Aflatoxin Resistance in Food and Feed Crop Plants

Progress and Prospects in Classical and Molecular Approaches

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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 (how and why the fungus makes the toxin and how to prevent it, fungal development and Aflatoxin Biosynthesis, Regulation of Secondary Metabolism, Genomics)

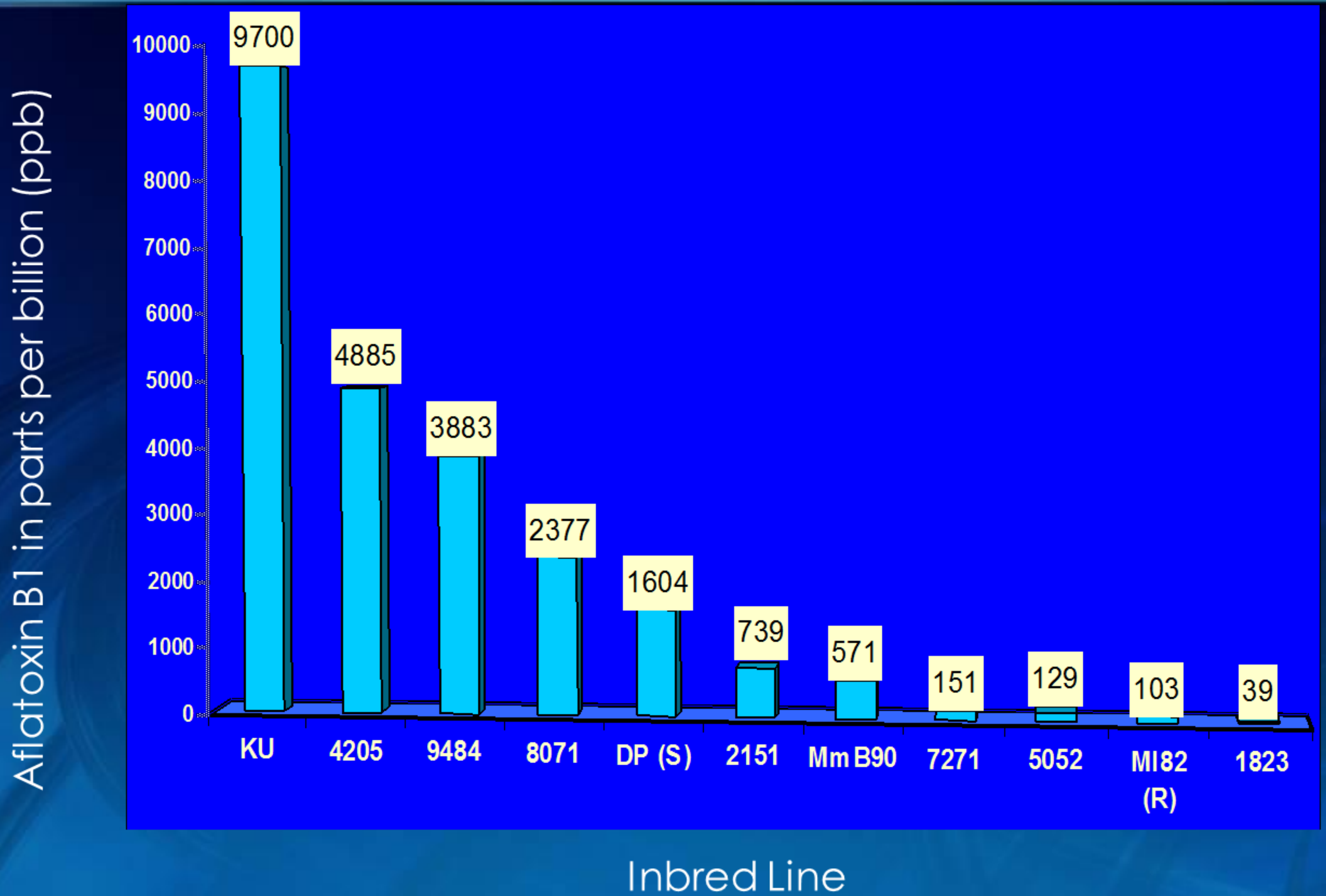
3. **Prevent the fungus from invading the crop and producing aflatoxin (changes in physiology of susceptible crops, enhancing Host Plant Resistance)**

Breeding for Resistance in Corn

Aspergillus flavus mycelial growth and sporulation on infected corn kernel

CIMMYT, 1987

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



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

Development of Resistant Maize Lines

IITA Lines crossed with US Lines

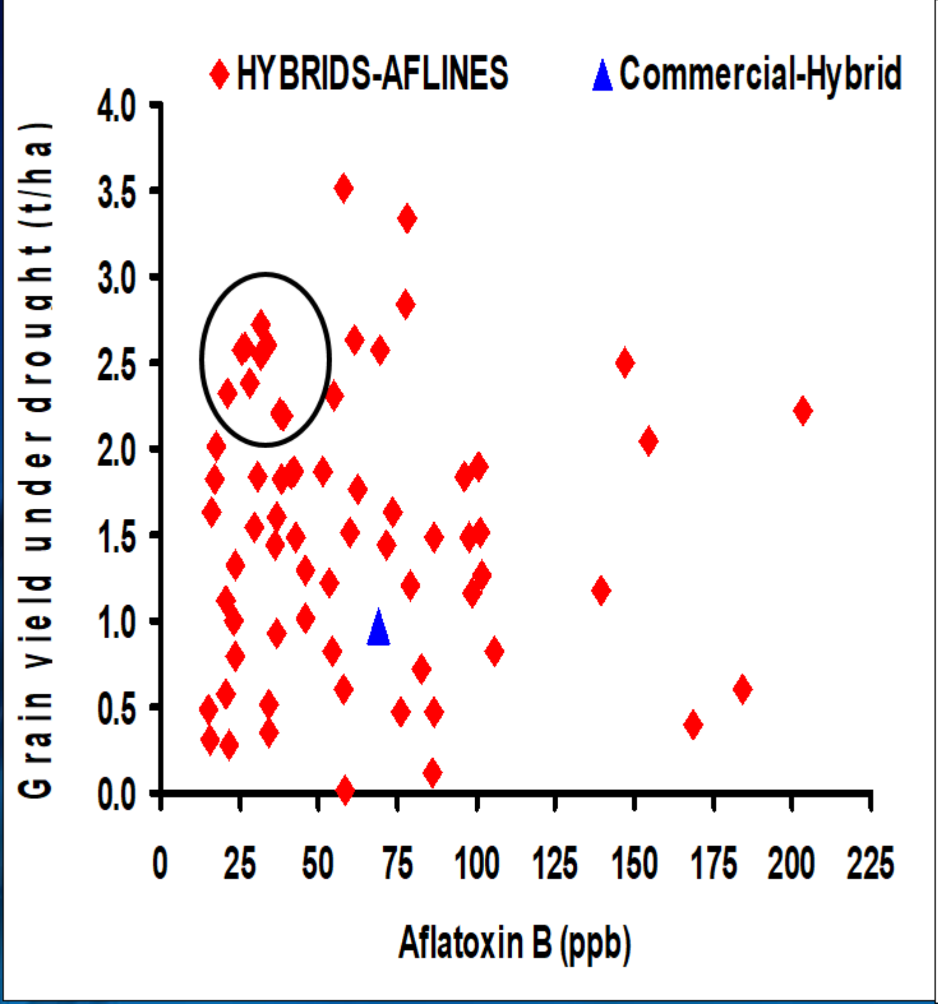
↓ 6 generations (agronomic characteristics)

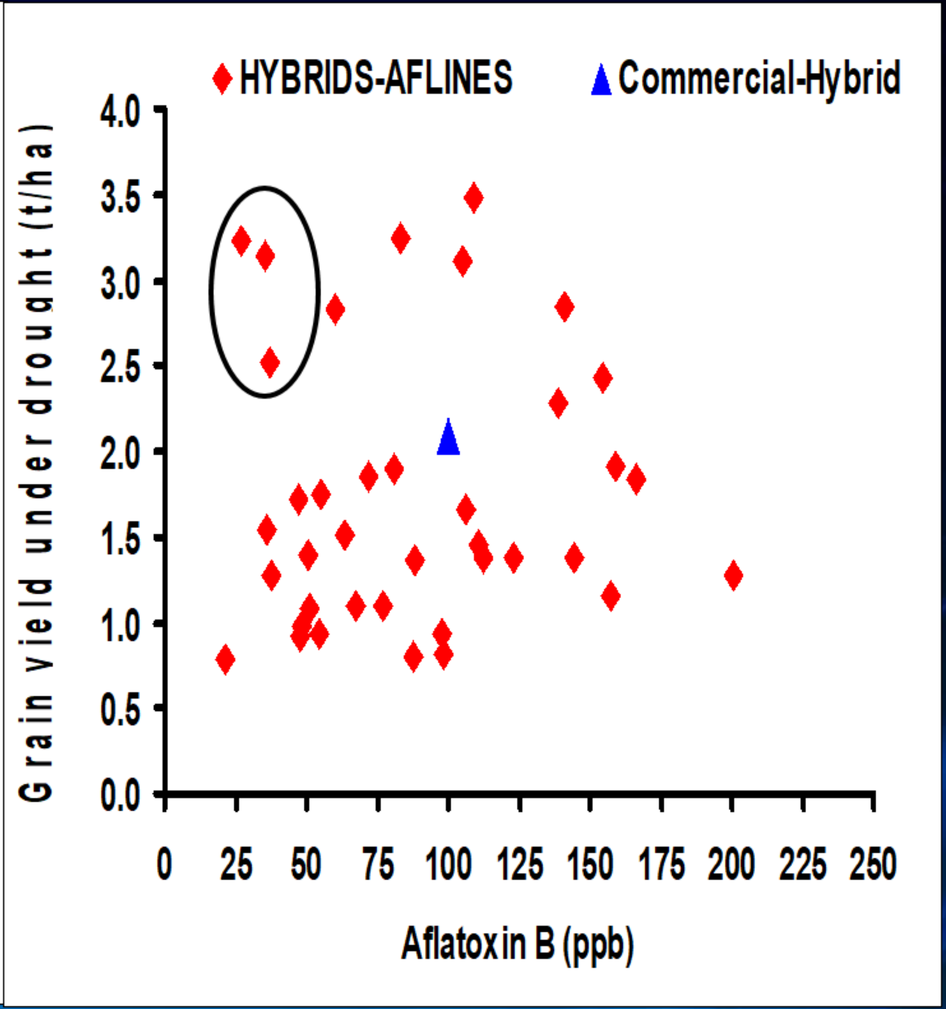
↓ 10 generations

Release of six resistance lines (TZAR 101-106)

Product Development: Breeding for resistance to aflatoxin accumulation

Mean aflatoxin values of hybrids formed from aflatoxin resistant lines with some levels of drought tolerance averaged over four locations in two trials.





