

AGRICULTURE

Aflatoxin-free transgenic maize using host-induced gene silencing

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Aflatoxins, toxic secondary metabolites produced by some *Aspergillus* species, are a universal agricultural economic problem and a critical health issue. Despite decades of control efforts, aflatoxin contamination is responsible for a global loss of millions of tons of crops each year. We show that host-induced gene silencing is an effective method for eliminating this toxin in transgenic maize. We transformed maize plants with a kernel-specific RNA interference (RNAi) gene cassette targeting the *afIC* gene, which encodes an enzyme in the *Aspergillus* aflatoxin biosynthetic pathway. After pathogen infection, aflatoxin could not be detected in kernels from these RNAi transgenic maize plants, while toxin loads reached thousands of parts per billion in nontransgenic control kernels. A comparison of transcripts in developing aflatoxin-free transgenic kernels with those from nontransgenic kernels showed no significant differences between these two groups. These results demonstrate that small interfering RNA molecules can be used to silence aflatoxin biosynthesis in maize, providing an attractive and precise engineering strategy that could also be extended to other crops to improve food security.

INTRODUCTION

Aflatoxins are potent carcinogenic metabolites produced by the *Aspergillus* species *Aspergillus flavus* and *Aspergillus parasiticus*. Ingestion of this mycotoxin by humans and animals can result in hepatotoxicity, liver cancer, kwashiorkor, Reye's syndrome, and impaired growth (1–4). These fungi infect a wide range of crops and are responsible for massive agricultural losses worldwide. For example, 16 million tons of maize is lost globally each year to aflatoxin contamination (5). In the United States alone, contamination of food and animal feed by aflatoxin results in an annual estimated agricultural loss of \$270 million (1). The presence of aflatoxins in the food chain therefore threatens people's livelihoods, agricultural development, food security, and human health. Consequently, more than 100 countries have legislative restrictions on the maximum level of aflatoxins in food and feed (6). Current prevention strategies include breeding disease-resistant crops (7), agronomic practices to decrease the ability of the fungus to grow, biocontrol with atoxigenic *Aspergillus* strains (8), improved postharvest storage methods (9), and the use of trapping agents to block toxin uptake (10), but these strategies are all proving inadequate.

Biotechnological approaches, such as host-induced gene silencing (HIGS), which involves the expression of double-stranded RNA molecules in plants to silence genes expressed by pests and pathogens, offer a viable alternative for alleviating aflatoxin contamination. HIGS has been used successfully in plants to limit insect (11, 12) and parasitic nematode (13) feeding and to retard growth of pathogenic fungi (14–17). Thus, we reasoned that HIGS technology could be applied in maize as well to suppress aflatoxin production in contaminating *Aspergillus* species.

At least 20 aflatoxins are known, but aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) are the four found in food (18), and their biosynthetic pathway is well characterized at both the genomic and biochemical levels (19, 20). The first step in the synthesis of all four of these aflatoxins is the formation of a

polyketide backbone structure from acetate (Fig. 1A), which is catalyzed by three enzymes: two fatty acid synthases and the polyketide synthase. The polyketide synthase *afIC* (*pkSA*) is a unique enzyme in this pathway and therefore is an ideal candidate to target by RNA interference (RNAi). The polyketide synthase *afIC* gene has been cloned from both *A. flavus* and *A. parasiticus* and encodes a 2109–amino acid protein. Here, we report the silencing of aflatoxin biosynthesis in *Aspergillus* through the novel use of HIGS targeted to the fungal *afIC* transcript in infected maize kernels. We selected maize as our model system because it is one of the crops most severely affected by aflatoxin contamination. We show that HIGS provides a viable means to reduce this toxin in maize and potentially in other food crops as well.

RESULTS

An *afIC*-silencing RNAi cassette was expressed in transgenic maize kernels

To knock down *afIC* expression (Fig. 1A), we first constructed an RNAi cassette consisting of three head-to-tail sections of the *Aspergillus afIC* gene to ensure that the fungal transcript was uniquely targeted and fully silenced (Fig. 1B). A 1.1-kb 27-kDa γ -zein endosperm-specific promoter was used to direct expression of the RNAi cassette targeting the silencing of the fungal polyketide synthase gene. The kernel-specific gene expression cassette was placed in a vector having bialaphos resistance as a plant selectable marker. Maize transgenic plants were obtained via *Agrobacterium tumefaciens*-mediated transformation of a B73 \times A118 hybrid line. Three transgenic maize lines were grown to the T₃ homozygous generation by repeated rounds of self-pollination and screened to confirm the expression of the selectable marker by glufosinate leaf-painting assays, and the presence of the inserted RNAi cassette by polymerase chain reaction (PCR) on genomic DNA.

