

Video Article

Inhibition Of *Aspergillus Flavus* Growth And Aflatoxin Production In Transgenic Maize Expressing The α -amylase Inhibitor From *Lablab Purpureus* L.

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Abstract

Aflatoxin contamination in food and feed crops is a major challenge worldwide. Aflatoxins, produced by the fungus *Aspergillus flavus* (*A. flavus*) are potent carcinogens that substantially reduce crop value in maize and other oil rich crops like peanut besides posing serious threat to human and animal health. Different approaches, including traditional breeding, transgenic expression of resistance associated proteins, and RNA interference (RNAi)-based host-induced gene silencing of critical *A. flavus* gene targets, are being evaluated to increase aflatoxin resistance in susceptible crops. Past studies have shown an important role of α -amylase in *A. flavus* pathogenesis and aflatoxin production, suggesting this gene/enzyme is a potential target to reduce both *A. flavus* growth and aflatoxin production. In this regard, the current study was undertaken to evaluate heterologous expression (under control of the constitutive CaMV 35S promoter) of a *Lablab purpureus* L. α -amylase inhibitor-like protein (AILP) in maize against *A. flavus*. AILP is a 36-kDa protein, which is a competitive inhibitor of *A. flavus* α -amylase enzyme and belongs to the lectin-arcelin- α -amylase inhibitor protein family in common bean. In vitro studies prior to the current work had demonstrated the role of AILP in inhibition of *A. flavus* α -amylase activity and fungal growth. Fungal growth and aflatoxin production in mature kernels were monitored in real time using a GFP-expressing *A. flavus* strain. This kernel screening assay (KSA) is very simple to set up and provides reliable and reproducible data on infection and the extent of spread that could be quantified for evaluation of germplasm and transgenic lines. The fluorescence from the GFP strain is closely correlated to fungal growth and, by extension, it is well-correlated to aflatoxin values. The goal of the current work was to implement this previous knowledge in a commercially important crop like maize to increase aflatoxin resistance. Our results show a 35%–72% reduction in *A. flavus* growth in AILP-expressing transgenic maize kernels which, in turn, translated into a 62%–88% reduction in aflatoxin levels.

Introduction

Mycotoxin contamination by the fungal genera, *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* is a major problem of food and feed crops grown worldwide^{1,2,3}. Among these phytopathogenic fungi, *Aspergillus* has the highest adverse impact on crop value and human and animal health. *Aspergillus flavus* (*A. flavus*) is an opportunistic plant pathogen that infects oil rich crops such as maize, cottonseed and peanut and produces the potent carcinogens, aflatoxins, as well as numerous toxic secondary metabolites (SMs). Maize is an important food and feed crop grown worldwide and is highly susceptible to contamination by *A. flavus*. The economic impact of aflatoxin contamination on losses and reduced value in maize can be as much as \$686.6 million/year in the U.S.² With predicted changes in global climate, the impact of aflatoxins could result in greater economic losses in maize with estimates as high as \$1.68 billion/year in the near future². Given the adverse economic and health effects of aflatoxins in humans and livestock, pre-harvest aflatoxin control in maize might be the most efficient way to prevent aflatoxin contamination in food and feed products.

The major pre-harvest control approach for aflatoxin resistance in maize that has been used extensively in the last few decades is primarily through breeding, which requires a significant amount of time⁴. Recently, biocontrol has had some success in aflatoxin reduction in large scale field applications^{5,6}. Besides biocontrol, application of cutting-edge molecular tools such as 'Host Induced Gene Silencing' (HIGS) through RNAi and transgenic expression of resistance-associated proteins has had some success in reduction of *A. flavus* growth and aflatoxin production in small scale laboratory and field studies. These approaches are currently being optimized in addition to identifying new potential *A. flavus* gene targets for future manipulation.

Besides genes that are directly involved in mycotoxin production as potential targets of transgenic control strategies, fungal amylases have been shown to play a critical role in maintaining successful pathogenesis and mycotoxin production during early stages of host plant infection. A few examples include *Pythium pleroticum* (causal agent of ginger rhizome rot), *Fusarium solani* (causal agent of cauliflower wilt), where positive correlations between pathogenicity and α -amylase expression and activity were observed^{7,8}. Inhibition of α -amylase activity either through gene knockout or knockdown approaches negatively affects fungal growth and toxin production. An α -amylase knockout mutant of *A. flavus* was unable to produce aflatoxins when grown on starch substrate or degermed maize kernels⁹. Similarly, in *Fusarium verticillioides* an α -amylase knockout strain failed to produce fumonisin B₁ (mycotoxin) during infection of maize kernels¹⁰. In a more recent study, Gilbert et al.

(2018) demonstrated that an RNAi-based knock down of *A. flavus* α -amylase expression through HIGS significantly reduced *A. flavus* growth and aflatoxin production during maize kernel infection¹¹.

Specific inhibitors of α -amylase activity have also produced similar results as obtained from down-regulation of α -amylase expression. The first report on the role of an α -amylase inhibitor in fungal resistance came from the isolation and characterization of a 14-kDa trypsin- α -amylase inhibitor from maize lines resistant to *A. flavus*¹². Further screening of several hundreds of plant species by Fakhoury and Woloshuk led to the identification of a 36-kDa α -amylase inhibitor-like protein (AILP) from the seeds of hyacinth beans, *Lablab purpureus* L.¹³. The peptide sequence of AILP resembled lectins belonging to the lectin-arcelin- α -amylase inhibitor family reported in common bean^{14,15}. Purified AILP does not exhibit any inhibitory activity towards mammalian trypsin and further in vitro characterization showed significant inhibition of *A. flavus* growth and conidial germination¹³. The reports presented here clearly shows α -amylase can serve as a target in control approaches to restrict pathogens or pests that depend on starch mobilization (through α -amylase activity) and acquisition of soluble sugars as an energy source during their pathogenic interaction with host plants.