Abebe Menkir, IITA

Combined resistance to aflatoxin and fumonisin production

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

	Fumonisin (ppm)	Aflatoxin-SRRC-USDA-KSA (ppb)		Aflatoxin-IITA-KSA (ppb)
Lines	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

Abebe Menkir, IITA

Aflatoxin Resistance in Cotton

Unlike maize, no known resistance mechanism in cotton germplasm

Options via Biotechnology is highly desired

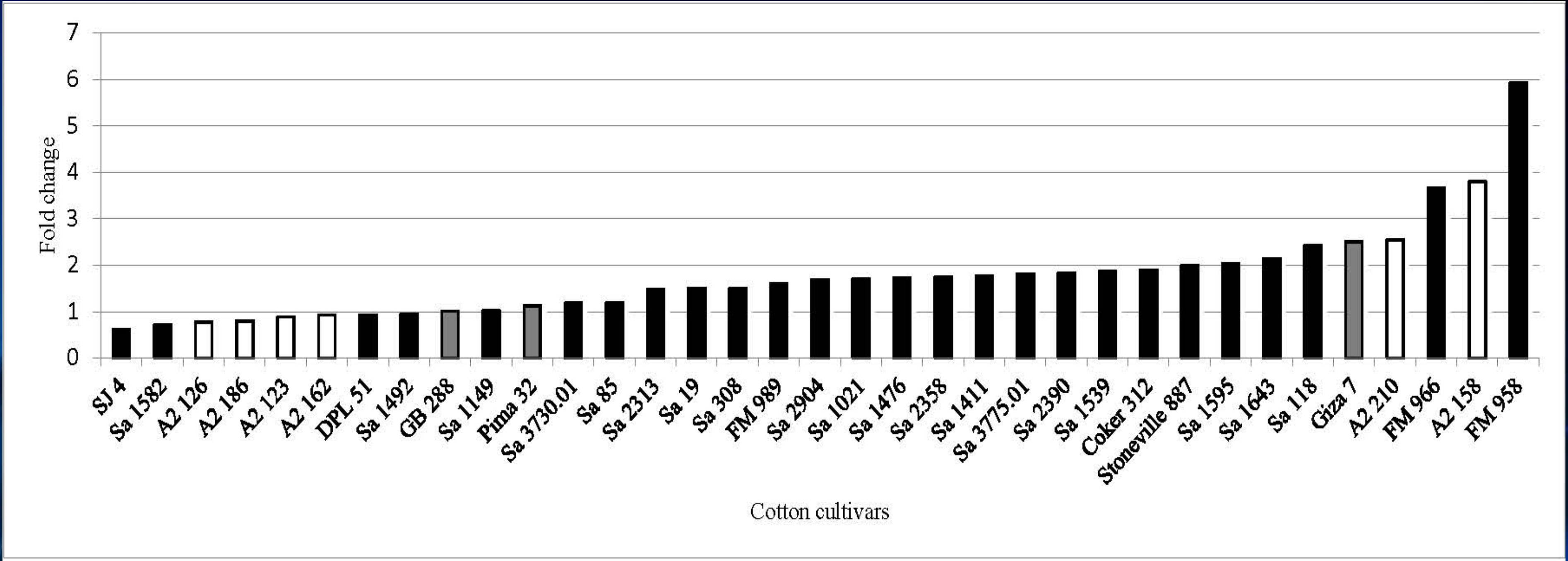


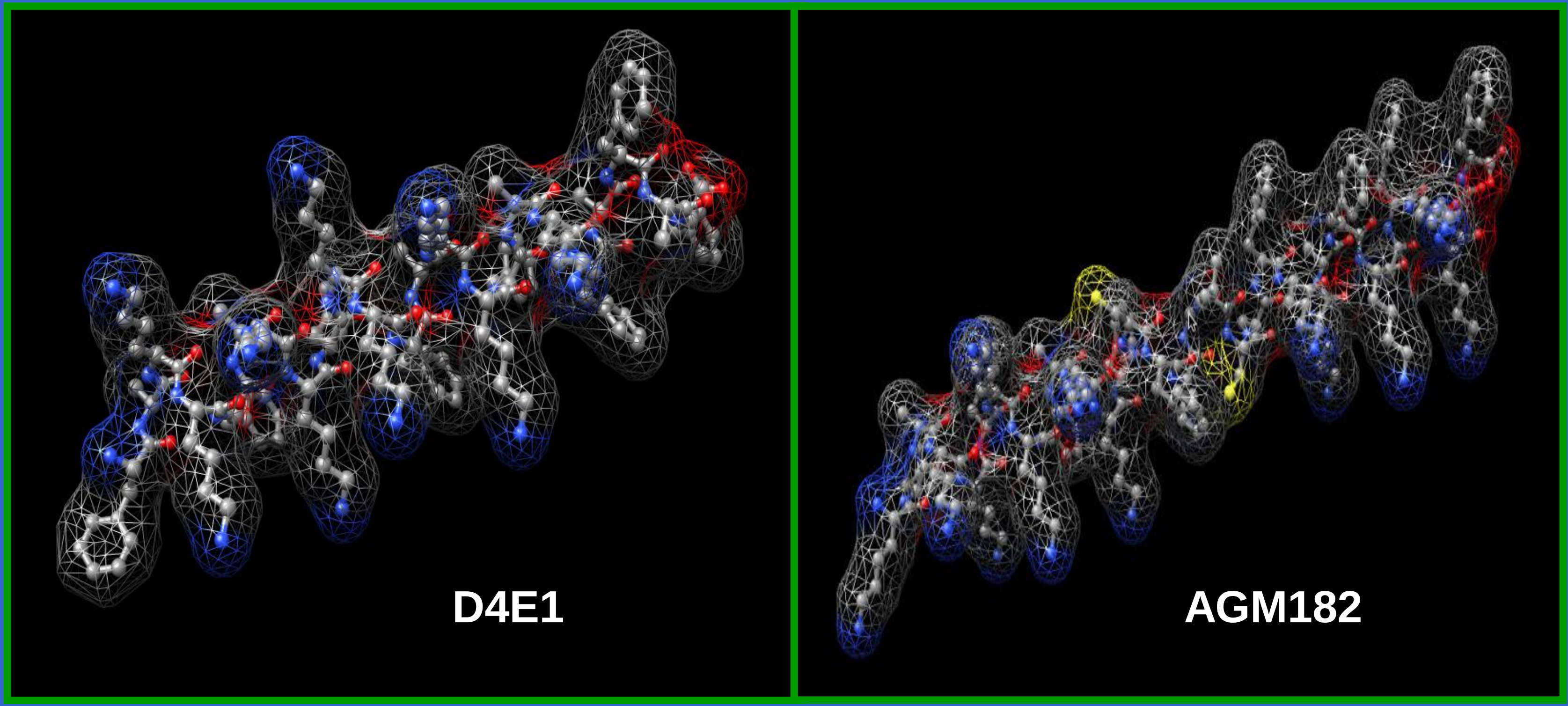
Figure 1: Preliminary screening of 36 varieties of cottonseeds to determine resistance and susceptibility to *A. flavus* 70-GFP infection. Varieties shown are from three different species, *G. arboreum* white bars, *G. barbadense* grey bars and *G. hirsutum* black bars. Fold change was determined by dividing the fluorescence values from infected cell extracted by that of control cell extract fluorescence.

Moore, Chlan

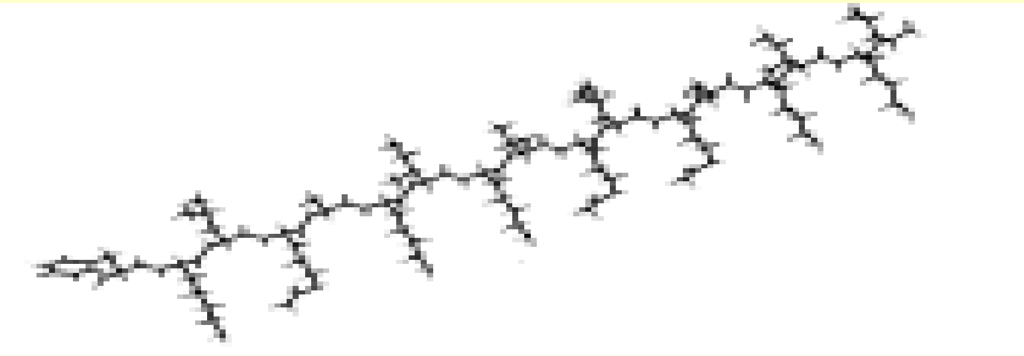
Incorporation of Aflatoxin Resistance into Crops

Control of saprophytic, opportunistic Aspergillus flavus requires unusual, novel genes

Synthetic peptides



D4E1



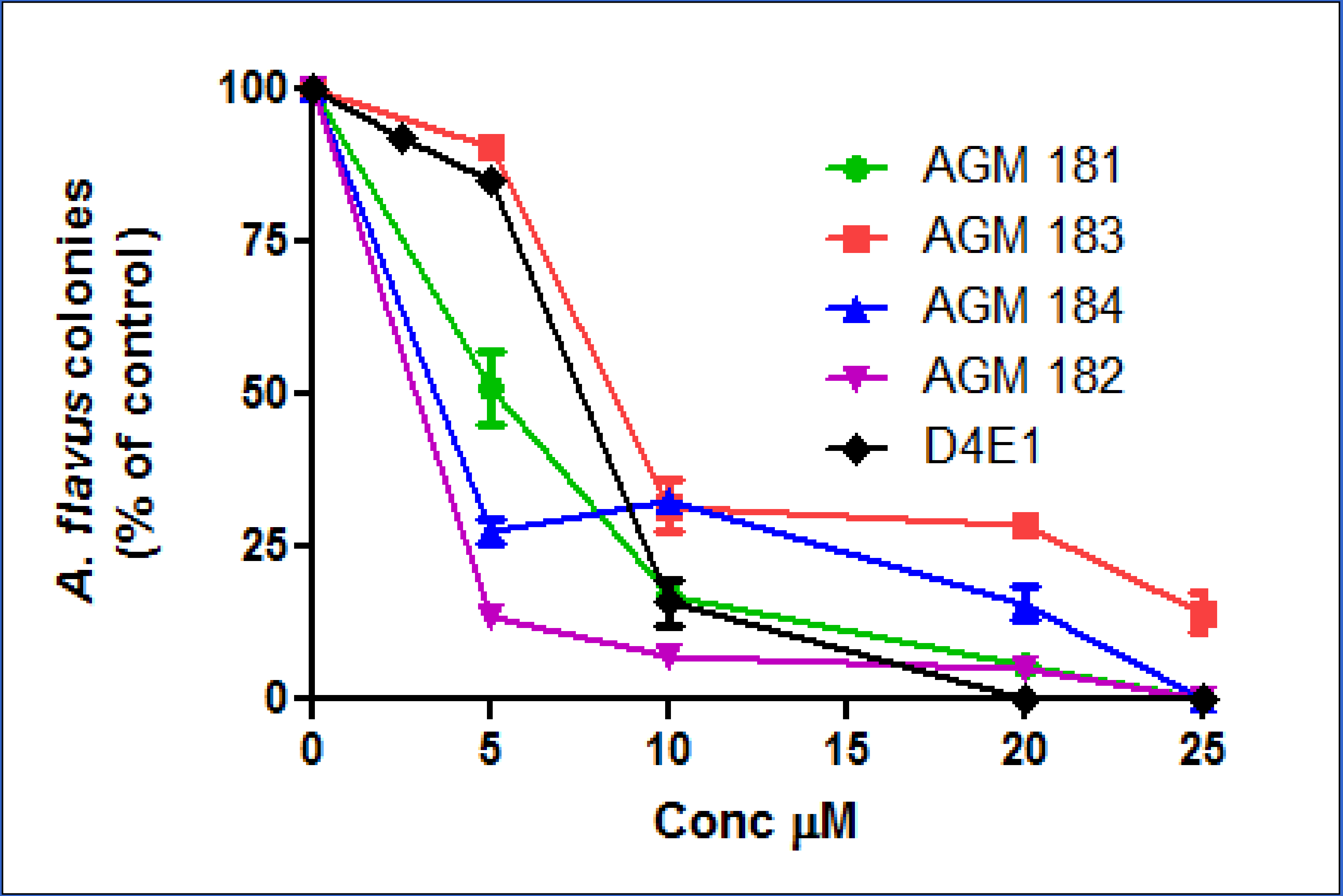
- Synthetic, linear, amphipathic, lytic peptide
- ~60 bp gene; product 17 amino acid peptide
- Effective *in vitro* and *in planta* against several microbial pathogens including *A. flavus*
- Fairly resistant to protease activity
- non-toxic, non-hemolytic

