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TITLE:

Inhibition of *Aspergillus flavus* Growth and Aflatoxin Production in Transgenic Maize Expressing the *A*-Amylase Inhibitor from *Lablab purpureus* L.

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KEYWORDS:

α-amylase inhibitor, aflatoxin, *Aspergillus flavus*, corn, kernel screening assay, *Lablab purpureus*, maize, transgenic

SUMMARY:

Here we present a protocol to analyze *Aspergillus flavus* growth and aflatoxin production in maize kernels expressing an antifungal protein. Using a GFP-expressing *A. flavus* strain we monitored the infection and spread of the fungus in mature kernels in real time. The assay is rapid, reliable, and reproducible.

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[1-3]. 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 loses 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 future[2]. 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 time[4]. 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 kernels[9]. Similarly, in *Fusarium*

verticillioides an α-amylase knockout strain failed to produce fumonisin B_1 (mycotoxin) during infection of maize kernels[10]. 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 infection[11].

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 germination[13]. 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[9–11], 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

1.1. PCR amplify Lablab *AILP* insert using the primers 5’-TATCTAGAACTAGTGATTACCATGGCTCC-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.

1.2. Transform *Agrobacterium* strain EHA101 with the final vector construct (**Figure 1**) as earlier described [16].

1.3. 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].

1.4. Grow T_0 plants in the greenhouse (26–29 °C; 16/8 h photoperiod supplemented with High Pressure Na-lamps) and repeatedly self-pollinate to obtain T_6 generation to achieve homozygosity for the transgenic trait.

2. Spore germination assay

2.1. 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 µL of protease inhibitor, and place tubes on ice.

2.2. Centrifuge tubes for 10 min at 16,000 x g. Transfer 225 µL of plant extract to 0.5 mL microcentrifuge tubes and place on ice.

2.3. 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 10^5 spores/mL with a haemocytometer.

CAUTION: *A. flavus* produces aflatoxins, all work with this fungus should be conducted in a biological safety cabinet.

2.4. Incubate in PDB at 28 °C until culture achieves 50% initiation of spore germination as indicated by bulging of spores.

2.5. Vortex and aliquot 25 µL spore solution to the 225 µL plant extract. Incubate samples for 20 hours at 28 °C with intermittent shaking.

2.6. Aliquot 25 µL 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)

3.1. 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**).

3.2. In a sterile biological safety cabinet, spray a square bioassay tray (24 cm x 24 cm) with 70% ethanol:H_2O (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.

3.3. 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 H_2O.

3.4. Prepare a spore suspension of *Aspergillus flavus* 70 – GFP[17] from a 6 day old culture grown on V8 medium (5% V8 Juice, 2% agar, pH 5.2).

3.4.1. Add 20 mL sterile 0.02% Triton X-100/deionized H_2O (v/v) and scrap off spores with a sterile loop. Pipet off the inoculum and place in a 300 mL sterile beaker.

3.4.2. 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 10^6 spores/mL.

3.5. 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.

3.6. 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.

3.7. Prepare kernels for photography.

3.7.1. 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.

3.7.2. Take pictures of kernels after seven days of inoculation with *A. flavus* (**Figure 3**).

3.7.3. Clean exterior of kernels with a soft tissue and deionized water. Perform longitudinal sections of kernels and immediately take photographs under the fluorescent microscope.

3.8. Prepare kernels for analysis.

3.8.1. Clean exterior of remaining kernels with a soft tissue and deionized water.

3.8.2. 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.

3.8.3. 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

4.1. 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.

4.2. 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).

4.3. Use forward primer 5'-TATACCACCCCCATCCGTGT-3' and reverse primer 5'-AGCTCGGAAGCAAAAGACCA-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

5.1. 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 modification[18]. 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.

5.2. Prepare cDNA using a cDNA synthesis kit according to the manufacturer's protocol [18].

5.3. Use 0.5 µL of undiluted cDNA per PCR reaction for semi-quantitative RT-PCR as earlier described[18]. 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'-GGCTTGGCTTAAAGGAAGGT-3' and qRib-R 5'-TCAGTCCAACTTCCAGAATGG-3' respectively for expression of maize ribosomal structural gene (Rib), GRMZM2G024838[19] as a house-keeping gene.

6. GFP quantitation

6.1. Prepare 50 mL each of 0.2 M monobasic sodium phosphate (NaH_2PO_4) and 0.2 M dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$) in deionized H_2O.

6.2. Make up 100 mL of pH 7.0 Sorenson's phosphate buffer. For pH 7.0, add 19.5 mL NaH_2PO_4 stock, 30.5 mL $NaHPO_4 \cdot 7H_2O$ stock, and 50 mL deionized H_2O.

6.3. 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.

6.4. 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

7.1. Sample processing and aflatoxin extraction

7.1.1. 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.

7.1.2. In a fume hood, add 25 mL methylene chloride to each flask.

CAUTION: Methylene chloride is highly volatile and is considered hazardous (irritant, carcinogen).

7.1.3. 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.

7.1.4. 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.

7.2. Aflatoxin analysis

7.2.1. Add 4.0 mL 80% methanol:deionized H_2O (v/v) to vials.

7.2.2. 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 LEGENDS:

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.

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.

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.

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*), GRMZM2G024838[19] was used as a house-keeping gene (NEB 2-Log DNA ladder: DNA marker).

