Peanut and Mycotoxin Innovation Lab
Full Proposal

Project Title (if title is longer than 8 words, provide a short title of 8 words or less)
Silencing of aflatoxin synthesis through RNA interference (RNAi) in peanut plants
Short Title: Silencing aflatoxin by RNA interference in peanut

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Geographical Locations (list all countries where the research will be conducted and/or initially targeted)

Kenya
Malawi
Mozambique
Uganda
United States of America
Zambia

Project Duration

Four years, September 2013 to September 2017

Executive Summary

We have obtained preliminary data using RNA interference (RNAi) where aflatoxin levels were reduced by 74 to 99% lower than the control (Arias et al. 2013a). However, much needs to be done to understand the process, to reduce variability of the results, and to improve the overall silencing efficiency. Thus, several RNAi constructs will be made in order to analyze their effectiveness. Genetic transformation of peanut with RNAi constructs and aflatoxin analysis by UHPLC will be done at the National Peanut Research Laboratory (NPRL). Several aspects of silencing will be analyzed, including distance of movement, dose effect and half life of small interfering RNA (siRNA) molecules. Also, if exposure of aflatoxigenic *Aspergillus* spp. to plants harboring siRNA results in reduced aflatoxin we will evaluate the silencing by quantifying expression of aflatoxin synthesis genes using RT-PCR.

An extremely important condition for the effectiveness of RNAi, is the RNA sequence specificity. Since our original RNAi work is based on the genome sequencing of *A. flavus* strain NRRL 3357, understanding the genetic variations of *A. flavus* and *A. parasiticus* in peanut growing areas of Sub-Saharan countries will be essential to develop the suitable RNAi technology for those areas. For several years we have been collaborating with the peanut research group from ICRISAT in Lilongwe, Malawi; giving them training and technical support in the areas of mycology and aflatoxins. Continuing with that support, we will perform a survey of the genetic diversity of the aflatoxin-synthesis genes in isolates of *Aspergillus flavus* and *Aspergillus parasiticus* from the peanut growing areas involved in this project (including Zambia, Mozambique, Kenya and Uganda). A similar survey will be done in peanut growing areas of the United States. Based on the results of the genetic diversity studies of *A. flavus* and *A. parasiticus*, modifications to the RNAi constructs will be made to increase their chances of being effective in geographically diverse areas.

Project Description

Goal

The goal is to develop RNA interference technology to silence *Aspergillus flavus* and *Aspergillus parasiticus* aflatoxin synthesis genes, thus preventing accumulation of these mycotoxins in peanut.

Relevance and Justification

Contamination of agricultural products with aflatoxins has been known for over 50 years (Blount 1961). Aflatoxins are among the most powerful carcinogens (AACR 1980), these polyketide-derived mycotoxins cause acute hepatotoxicity and immunosupression (Eaton and Groopman 1994; Pier et al. 1977), in addition to stunting in children (Gong et al. 2003). Aflatoxins are produced primarily by two phytopathogenic fungi, *Aspergillus flavus* Link, and *Aspergillus parasiticus* Speare. Though much has been done to mitigate their effect (Cotty 1990; Dorner 2010), there are no methods yet to consistently and/or effectively control aflatoxins. In tropical and subtropical developing countries up to 80% of liver cancer cases are related to consumption of aflatoxin contaminated food (Feitelson et al. 2002; Villar et al. 2012). In the USA, aflatoxins cost the peanut industry $26-58M annually (Lamb and Sternitzke 2001; Leidner 2012) depending on droughts, while the cost to human health from the chronic ingestion of low doses of mycotoxins is unknown. Droughts trigger preharvest accumulation of aflatoxins, and drought frequency has increased over the last decade (Craufurd et al. 2006; Dorner et al. 1989). Thus, levels higher than 1000ppb have been detected at peanut shellers' plants in recent years (N'Dede 2009), whereas the maximum limit established by the FDA for edibility is 20ppb. Our group is currently working to understand pathogen invasion under drought conditions (Arias et al. 2013b), on identifying genes related to drought resistance in peanut plants (Dang et al. 2012), and on incorporating traits to generate new peanut varieties (Chen et al. 2013). The peanut industry in USA has expressed interest in transgenic peanut as a method to control aflatoxins, and has financially supported the creation of a biotechnology program at the National Peanut Research

Laboratory (NPRL). Currently, several biotechnology programs are being established in Sub-Saharan African countries to improve the quality and safety of peanuts through genetic modification, including Uganda, Kenya and Mali.

The ribonucleic acid interference (RNAi) machinery, discovered by Fire and Mello in 1998 (Fire et al. 1998), induces gene silencing in many eukaryotes including plants, animals and fungi (Agrawal et al. 2003; Bernstein et al. 2001; Frizzi and Huang 2010). Silencing signals of RNAi in plants can travel systemically, and it has been shown that both small RNAi and high molecular weight RNA are responsible for the systemic posttranscriptional gene silencing in plants (Fagard and Vaucheret 2000; Klahre et al. 2002). Mobility of RNA cell-to-cell or long distance within the organism has been demonstrated (Ding and Wang 2009; Wang and Ding 2010), and the fact that RNAi molecules can move between host plants and their parasites, opens great opportunities for pathogen control. A parasite in close contact with a plant producing RNAi can receive a signal and undergo silencing of the target gene (Nowara et al. 2010; Tinoco et al. 2010; Tomilov et al. 2008; Yin et al. 2011). Silencing of fungal genes by plant RNAi has shown efficiencies between 62 and 97% (Nowara et al. 2010; Tinoco et al. 2010; Yin et al. 2011). In 2013, the company Venganza was granted a patent for the use of RNAi technology against plant pathogens by silencing essential genes in the pathogens (http://www.genomeweb.com/rnai/plant-biotech-firm-venganza-secures-us-patent-rnai-technology). From the ecological point of view, we believe that a longer term control of aflatoxins would be achieved by targeting only aflatoxin synthesis genes instead of essential genes, since the latter could result in high selective pressure allowing resistant/mutant strains to proliferate. Thus, our approach is different than the one pursued by that company.