Alpha-amylase is known to be critical in *A. flavus* pathogenicity^{9,10,11}, and given the importance of AILP as a potent anti-*A. flavus* agent (α -amylase inhibition/antigrowth)¹³, we generated transgenic maize plants expressing Lablab AILP gene under the constitutive CaMV 35S promoter. The goal was to investigate if heterologous expression of this α -amylase inhibitor in maize is effective against *A. flavus* pathogenesis and aflatoxin production during maize kernel infection. Our results demonstrate that transgenic maize kernels expressing AILP significantly reduced *A. flavus* growth and aflatoxin production during kernel infection.

Protocol

1. Plasmid constructs and maize transformation

1. PCR amplify Lablab AILP insert using the primers 5'-TATCTAGAAGTAGTATTACCATGGCTCC-3' and 5'-ATACTGCAGGATTGCATGCAGAGTAGTACTG-3'. The PCR conditions include an initial denaturation step at 98 °C for 30 s (step 1), followed by denaturation at 98 °C for 10 s (step 2), annealing at 55 °C for 30 s (step 3), elongation at 72 °C for 20 s (step 4), 31 cycles of step 2 to step 4, and a final elongation step at 72 °C for 5 min. Clone the PCR product into a modified pCambia 1300 vector using *Xba*I and *Pst*I restriction sites. Sequence the final plant destination vector to confirm the orientation and sequence of the cloned AILP gene in the vector.
2. Transform *Agrobacterium* strain EHA101 with the final vector construct (**Figure 1**) as earlier described¹⁶.
3. Use transformed *Agrobacterium* containing the final plant destination vector to transform immature maize (*Zea mays* L. Hi-II) embryos (performed at the Plant Transformation Facility of Iowa State University)¹⁶.
4. Grow T₀ plants in the greenhouse (26–29 °C; 16/8 h photoperiod supplemented with High Pressure Na-lamps) and repeatedly self-pollinate to obtain T₆ generation to achieve homozygosity for the transgenic trait.

2. Spore germination assay

1. Harvest leaf samples and store at -80 °C. Grind leaf samples with a mortar and pestle using liquid nitrogen. Weigh out 0.5 g in 2 mL microcentrifuge tubes, add 17 μ L of protease inhibitor, and place tubes on ice.
2. Centrifuge tubes for 10 min at 16,000 x g. Transfer 225 μ L of plant extract to 0.5 mL microcentrifuge tubes and place on ice.
3. In a 15 mL centrifuge tube, prepare a 5 mL spore suspension of *Aspergillus flavus* 70 – GFP in 1% potato dextrose broth (w/v). Vortex and adjust spore concentration to 10⁵ spores/mL with a haemocytometer.
CAUTION: *A. flavus* produces aflatoxins, all work with this fungus should be conducted in a biological safety cabinet.
4. Incubate in PDB at 28 °C until culture achieves 50% initiation of spore germination as indicated by bulging of spores.
5. Vortex and aliquot 25 μ L spore solution to the 225 μ L plant extract. Incubate samples for 20 hours at 28 °C with intermittent shaking.
6. Aliquot 25 μ L of spore solution on a microscope slide and measure the length of germ tubes spores using the digital camera software. Use a minimum of 20 replicate measurements for each line.
NOTE: At this stage, place all cultures at 4 °C to stop colony growth until ready to count.

3. Kernel Screening Assay (KSA)

1. Construct KSA caps by gluing 4 snap caps (22 mm) in a 60 x 15 mm petri dish. Allow glue to dry for 48 H before using caps. Each KSA cap constitutes one rep. (**Figure 2**).
2. In a sterile biological safety cabinet, spray a square bioassay tray (24 cm x 24 cm) with 70% ethanol:H₂O (v/v) and let air dry. Add sterile chromatography paper to the tray. Spray 9 KSA caps with 70% ethanol, let air dry, and place in the bioassay tray.
3. For each transgenic maize line being tested, select 20 undamaged kernels and place in a 50 mL centrifuge tube. Add 70% ethanol and let sit for 4 minutes. Gently shake tubes during sterilization. Pour off the ethanol and rinse kernels three times in sterile deionized H₂O.
4. Prepare a spore suspension of *Aspergillus flavus* 70 – GFP¹⁷ from a 6 day old culture grown on V8 medium (5% V8 Juice, 2% agar, pH 5.2).
 1. Add 20 mL sterile 0.02% Triton X-100/deionized H₂O (v/v) and scrap off spores with a sterile loop. Pipet off the inoculum and place in a 300 mL sterile beaker.
 2. Prepare a 5-fold dilution with 0.02% Triton X-100, then perform a spore count with a haemocytometer. Dilute again if necessary to obtain 100 mL with a concentration of 4 x 10⁶ spores/mL.
5. Place kernels in a sterile 300 mL beaker with a stir bar. Add inoculum to the beaker.
CAUTION: Adding kernels to inoculum will cause solution to splash. Place beaker on a stir plate for 3 minutes. After 3 minutes, pour off inoculum into an empty beaker.
6. Using a forceps, place kernels in the bioassay dish (1 kernel/cap). Add 30 mL deionized water to the bottom of each bioassay tray and incubate at 31 °C in the dark for 7 days.

7. Prepare kernels for photography.
 1. Set aside 4 kernels from each line for microscopic analysis and photography.
NOTE: Kernels can be stored at 4 °C for no longer than 5 days before completing photography.
 2. Take pictures of kernels after seven days of inoculation with *A. flavus* (Figure 3).
 3. Clean exterior of kernels with a soft tissue and deionized water. Perform longitudinal sections of kernels and immediately take photographs under the fluorescent microscope.
8. Prepare kernels for analysis.
 1. Clean exterior of remaining kernels with a soft tissue and deionized water.
 2. Place 4 kernels, constituting 1 rep, in a 15 mL screw cap polycarbonate vial containing 2 stainless steel balls. Immediately freeze vials in liquid nitrogen and store at -80 °C until further processing and analysis.
 3. Remove vials from -80 °C freezer and grind kernels in a homogenizer at 1500 rpm for 3 minutes.
NOTE: Keep kernels frozen with liquid nitrogen.

