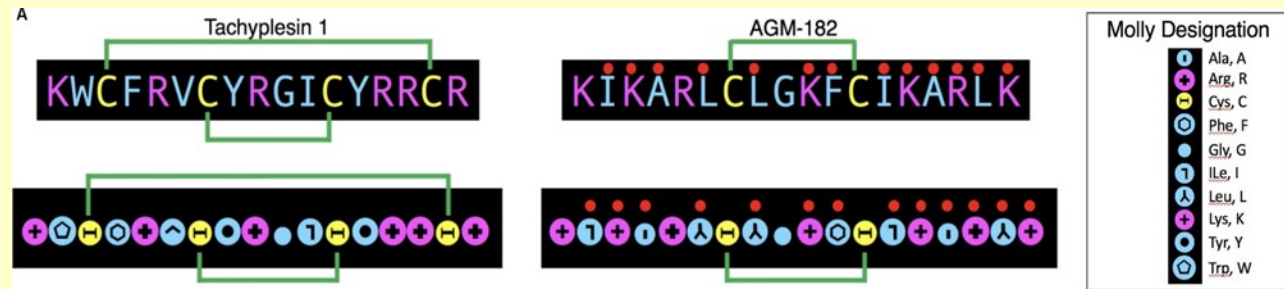
Transformation of Maize

with the synthetic peptide gene
AGM182

In-house Maize Transformation and Regeneration



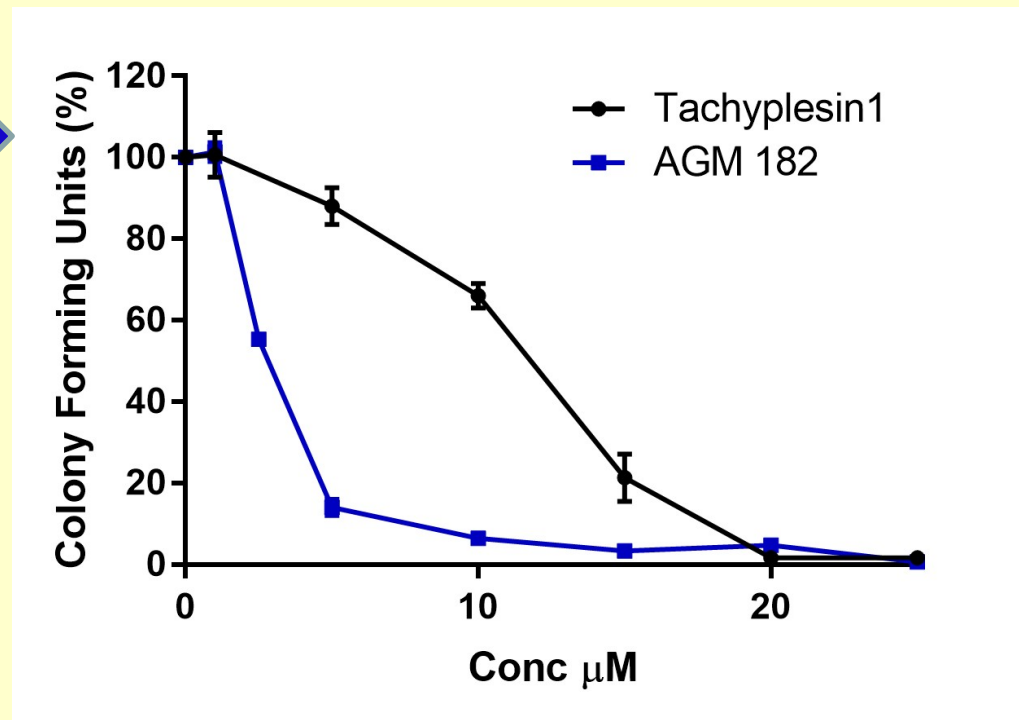
AGM182



- Synthetic, linear, amphipathic, β -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 μM concentrations
- 18 amino acids – 5 lysine
- Potential to improve protein nutrition of kernel