Broad spectrum antimicrobial activity of D4E1 *in vitro*

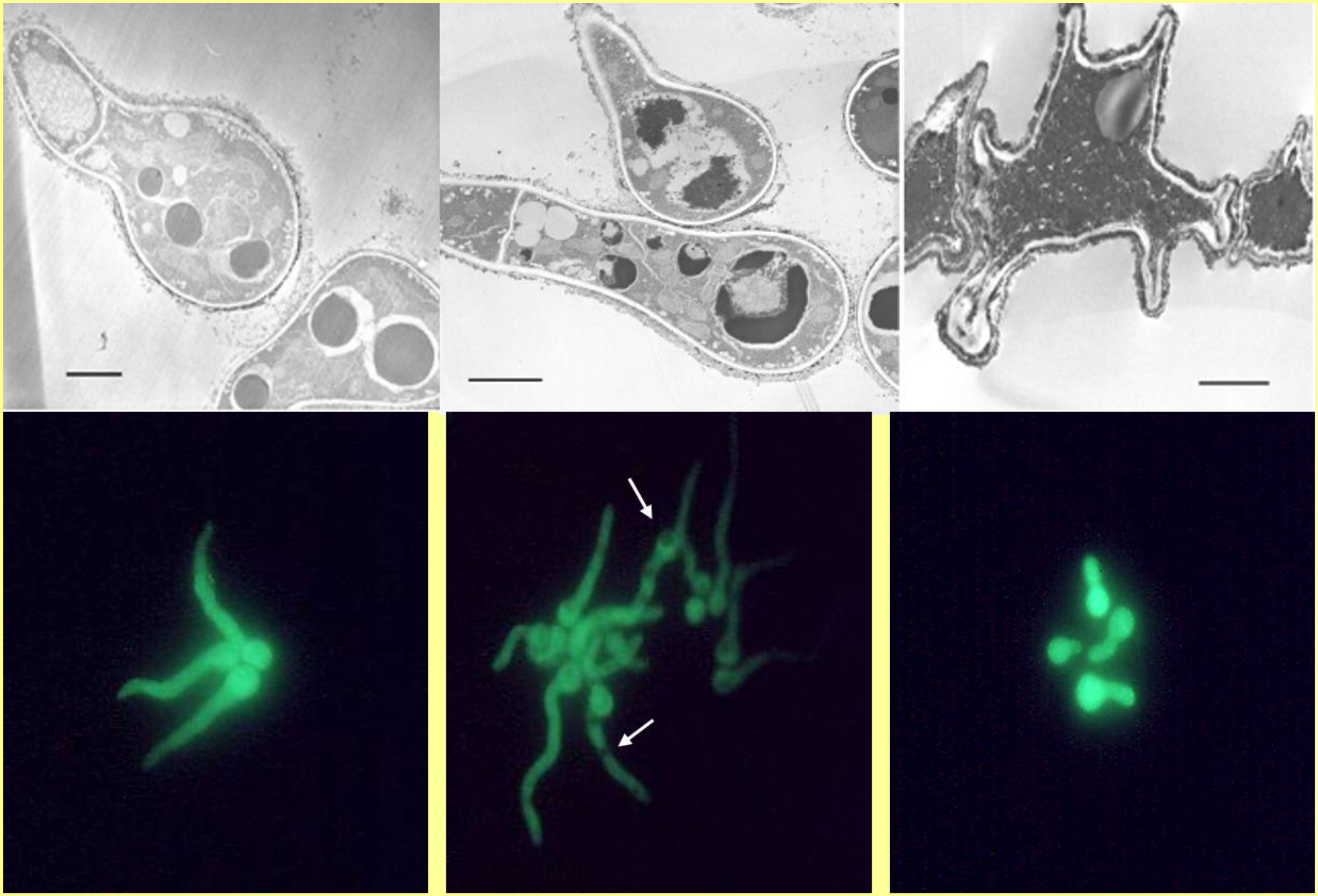
Phytopathogen	IC_{50} (μM)	MIC (μM)
Alternaria alternata	12.39	>25.0
Aspergillus flavus	7.75	25.0
Aspergillus flavus 70-GFP	11.01	25.0
Cercospora kikuchii	8.67	>25.0
Colletotrichum destructivum	13.02	>25.0
Claviceps purpurea	1.60	20.0
Fusarium graminearum	2.10	25.0
Fusarium moniliforme	0.88	12.5
Fusarium oxysporum	2.05	12.5
Penicillium italicum	5.92	>25.0
Phytophthora cinnamomi	nd	4.67
Phytophthora parasitica	nd	4.67
Pseudomonas syringae pv. *tabaci*	0.52	2.25
Pythium ultimum	nd	13.33
Rhizoctonia solani	nd	26.7
Thielaviopsis basicola	0.52	6.0
Verticillium dahliae	0.60	5.25
Xanthomonas campestris pv. *malvacearum*	0.19	1.25

nd = not determined

AGM and D4E1 on *Aspergillus flavus*

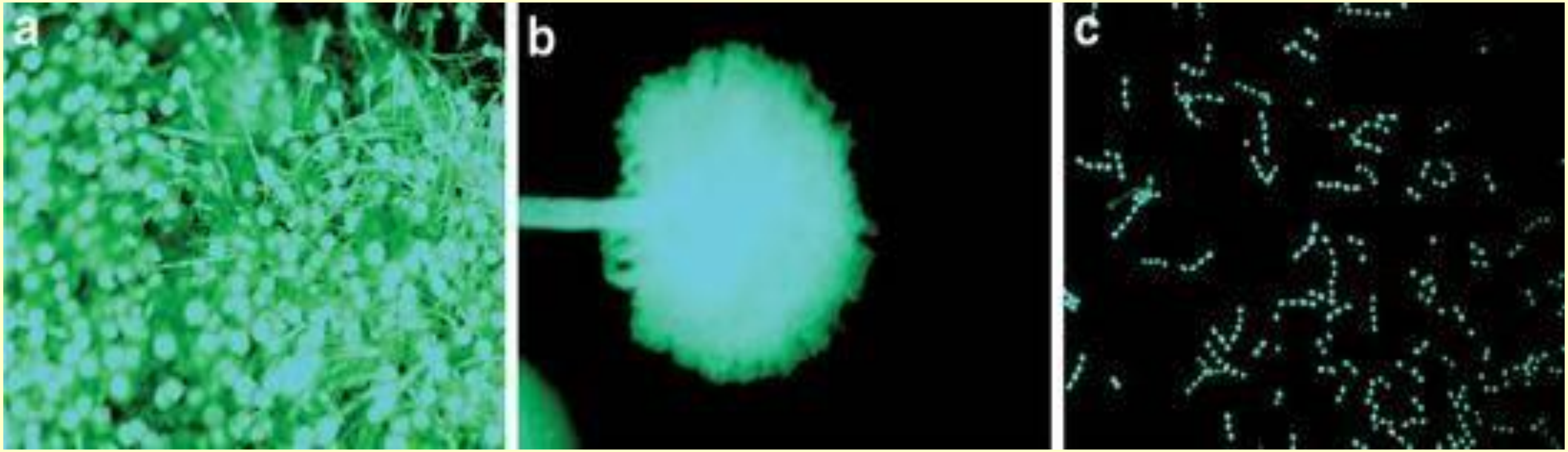


Effects of D4E1 on germinating *A. flavus* conidia

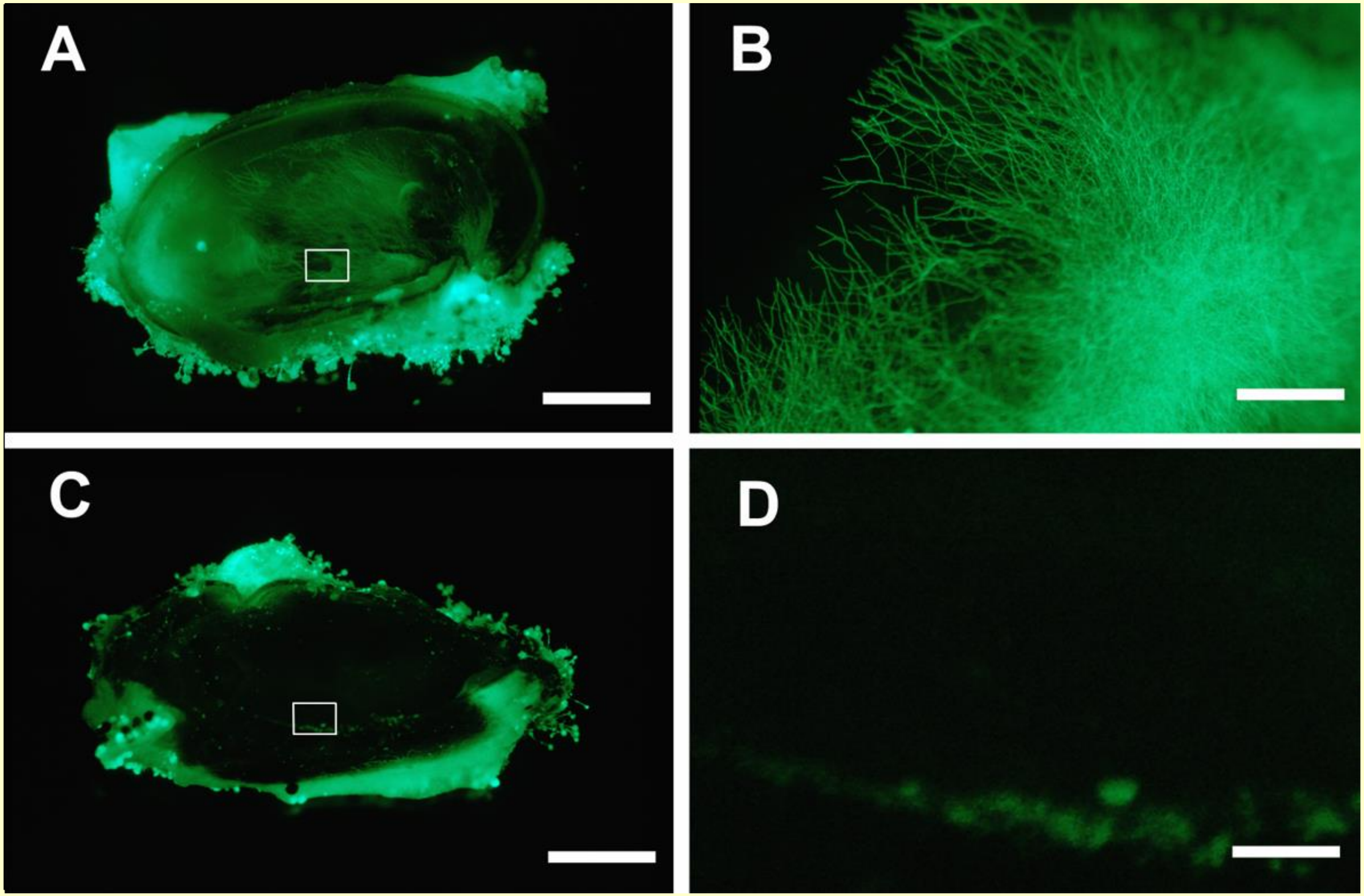


0 μM　　12.5 μM　　25.0 μM

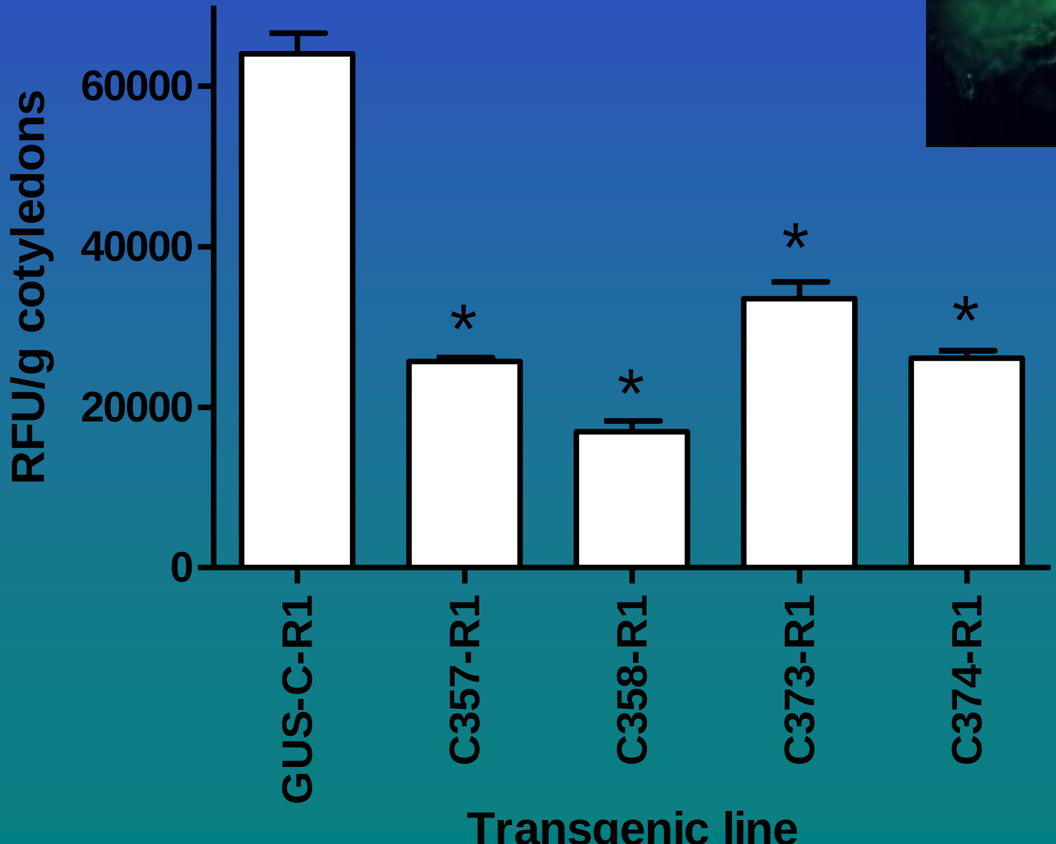
GFP-*A. flavus* to evaluate resistance

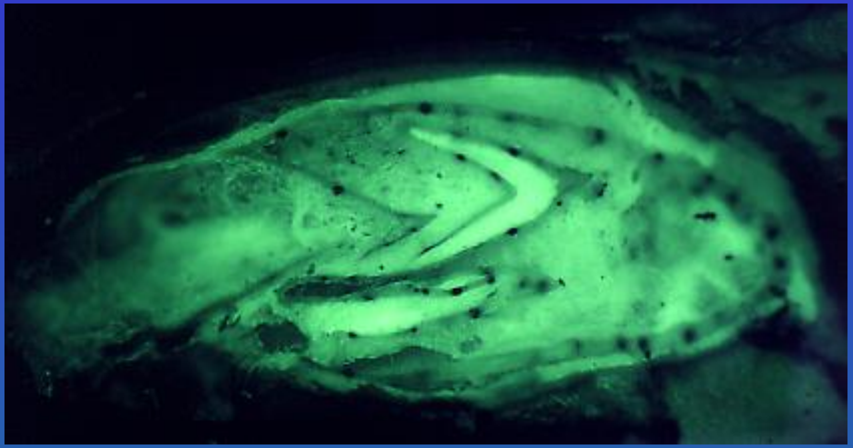


Growth of *A. flavus* 70-GFP on inner seed coat of cotton transformed with antimicrobial peptide D4E1