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.

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.

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.

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**.

Supplemental Figure 2: Close correlation between GFP-RFU values (=fungal growth) and aflatoxin levels ($r^2 = 0.86$) reported in the KSA.

DISCUSSION:

Yield losses in agricultural crops due to pathogens and pests is a global problem[20]. 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 health[21]. 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 parameters[4]. 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 (**Figures 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 production[23]. However, the GFP gene in this particular strain is placed under the control of the constitutively expressed glyceraldehyde phosphate dehydrogenase (*gpd*A) gene promoter [24] which is more apt for tracking fungal growth. However, using this stain 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 *omt*A or *ver*-1[25,26] will be more suitable than *gpd*A. 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 contamination[27].

The KSA is very useful in our laboratory to screen classically-bred new varieties[4] 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 activity[13]. 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*[28–31]. Alpha amylase inhibitors are not only effective against fungi and bacteria but were also shown to be effective against insect pests[32]. 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[12,33–35]. 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 expansion[28]. 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 AFB_1 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 production[38]. Other soluble sugars such as glucose and maltose also positively contribute to aflatoxin production both in artificial media and seed substrates[38]. 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 B_1 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 infection[11]. 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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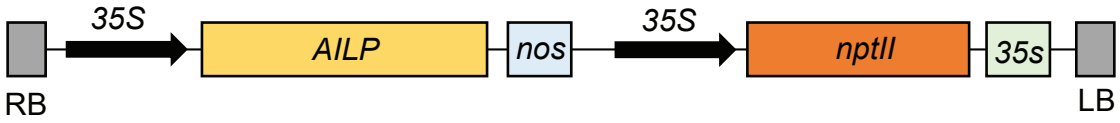

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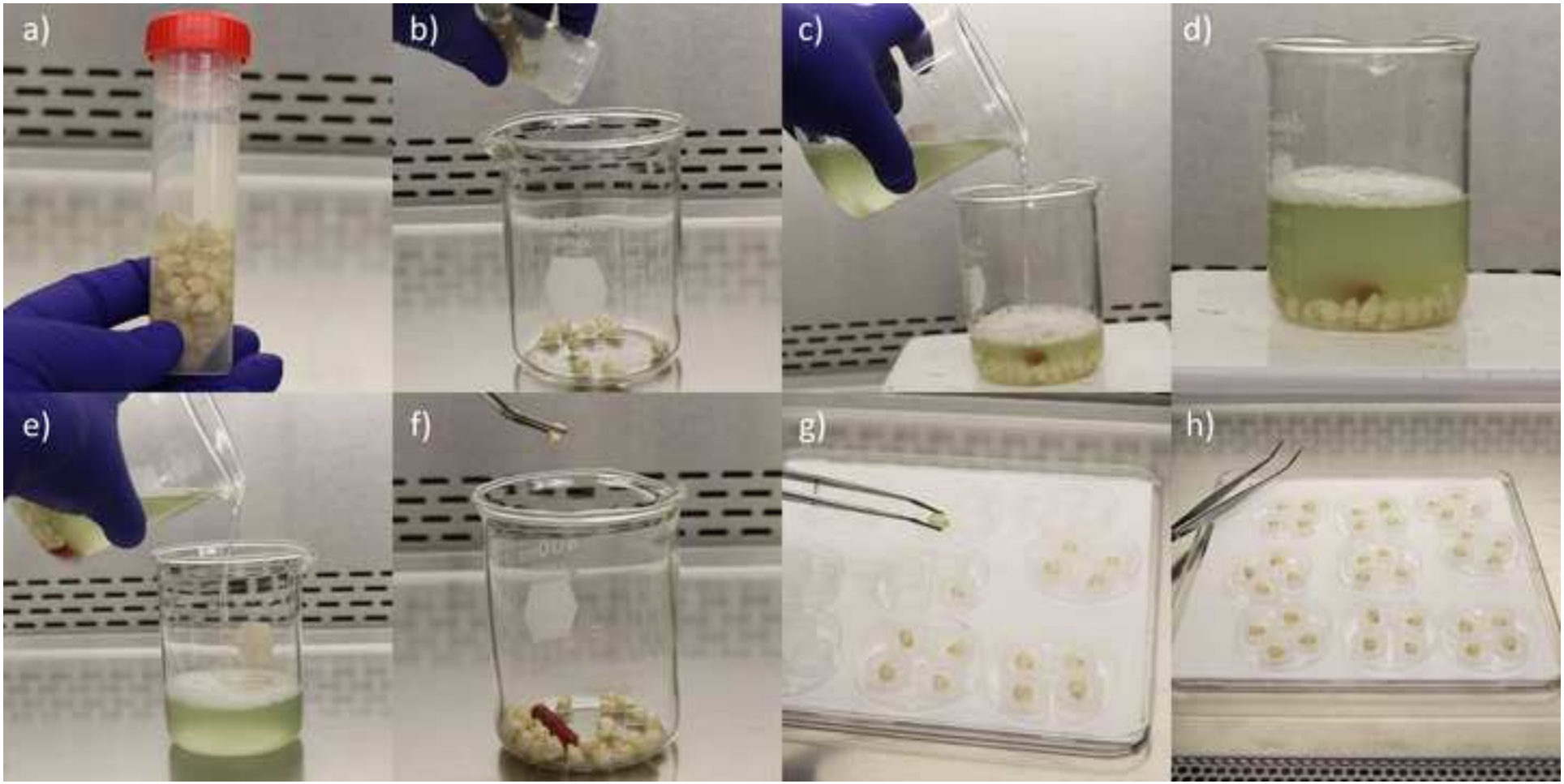