4. PCR screening of transgenic maize kernels

1. Use DNA isolation kit to isolate genomic DNA (gDNA) from pulverized maize kernels infected with *A. flavus* according to the manufacturer's instructions. Briefly, add 280 µL buffer F, 20 µL protease, and 3 µL dithiothreitol (DTT) to 10-15 mg of pulverized maize kernels. Incubate and shake in a thermomixer (56 °C, 1200 rpm, 30 min). Centrifuge at 10,000 x g for 1 min and transfer the clear supernatant to equilibrated column. Incubate at room temperature for 3 min and centrifuge at 700 x g for 1 min to elute gDNA.
2. Take 0.8 µL of extracted gDNA to set up a 20 µL PCR reaction for each sample using a PCR kit. Follow thermocycling conditions as recommended in the manufacturer's protocol. The PCR conditions include an initial denaturation step at 98 °C for 5 min (step 1) followed by denaturation at 98 °C for 5 s (step 2), annealing at 55 °C for 5 s (step 3), elongation at 72 °C for 20 s (step 4), 40 cycles of step 2 to step 4, and a final elongation step at 72 °C for 1 min (step 5).
3. Use forward primer 5'-TATACCACCCCATCCGTGT-3' and reverse primer 5'-AGCTCGGAAGCAAAGACCA-3' to confirm the presence of the *Lablab AILP* gene in the transgenic maize kernels.

5. RNA isolation, cDNA synthesis, and semi-quantitative RT-PCR

1. Take homogenized *A. flavus* infected maize kernels previously stored at -80°C for RNA extraction. Extract RNA using a total RNA isolation kit according to the manufacturer's protocol but with slight modification¹⁸. After adding 50 mg of homogenized maize kernel powder in the extraction buffer, mix it well (without vortexing) using a 1 mL pipet tip and leave it on ice for 5-6 minutes before proceeding to subsequent steps as described in the manufacturer's protocol.
2. Prepare cDNA using a cDNA synthesis kit according to the manufacturer's protocol¹⁸.
3. Use 0.5 µL of undiluted cDNA per PCR reaction for semi-quantitative RT-PCR as earlier described¹⁸. Use forward and reverse primers qAILP-F 5'-TCCCAACAAGGCAACTACTG-3' and qAILP-R 5'-CGGTGTCGAAGAGACCTAGATA-3' respectively to detect *Lablab AILP* gene expression, and forward and reverse primers qRib-F 5'-GGCTTGCTTAAAGGAAGGT-3' and qRib-R 5'-TCAGTCCAACCTCCAGAATGG-3' respectively for expression of maize ribosomal structural gene (Rib), GRMZM2G024838¹⁹ as a house-keeping gene.

6. GFP quantitation

1. Prepare 50 mL each of 0.2 M monobasic sodium phosphate (NaH₂PO₄) and 0.2 M dibasic sodium phosphate (Na₂HPO₄·7H₂O) in deionized H₂O.
2. Make up 100 mL of pH 7.0 Sorenson's phosphate buffer. For pH 7.0, add 19.5 mL NaH₂PO₄ stock, 30.5 mL NaHPO₄·7H₂O stock, and 50 mL deionized H₂O.
3. Weigh out 25 mg ground kernel material (fresh weight, FW) and place in a 1.0 mL microcentrifuge tube. Add 500 µL phosphate buffer to the centrifuge tube and vortex for 30 s.
4. Centrifuge samples for 15 minutes at 16,000 x g. Pipet 100 µL supernatant into a black 96 well plate. Read the samples with a fluorometer (excitation 485 nm, emission 528 nm).

7. Total aflatoxin analysis

1. Sample processing and aflatoxin extraction
 1. Dry kernel samples in a forced air oven for 2 days at 60 °C. Weigh samples and place in a 50 mL Erlenmeyer flask with a glass stopper.
 2. In a fume hood, add 25 mL methylene chloride to each flask.
CAUTION: Methylene chloride is highly volatile and is considered hazardous (irritant, carcinogen).
 3. Shake samples for 30 min on a wrist action shaker. After 30 min, slowly pour the extract through a fluted filter paper circle into an 80 mL beaker. Let the beakers dry overnight in a fume hood.
 4. The next day, squirt methylene chloride (approximately 5 mL) around the inside rim of the dry beakers, swirl around, and pour into 2 dram glass vials. Leave vials open to dry in a fume hood overnight.
2. Aflatoxin analysis
 1. Add 4.0 mL 80% methanol:deionized H₂O (v/v) to vials.

2. Use an aflatoxin extraction kit to filter/purify methanol solution with the provided column. Analyze total aflatoxin levels with a fluorometer, according to the manufacturer's instructions.

Representative Results

Maize transformation and molecular screening of transgenic plants

Immature embryos of maize Hi-II lines were transformed using *Agrobacterium tumefaciens* EHA101 strain containing the final plant destination vector expressing the *Lablab purpureus AILP* gene under the control of CaMV 35S promoter. Five independently transformed maize lines were advanced to the T6 generation for subsequent studies. The transgenic maize plants were much smaller and less vigorous but did not show any other abnormalities compared to the isogenic negative control plants. PCR amplification of the target *AILP* gene showed a 548 bp amplicon, observed only in the transgenic maize lines as compared to the control plants (**Figure 4A**). The *AILP* gene showed expression in the transgenic lines whereas no *AILP* expression was observed in the control plants (**Figure 4B**).

Aspergillus flavus spore assay with leaf extracts

Crude leaf extracts from young transgenic maize plants were prepared by grinding in liquid N and centrifuging at 16,000 x g. Early stage germinating spores were exposed to the leaf extract for 20 hours and the mean spore hyphal length was recorded from 20 samples. Hyphal length from spores exposed to transgenic leaf extracts showed a 58%–80% reduction compared to the negative control (**Figure 5**).