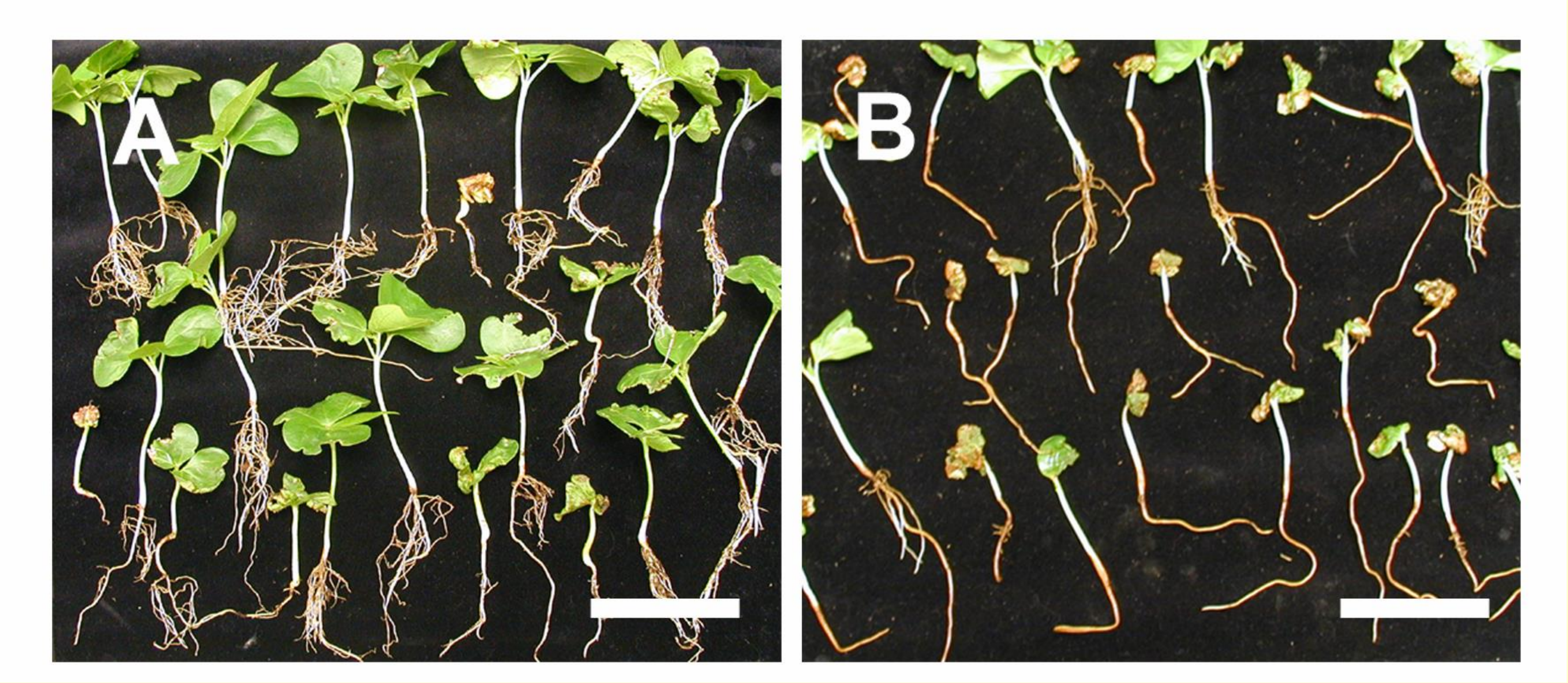
GFP-*A.flavus* 70 infected cotyledons





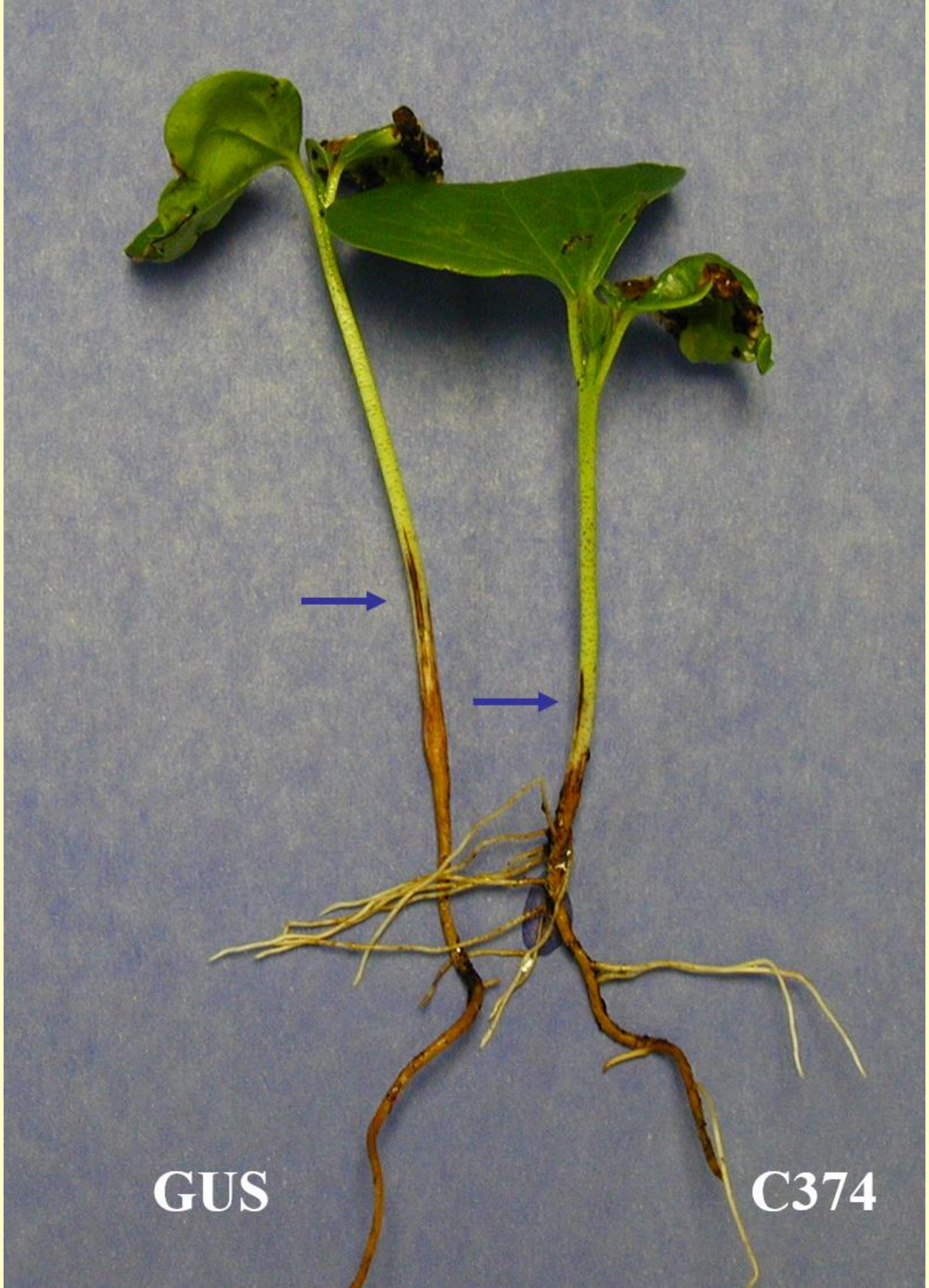
Fluorescence is highly correlated to aflatoxin production in this strain

In planta transgenic cotton seedling assay for resistance to *Thielaviopsis basicola*



C374 **GUS**

Black Root rot by *Thielaviopsis basicola*



Field trials of transgenic D4E1 cotton in Shafter, CA Tuskegee, AL and Maricopa, AZ

Tolerance to seedling disease complex demonstrated in these locations

Cotton - Summary

- **Synthetic peptides offer broad-spectrum control of microbial phytopathogens.**
- **Developing tolerance to saprophytic *Aspergillus* (and preharvest aflatoxin contamination) means resistance to several other pathogens as well in field tests.**
- **Non-target organisms are not affected.**
- **Other potential genes identified through genome-wide transcriptomic analysis through RNAseq are under evaluation** (e.g., spot11 catalase).

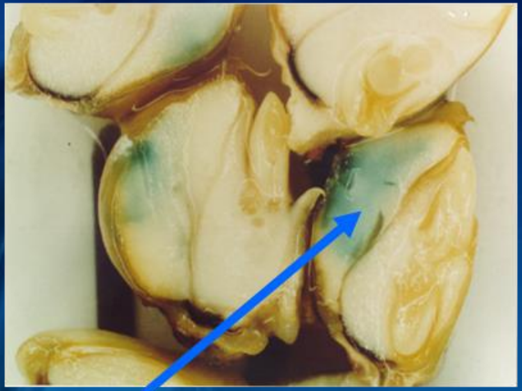
Resistance to Aflatoxin Contamination in Corn

- Identify native antifungal proteins by proteomics, or genome-wide transcriptomics.
- Evaluate the roles of antifungal proteins by silencing. Overexpress the promising candidate proteins
- Introduce safe, heterologous proteins from other species including synthetic peptides
- Improve host resistance through host induced gene silencing of *flavus* genes

CORN PROTEOMICS
(ARS-SRRC/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

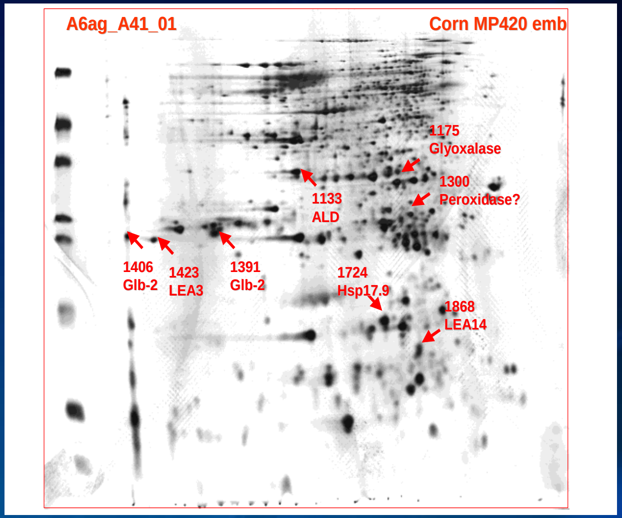
Susceptible



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.