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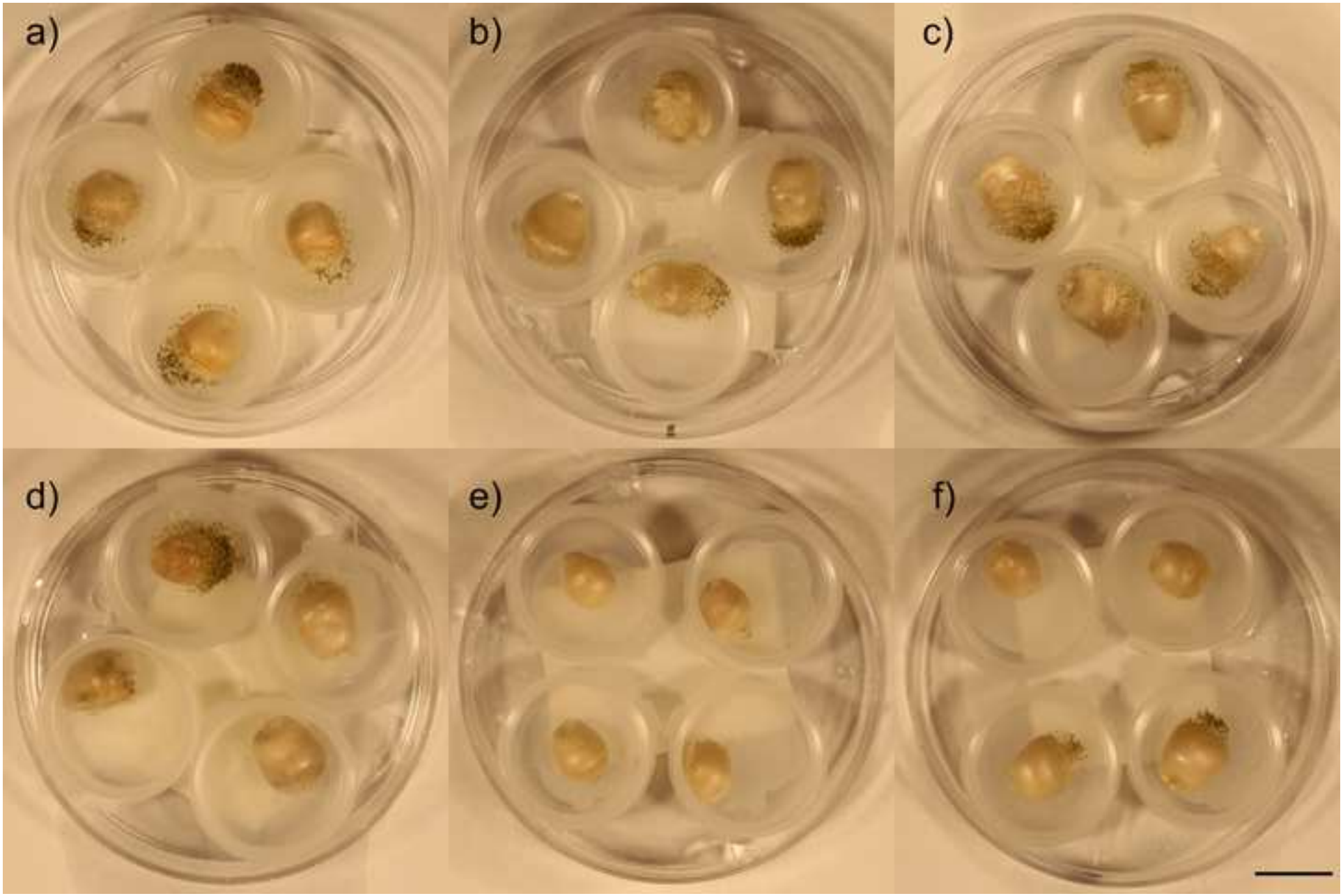