AGM182 Transgenic maize

Reduced *flavus* growth in AGM182 transgenic kernels

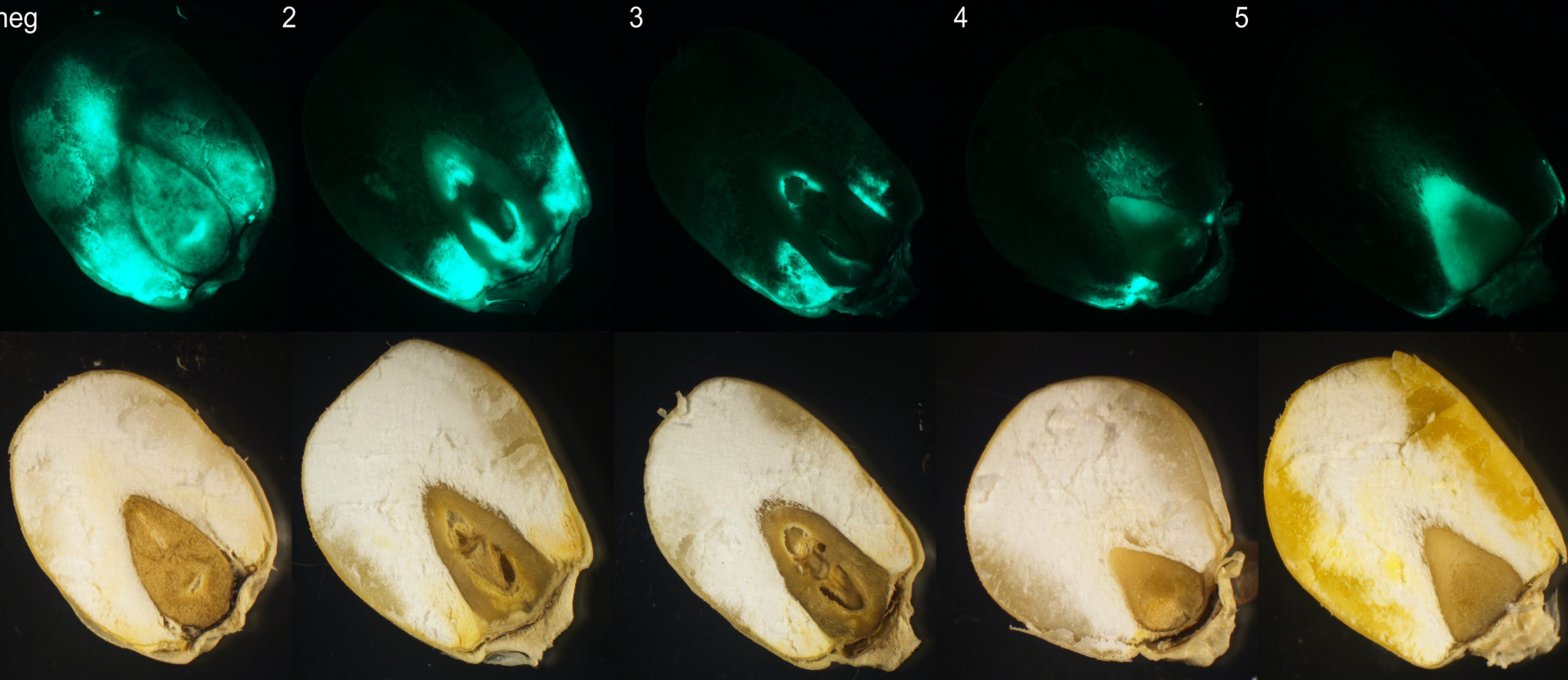
neg

2

3

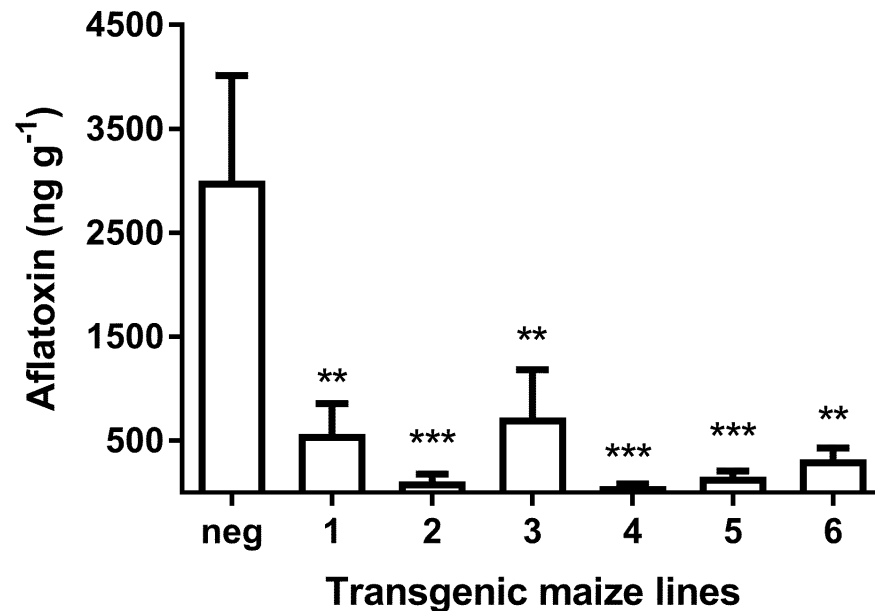
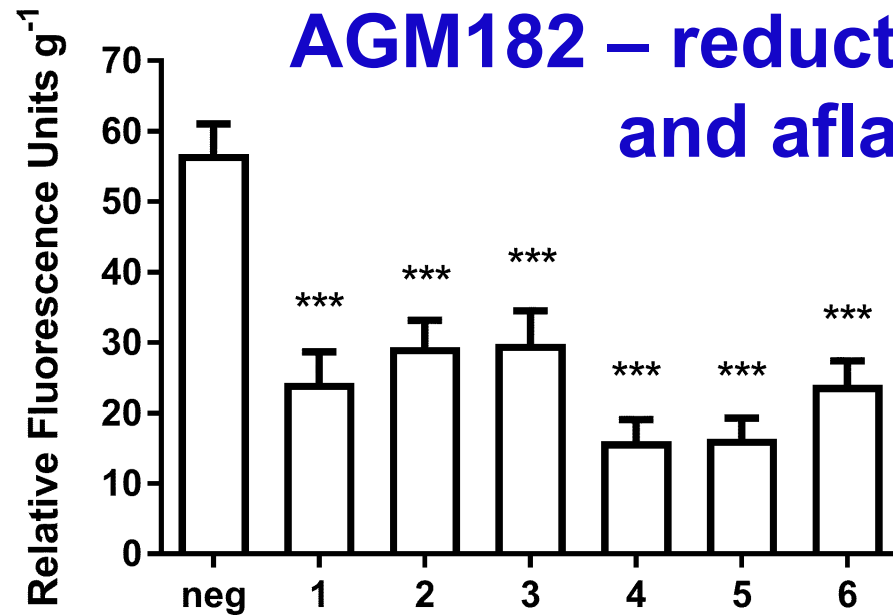
4

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

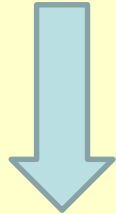
Transformation of Maize

with an α -amylase inhibitor from
Lablab purpureus L.
(hyacinth bean)