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*

Evaluation of native antifungal genes by gene silencing

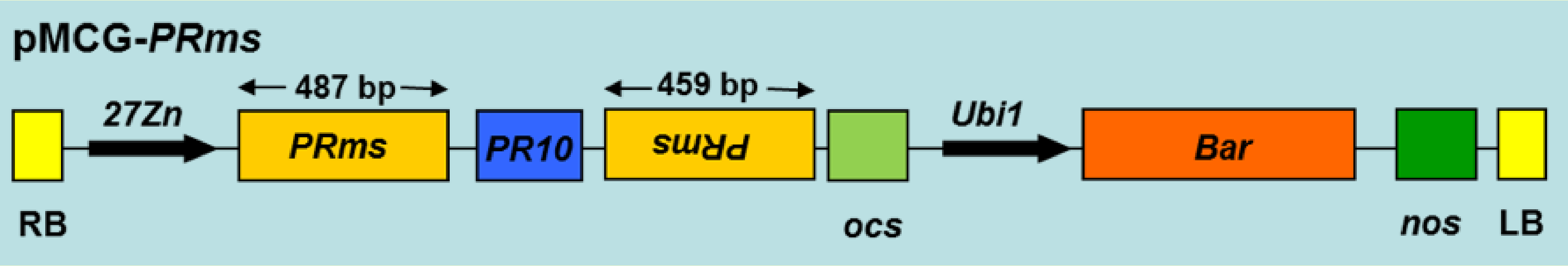
14 kDa TI

PR-10

β-1,3-glucanase (PR-2)

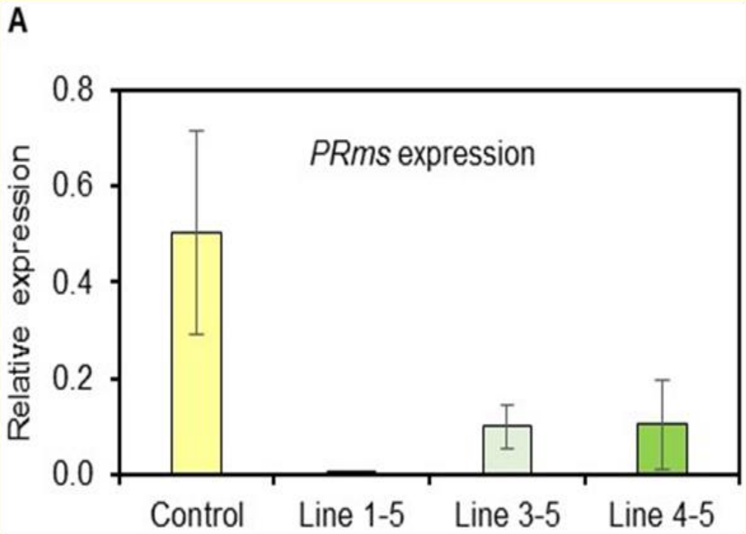
PRms

Maize *PRms* gene and *PRms*-RNAi construct design

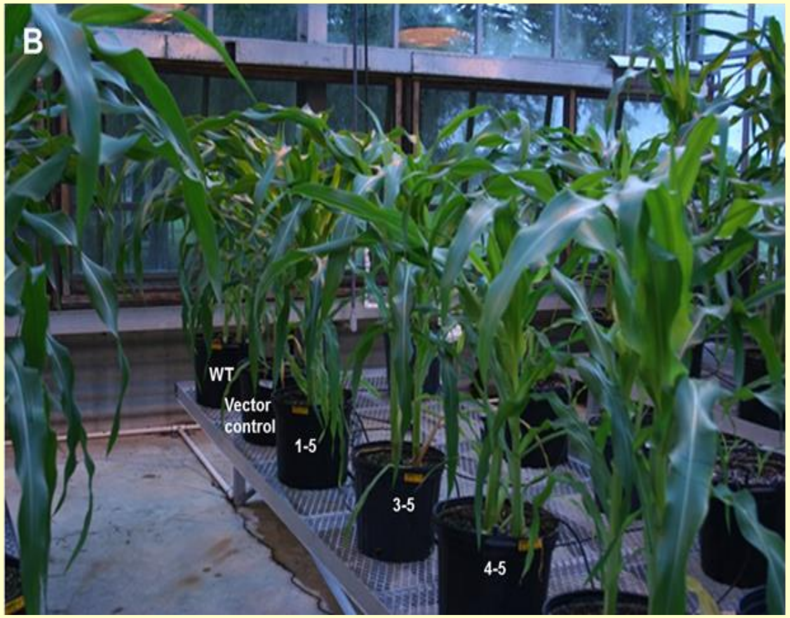


27*Zn* - corn endosperm specific promoter, PR10 - intron, *Ubi*1 - constitutive promoter, *Bar* - bialaphos resistance gene, *ocs* and *nos* - terminators.

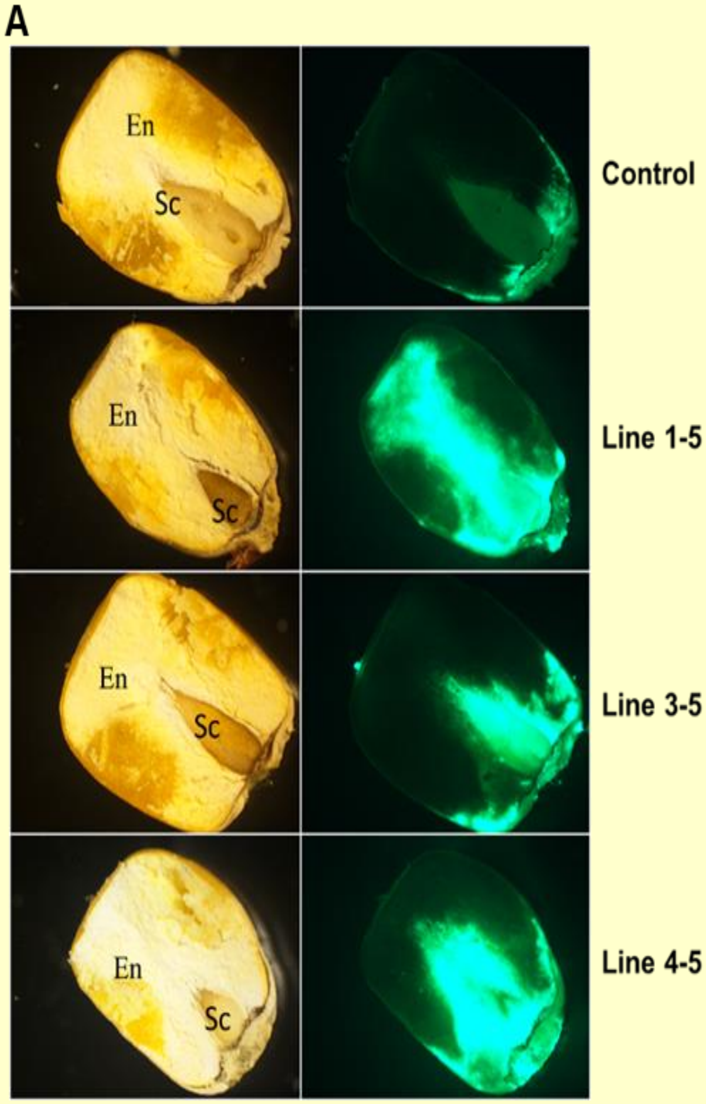
Seed specific silencing of *ZmPRms* gene does not have any negative effect on plant growth or kernel phenotype

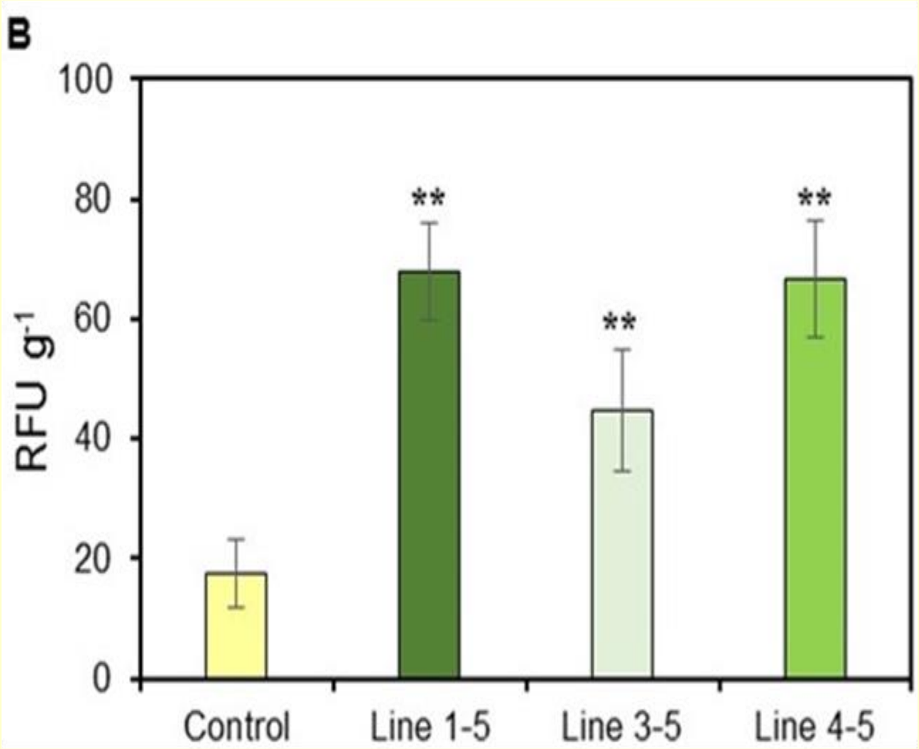


Plants	Seed wt (g/ seed)
WT	0.285 ± 0.013
Vector control	0.249 ± 0.011
Line 1-5	0.288 ± 0.010
Line 3-5	0.362 ± 0.008
Line 4-5	0.284 ± 0.009

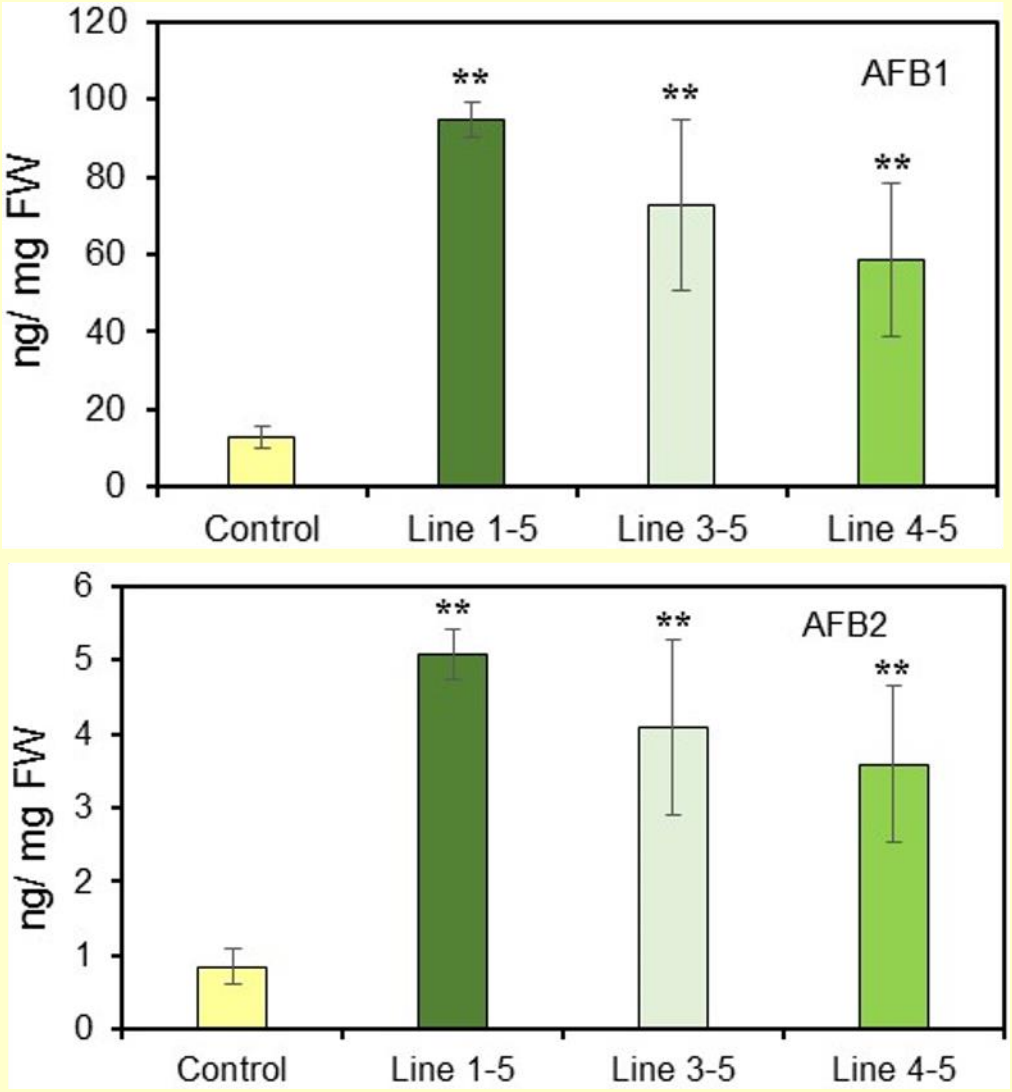


Silencing of *ZmPRms* in kernels significantly increases fungal growth in *PRms*-RNAi lines