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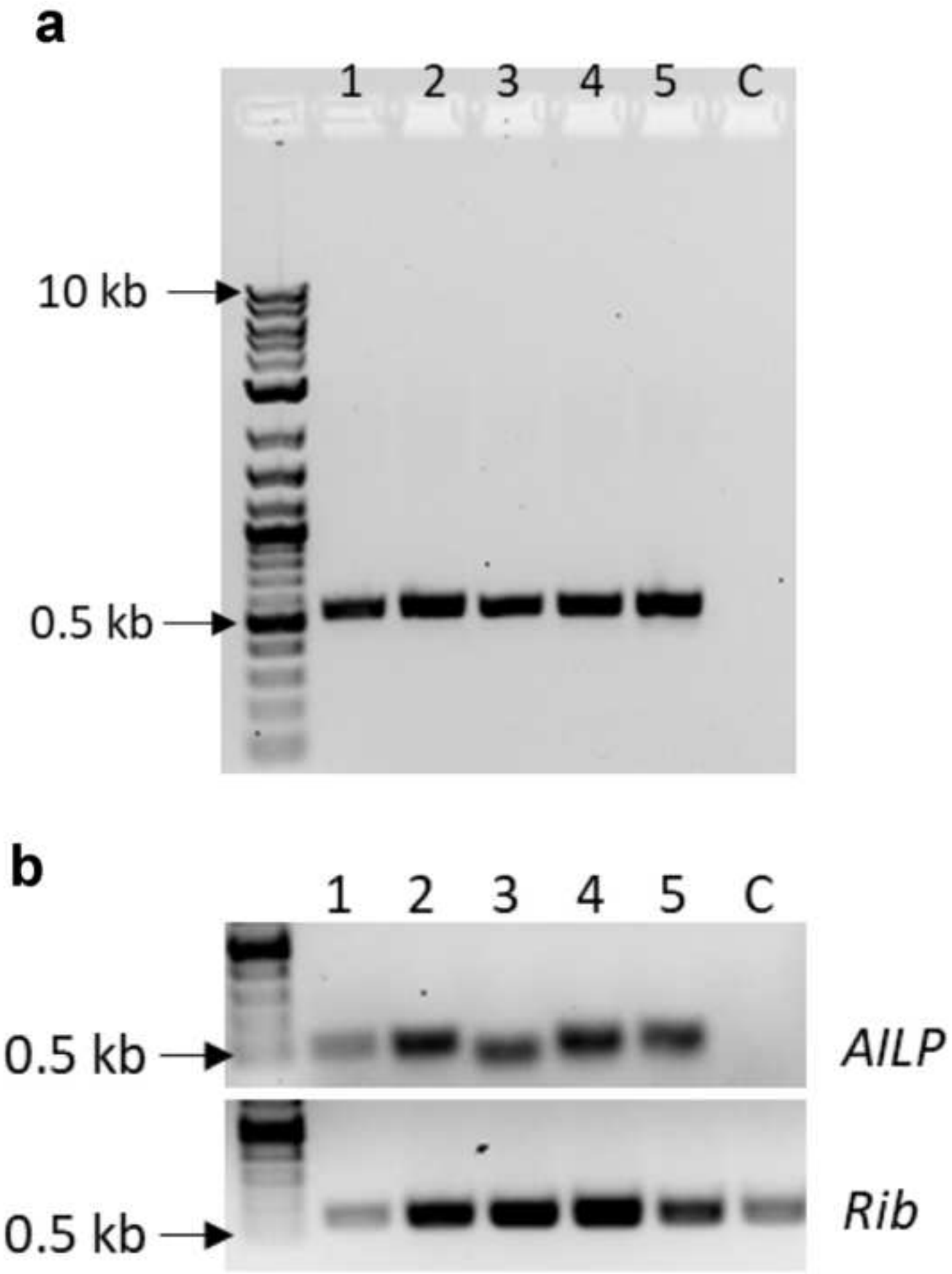

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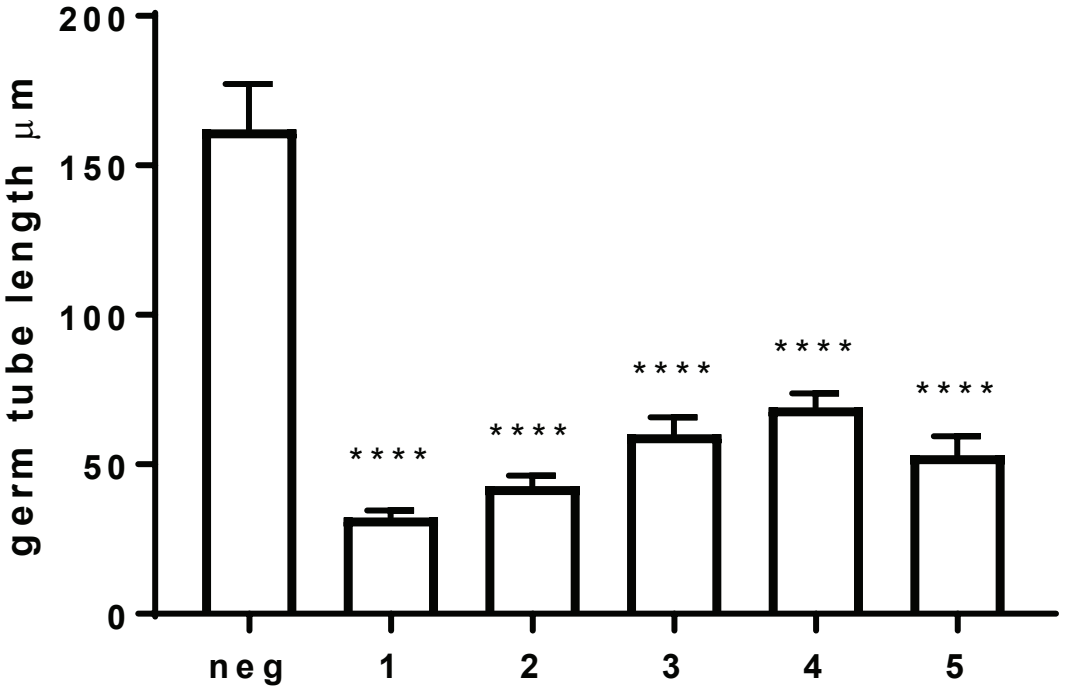