We then conducted reverse transcription PCR (RT-PCR) analysis to determine whether the RNAi cassette was expressed (Fig. 2A). Total RNA extracted from kernels 10 to 12 days after pollination (DAP) was used to produce complementary DNA (cDNA) and to investigate the expression of the inserted RNAi aflatoxin (RNAiAFL) cassette. Our results show that the RNAi cassette was transcribed in the three RNAiAFL samples tested (AFL4, AFL5, and AFL20), but not in the control segregating nontransgenic samples (Null) (Fig. 2A, upper panel). Figure 2A shows the presence

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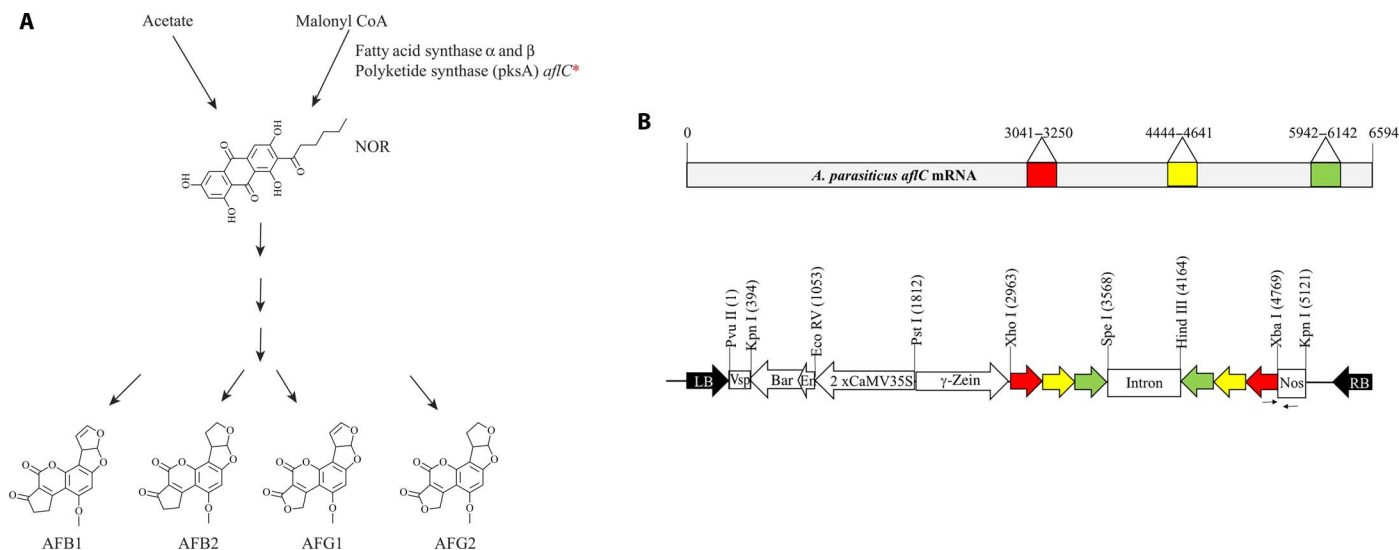


Fig. 1. Construction of an RNAi cassette to silence aflatoxin synthesis. (A) Overview of the fungal biosynthesis pathway of all four aflatoxin variants (AFB1, AFB2, AFG1, and AFG2) from the common precursor metabolite norsolorinic acid (NOR). The polyketide synthase *aflC* (pksA) step targeted for silencing in this report is denoted by “*.” (B) Map of transformation vector depicting the RNAi cassette under the control of an endosperm-specific promoter (γ -zein) and with three tandem 200–base pair (bp) fragments homologous to three different regions of the targeted aflatoxin biosynthetic enzyme *aflC* 6594-bp transcript. En, tobacco etch virus translational enhancer; Vsp, soybean vegetative storage protein terminator; LB, left border; RB, right border; Nos, nopaline synthase gene terminator; CaMV35S, cauliflower mosaic virus promoter; Bar, bialaphos resistance gene. The two small arrows depict the annealing site for primers used in the RT-PCR for detecting the expression of the RNAi-inserted cassette.