Aspergillus flavus α -amylase

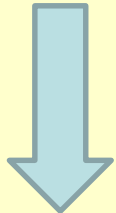


Starch



α -amylase
A. flavus

Glucose



Aflatoxin

Starch



Glucose

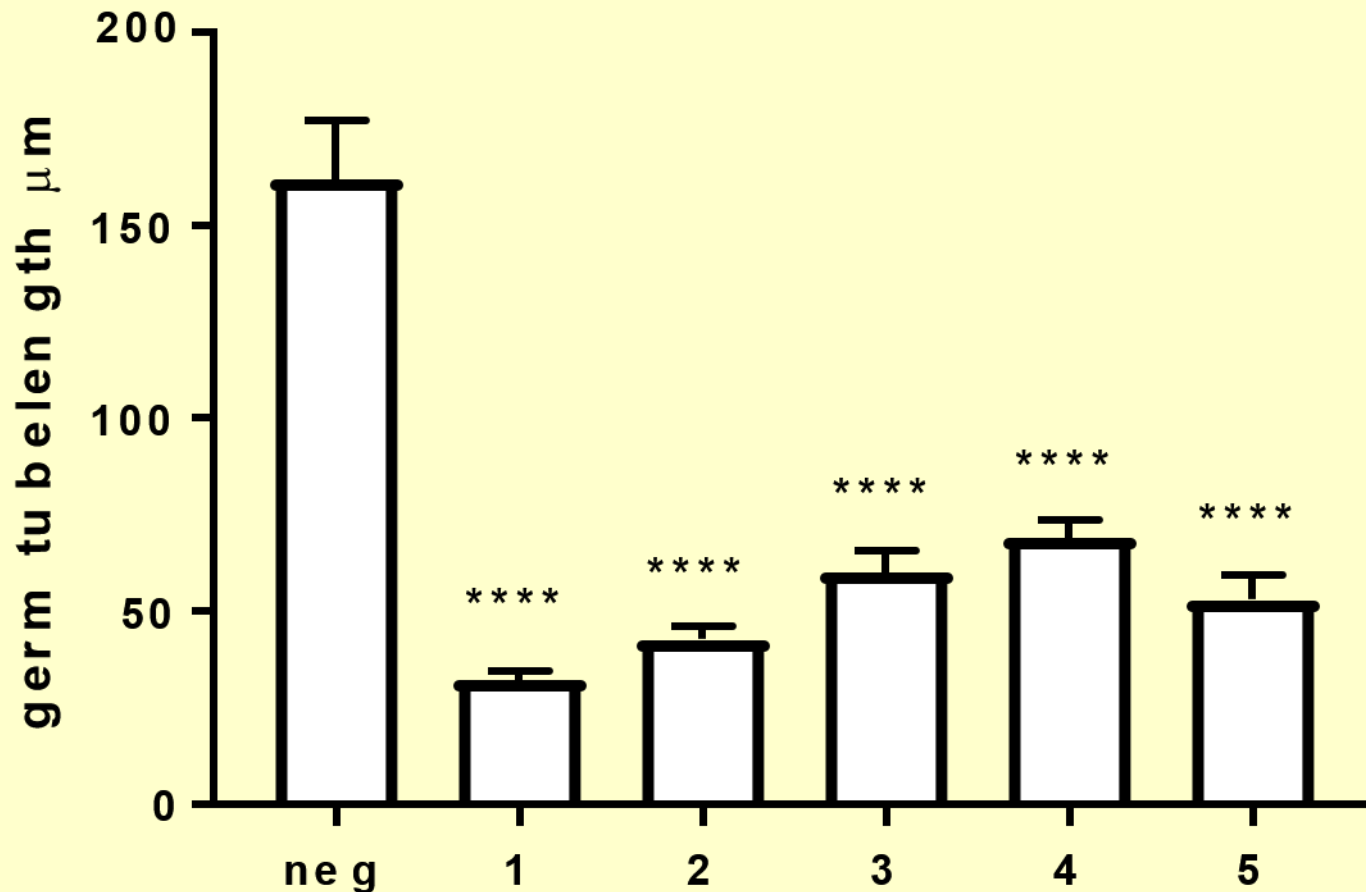


No aflatoxin

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

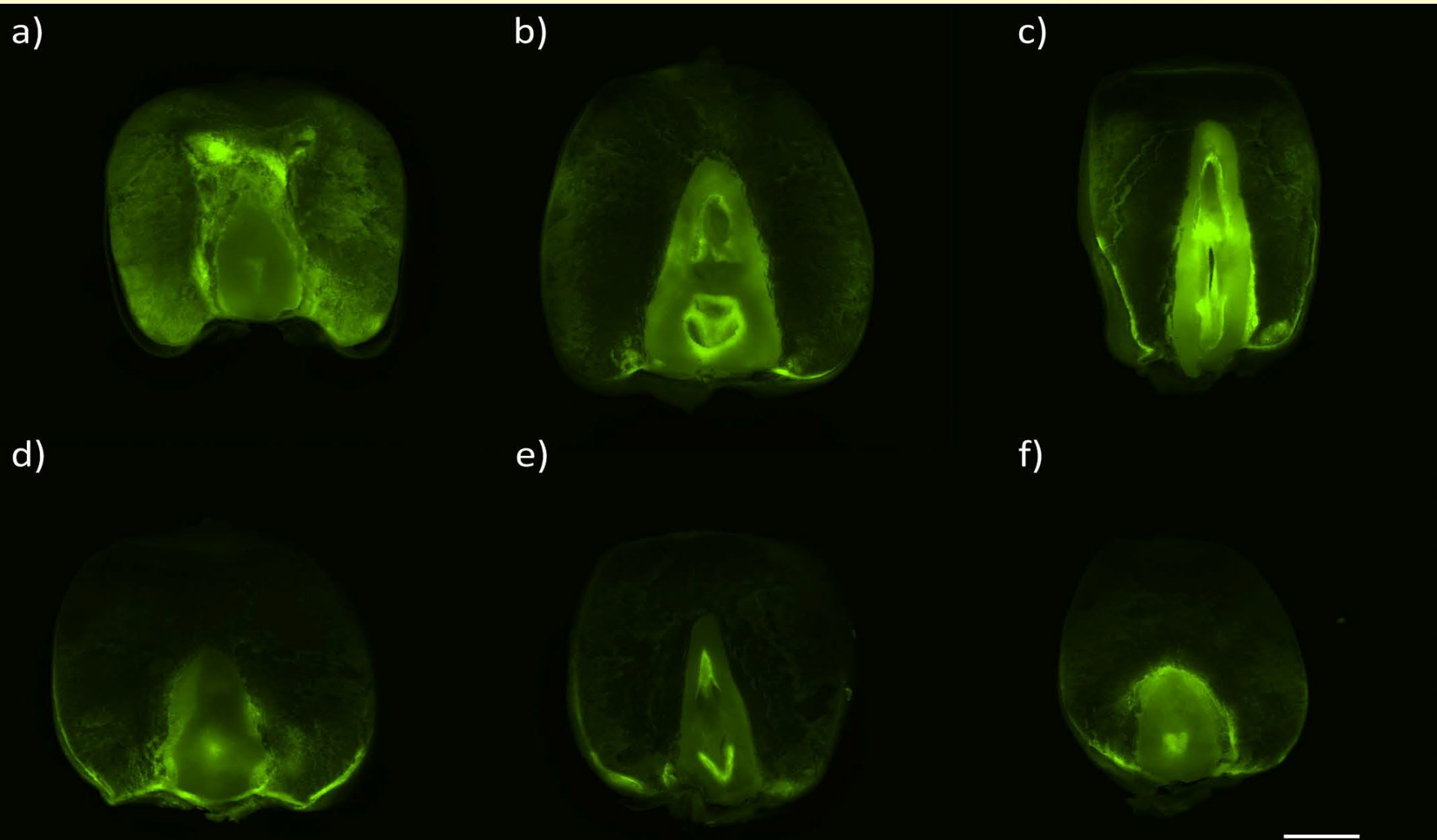
Transgenic Corn Expressing the α -Amylase Inhibitor (AIP)