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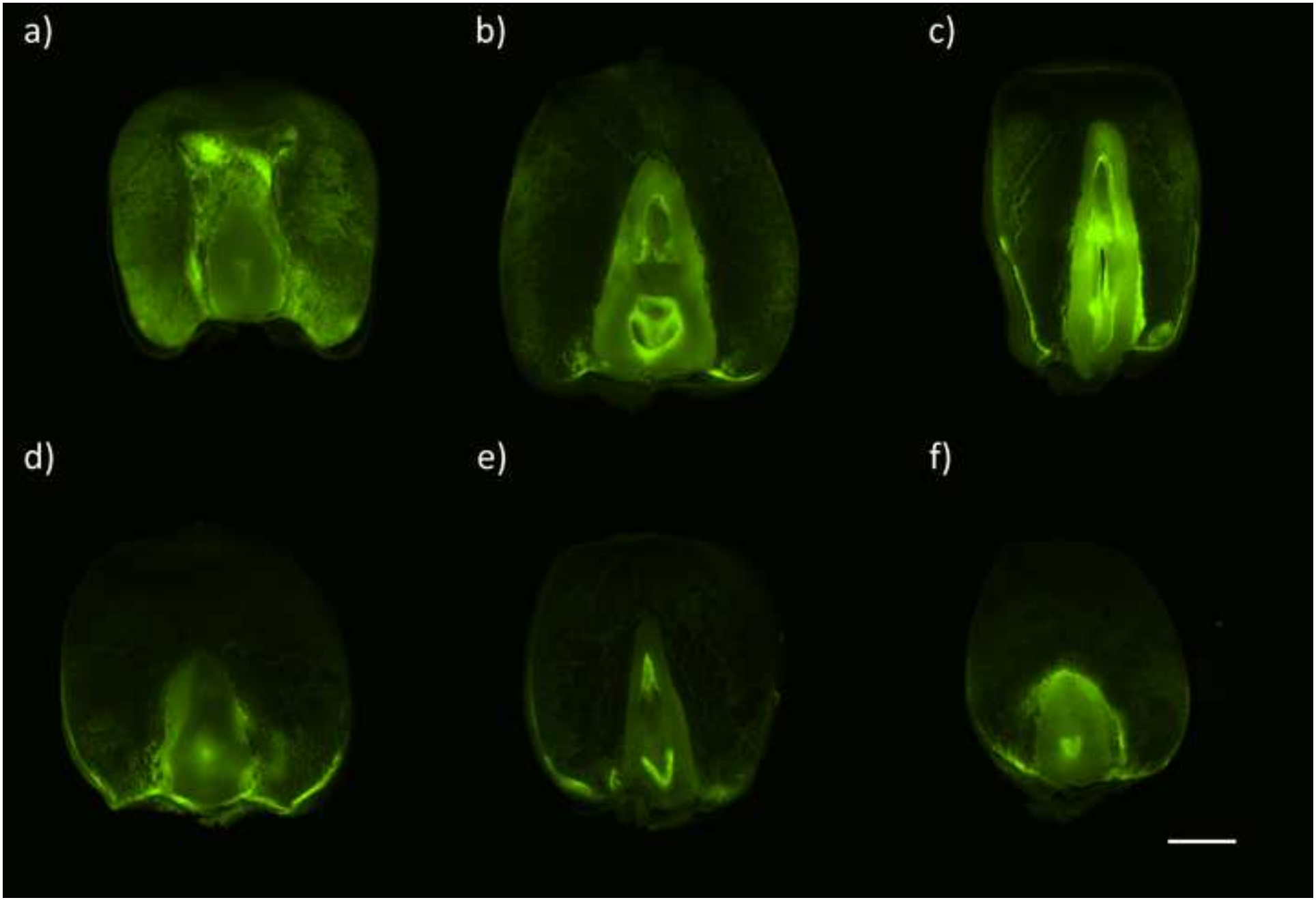
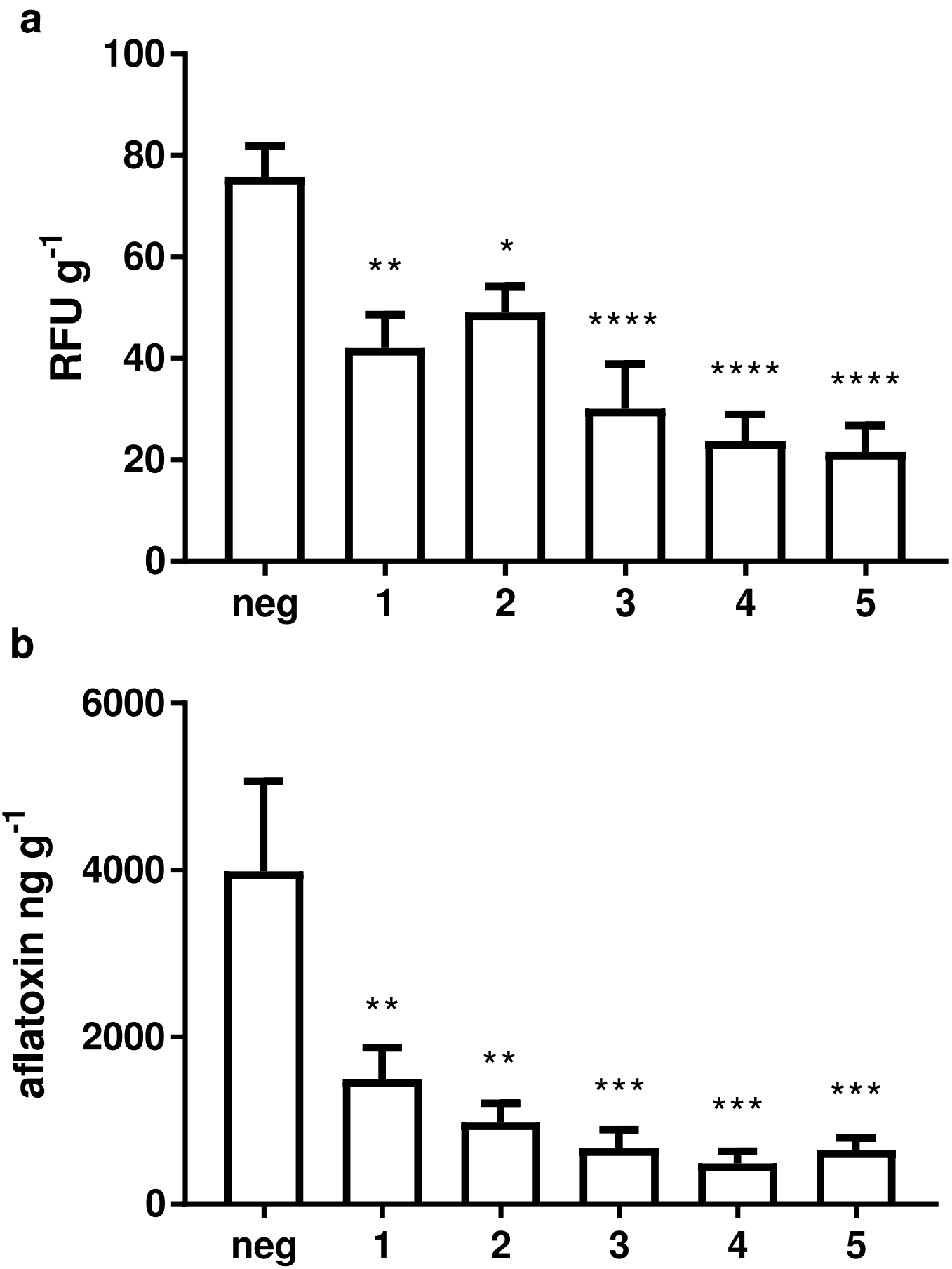