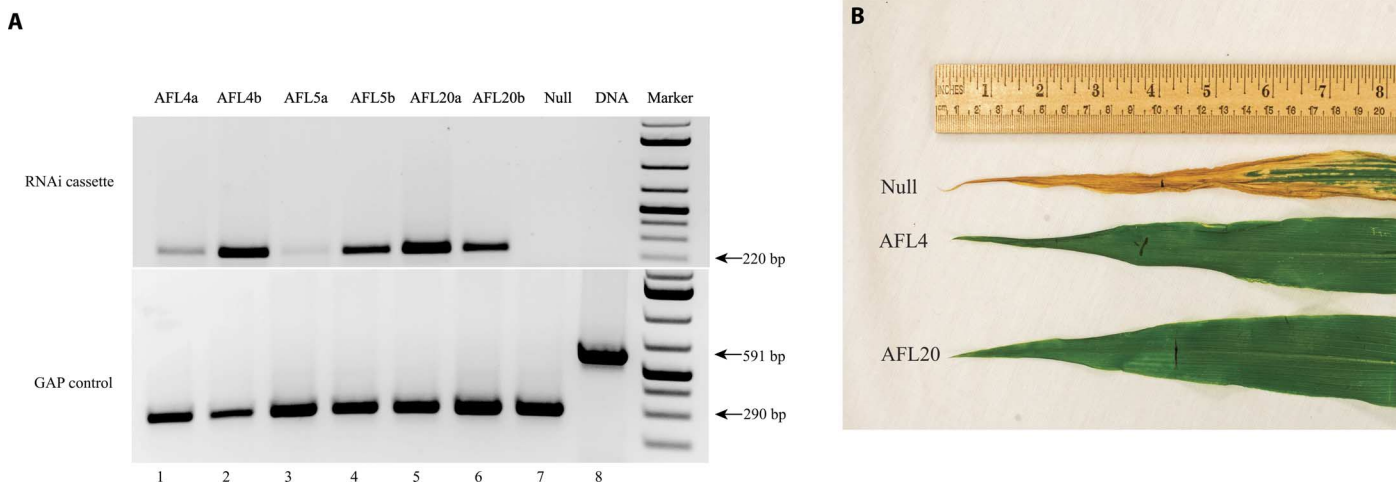


Fig. 2. Expression of RNAi cassette in maize kernels. (A) RT-PCR analysis of total RNA isolated from developing kernels to assess whether the RNAiAFL cassette is expressed in transgenic kernels. cDNA produced from RNA isolated from two plants from each of the three transgenic RNAiAFL lines (AFL4, AFL5, and AFL20) and from a segregating null plant was used to amplify a 220-bp segment of the inserted RNAiAFL cassette (upper panel) and amplify a 290-bp endogenous maize gene (GAPDH; lower panel; lanes 1 to 6 cDNA from transgenics and lane 7 cDNA from null). The presence of genomic DNA in the cDNA preparation would be detected with the primers used as amplification from genomic DNA, which would produce a 591-bp band (lane 8 genomic DNA from null). The presence of the RNAi cassette amplicon (upper panel) indicates its expression in the transgenic lines tested (lanes 1 to 6) and not in the null control (lane 7). The differential size of GAPDH control amplification indicates that cDNA was amplified in transgenic samples, not genomic DNA. (B) Leaf-painting assay results using glufosinate ammonium solution (3 mg/ml) and observed after 7 days. Necrotic tissue in null control indicates the lack of expression of the *Bar* plant selectable marker, whereas RNAiAFL lines (AFL4 and AFL20) were resistant.

of a 220-bp segment in all the RNAiAFL transgenic samples tested and not in segregating null control kernels. An internal maize gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified as a constitutively expressed control (Fig. 2A, lower panel). The designed GAPDH primers flank an intron, so they amplify a 591-bp fragment from genomic DNA (Fig. 2A, lane 8) and amplify a 290-bp fragment from cDNA (Fig. 2A, lanes 1 to 7). The 290-bp amplicon present in the

RNAiAFL transgenic samples, with the absence of the 591-bp segment, indicates that there was no contaminating genomic DNA present in the samples. Together, these results show that the inserted RNAiAFL silencing cassette is expressed in the three transgenic maize RNAi lines tested (AFL4, AFL5, and AFL20). Because RNAiAFL5 line showed weak expression in plant AFL5a (Fig. 2A), this line was excluded from further assays. The three transgenic lines (AFL4, AFL5, and AFL20) were screened

both by PCR (to assay for the presence of the inserted RNAi gene cassette) and by leaf-painting assays [glufosinate ammonium (3 mg/ml)] (to determine the expression of the bialaphos-resistant selectable marker) (Fig. 2B) for repeated generations until homozygous lines were obtained; these homozygous lines were then used for the rest of the experiments.

Aflatoxin was undetectable in RNAiAFL-expressing maize kernels

Transgenic RNAi-expressing lines were infected with the *A. flavus* AF13 isolate, which is capable of producing very high amounts of aflatoxins in maize (8). To assess the extent to which the inserted RNAi cassette was capable of silencing aflatoxin production, we wound-inoculated developing maize kernels on cobs born on plants growing in a contained greenhouse with AF13. Briefly, 10 μ l of spore suspension containing approximately 100,000 spores of *A. flavus* AF13 was inoculated into 3-mm holes cut into developing cobs 10 DAP (R2 development stage) with a cork borer. Two homozygous RNAiAFL lines (AFL4 and AFL20) and three null B73 hybrid controls (Null A, Null B, and Null C) were infected, and inoculated cobs were harvested 30 days after infection (Fig. 3A). Six to eight kernels surrounding the infection point were combined and assayed for aflatoxin load by thin-layer chromatography (TLC) with fluorescence densitometry.

Kernels from the RNAiAFL transgenic maize plants contained nondetectable levels of aflatoxin after *Aspergillus* infection, in contrast to the high levels of toxin detected in kernels from nontransgenic null controls (Fig. 3B). Quantification of aflatoxin in the individual samples is shown in table S1. The limit of detection for TLC fluorescence quantification used in this study was approximately 93 parts per billion (ppb). Three RNAiAFL4 plants (AFL4a, AFL4b, and AFL4c) were infected and assayed along with two RNAiAFL20 plants (AFL20a and AFL20b) and three segregating nontransgenic plants of the same cultivar (Null A, Null B, and Null C). For each plant tested, there were three to four infections on each cob. Although there was variability from plant to plant in the extent of toxin accumulation in the nontransgenic control samples, the results consistently show that null plants became contaminated with aflatoxin concentrations higher than 1000 ppb, whereas aflatoxins were not detected in any of the RNAiAFL transgenic lines.