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

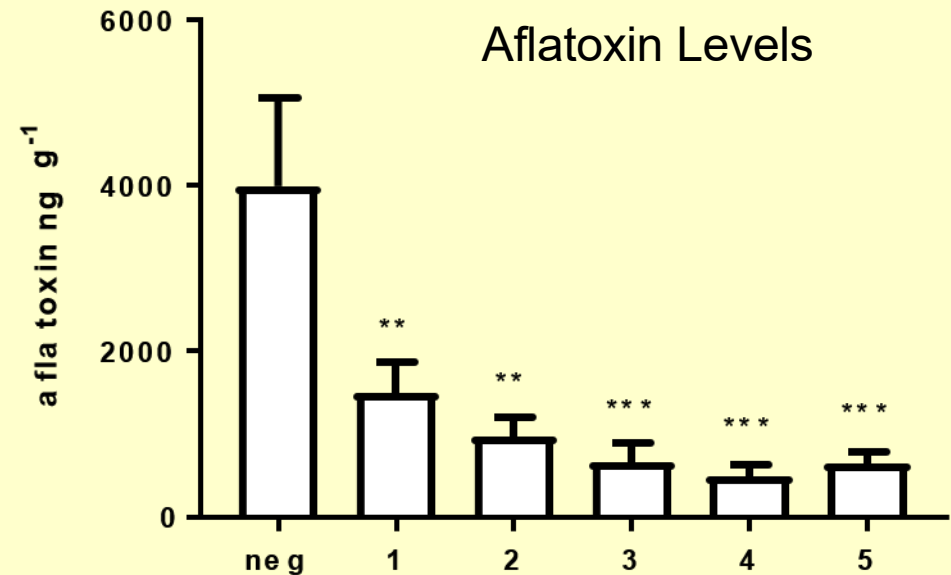
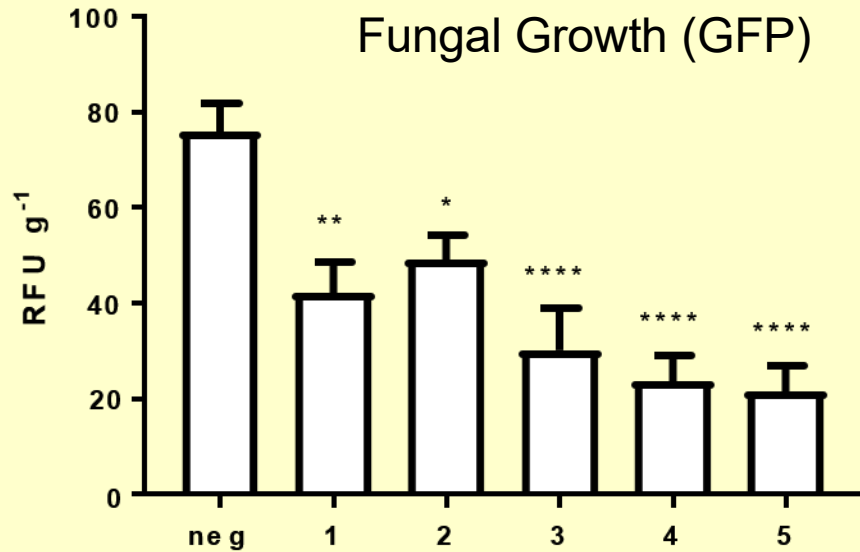


Transgenic Corn Expressing the α -Amylase Inhibitor (AILP)

Fungal Growth (=GFP)



Transgenic Corn with Hyacinth Bean α -Amylase Inhibitor (AILP)



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

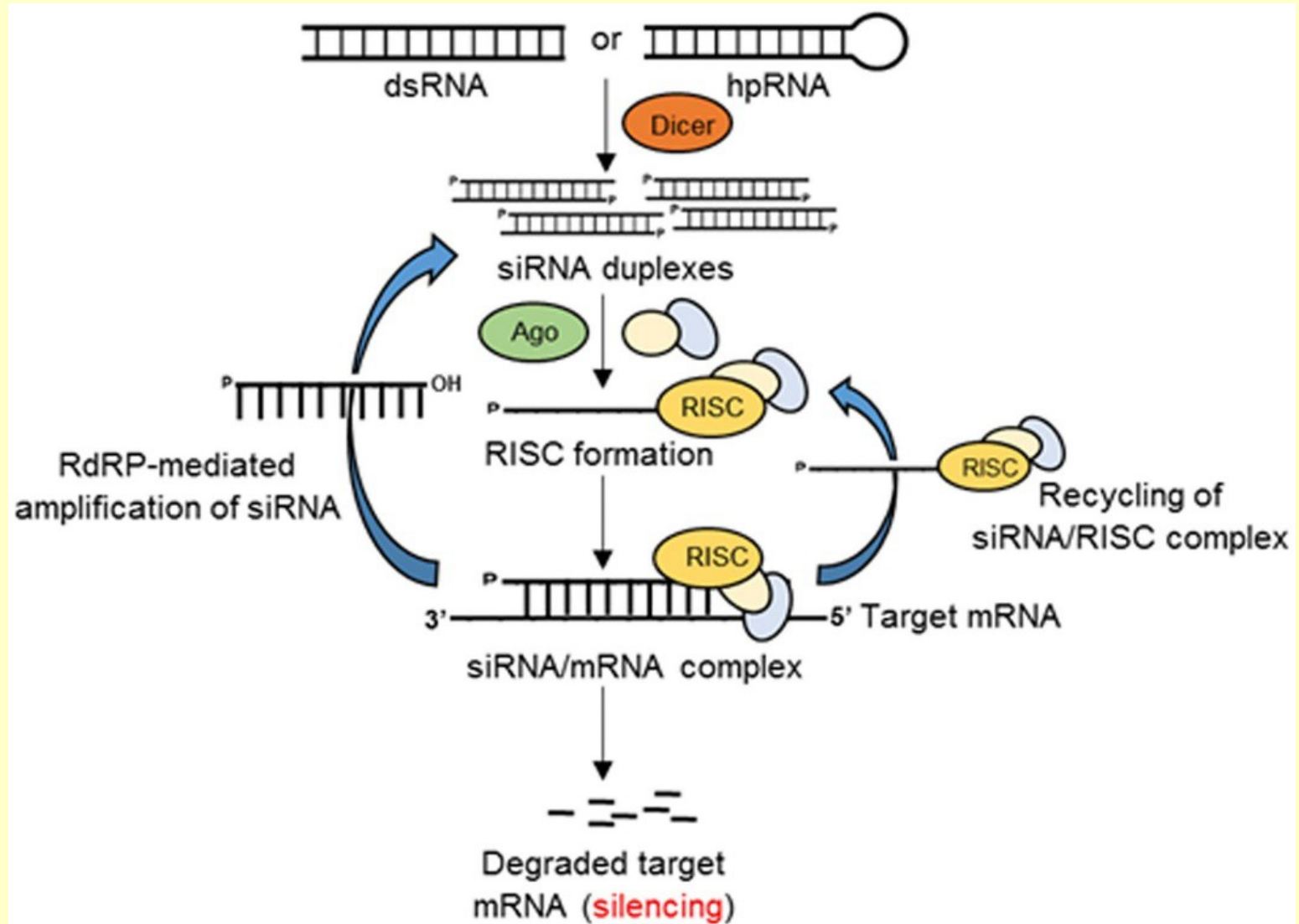
14 kDa TI

PR-10

β -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)

