Using Biotechnology to Eliminate Mycotoxins

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Mycotoxins are compounds produced by certain fungi that are deleterious to the health of animals. They are inadvertently ingested by both livestock and humans when crops are infected with a toxin-producing fungus. Food and Agriculture Organization estimates one quarter of the world's crops are contaminated with a mycotoxin[1]. Perhaps the most problematic and widespread is the mycotoxin produced from certain species of the fungus *Aspergillus* (*A. flavus* and *A. parasiticus*) called aflatoxin.

Health concerns of aflatoxin contamination

Four aflatoxin compounds produced by some *Aspergillus* fungi are a major health concern and are responsible for massive agricultural losses world-wide. It is estimated that approximately 4.5 billion people are chronically exposed to aflatoxin through the ingestion of contaminated food items[2]. Aflatoxins are known carcinogenic compounds and have been extensively linked to liver cancer[3]. Liver cancer is the sixth leading cause of cancer worldwide with a prevalence 16-32 times higher in developing countries[3]. Additionally aflatoxin has been associated with growth impairment in children[4] and immunosuppression (for review[5]), the latter likely leading to increased incidence of secondary infections such as HIV and malaria. Populations at high risk for aflatoxin contamination are communities consisting of rural subsistence farmers in developing nations, not only because hot and humid climate conditions are ideal for fungal growth, but also because of both low risk awareness and insignificant enforcement of regulatory consumption limits. As a consequence, blood samples collected from regions of West Africa and Guangxi Province of China were over 90% positive for the aflatoxin biomarker[6].

Economic losses due to aflatoxin contamination

Aflatoxin ingestion is not considered a major health issue in developed countries as there are strict consumption limits enforced. Over 100 countries regulate the level of aflatoxin in food and animal feed for consumption. For example, the US Department of Agriculture regulates the allowable level of aflatoxin in corn destined for human food and dairy cattle feed at their most rigorous limit of 20 parts per billion (ppb). To put this number into perspective, 1 ppb is equivalent to a single drop of water in a 21,700 gallon (82,135 liter) swimming pool or from a time perspective, 1 second in 31.7 years. If a crop is measured above all allowable limits, then it is not permitted to move forward in the production stream and likely is incinerated. In the US alone, aflatoxin contamination of food/feed results in an estimated $270M agricultural loss every year[7]. Conservative estimates are that fungal toxins cost the US between $500 million to $1.5 billion a year in lost crop revenues and expenses in monitoring[8].

Many crops such as peanuts, grains, and nuts are susceptible to *Aspergillus*-infection with subsequent aflatoxin contamination. Current aflatoxin prevention mechanisms involve breeding for fungal resistant crops[9], agronomic practices to lower the ability of the fungus to grow, biocontrol with atoxigenic fungal strains[10], improved post-harvest storage methods[11], and use of trapping agents to block uptake of aflatoxins[12]. These measures are inadequate, as millions of tons of crops are lost due to this toxin each year. Corn is the crop that suffers the most losses due to fungal toxin contamination, with an annual estimated loss of 16 million tons worldwide[13].

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Biotechnology used to alleviate aflatoxin

We have genetically engineered corn to give it the ability to turn off the toxin-producing biosynthesis pathway in *Aspergillus* when the fungus infects the corn kernel[14]. This research was based on a few genetic charachertistics. First is the fact that sequence information within eukaryotic cells flows from double stranded DNA to single stranded RNA to encode for protein. Second is the knowledge that eukaryotic cells, including fungal cells, have an inherit mechanism whereby double stranded RNA molecules are deemed foreign and subsequently degraded by cells[15]. The third principle is that a somewhat new discovery that small RNA molecules readily pass between a host and

its pathogen upon infection[16].

Using this collective information, we inserted a DNA cassette that is expressed only in the edible kernels of the corn plant. In our research, the inserted gene cassette directs the corn to produce a hairpin RNA molecule that would be degraded by the cell's own machinery into small RNA molecules. The small RNA molecules were directed to the corn kernels where they would express a sequence similar to that of a fungus *Aspergillus* gene that encodes for a key enzyme needed for aflatoxin biosynthesis. The corn kernels produced the small RNA molecule throughout corn development, and only when infected with *Aspergillus* does the small RNA molecule gain entrance into the infecting fungal cell. In the fungal cell, it will find its matching RNA sequence to the fungal toxin-encoding full RNA transcript. When the expressed small RNA is in the fungal cell, it finds and pairs with the endogenous fungal RNA sequence. The double RNA structure is recognized by the fungal cell as foreign and the fungal cell's own cellular machinery degrades it. With the fungal RNA degraded, it is not available to encode for the enzyme necessary in aflatoxin biosynthesis.