Total RNA isolated from fungus-infected maize tissue was used in quantitative RT-PCR (qRT-PCR) assays to determine whether the RNAiAFL cassette expressed in transgenic maize kernels could silence the targeted *afIC Aspergillus* transcript. Figure 3C shows the presence of the *afIC* transcript in two null control lines but very low levels in two biological replicates of the two transgenic RNAiAFL lines (AFL4 and AFL20). The reduction of this transcript in both replicates of the two RNAiAFL-expressing lines AFL4 and AFL20 indicates that the inserted RNAi cassette silences the targeted fungal gene. The targeted *Aspergillus afIC* gene does not contain an intron, and so it is not possible to tell whether it is cDNA or contaminating genomic DNA that is being amplified in these experiments. Therefore, the intron-containing *Aspergillus tubulin* gene was used as a control to ensure that cDNA was being amplified and that the qRT-PCR experiments are therefore a true reflection of *afIC* expression levels (fig. S1). Because the *afIC* transcript was significantly suppressed in all RNAiAFL samples tested compared to null control transcript levels (Fig. 3C), the inserted RNAiAFL cassette is sufficient to silence the *afIC* transcript during *Aspergillus* infection of transgenic kernels.

We used qRT-PCR to investigate the presence and quantity of *Aspergillus* in infected maize kernels. As with the *afIC* qRT-PCR assays described above, RNA was isolated from infected maize tissue and used as a template. Expression of the *Aspergillus* chitin synthase C gene was measured and normalized relative to the expression levels of the maize endogenous GAPDH gene (as above). The expression of a fungal gene compared with the expression of a maize gene should indicate the amount of fungal tissue present in each of the infected maize cobs that were subsequently tested for aflatoxin accumulation. We found comparable amounts of fungal chitin synthase gene transcription present in both segregating toxin-accumulating null maize kernels and RNAiAFL transgenic maize kernels in which no toxin was detected (Fig. 3D). Combining these results with those that show the silencing of the fungal *afIC* transcript in the RNAiAFL transgenic maize kernels indicates that RNAi silencing cassettes can be expressed in maize to selectively target actively growing *Aspergillus*.

RNAiAFL-expressing maize kernels demonstrate no substantial expression alterations

Because the effectiveness of RNAi technology relies on a basic eukaryotic cellular mechanism, there is an inherent risk of collateral off-target gene silencing. We therefore investigated whether the RNAiAFL-silencing strategy resulted in the inadvertent suppression of nontargeted maize genes. If other genes were affected in the maize RNAiAFL transgenic plants, it might result in adverse agronomic traits. The RNAiAFL transgenic plants and kernels exhibited the same growth and development as null control plants (fig. S2). We hypothesized that if the inserted expressed RNAiAFL cassette affected gene transcription in the maize kernels, there would consistently be significantly differentially expressed transcripts in the pairwise comparisons between any RNAiAFL transgenic kernel and any nontransgenic null kernel. Because the RNAiAFL cassette is driven by a kernel-specific promoter, we compared the transcript profiles from RNA isolated from three immature (12 DAP) kernels from plants grown to the same developmental stage from three RNAiAFL-expressing transgenic lines (AFL4, AFL5, and AFL20) and two segregating nontransgenic controls (Null A and Null B) grown simultaneously side by side under greenhouse conditions. Transcripts were assembled, and their abundance was estimated using Cufflinks software. Analysis of differentially expressed transcripts was performed using Cuffdiff to both assemble and quantify transcripts from two samples (21, 22). The number of transcripts detected within all five samples was comparable, with Null A and Null B having 84,830 and 84,430, respectively, and the three RNAiAFL lines having 84,223 for AFL4, 86,241 for AFL5, and 84,956 for AFL20. All six possible pairwise comparisons were generated with the three RNAiAFL transgenic lines and two null samples [National Center for Biotechnology Information (NCBI) BioProject PRJNA319828]. The transcripts common to a pairwise comparison with putative functions in all the six RNAiAFL transgenic to nontransgenic null comparisons were determined (http://de.iplantcollaborative.org/dl/d/24DC6B1C-EF2C-4A00-9EBF-85B43234E88C/Pairwise_Sig_Transcripts_with_Function.xlsx), and the matrix intersections of the comparisons are shown in Fig. 4. Comparison of the transcripts at $P < 0.05$ revealed 70 to 100 significantly differentially expressed transcripts depending on which individual transgenic RNAiAFL data set was compared to which individual null control data set (Fig. 4, orange horizontal bars). Further analysis of the transcript data sets revealed that no single significantly differentially expressed transcript intersected with all six pairwise comparisons (Fig. 4, green vertical bars)—that is, no