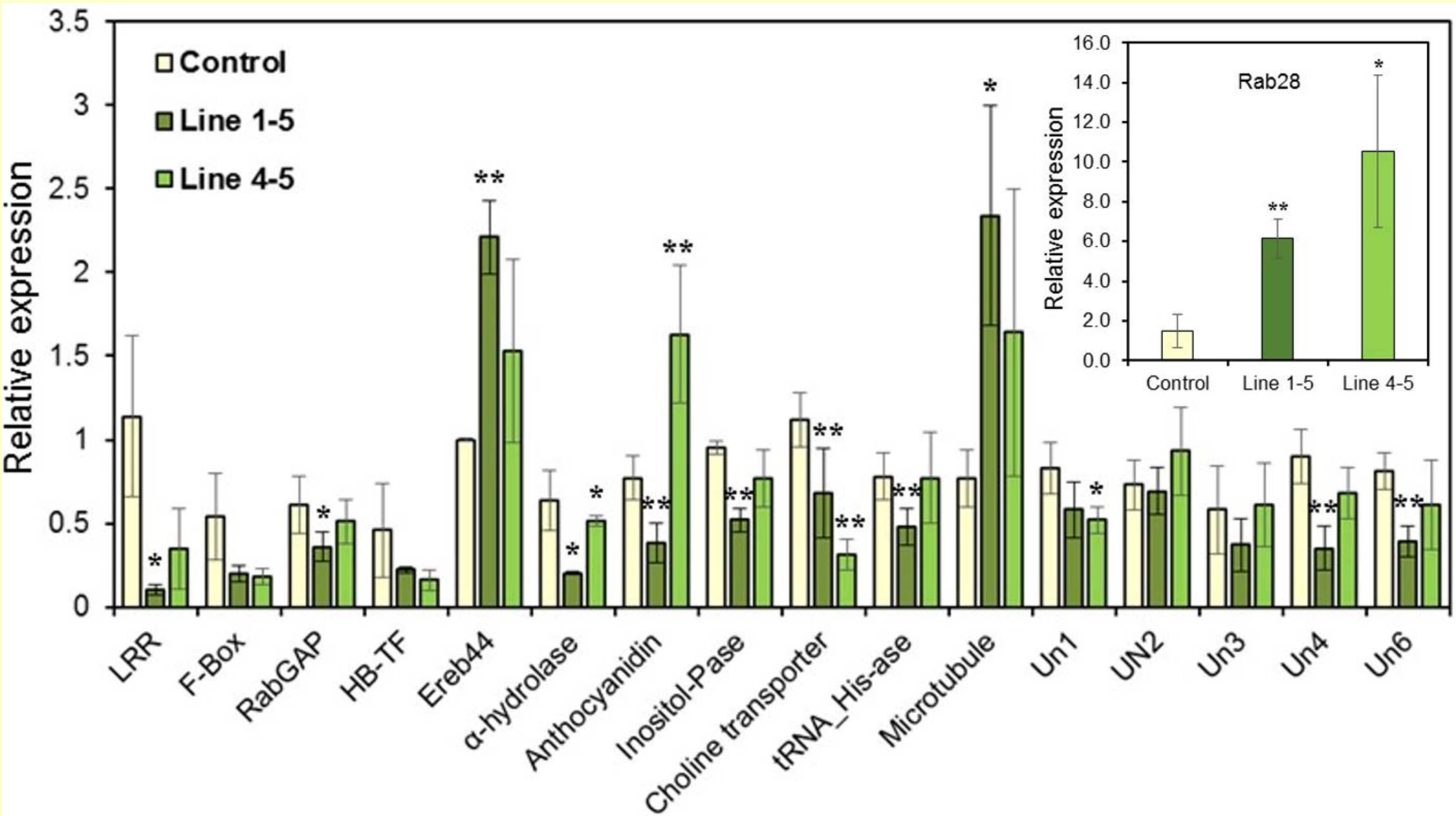




Aflatoxins are significantly increased in kernels of *ZmPRms*-RNAi lines vs. control upon *A. flavus* infection



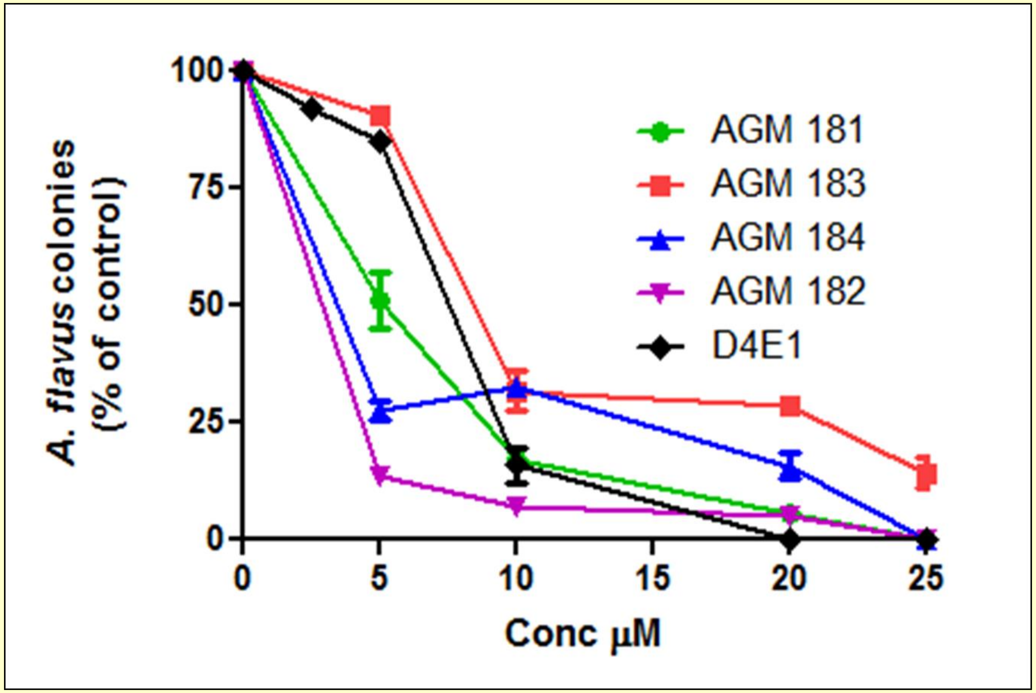
Gene regulatory network analysis: identification and validation of predicted *ZmPRms* regulated downstream genes in the *ZmPRms*-RNAi lines vs. control



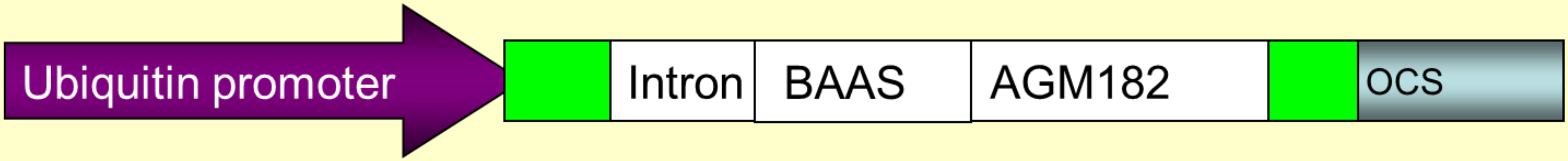

Transgenic maize to express heterologous genes

New Generation of Designed Peptide AGM 182

- Effective at lower concentrations than D4E1
- Killed germinated spores of *Fusarium verticillioides* and *Verticillium dahliae* at 5-20 µM concentrations
- 18 amino acids – 5 lysine
- Potential to improve protein nutrition of kernel

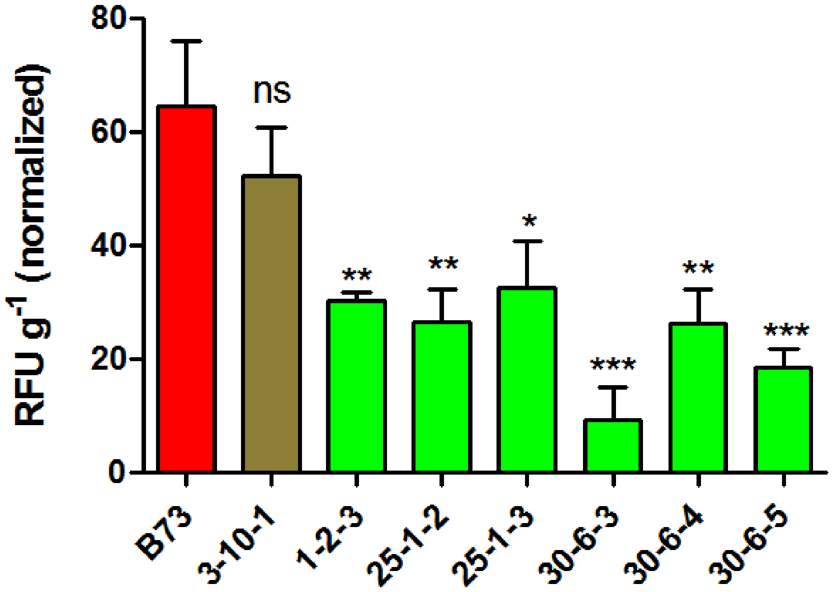


Next Generation Peptide AGM182



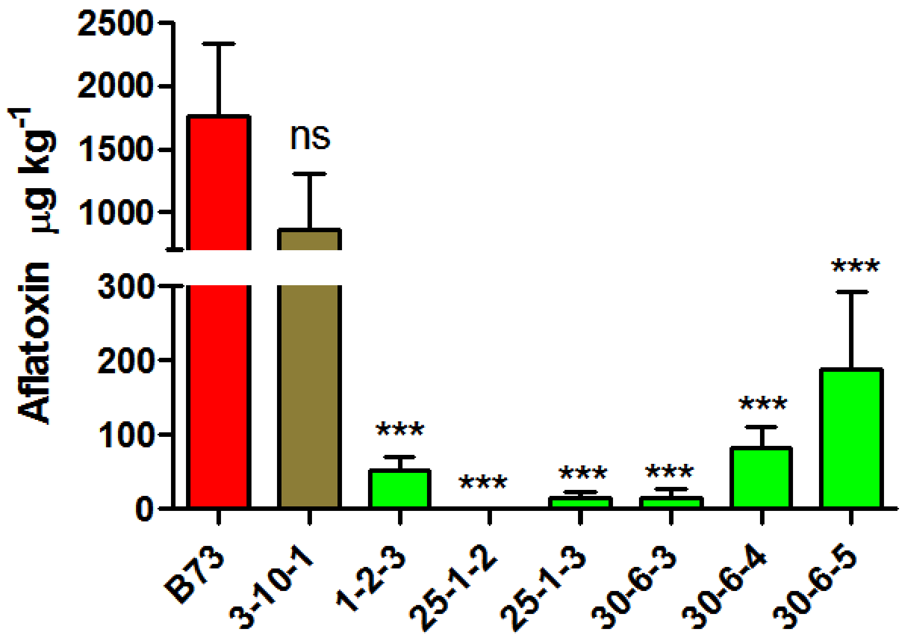
- **<u>pMCG1005 with Ubiquitin promoter and intron (Adh1)</u>**
  - Codon optimized for expression in corn
  - Improved expression in monocots
  - Expression in silks and husk tissues
  - Ease of early stage testing
  - (BAAS) for excretion to the apoplast
  - Potential for resistance to foliar and root pathogens