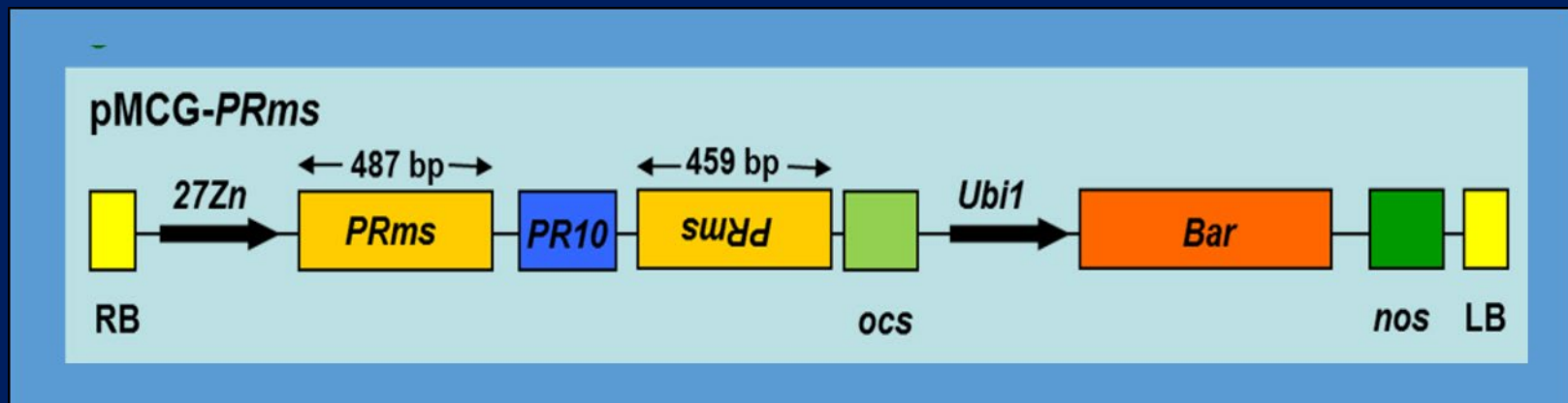


RNAi-based Silencing of Maize PRms

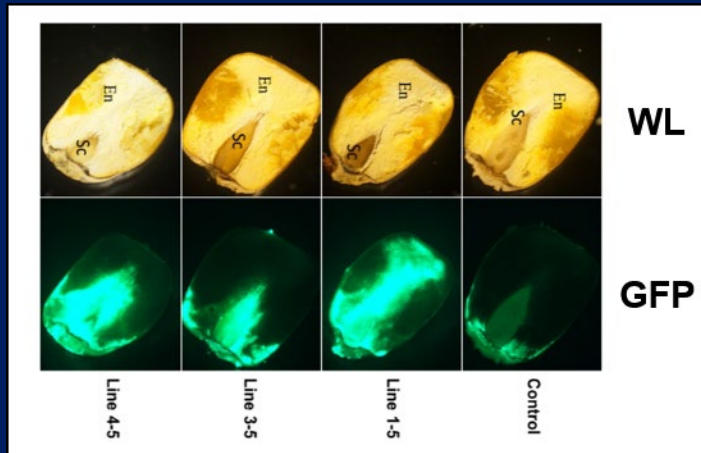
Why PRms?

- 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](#)

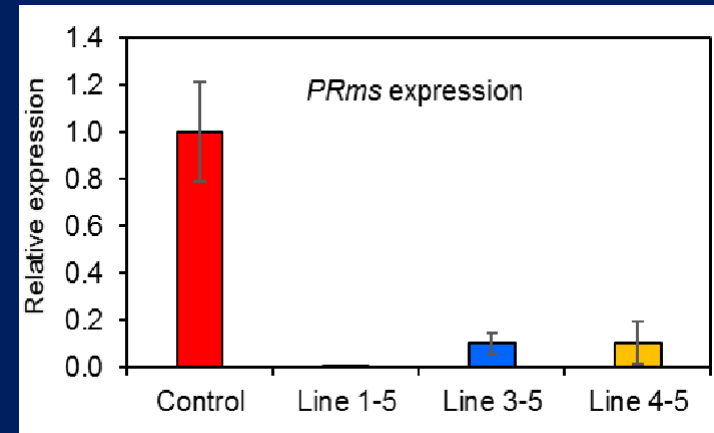
Construction of PRms Silencing Binary Vector