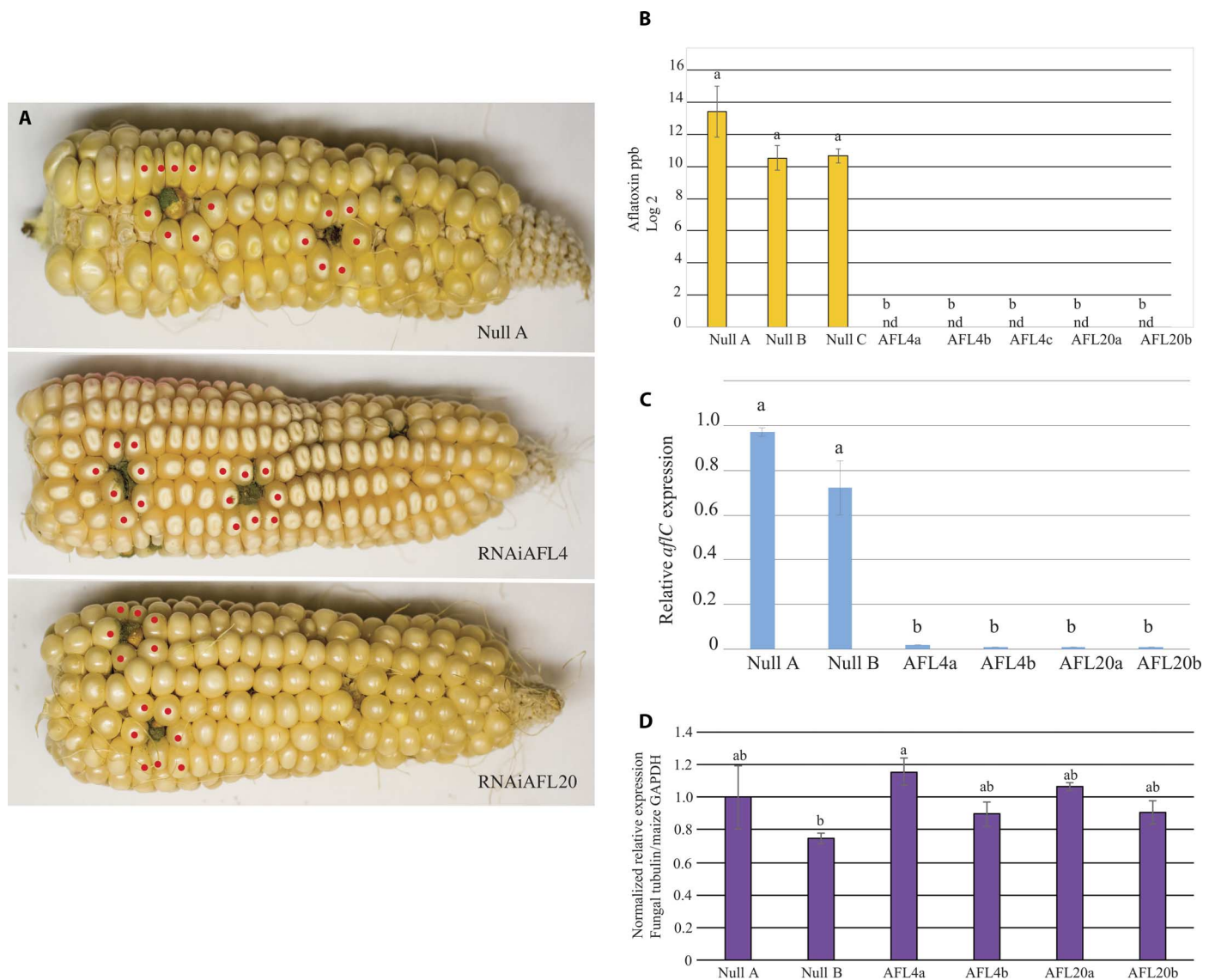


Fig. 3. *Aspergillus* infection of transgenic RNAiAFL cobs and toxin assay. (A) Toxin-producing *A. flavus* was injected into 8- to 10-DAP maize cobs and allowed to grow for 30 days. Infected maize cobs were subsequently harvested, and kernels surrounding each infection (denoted by red dots) were combined and assayed for toxin. (B) Aflatoxin levels (as log₂ ppb) in three biological replicates of the transgenic RNAiAFL 4 line (AFL4a, AFL4b, and AFL4c), two biological replicates of the transgenic RNAiAFL 20 line (AFL20a and AFL20b), and three Null control (A, B, and C) cobs, as determined by TLC analysis. nd, nondetectable. (C) Transcript levels of the *Aspergillus aflC* gene relative to the fungal endogenous tubulin gene were determined by qRT-PCR analysis performed on RNA isolated from *Aspergillus*-infected maize kernels. (D) Relative expression of the *Aspergillus* chitin synthase gene was determined and normalized to the expression of the maize GAPDH gene. As above, RNA from fungal-infected maize cobs was used to extract RNA and perform qRT-PCR. Analysis showing that only cDNA, not contaminating genomic DNA, was amplified in (C) and (D) reactions is shown in figs. S1 and S4, respectively. All means and SEs were calculated from at least three replicates. Means with the same lowercase letter do not display a significant difference [$P < 0.05$; analysis of variance (ANOVA)].

transcript was seen to be consistently differentially expressed in all six pairwise comparisons involving a transgenic line and a nontransgenic null. Maize transcripts that have sequence homology to the hairpin portion of the inserted RNAiAFL cassette should exhibit differential gene expression in transgenic kernels compared to nontransgenic null controls. Our transcriptional analysis indicates that the RNAiAFL cassette did not have sufficient sequence homology to any kernel maize transcript to result in suppression because the analysis did not show a single significantly differentially expressed transcript common to all the transgenic and nontransgenic comparisons. This

suggests that the inserted and expressed RNAi cassette to silence the fungal aflatoxin biosynthesis pathway did not target other genes in the maize kernels.