This aflatoxin-silencing genetically engineered corn provides proof-of-concept that a genetic suppression strategy is an effective means to prevent aflatoxin contamination in crops.

In essence, the small RNA molecules expressed in the corn enter the fungal cell and stop the fungal RNA from synthesizing a necessary toxin-producing enzyme. Corn engineered to express the RNA that was designed to target the toxin-producing enzyme was infected with toxin-producing *Aspergillus* and incubated for one month under controlled greenhouse conditions. There was no toxin detected in engineered kernels (below levels of detection in our methods) compared to control non-engineered kernels exhibiting at least 1,000s ppb toxin. Further molecular analysis showed that the targeted fungal RNA molecule was significantly suppressed in the engineered lines compared to control non-engineered kernels.

To summarize, the engineered corn kernel is directed to produce a small RNA molecule after infection by *Aspergillus*. The small RNA molecule enters the contaminating fungal cell, finds its matching RNA sequence and, in so doing, stops that transcript from encoding for a pivotal enzyme in the aflatoxin biosynthetic pathway. Research involving the use of a host organism, in this case corn plants, to suppress or silence gene expression of an infecting pathogen, such as *Aspergillus*, is known as Host Induced Gene Silencing (HIGS). HIGS has been used successfully to suppress pathogen growth in crops previously (for example[17]), but this is the first report of its successful use of the suppression of a pathogen-produced toxin.

The success of HIGS technology relies heavily on the specificity of the RNA transcript targeted for degradation. The RNA transcript that encodes for the toxin-producing enzyme that we targeted for degradation in our research was the *Aspergillus* 7 kb polyketide synthase gene. Initially we performed a preliminary sequence search of this fungal transcript in other databases, such as in corn, humans, and pigs, as it was essential to find unique regions within the fungal transcript that we could use as the introduced expressed small RNA molecules. We chose three 200 bp regions from the single fungal transcript to introduce into corn for the production of the small RNA molecules. Simultaneously targeting three areas of the one fungal RNA transcript served two purposes: (1) it would enhance the likelihood that the fungal polyketide synthase RNA transcript would be targeted for complete degradation, and not merely be truncated; and (2) it would severely decrease the probability that the *Aspergillus* fungus could evolve resistance to this genetically engineered corn, as the fungus would have to simultaneously mutate three separate sections of the polyketide synthase gene while still encoding for a functional toxin-producing enzyme.

Substantial equivalence at transcript level

Because this research involves the production of an engineered corn plant, we performed preliminary transcript analysis of the corn kernels to assay for substantial equivalence. The trait inserted in the engineered corn plants was targeted only to kernels during their development. No gross morphological differences were observed in the kernels or whole plant of the engineered plants compared to the non-engineered control plants grown side-by-side under greenhouse conditions. Because the inserted trait was a small RNA molecule expressed in the engineered corn kernels, we performed RNA transcript analysis

of RNA isolated from the kernels of three engineered events and two non-engineered controls. We were particularly interested to know if the inserted cassette expressed in corn kernels was causing any 'off target' affects within the overall gene expression in corn kernels. That is, did the sequence of the introduced expressed small RNA molecule targeted to the fungal toxin gene have enough sequence similarly to match RNA transcripts within the corn kernels? If so, the matched RNA would again first form double RNA structures and then cause the degradation of the matching corn transcript and, in turn, perhaps cause many undesired altered characteristics.

We compared transcripts from three engineered kernels to the transcripts from two control kernels in six pairwise comparisons. We did not detect a single transcript that was consistently and significantly expressed differentially in the engineered samples compared to the controls*. This transcript analysis indicates that the introduced fungal-targeted small RNA molecule is specific to the contaminating fungus and does not appear to alter gene expression in the corn kernels.

HIGS as a promising biotechnology tool to combat all mycotoxins

This research shows that fungal-produced toxins can be suppressed or silenced using HIGS biotechnology in corn kernels. This aflatoxin-silencing genetically engineered corn provides proof-of-concept that a genetic suppression strategy is an effective means to prevent aflatoxin contamination in crops. This technology could be incorporated into other aflatoxin-susceptible crops and in the future could be extrapolated to target other mycotoxins in crops grown globally. Mycotoxin contamination in crops threatens agricultural development, trade, food security, and human health. Our research shows biotechnology is a viable option to alleviate fungal toxins from contaminated crops.

Footnote: *This analysis was performed on full RNA transcripts that will encode for proteins to carryout numerous cellular functions and structural roles. Small RNA molecules, including the one introduced in our research, are not detected in this assay.

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