Analysis of PRms-RNAi Lines

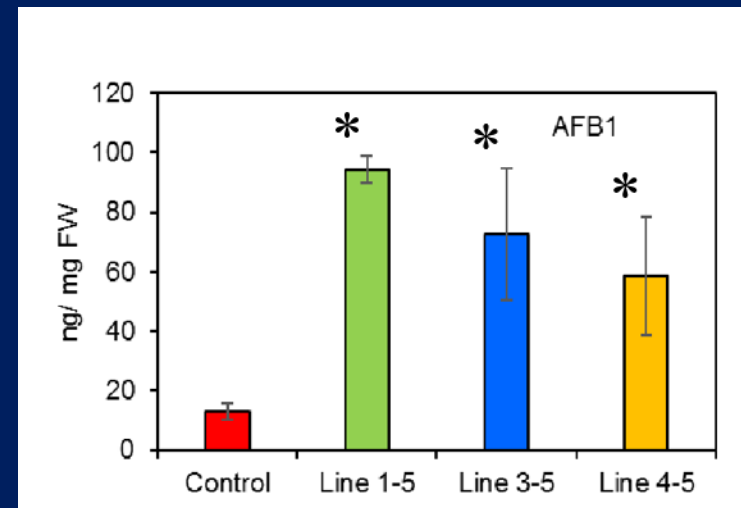
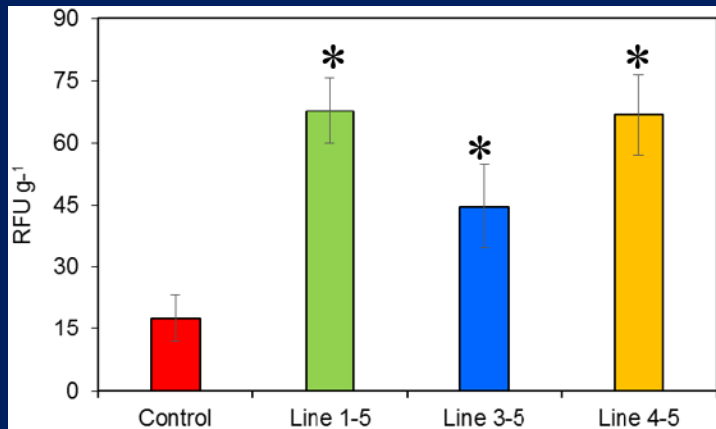