A. flavus growth / Aflatoxin production

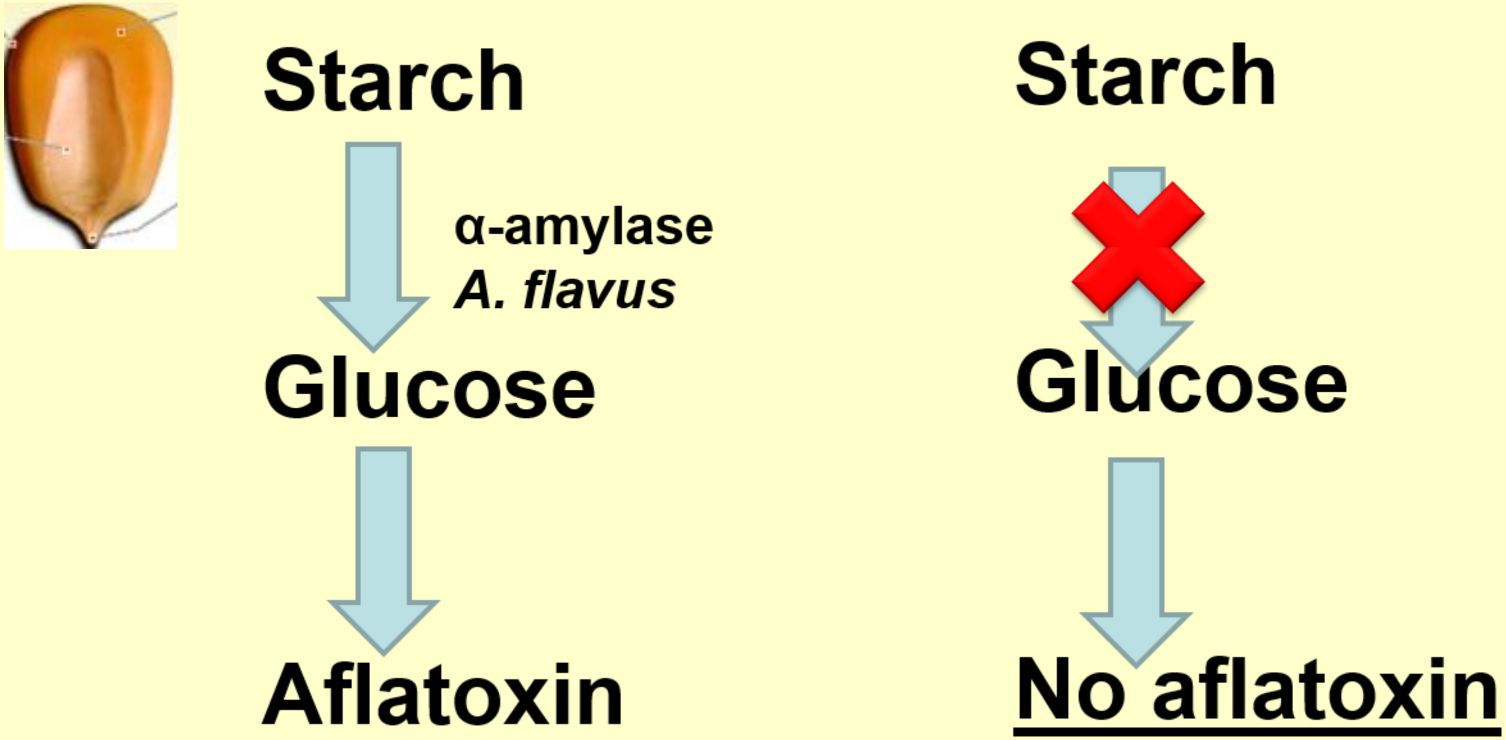


A. flavus growth

Aflatoxin production

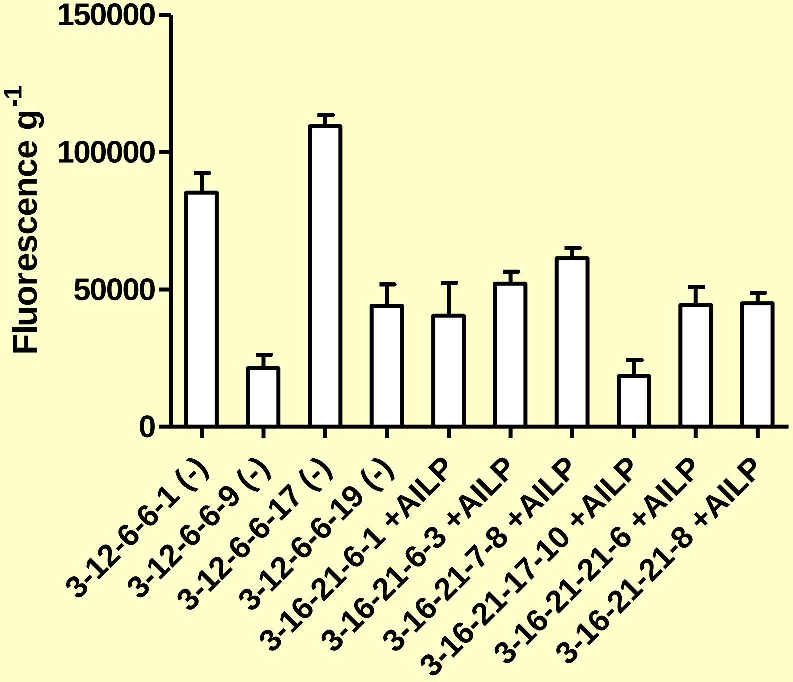


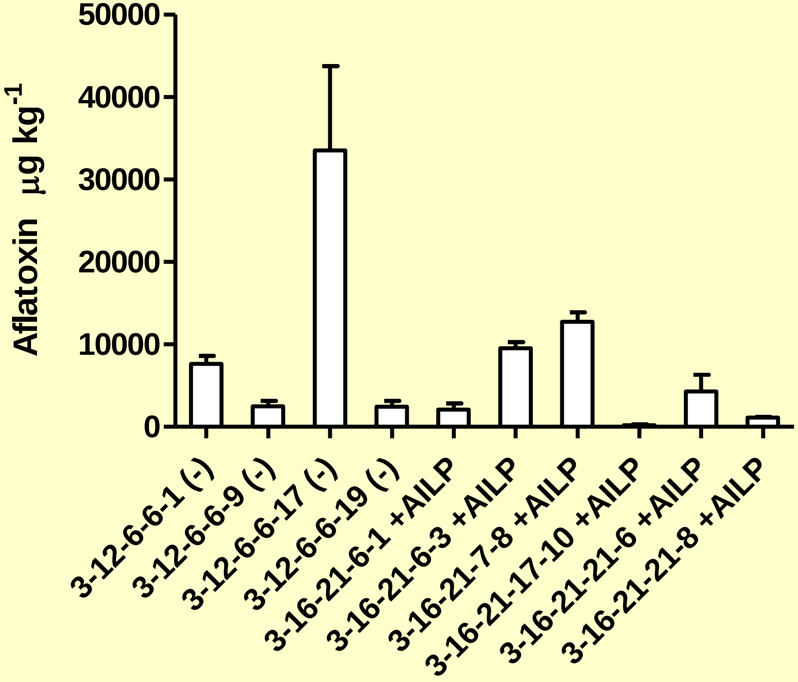
Aspergillus flavus α-amylase



- 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 - Fakhoury, Woloshuk et al. 1999

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

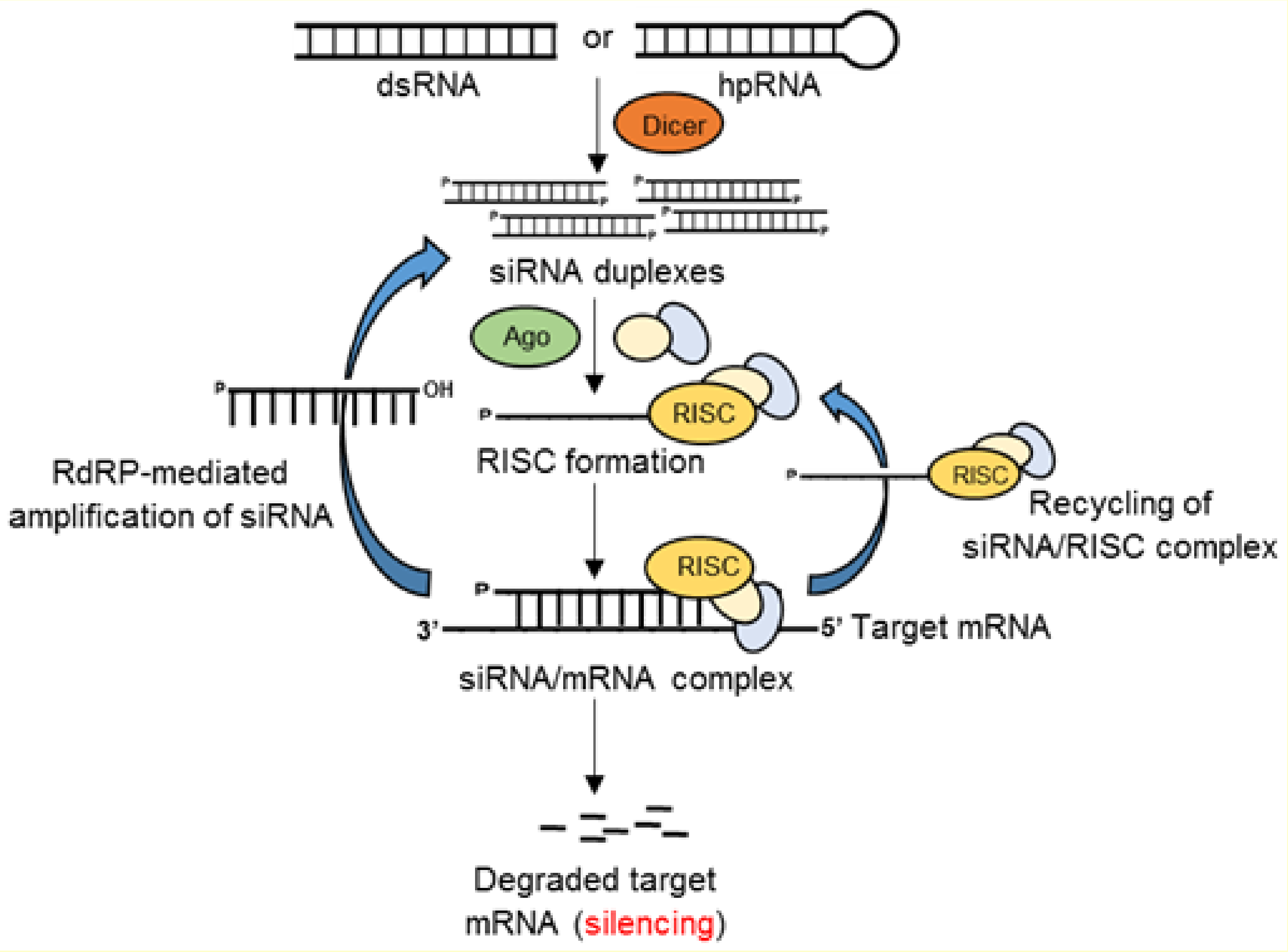




Host Induced Gene Silencing (HIGS)

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

Host Induced Gene Silencing (HIGS)