Aspergillus flavus growth during kernel infection

A Kernel Screening Assay (KSA) was performed to examine the growth of *A. flavus* in the transgenic and control kernels. Expression of the *AILP* gene in transgenic kernels negatively affected *A. flavus* growth. The *A. flavus* strain used in the current study contains a GFP reporter which enables the monitoring and quantification of fungal growth in real-time. Significant reduction in GFP fluorescence was observed in transgenic *AILP* kernels compared to the isogenic negative control (**Figure 6**). *AILP* Lines 4 and 5 showed a significant reduction in fungal growth, 69% and 72%, respectively, followed by the other lines where a 35%–60% reduction was observed in comparison to the control kernels (**Figure 7A**).

Aflatoxin production in the infected kernels

Heterologous expression of the *AILP* gene in maize resulted in significant reduction of aflatoxin content in the transgenic lines vs. control. The aflatoxin data provided here is the total aflatoxin content detected in the infected kernels. A 62%–88% reduction in aflatoxin content was observed in the *AILP* kernels as compared to the control (**Figure 7B**). Among the 5 different *AILP* lines, Line 4 showed the lowest aflatoxin content (486 ng/g DW), followed by other lines ranging between 640–1498 ng/g DW in the kernels vs. control (3986 ng/g DW).

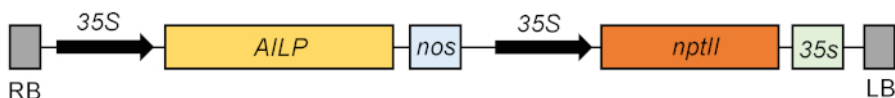


Figure 1: Vector design for transgenic expression of *Lablab AILP* gene in maize. 35S = Cauliflower mosaic virus or CaMV constitutive promoter; RB = right border; LB = left border; *nptII* = neomycin phosphotransferase-kanamycin resistance gene; *nos* and 35s = transcription terminators. [Please click here to view a larger version of this figure.](#)

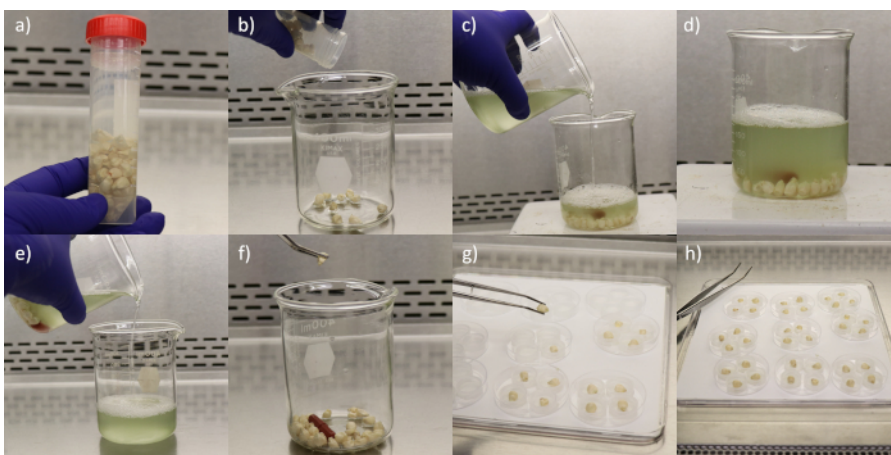


Figure 2: Kernel Screening Assay. (A,B) Sterilize kernels and pour into a sterile beaker in a biological safety cabinet. (C–E) Inoculate kernels with *A. flavus* spore suspension. (F–H) Using a sterile forceps, place kernels in bioassay dish. [Please click here to view a larger version of this figure.](#)

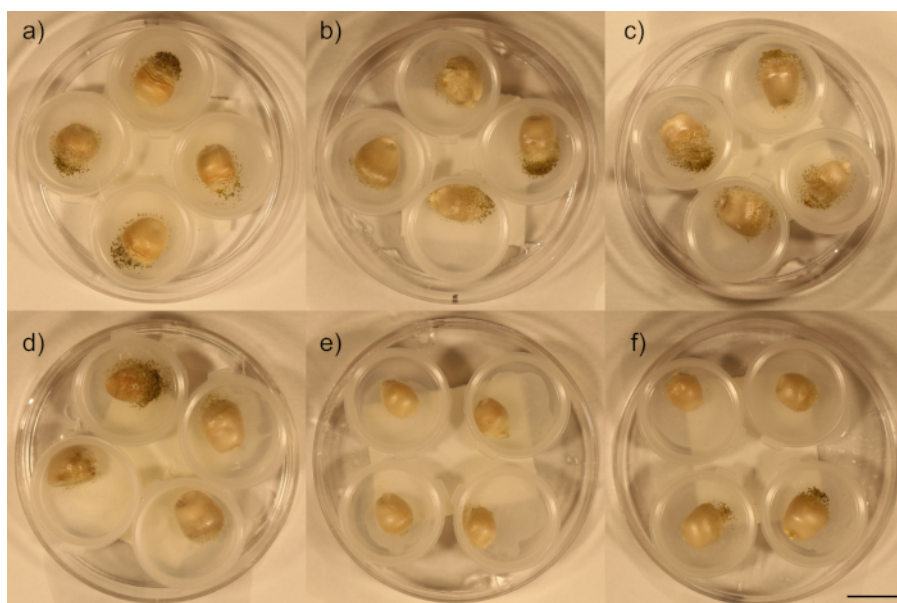


Figure 3: Light micrographs of *A. flavus* growth. (A) Isogenic negative control. (B–F) Transgenic kernels expressing AILP (lines 1–5) one week after inoculation. Scale bar = 1 cm. [Please click here to view a larger version of this figure.](#)