Kernels infected with AF70-GFP strain

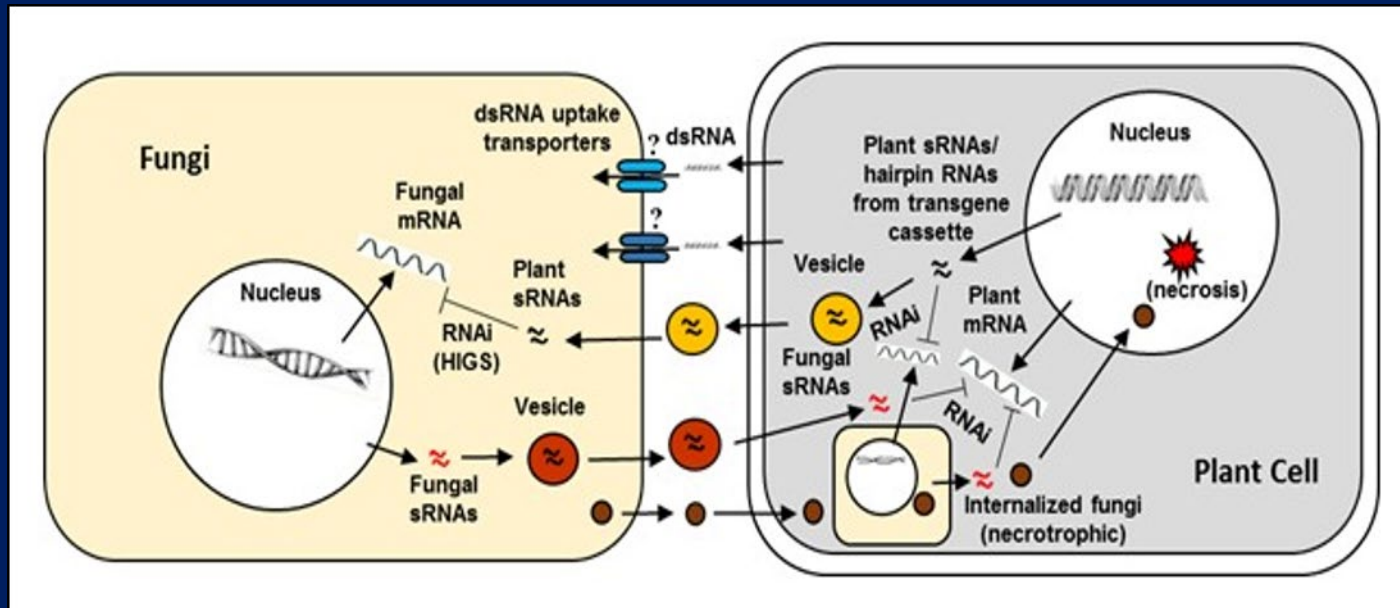


AFB1 Production

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

α -amylase- starch degradation

Alkaline protease- protein degradation

CYP51- ergosterol biosynthesis

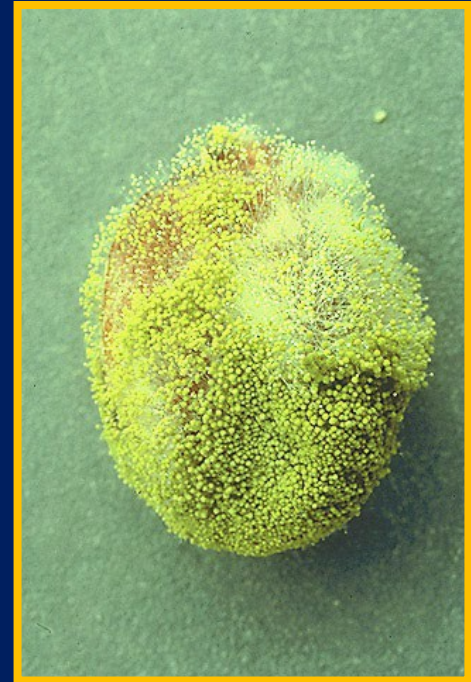
Toxin Production

VeA- global regulator of aflatoxin production

NsdC- global regulator of aflatoxin production

aflR- aflatoxin pathway- specific regulator

aflC (pksA)- aflatoxin biosynthetic gene



Aspergillus flavus α -amylase

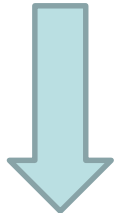


Starch



α -amylase
A. flavus

Glucose



Aflatoxin

Starch



Glucose

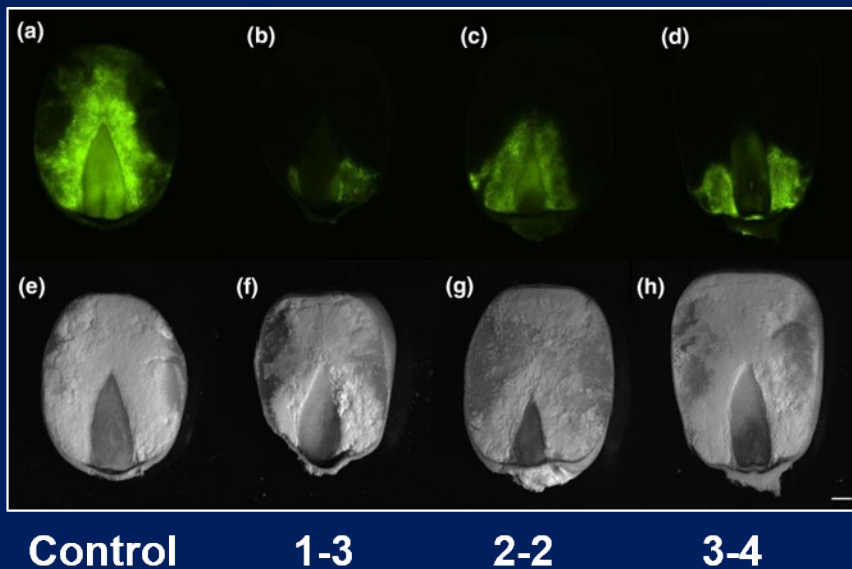


No aflatoxin

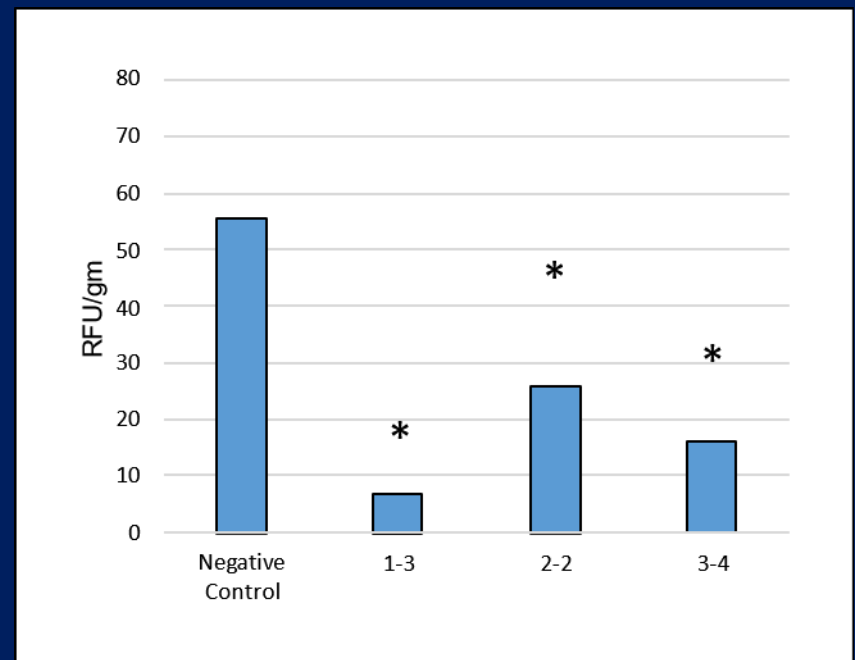
- α -amylase is an enzyme necessary for the breakdown of starch into glucose
- Mutant *A. flavus* strain lacking α -amylase cannot infect maize kernels or produce aflatoxin