Majumdar et al. Frontiers in Plant Science 2017

Aspergillus flavus α-amylase

Starch

↓ α-amylase *A. flavus*

Glucose

↓

Aflatoxin

Starch

✗

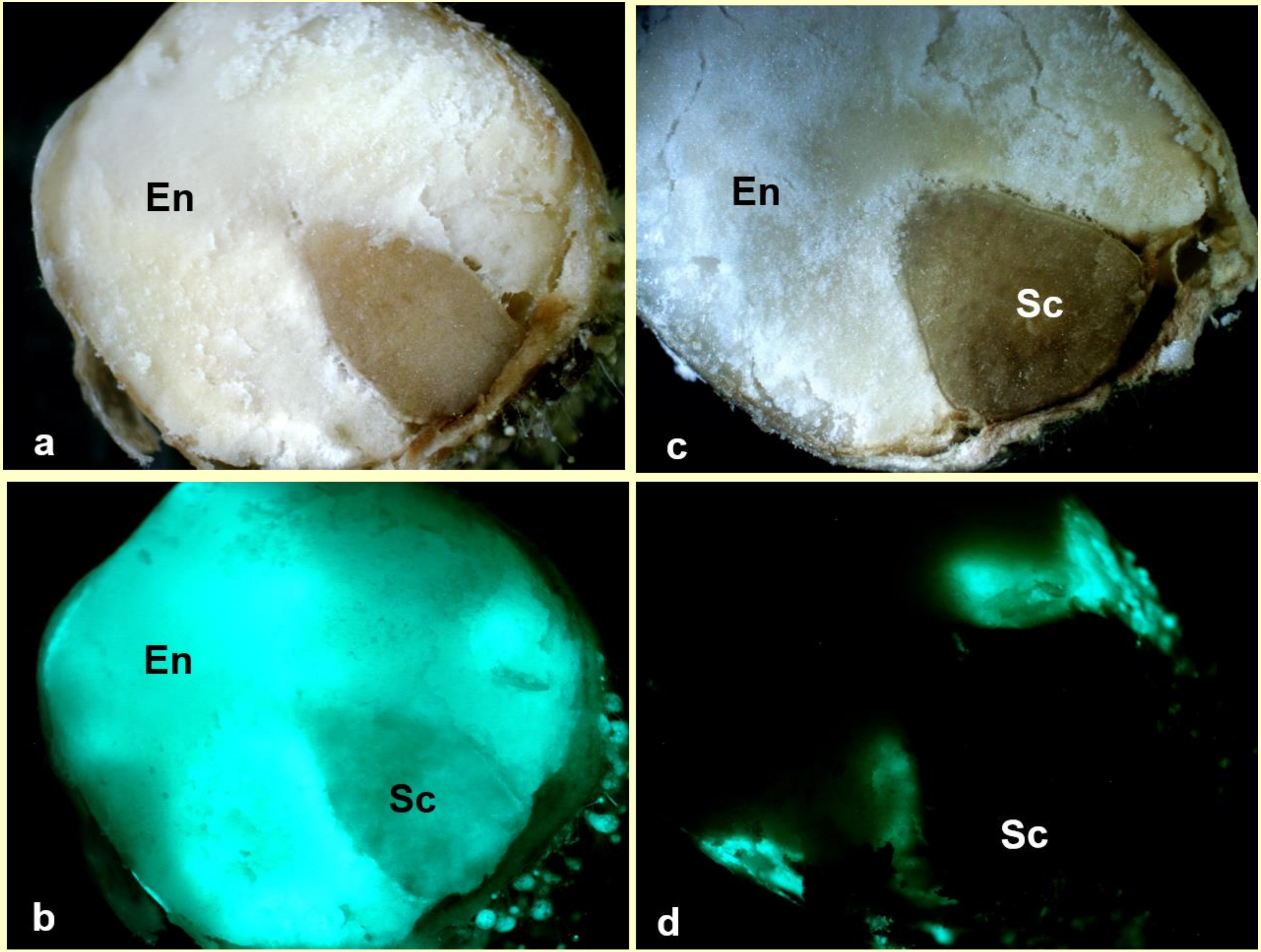
Glucose

↓

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 - Fakhoury, Woloshuk et al. 1999

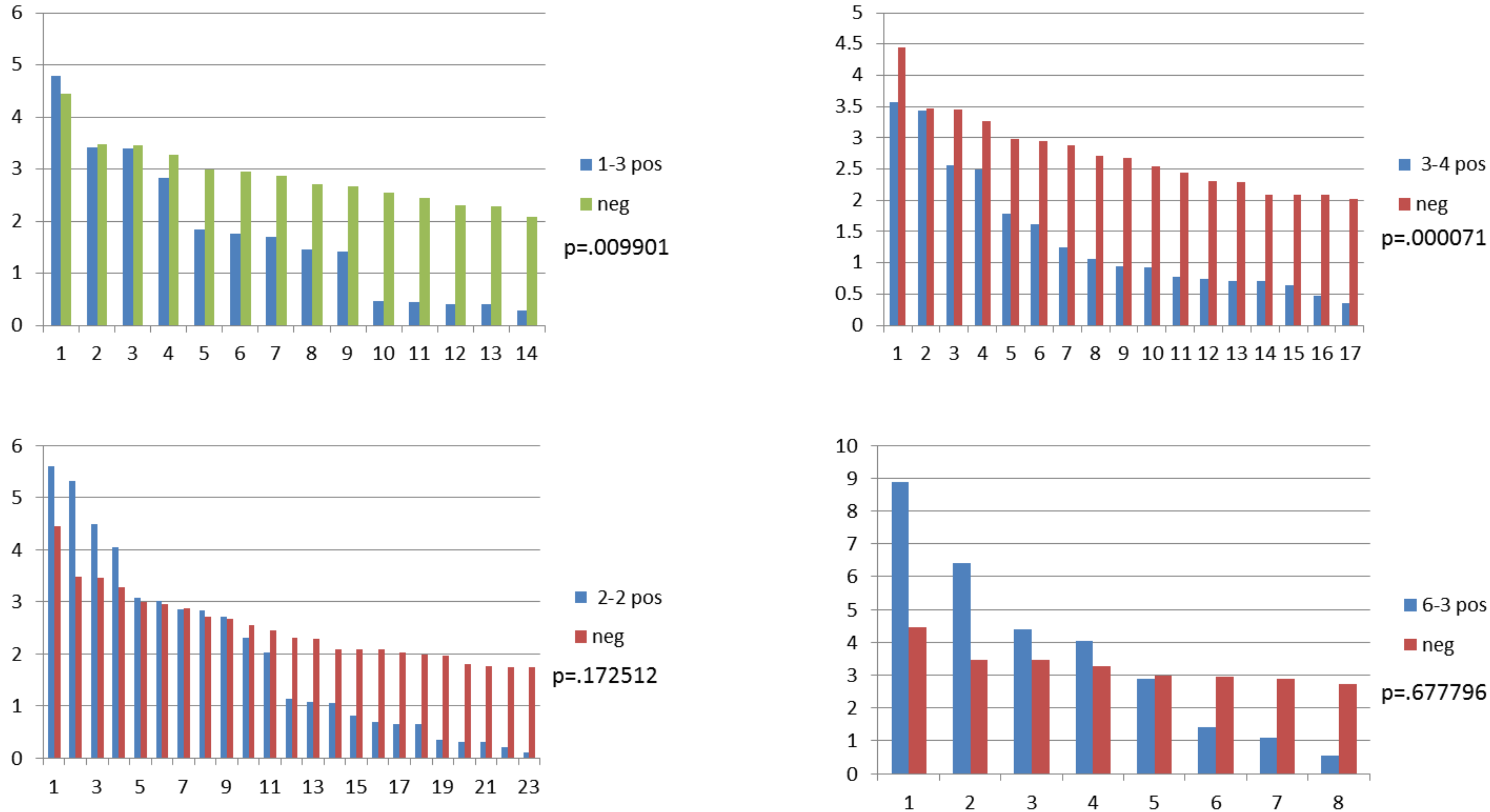
Fungal Growth in Transgenic Kernels (*amy*-RNAi)



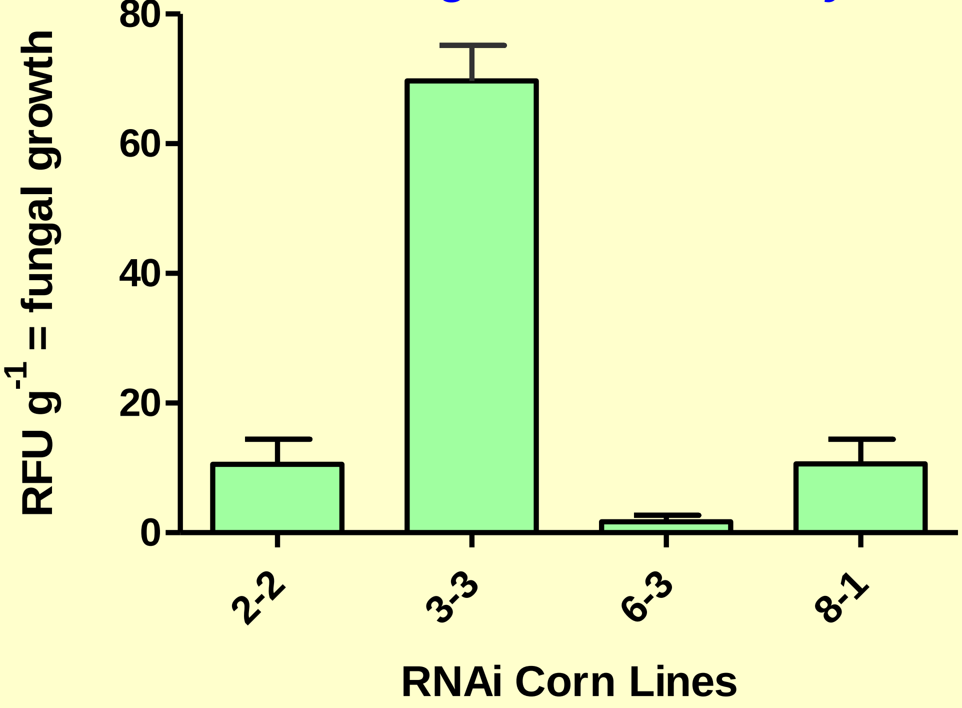
Negative Control (3-3)

Transgenic Corn (8-1)

Reduced α-amylase expression (qPCR) in RNAi transgenic kernels

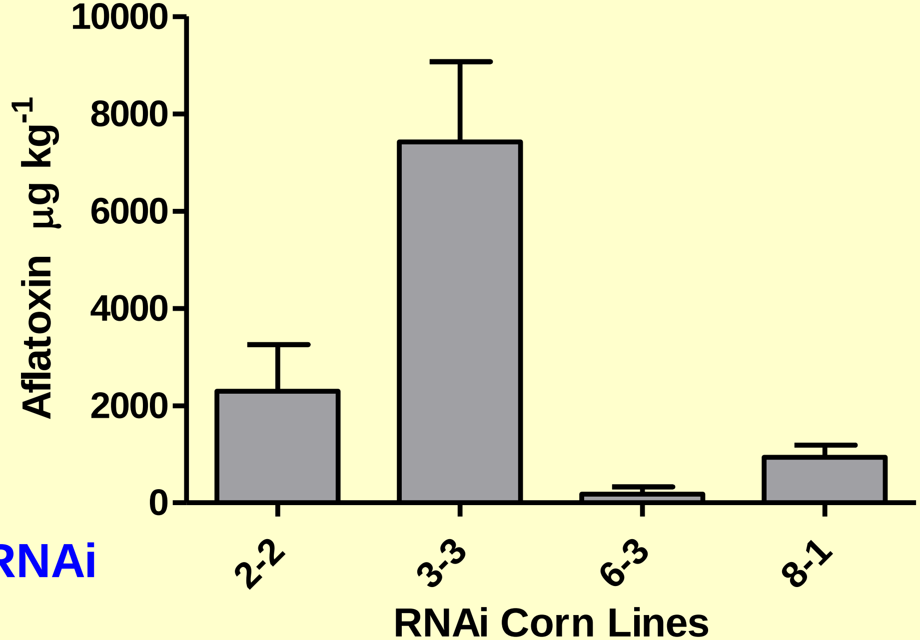


Fungal Growth in *amy*-RNAi Transgenic Kernels



3-3 Negative Control

2-2, 6-3, 8-1 Transgenic lines



Aflatoxin Production in *amy*-RNAi Transgenic Kernels

Other *flavus* genes

The following *flavus* genes are also being evaluated in transgenic maize via HIGS either alone or in combination

*ve*A, *nsdC, afl*C *(pks*A*), afl*R, *afl*M *(ver-1)*

Simultaneous silencing of five aflatoxin biosynthetic, transport, or non-ribosomal peptide synthetase (NRPS) related genes (*afl*R, *afl*S, *afl*C, *aflep*, and *pes1*) by RNAi resulted in 100% reduction of aflatoxin B_1 and B_2 in peanuts (Arias et al. 2015)

Silencing of *nsd*C has resulted in reduction of aflatoxin production in peanuts (Sharma, Bhatnagar-Mathur et al, 2017*).*

Maize - 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 *ve*A, *nsd*C, *afl*R, *afl*M, either alone or in combination are under evaluation for fungal infection and/or toxin production in transgenic maize lines.

Collaborators

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David Ambrogio

Darlene Downey

Stephanie Gross

Jonte Ellison

In Memoriam

Dedicated to our colleague, Dr. Robert L. Brown (1947-2017)