Figure 7



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Agar	Caisson		
Amazing Marine Goop	Eclectic Products		
C1000 Touch CFX96 Real-Time System	Bio-Rad		
Corning Falcon Tissue Culture Dishes, 60 mm	Fisher Scientific	08-772F	
Eppendorf 5424 Microcentrifuge	Fisher Scientific		
Erlenmeyer flask with stopper, 50 mL	Ace Glass	6999-10	
Ethanol			
FluoroQuant Afla	Romer Labs	COKFA1010	
Fluted Qualitative Filter Paper Circles, 15 cm	Fisher Scientific	09-790-14E	
Force Air Oven	VWR		
FQ-Reader	Romer Labs	EQFFM3010	
Geno/Grinder 2010	OPS Diagnostics	SP 2010-115	
Innova 44 Incubator Shaker	Brunswick Scientific		
iScript cDNA Synthesis Kit	Bio-Rad	1708890	
liquid Nitrogen			
Low Form Griffin Beakers, 100 mL	DKW Life Sciences	14000-100	
Methanol			
Methylene Chloride			
Nexttec 1-step DNA Isolation Kit for Plants	Nexttec	47N	
Nikon Eclipse E600 microscope with Nikon DS-Qi1 camera	Nikon		
Nikon SMZ25 stereomicroscope with C-HGFI Episcopic Illuminator and Andor Zyla 4.2 sCMOS camera	Nikon		
Nunc Square BioAssay Dishes	ThermoFisher Scientific	240835	
Phire Plant Direct PCR Kit	ThermoFisher Scientific	F130WH	
Polycarbonate Vials, 15 ml	OPS Diagnostics	PCRV 15-100-23	
Potato Dextrose Broth			
Snap Cap, 22 mm	DKW Life Sciences	242612	
Sodium Phosphate dibasic heptahydrate	Sigma-Aldrich		
Sodium Phosphate monobasic	Sigma-Aldrich		
Spectrum Plant Total RNA Kit	Sigma-Aldrich	STRN50	
Stainless Steel Grinding Balls, 3/8''	OPS Diagnostics	GBSS 375-1000-02	
Stir Plate			

Synergy 4 Fluorometer	Biotek	
T100 Thermal Cycler	Bio-Rad	
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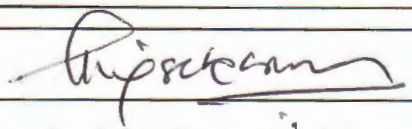
Signature:	
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Nov 09, 2018

Dear Dr. Steindel,

Thank you for your letter of October 22, 2018 with editorial and peer two reviews of our Manuscript No. JoVE59169 "Inhibition of *Aspergillus flavus* α-amylase in maize expressing the *Lablab purpureus* AILP gene: analysis of fungal growth and aflatoxin production", by Kanniah Rajasekaran, Ronald J. Sayler, Christine Sickler, Rajtilak Majumdar, and Jeffrey W. Cary. The title of the revised manuscript is "**Inhibition of *Aspergillus flavus* growth and aflatoxin production in transgenic maize expressing the *Lablab purpureus AILP* gene".** Thank you again for approving additional time to complete our revision.