Fungal Growth on *amy*-RNAi Transgenic Maize Seed

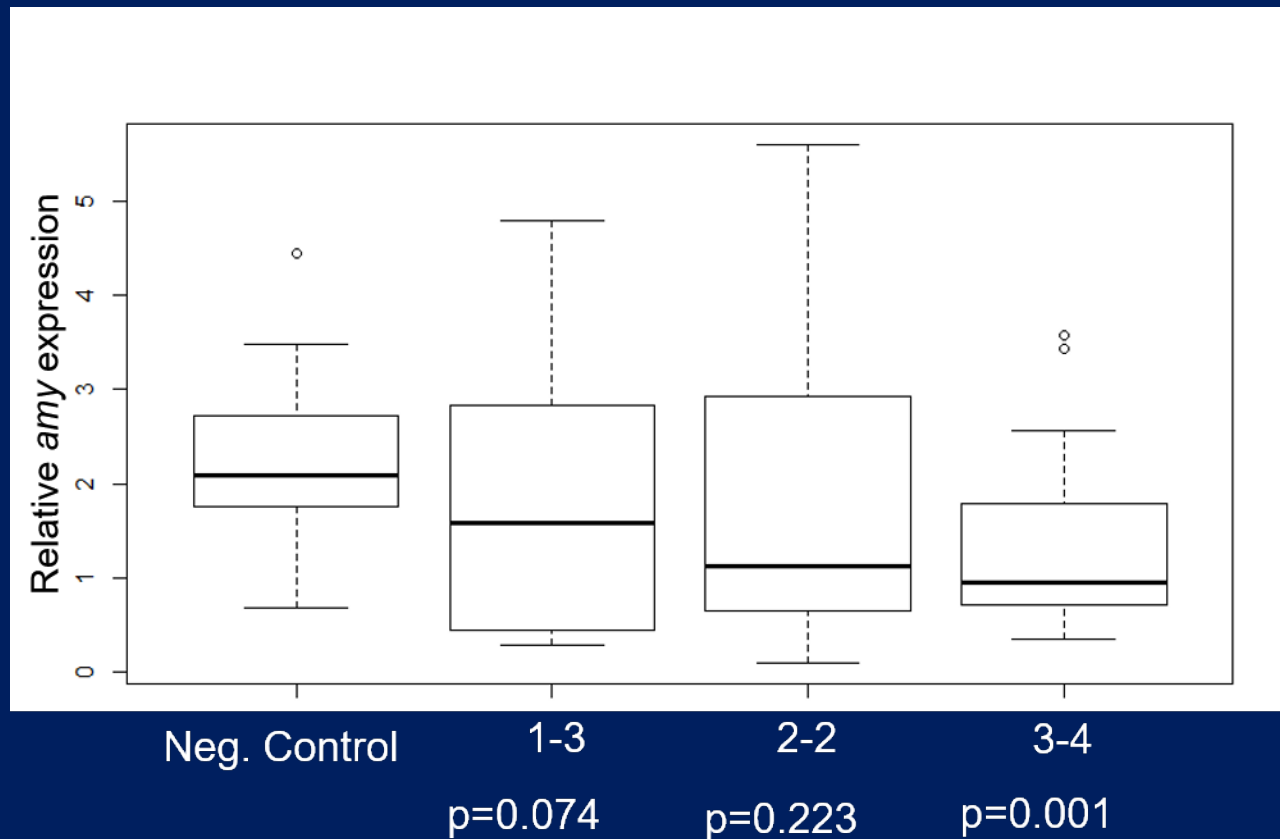
GFP Microscopy



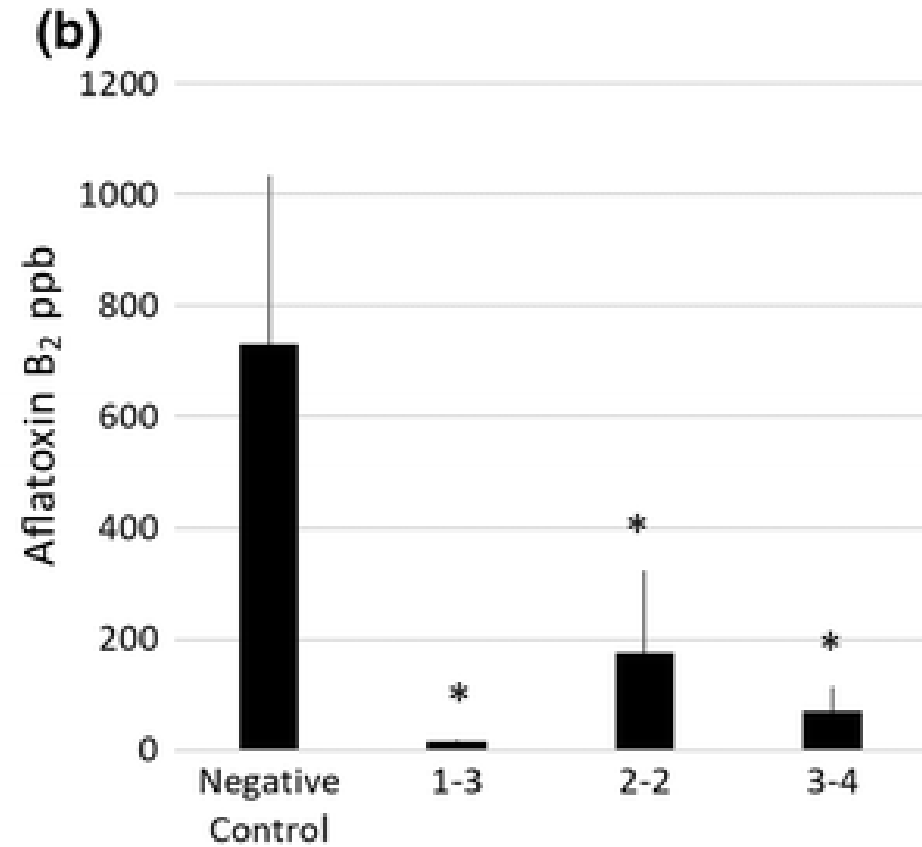
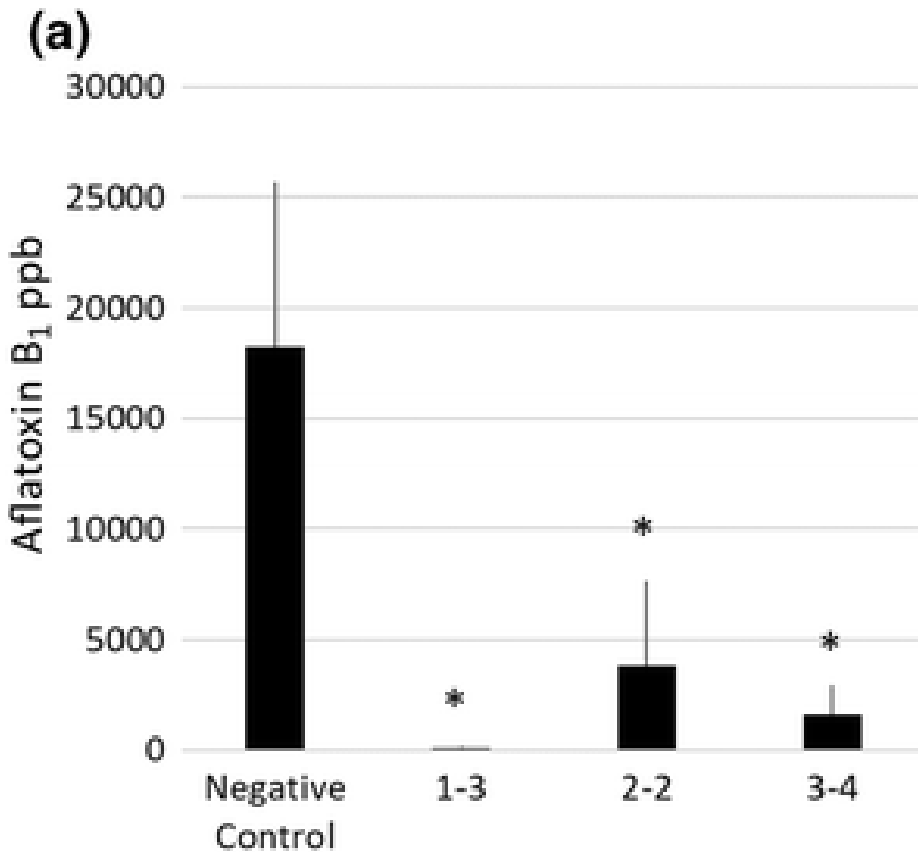
GFP Fluorometric Analysis



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



Reduced Aflatoxin levels in *amy*-RNAi transgenic kernels



Summary

1. Demonstrated the antifungal effects of α -amylase inhibitor protein from *Lablab purpureus* L. (AILP) in transgenic maize and its progenies against *Aspergillus flavus* infection resulting in reduced aflatoxin production.
2. Tachyplesin-based synthetic peptide AGM-182, when expressed in transgenic corn lines, demonstrated significant reduction in infection of *A. flavus* and aflatoxin levels.
3. Several native antifungal genes have been evaluated by gene silencing (e.g., PR-10, PRms, 14kDa TI) and their role(s) in imparting resistance to *A. flavus* confirmed.
4. Reduction in *A. flavus* growth and aflatoxin production correlated with the reduced expression of amylase in RNAi-*amy* transgenic corn lines.
5. HIGS of *A. flavus* genes *veA*, *nsdC*, *aflR*, *aflM*, either alone or in combination are under evaluation for fungal infection and/or toxin production in transgenic maize lines.

SRRC New BSL 2 Greenhouse



NEW ORLEANS, LA

USDA

SRRC
GREENHOUSE

SOUTH
AERIAL VIEW

WITH ROOF



JOHNSON McADAMS FIRM, PA



SHADOW TIME: 11:00 AM

Thanks to NPL, SEA Dir, and SRRC Dir



18 Dec 2018

Collaborators

Niranjan Baisakh, LSU

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