The intersection involving groupings of fewer than all six transgenic-to-null pairwise transcript data sets did have some significantly differentially expressed transcripts in common. These variations are probably attributable to different genome insertion sites of the transgene between RNAiAFL lines, slight changes in kernel development, and/or microenvironments as opposed to an alteration of transcription attributable to the inserted gene cassette because they do not consistently correlate

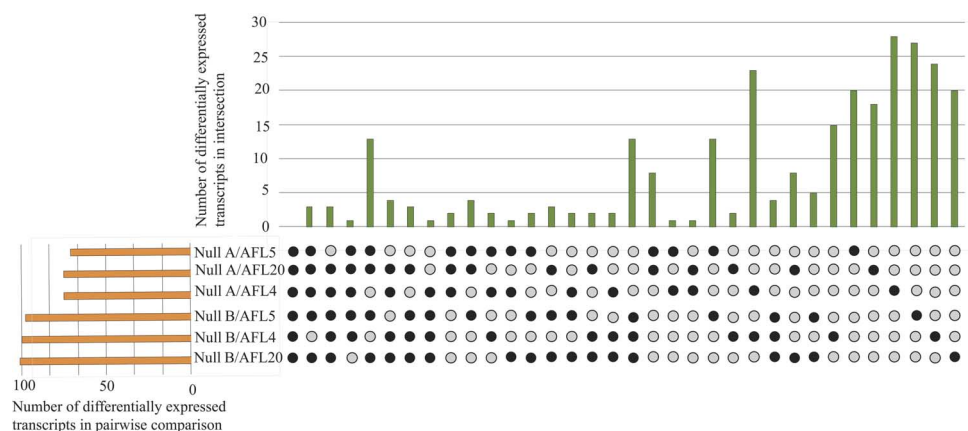


Fig. 4. Matrix for intersections of differentially expressed transcripts between RNAiAFL transgenic and null kernels. The intersection of six pairwise comparisons between two nontransgenic (Null A and Null B) samples and three transgenic (AFL4, AFL5, and AFL20) samples are shown. Set size was sorted by the number of significantly differentially expressed transcripts within a given pairwise comparison (horizontal orange bars). Dark circles in the matrix indicate sets of transcripts that are part of an intersection. The number of intersecting transcripts that are differentially expressed within the given grouping is shown in green. The matrix intersection of the comparison of the three RNAiAFL transgenic lines among themselves and the two Null controls shows similar or larger intersections of the number of differentially expressed transcripts (fig. S3). A generated table shows the significantly expressed transcripts detected in all six pairwise comparisons used to generate this matrix (available online at http://de.iplantcollaborative.org/dl/d/24DC6B1C-EF2C-4A00-9EBF-85B43234E88C/Pairwise_Sig_Transcripts_with_Function.xlsx).

with the expression of the transgenic cassette. Similarly, a small number of transcripts were also seen to differ in comparisons of RNAiAFL transgenic data sets and also the pairwise comparison of the two nontransgenic null data sets (fig. S3). This further suggests that these minor transcript differences are caused by natural variations in gene expression. In summary, these transcript results demonstrate that in the total gene expression profile state, there are no significant differences in the transgenic RNAiAFL kernels compared with the nontransgenic kernels.

DISCUSSION

Our studies show that HIGS is highly effective for reducing aflatoxin levels in engineered maize kernels to below current U.S. and international regulatory thresholds without either morphological or gene transcription alterations of the transgenic kernels. Previous research efforts to use RNAi to silence toxin production resulted in a mere 5- to 10-fold reduction and impaired the growth of transgenic plants, presumably because the RNAi cassette targeted a fungal transcription factor gene that may have had other silencing effects (23). Before embarking on this project, a bioinformatics analysis determined that the *Aspergillus* polyketide synthase gene does not have any notable DNA sequence homology with the maize genome. In addition, because *aflC* is quite large (6594 bp in *A. parasiticus*), three approximately 200-bp regions could be selected for use in the RNAi cassette on the basis of their lack of homology to the maize genome. This is probably why the RNAiAFL maize plants seem overtly similar to nontransgenic plants and that no substantial differences could be detected in the kernel transcript analysis. The targeting of three separate areas of the polyketide synthase gene all contributed to its silencing and thereby to no-toxin production, as opposed to other research efforts that achieved only a slight transcript reduction and toxin production.

Gene expression silencing by RNAi technology has been extensively used both in functional genomics studies [for example, (24, 25)] and to produce desired plant phenotypes [for example, (26–28)]. With recent advances in plant genome-editing technologies, particularly through the CRISPR (clustered regularly interspaced short palin-

dromic repeats)/Cas system (29, 30), the use of RNAi for commercial trait production will probably decline. CRISPR/Cas has the advantage of producing a targeted mutation that can be designed to silence a gene without incorporating any foreign DNA, but this results in the production of a trait that might not be a regulated transgenic event (31, 32). RNAi technology will still be needed as the enabling technology for HIGS because it is currently the only known means to produce small interfering RNA molecules in plant tissue that could be used to mitigate transient biotic stresses, including the mycotoxins detailed here, and to target plant pathogens (12–14). However, with HIGS, there is the possibility of silencing off-target gene expression and, consequently, producing unintended phenotypes in the host species. In the maize-kernel transcript analysis presented here, we found no consistent difference in transcripts corresponding to the expression of the inserted aflatoxin-silencing RNAi cassette, suggesting that no off-target gene expression events occurred in the transgenic maize kernels. This result is crucial because for a HIGS transgenic plant to receive regulatory approval, it must be substantially equivalent to the nontransgenic material except for the inserted phenotype(s).