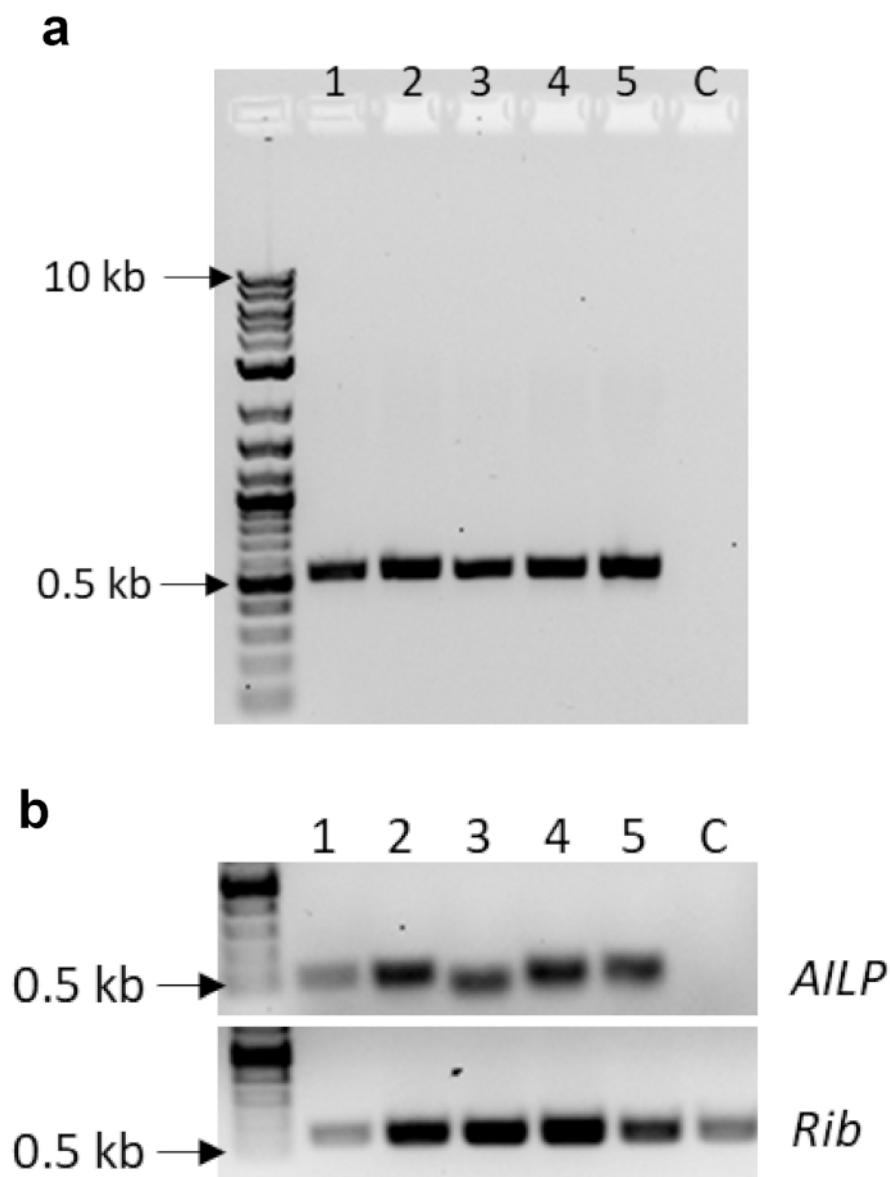


Figure 4. Molecular screening of transgenic plants. (A) PCR confirmation of transgenic maize plants (lanes 1-5) containing *Lablab AILP* (548 bp diagnostic DNA fragment; C = isogenic negative control; NEB 2-Log DNA ladder used as a DNA marker). (B) Expression of *Lablab AILP* gene in transgenic maize lines (lanes 1-5) using RT-PCR. Lane C represents isogenic negative control. Maize ribosomal structural gene (*Rib*), GRMZM2G024838¹⁹ was used as a house-keeping gene (NEB 2-Log DNA ladder: DNA marker). [Please click here to view a larger version of this figure.](#)

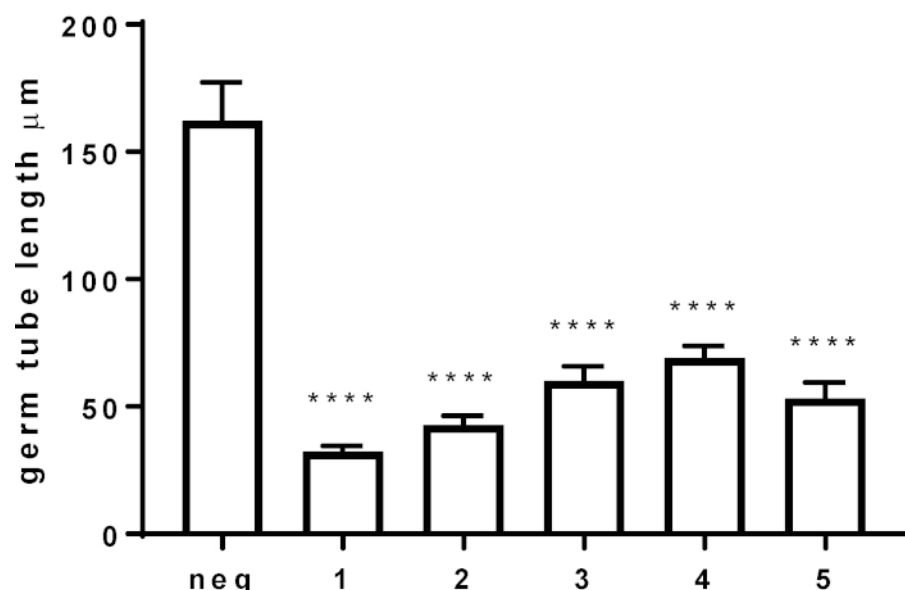


Figure 5. Effect of crude leaf extracts from transgenic AILP maize plants on *A. flavus* spore germination as compared with an isogenic negative control (neg). Mean separation was done by Dunnett's posttest after ANOVA. Levels of significant reduction are denoted by asterisks (**** $P \leq 0.0001$). Error bars indicate standard error of means for twenty samples. [Please click here to view a larger version of this figure.](#)