We appreciate the editorial and reviewers' comments and have revised the manuscript in accordance with the suggestions. They have definitely helped us improve the paper. We took this opportunity to thoroughly proofread our manuscript to correct syntax and typographical errors. Our revision has been highlighted in Red in the manuscript text as well (no track editing to avoid clutter). The following is our item-by-item disposition of the comments and suggestions (in Red):

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
DONE

2. Please revise lines 319-321 to avoid previously published text.
DONE (lines 373-376)

3. Please revise the title to be more concise and avoid punctuation.
The title is revised to read "**Inhibition of *Aspergillus flavus* growth and aflatoxin production in transgenic maize expressing the *α*-amylase inhibitor from *Lablab purpureus* L."**

4. Please provide an email address for each author.
DONE

5. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
Revised Summary is provided

6. Abstract: Please include an overview of the presented method and a summary of its advantages, limitations, and applications.
We have included an overview of the Kernel Screening Assay and highlighted its advantages, limitations and applications

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For

example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
DONE

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
We have indeed provided additional details as pointed out

9. Line 100: Please also describe PCR conditions.
DONE (lines 113-118)

10. Line 111: Please specify growth conditions (temperature, light conditions).
DONE (lines 126-127)

11. Line 172: Please add more specific details about DNA isolation or provide relevant references.
DONE (lines 185-190)

12. Line 174: Please list PCR primers and conditions.
DONE (lines 192-195)

13. Line 182: Please add more specific details about RNA isolation or provide relevant references.
DONE (lines 202-205)

14. Line 187: Please add more specific details about preparing cDNA or provide relevant references.
DONE (line 206)

15. Lines 198-199: Please list an approximate volume of solutions to prepare.
DONE (line 215)

16. Line 223: Please provide more specific details.
We do not know the specific details of the proprietary information. However, we have given the information as available from the manufacturers, Romer Labs. (lines 240-242)

17. Please include single-line spaces between all paragraphs, headings, steps, etc.
DONE

18. Please remove commercial language (TriDye™).
DONE

19. Line 274: Please use a superscripted number for the reference.

DONE (line # 301 in the revision)

20. Figure 4: Please explain what different lanes are. Please consider combining Figure 4 and Figure 5 to reduce the total number of figures
DONE – see Figure Legends

21. Figure 6: Please explain what different bars represent. Different samples?
DONE

22. Figure 8 and Figure 9: Please consider combining Figure 8 and Figure 9 to reduce the total number of figures.
DONE - see Figure Legends

23. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the Name of Material/ Equipment.
DONE – sorted the list according to Name of Material/Equipment

24. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We have added three paragraphs in the Discussion with citations to cover all the details suggested above.

25. References: Please do not abbreviate journal titles.
DONE

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript reports a protocol that should be of interest to research scientists interested resistance to Aspergillus flavus infection and aflatoxin contamination. It likely has applicability to other fungi as well with appropriate modifications.

Minor Concerns: The germplasm line Hi-II is highly susceptible to aflatoxin contamination. The effects of AILP would be better determined in lines better adapted to field conditions. I encourage the evaluation of the effects of AILP in other genetic backgrounds when future

experiments are undertaken.

Thanks to this reviewer for the comments. We agree that Hi-II hybrid is a susceptible to aflatoxin contamination. However, we worked with this variety for two reasons: 1) this is one of the few easily regenerable maize variety available when we started this work seven years ago; 2) any improvement in antifungal resistance can easily be measured in this susceptible line after prolonged selfing, as we have demonstrated in this paper. However, we agree that we will be using other promising inbred lines in our future experiments so that we could transfer the trait to commercial lines.

Reviewer #2:

Manuscript Summary:
Paper is interesting main results, well described and straightforward. I've some minor concerns and some point I'd be glad authors discuss further

Minor Concerns:
Please provide the data on af inhibitions normalized on fungal growth. The inhibition rate is meaningful but should be adjusted upon fungal growth inhibiting effect 'bias'.

The graph that we generated showing ng/g (ppb) levels of aflatoxin is one of the standard way of presentation in the scientific literature and also by the corn farming/marketing community. For these simple reasons, we would like to keep the graph as is; however, as per the reviewer's suggestions we have recalculated the inhibition rate devoid of bias due to fungal growth inhibition and is provided as a supplemental file. We have also provided a regression coefficient analysis, as a supplemental file, linking a close correlation between GFP values (=fungal growth) and aflatoxin levels.

Please provide a study on the omologues or orthologues presenting consistent match to your amylase inhibitor. The effect on maize growth - reduction - could fit better if you experience some cross inhibitory effects on maize omo/orthologues.

This is a relevant question. Production of α- amylase inhibitors targeting pathogen/pests are reported in a wide variety of plant species. This serves as an important natural defense component in plants. The substrate binding specificity of the plant derived inhibitors of pathogen's α- amylase is unique and only targets and inhibits α- amylases from pathogen/pests. Several investigators (Woloshuk and others cited in our manuscript) demonstrated previously the uniqueness of α-amylase inhibitor gene *AILP* from *Lablab purpureus* against *A. flavus* and other fungal pathogens. We also observed that transgenic expression of the *AILP* gene in maize did not negatively affect phenotype, growth or seed set in transgenic maize plants suggesting that the *AILP* gene does not affect maize native α- amylases.