The Food and Agriculture Organization estimates that 25% of the world's food crops are affected by fungal toxins (33), often in areas that experience significant food security challenges. In particular, aflatoxins create broad economic and health problems that have an effect on the consumption of maize and several other crops. These toxins become more prevalent and thus become more of a food safety concern during severe heat and drought, because these conditions are optimal for the fungal invasion of crops. Aflatoxin-contaminated products cause significant economic and trade problems at almost every stage of production and marketing. Our study shows that HIGS is a viable control mechanism to alleviate aflatoxins in maize and could be applicable to other crops. Furthermore, we have shown that, by targeting the mycotoxin biosynthetic pathway, aflatoxin levels can be effectively reduced below the regulatory threshold without producing any overt off-target effects on the host crop plant. In a wider context, metabolic-targeted HIGS could be effective at eliminating a broad range of adverse bioactive compounds in plants and their pests.

MATERIALS AND METHODS**RNAi polyketide synthase (*aflC*) cassette**

Synthetic DNA (Celtek) that incorporates three tandem sections of the polyketide synthase (*aflC*) mRNA from *A. parasitus*, which constitute the arms of the RNAi silencing cassette, was produced. A 605-bp chimeric synthetic fragment homologous to the *aflC* gene consisted of 5' restriction enzyme sites Xba I and Xho I, followed by a 209-bp fragment from nucleotide regions 3041 to 3250, 197 bp from regions 4444 to 4641, and 200 bp from regions 5942 to 6142 (numbering according to GenBank accession L42766), with 3' Hind III and Spe I restriction sites. The synthetic DNA was cloned in inverted repeats around an intron in plasmid pKan-intron, as previously described (24). The hairpin cassette and the 1.1 kb γ -zein promoter from maize (34) (provided by K. Wang, Iowa State University) were cloned into vector pMON999. Correct orientation with respect to the regulatory elements was independently confirmed by sequencing using both a γ -zein promoter primer and a nopaline synthase (Nos) terminator primer (table S2). The γ -zein promoter-regulated RNAi cassette was then cloned into plasmid pTF101.1, an *Agrobacterium* transformation vector harboring *bar* resistance (phosphinothricin acetyltransferase) under the enhanced 35S CaMV promoter (Gateway). The resultant cassette was hereafter referred to as pRNAiAFL (Fig. 1B). All enzymes were used according to the manufacturer's instructions and were purchased from New England Biolabs.

Transgenic maize production

Maize (*Zea mays* Hi II hybrid A188 and B73 background) was transformed with the pRNAiAFL construct via *A. tumefaciens*-mediated transformation (35) by the Iowa State University Plant Transformation Facility (<http://agron-www.agron.iastate.edu/ptf/>). Nine bialophos-resistant maize lines were received as plantlets and regenerated and screened both by PCR to detect the γ -zein::RNAiAFL construct within the genomic DNA and by leaf-painting assays. For painting assays, an approximately 3.8 cm adaxial tip surface of the fully expanded leaves of 8- to 10-day-old plants was sprayed with a glufosinate ammonium (3 mg/ml; Oakwood Products) solution with the treated area marked. Glufosinate resistance was visually scored 7 days after treatment. Although all nine transgenic lines expressed the *bar* selection gene, only three lines were grown to T₃ homozygosity by both repetitive self-pollination and glufosinate leaf-painting assays for use in the subsequent experiments.

RNAiAFL expression in transgenic maize

For the expression analysis of the RNAiAFL transcript, RNA was extracted using TRIzol (Fisher Scientific) from 10 to 12 DAP kernels harvested from three homozygous transgenic lines (RNAiAFL4, RNAiAFL5, and RNAiAFL20) and a nontransgenic (Null) control. First-strand cDNA was synthesized using 1 μ g of total RNA per sample, 9 μ l of 2 M betaine monohydrate (36) (Sigma-Aldrich), and random primers using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions.

PCR was performed using primers specific to both the inserted RNAi cassette and the maize GAPDH gene (GenBank accession X15596.1) (primer sequences in table S2). The reaction mixture included 1 \times PCR buffer, 0.4 μ M of each primer, 0.2 mM of each deoxynucleotide triphosphate, 2.5 U of Taq DNA polymerase (New England Biolabs), and 50% volume of 2 M betaine. The PCR cycles were set at 94°C for 2 min, followed by 34 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min. The amplified products were separated

on a 1% agarose gel (Sigma-Aldrich) mixed with ethidium bromide (0.5 μ g/ml) (Sigma-Aldrich) along with GeneRuler 1 kb Plus DNA Ladder (Fisher Scientific).

***Aspergillus* infection and aflatoxin quantification**

An *A. flavus* AF13 strain (37) spore suspension from water vial stocks was used to grow fresh cultures. A 15- μ l suspension was loaded in a hole made at the center of 5/2 agar medium [5% V-8 vegetable juice and 2% agar (pH 5.2)] in 9-cm-diameter plates and incubated in the dark at 31°C for 5 to 7 days (38). On the day of infection, the surface growth containing mostly spores was picked up using a sterile cotton bud, suspended in 20-ml vials containing 10 ml of sterile 0.02% Tween 80, and vortexed. The spores were counted in nephelometric turbidity units (NTUs) from 12 ml of a 1:10 dilution of spores in 50% ethanol (that is, 1.2 ml of the suspension and 10.8 ml of 50% ethanol) in a marked glass sample vial (1 NTU = 49,937 spores) using a calibrated turbidimeter (Orbeco-Hellige Farmingdale NY model 965-10). The spore suspension was diluted to 1.0 \times 10⁷/ml (100,000 spores/10 μ l) in sterile distilled water.