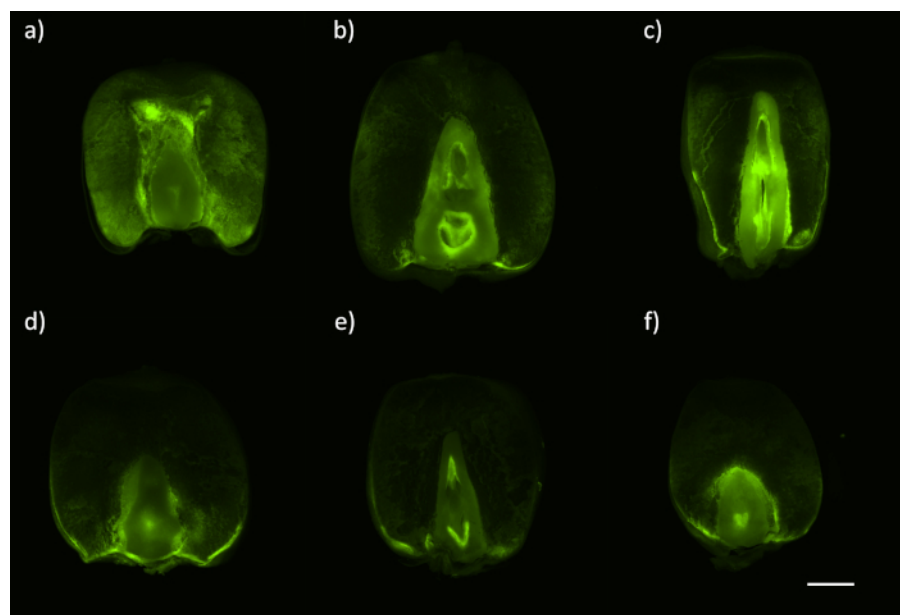


Figure 6. GFP fluorescence emanating from the *A. flavus* strain 70-GFP in transgenic kernels expressing AILP after one week of bioassay. Isogenic negative controls (A) were used to evaluate the fungal infection and spread in transgenic kernels (B-F representing transgenic lines 1-5). At least four kernels were screened for each line under the fluorescent microscope. Scale bar = 2 mm. [Please click here to view a larger version of this figure.](#)

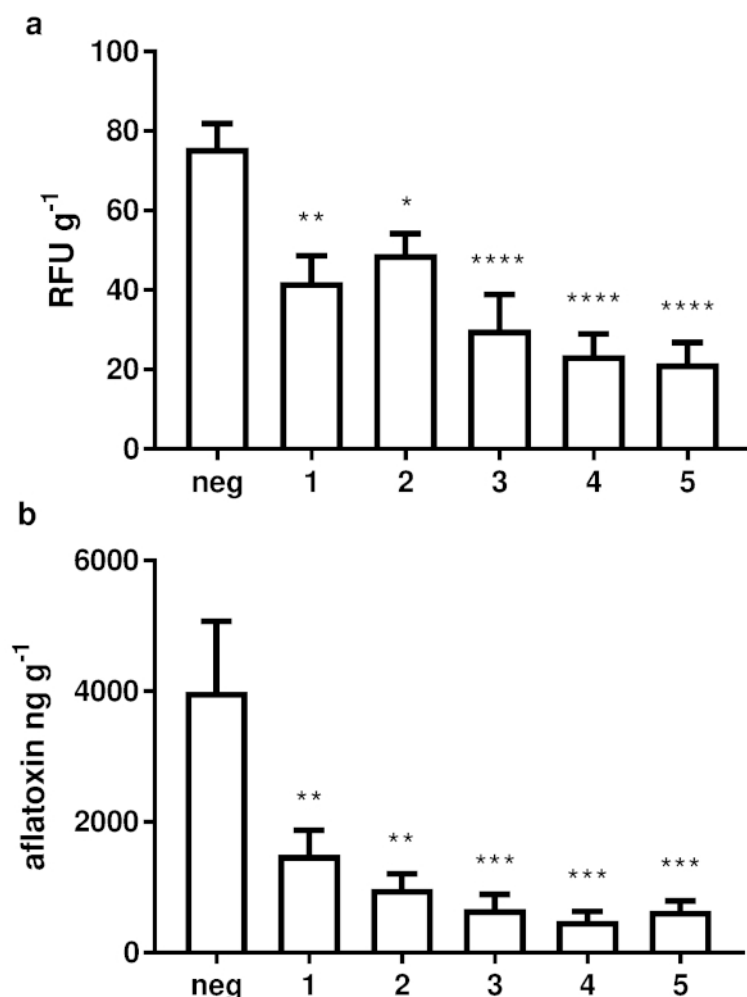
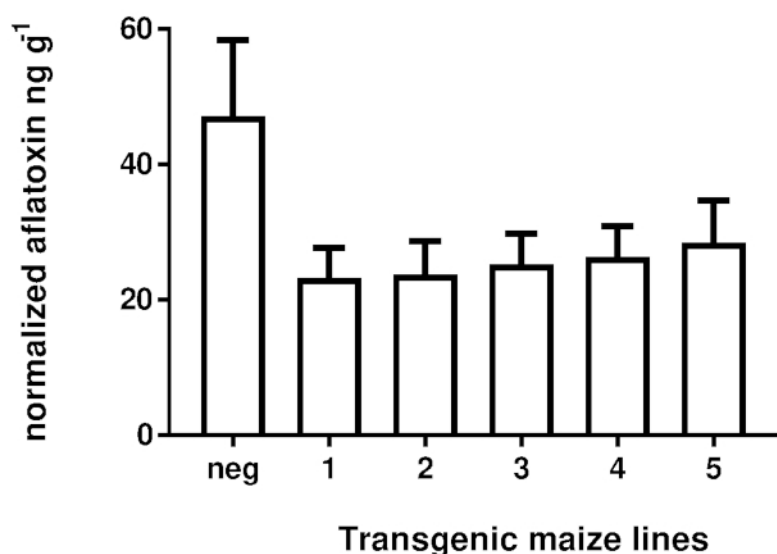
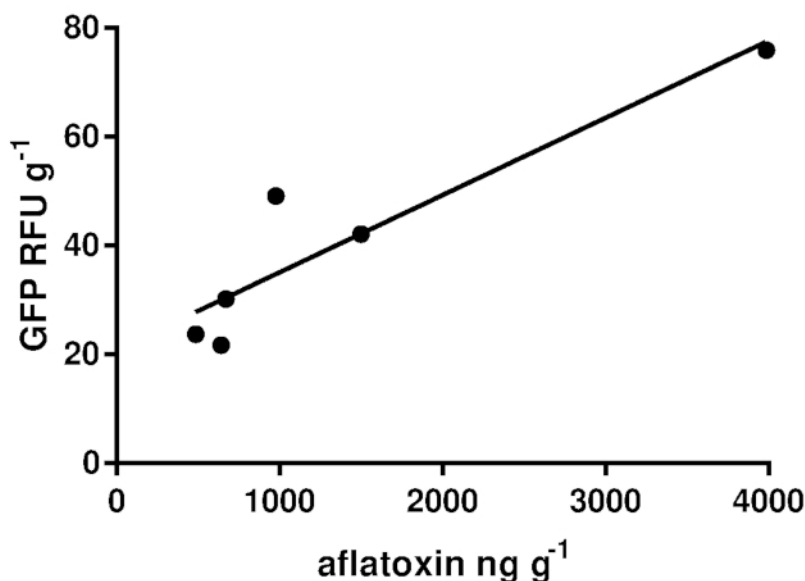