If some protein sharing partly the same sequence and/or function exists, it could be possible to propose a genome editing (crisp cas9) approach instead of "classic" transgender strategy. It could

be more 'palatable' even for countries experiencing the AF problem but with strict legislation on the US of gmo. This point is solely intended for discussion.

We agree that CRISPR/Cas9 gene editing is favorable to transgenes from various sources. However, it is essential to first identify the gene(s) that we can go after for gene editing. As we know now, resistance to this saprophytic *A. flavus* is multigenic in maize other susceptible crops and it is difficult to identify native genes. Further transcriptomic/metagenomic analyses may provide clues to identifying suitable target genes for editing, which we are currently undertaking. For example, if there are specific maize α- amylases that are targeted by the pathogen for successful pathogenesis that would be a prime target for editing to increase resistance (without compromising agronomic traits). Hopefully all our and collaborators' research efforts will lead to selecting the most efficient, consumer-friendly tool to incorporate resistance to this toxic fungal species.

Hopefully I have answered all the comments or suggestions to your satisfaction and that the revised manuscript is now acceptable for publication in JoVE.

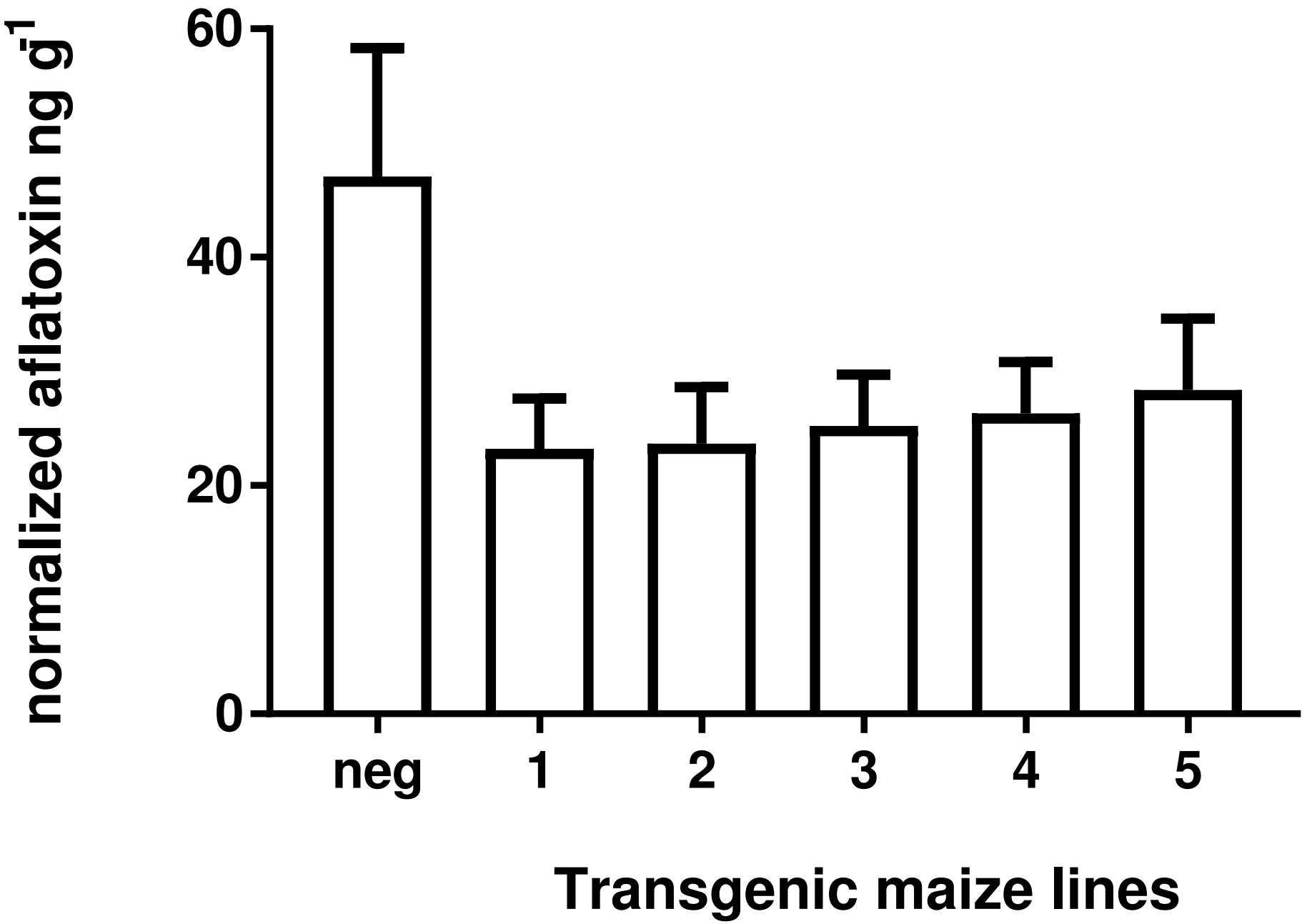
Thank you,

Best regards,

Kanniah Rajasekaran

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Normalized aflatoxin to fungal growth or GFP.eps



Supplemental Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);aflatoxin_GFP correlation.eps