At 8 to 10 DAP, the developing maize ears were wounded at three to four places by pushing a cork borer (3-mm diameter) through the cob husk to a depth of 5 mm (39). Each ear was inoculated by applying 10 μ l of the above conidial suspension. In each experiment, at least three cobs of each RNAiAFL transgenic line (AFL4 and AFL20) and nontransgenic segregate nulls (A, B, and C) were used. The infection was allowed to progress for 30 days. Harvested cobs were incubated at 45°C for 72 hours. Kernels surrounding the infected areas were harvested and pooled. Dry weights were measured for each set of pooled ground powder of kernels and then suspended in 70% methanol, and the toxin was quantified by TLC, as previously described (39, 40). Briefly, 4 μ l of extract was spotted on 20 \times 20-cm TLC glass plates (Silica Gel 60 F254, Millipore) 2 cm from the bottom. Sample extracts and aflatoxin standards consisting of a mixture of AFB1, AFB2, AFG1, and AFG2 (Sigma-Aldrich) with AFB1 at a concentration of 1 ng/ μ l were developed with diethyl ether/methanol/water (96:3:1). The presence or absence of AFB1 was scored visually under ultraviolet light (365 nm) and quantified using CAMAG TLC Scanner 3 (Camag Scientific Inc.) with winCATS 1.4.2 software (39, 40). Aflatoxin values were determined as ppb of dry weight.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from maize kernels 30 days after infection using the lithium chloride method (41). Three kernels surrounding each infection site from two cobs from each sample (Null A, Null B, RNAiAFL4, and RNAiAFL20) were used. As described previously, cDNA was produced, and qRT-PCR was performed using primers (42) specific to *A. flavus aflC* (GenBank accession AY510451.1). Primers flanking an intron and annealing to *A. flavus* β -tubulin gene transcript (GenBank accession M38265) were used as an expression control (primer sequences in table S2). The qRT-PCR reaction comprised 5 μ l of 0.1 \times diluted cDNA, 10 μ l of SYBR Select Master Mix (Fisher Scientific), and 2.5 μ l of 10 mM of each primer in 20 μ l of total reaction volume. The PCR reaction was performed in a realplex4 Mastercycler (Eppendorf) with realplex 2.0 software. The cycling parameters were 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 68°C for 40 s. For each reaction run, dissociation curves were performed. The baseline was autoselected, and the values of threshold

cycles (C_T) were determined. Transcript levels of *Aspergillus aflC* relative to the β -tubulin gene transcript of *A. flavus* were determined using the comparative C_T method (43). Similar qRT-PCR conditions were performed to determine the relative expression of a fungal endogenous gene, *A. flavus* chitin synthase C (GenBank accession XM_002375348) (primer sequences in table S2), compared with the expression of the endogenous maize GAPDH transcript from RNA isolated from infected maize kernels.

Transcript analysis

Three immature (10 to 12 DAP) kernels from three independent homozygous lines (RNAiAFL4, RNAiAFL5, and RNAiAFL20) and two segregating null plants (Null A and Null B) were used to extract total RNA. Extraction and transcript sequencing were performed by the University of Arizona Genetics Core. Sequencing was performed using Rapid Run SBS chemistry (Illumina) on a HiSeq 2500 machine. All reads were quality-trimmed, and adapters were removed using Trimmomatic 0.25 (44). Paired-end mRNA reads were mapped to the maize genome and its gene models as reference annotations (B73 RefGen_v3, www.maizegdb.org/assembly/) using TopHat (21, 22) with the Bowtie 2 algorithm (45). Transcripts were assembled and their abundance was estimated using Cufflinks, and the assembled and quantified transcripts from two samples were used to analyze differential expression with Cuffdiff (21, 22). Significantly differentially expressed transcript identifiers were analyzed using PhytoMine (<http://phytozone.jgi.doe.gov>) to assign putative gene functions. Intersections of transcript comparisons were determined using the interactive web browser UpSet (<http://vcg.github.io/upset/>) (46).

Statistics

All experiments were repeated with at least three replicates. Data are means \pm SE. To compare differences in treatments, ANOVA with Tukey post hoc test was performed at 95% confidence levels. Means displaying nonmatching lowercase letters are significantly different. *P* values of at least less than 0.05 were considered significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/3/3/e1602382/DC1>

table S1. Individual aflatoxin quantification reading by TLC on maize kernels after *Aspergillus* infection.

table S2. Primer sequences used in this study.

fig. S1. RT-PCR to detect the expression of the *Aspergillus aflC* transcript in fungus-infected maize kernels.

fig. S2. Side-by-side comparisons of RNAiAFL transgenics and null maize plants.

fig. S3. The intersection matrix of pairwise comparisons of significantly differentially expressed transcripts between three RNAiAFL transgenic plants (AFL4, AFL5, and AFL20) and two nontransgenic controls (Null A and Null B).

fig. S4. RT-PCR to detect the expression of the *Aspergillus* chitin synthase C transcript in fungus-infected maize kernels.

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Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper, and Supplementary Materials and transcript comparisons are in repository NCBI Bioproject PRJNA319828. Additional data related to this paper may be requested from the authors.

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