Figure 7: *A. flavus* growth and aflatoxin production. (A) *A. flavus* 70-GFP infection and growth in maize kernels after 7 days in a Kernel Screening Assay. Average of three independent experiments with four biological replicates each time. GFP quantification (in Relative Fluorescence Units, RFU) indicates fungal growth in transgenic maize lines expressing *AILP* to an isogenic negative control. Mean separation was done by Dunnett's posttest after ANOVA. Levels of significant reduction is denoted by asterisks (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). Error bars indicate standard error of means for four randomized biological replicates. (B) Aflatoxin production by *A. flavus* 70-GFP in maize kernels after 7 days in a Kernel Screening Assay. Average of 12 biological replicates and each replicate contained four kernels. Aflatoxin levels in transgenic *AILP* kernels (lines 1-5) as compared with an isogenic negative control (neg). Mean separation was done by Dunnett's posttest after ANOVA. Levels of significant reduction is denoted by asterisks (** $P \leq 0.01$; *** $P \leq 0.001$). Error bars indicate standard error of means for four randomized biological replicates. See Supplemental Figures to view the data in panel B devoid of bias due to fungal inhibition. Close correlation between GFP=RFU and aflatoxin values are also provided. [Please click here to view a larger version of this figure.](#)



Supplemental Figure 1: Data from Figure 7B redrawn to show aflatoxin production by *A. flavus* 70-GFP in maize kernels after 7 days in a Kernel Screening Assay to eliminate bias due to fungal growth inhibition. Aflatoxin values were divided by the fungal growth inhibition represented as GFP-RFU values in Figure 7B. [Please click here to view a larger version of this figure.](#)



Supplemental Figure 2: Close correlation between GFP-RFU values (=fungal growth) and aflatoxin levels ($r^2 = 0.86$) reported in the KSA. [Please click here to view a larger version of this figure.](#)

Discussion

Yield losses in agricultural crops due to pathogens and pests is a global problem²⁰. Currently, application of synthetic fungicides and pesticides is the predominant means for controlling plant pathogens and pests, but residual toxicity of these biochemicals in food and feed can pose serious threat to human and animal health²¹. Considering the economic importance of maize as a food and feed crop, reduction in or elimination of aflatoxin contamination is of utmost importance^{2,3,22}. Conventional breeding through resistant gene introgression into susceptible varieties is a time-consuming process and such resistance is not always stable as aflatoxin resistance is a polygenic trait and expression of participating genes vary significantly with changes in environmental parameters⁴. Alternative approaches are being evaluated to increase and stabilize host plant defense against *A. flavus* in an ecofriendly manner.

In the current study, we chose to evaluate the efficacy of the *AILP* gene from hyacinth beans in maize against *A. flavus*. As AILP is a competitive inhibitor of α -amylase and also possesses antifungal properties, we expressed this gene under a strong constitutive promoter CaMV 35S.

The goal was to increase the production of this heterologous protein in maize irrespective of developmental stage or tissue type. Significant expression of the α -amylase gene in the transgenic maize lines (**Figure 4A,B**) supports stable expression of this gene in a heterologous system. This negatively affected fungal growth as evident from the reduction in spatial distribution of GFP fluorescence inside the infected AILP kernels and reduction in overall GFP fluorescence in the kernels (**Figure 6** and **Figure 7A**).

The development of the Kernel Screening Assay (KSA) utilizes a genetically engineered *A. flavus* reporter strain expressing a gene encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*^{18,23}. To devise control strategies for any pathogen, it is necessary to understand the mode of infection, spread and colonization within the host plant or tissue. The colonization and spread of the aflatoxigenic saprophyte, *A. flavus* is not well understood because genetic regulation of resistance to the pathogen, if one exists, is not well understood. Use of the GFP-expressing *A. flavus* strain makes it possible to track in real time the infection process and colonization. The GFP strain and the wild-type did not differ significantly in pathogen aggressiveness and aflatoxin production²³. However, the GFP gene in this particular strain is placed under the control of the constitutively expressed glyceraldehyde phosphate dehydrogenase (*gpdA*) gene promoter²⁴ which is more apt for tracking fungal growth. However, using this strain on cottonseed and maize kernels, we have shown a close relationship between GFP fluorescence emanating from the fungus to fungal growth and aflatoxin levels^{18,23}.

To establish a close correlation with aflatoxin producing ability, *gfp* driven by aflatoxin gene promoters such as *omtA* or *ver-1*^{25,26} will be more suitable than *gpdA*. The GFP fluorescence is sensitive enough to allow for detection and measurement in real time of even small changes in fungal growth, both *in vitro* and *in planta*, and evaluation of inhibitory activities of unknown antifungal proteins and peptides such as AILP and others^{11,17,18,23}. In addition, results from the KSA correlates well with field performance of resistant or susceptible maize lines with regards to evaluation for aflatoxin contamination²⁷.

The KSA is very useful in our laboratory to screen classically-bred new varieties⁴ and transgenic lines^{11,18}. It is easy to set up and perform KSA in any laboratory. One needs to use undamaged clean kernels to perform this assay so that a clear understanding of host plant-fungal interaction could be evaluated. We routinely scan all the kernels that we use in our laboratory under a stereomicroscope before conducting the assay. It should be noted that the conditions that we employ for the KSA include high humidity (95% RH) and constant temperature (31 °C). With the onset of simulated germination process under these conditions, the assay provides ideal conditions to test the integrity of the seed coat, genetic makeup of the kernels including antifungal genes/proteins turned on during germination, and/or fungal infection. Fungal infection and aflatoxin production will always be higher in KSA compared to their occurrence in the field; however, a close correlation has been established^{4,27}.

We have demonstrated in this KSA that AILP reduces fungal growth in maize kernels. AILP possesses antifungal properties of plant lectins, and has been shown to inhibit α -amylase, decreasing starch breakdown and limiting fungal growth. *In vitro* studies demonstrated that AILP was capable of inhibiting *A. flavus* growth in sugar-rich potato dextrose broth (PDB) artificial growth medium, indicating its antifungal/growth inhibition activity was independent of its amylase inhibition activity¹³. Other plant lectins isolated from *Urtica dioica* (common nettle), *Hevea brasiliensis* (rubber tree), and *Solanum tuberosum* (potato) tubers also show similar antifungal properties and inhibited growth of *Botrytis cinerea*, *Pyrenophora tritici*, and *Fusarium oxysporum*^{28,29,30,31}. Alpha amylase inhibitors are not only effective against fungi and bacteria but were also shown to be effective against insect pests³². Alpha amylases are reported to be critical for normal growth and development of plant-feeding insects, and several insect α -amylase inhibitors have been isolated from plants such as wheat, common bean, and maize^{12,33,34,35}. A significant reduction in fungal hyphal length from spores exposed to crude leaf extracts (**Figure 5**), reduction in fungal growth in the AILP-expressing maize kernels as demonstrated by the reduction of GFP fluorescence both qualitatively (**Figure 6**) and quantitatively (**Figure 7A**) is possibly due to a combination of its α -amylase inhibition and antifungal properties. Experiments using these AILP-expressing maize plants against other pathogens and pests in the future will be of great interest to test its efficacy against diverse biotic stressors of seed and vegetative tissues. The antifungal and growth inhibition properties of lectin has been mainly attributed to its cross-linking with chitin and inhibition of hyphal tip expansion²⁸. In fact, *in vitro* AILP-mediated inhibition of α -amylases from diverse fungal and bacterial origins showed over 65% inhibition in fungal pathogens including *A. flavus*, *Magnaporthe grisea*, *Helminthosporium victoriae*, and 41% inhibition in the bacterial pathogen *Bacillus subtilis*¹³. The results on reduction of *A. flavus* growth presented here are in line with the role of α -amylase inhibitors as antifungal agents. The current study further demonstrates practical application of such useful information obtained from earlier *in vitro* studies.

Reduction in aflatoxin content (**Figure 7B**) in the AILP-expressing maize lines is possibly due to inhibition of *A. flavus* α -amylase activity, leading to diminished breakdown of stored starch, and reduced acquisition of sugars as an energy source from the maize kernels during pathogenic interaction. As fungal α -amylase plays an important role in breaking down kernel starches, any change in fungal α -amylase activity by AILP likely alters soluble sugar content during kernel infection. Content of soluble sugars, especially sucrose and glucose, has been highly correlated with increased production of AFB₁ and total aflatoxins in maize and other *A. flavus* susceptible crops such as peanut^{36,37}. Increasing sucrose content in artificial growth media increases *A. flavus* aflatoxin production³⁸. Other soluble sugars such as glucose and maltose also positively contribute to aflatoxin production both in artificial media and seed substrates³⁸. Inhibition of α -amylase activity and reduction in mycotoxin production in other fungi reinforces an important role of fungal α -amylases in toxin production during kernel infection. Alpha-amylase knockout mutants of both *F. verticillioides* and *A. flavus* failed to produce fumonisin B₁ and aflatoxins respectively following infection of maize kernels^{9,10}. Similarly, down-regulation of *A. flavus* α -amylase significantly reduced fungal growth and aflatoxin production during maize kernel infection¹¹. A 62%-88% reduction in aflatoxin in the transgenic lines are in accordance with earlier reports on the role of α -amylase in aflatoxin production. It is to be noted that the *in vitro* Kernel Screening Assay (KSA) is more stringent than normally found under natural conditions. Conditions during the *in vitro* assay are also highly conducive for aflatoxin production, so the percentage change in aflatoxin content in the transgenic lines might be more meaningful rather than the absolute numbers of aflatoxin content in the infected kernels. The results presented here are promising and future experiments under field conditions are important to examine the potential of these AILP-expressing maize lines for aflatoxin resistance under a natural scenario.

Disclosures

The authors have no conflict of interest.

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