In the control of human diseases, where RNAi technology has advanced faster than in agriculture, the need to take into account the genetic variability of human pathogens has been made evident. Thus, in RNAi therapies against HIV-1, combinations of multiple highly active and highly conserved short hairpin RNA (shRNA) had to be designed to control the emergence of resistant (mutant) strains of the virus (McIntyre et al. 2009). In the case of aflatoxins, the transcription activator *aflR*, which is necessary for the production of these mycotoxins in *A. flavus* and *A. parasiticus* (Woloshuk et al. 1994), could be considered as an interesting target for RNAi silencing. Indeed, transformation of *A. flavus* and *A. parasiticus* with inverted repeats of *aflR* resulted in gene silencing and minimal or no production of aflatoxins, showing the potential of RNA silencing as a mechanism to control these mycotoxins (McDonald et al. 2005). However, variations of the *aflR* gene among *Aspergillus* species and within *A. flavus* itself (Ehrlich et al. 2003; Lee et al. 2006) and high variability in the aflatoxin synthesis gene cluster in general (Chang et al. 2005), highlight the importance of knowing the genetic variations of natural populations of *A. flavus* and *A. parasiticus* to develop effective RNAi therapies.

Genomic analysis indicates that RNA mediated silencing may follow different pathways within the genus *Aspergillus.* For example, *Aspergillus nidulans* has lost a QDE-1 ortholog and does not require RNA-dependent-RNA polymerase (RDRP) for RNA-silencing induced by inverted-repeat transgenes, but that is not the case of *Aspergillus oryzae* or *Aspergillus fumigatus* (Hammond and Keller 2005). Thus, the various RNAi mechanisms need to be considered when designing RNAi silencing technology for *Aspergillus* spp. *A. flavus* produces at least 14 mycotoxins, 10 of them from the aflatoxin biosynthesis pathway (Georgianna et al. 2010) which is organized in a 70-kb-gene cluster on chromosome III (Chang and Ehrlich 2010). The feasibility of developing RNAi technology against mycotoxins is promising, since specific silencing of gene expression by RNAi has been shown by genetic transformation of several species of *Aspergillus* (Nemoto et al. 2009; Salame et al. 2011; Yamada et al. 2007). We have experience working with RNA interference and have shown how different silencing efficiency can be achieved by using transitive or inverted repeats (Filichkin et al. 2007). We also have experience determining genetic variations of fungi (Arias et al. 2011c) and other organisms (Arias et al. 2011a; Arias et al. 2011b; Arias et al. 2009)

Research Plan

Objective(s)

1. Develop a standardized method for accurate quantification of aflatoxins in single peanut kernels, to analyze transgenic plants.
2. Create RNAi constructs for various gene fragment combinations within the aflatoxin synthesis gene cluster, to target several intermediates. Analyze dose effect, and half life of silencing signal/s.
3. Give training to scientists/postdocs/technicians/graduate and undergraduate students from the various geographical locations involved in the project, to better prepare them to perform their tasks and to lead research in the future.
4. Transform peanut plants with each of the constructs, bring the plants to maturity under environmental conditions that promote aflatoxin accumulation, and analyze aflatoxin content in seeds. If aflatoxin reduction is consistently found, then start crosses with commercial peanut varieties.
5. Isolate *Aspergillus flavus* and *Aspergillus parasiticus* mainly from peanut growing areas, and determine the genetic diversity of the aflatoxin synthesis cluster in the isolates, in order to feed that information back into the design of RNAi molecular constructs.

Role of each scientist/partner

Arias: molecular cloning, DNA sequence analysis, RNAi construct design and building, plant propagation and PCR screening, Real-Time PCR, give training to scientists, technicians, graduate and undergraduate students, compile information for reports and manuscripts

Sobolev: Analysis of aflatoxins and other mycotoxins by UHPLC and LC-MS, develop methods when necessary, give training to visitor scientists and students

Dang: genetic transformation of peanut plants, bring plants to maturity in greenhouse, give training to visitor scientists and students

Njoroge: Collect peanut and soil samples from Malawi, Zambia and Mozambique; isolate *A. flavus* and *A. parasiticus*, extract DNAs and send them to Arias's laboratory for analysis, give training to visitor scientists/technicians from Uganda and/or Kenya

Sserumaga: Collect peanut and soil samples from Uganda and Kenya, bring them to Lilongwe, Malawi and help processing them for isolation of *A. flavus* and *A. parasiticus*

Chen: Research geneticist and peanut breeder will do crosses between peanut plants harboring RNAi and commercial varieties.

Scheffler: Collaborate with various high throughput sequencing platforms and robotics for the different parts of the project that require DNA sequencing and/or fingerprinting

Lamb: Provide vast expertise on all aspects of the peanut crop, its biology, industry needs and avenues for commercialization. Coordinate the use of resources at the NPRL to allocate personnel, building, greenhouse space and equipment to facilitate the project's success

Annual work plan, milestones and timeline

The framework is: to generate RNAi plasmid constructs that target two to five genes in the aflatoxin synthesis pathway, and use those constructs for transformation of peanut plants. Then, infect the transgenic plants with aflatoxigenic strains of *Aspergillus*, and evaluate the potential silencing of aflatoxin-synthesis genes in the pathogen, as well as quantify aflatoxin accumulation in transgenic peanut kernels. At the same time, perform a survey of the genetic diversity of the aflatoxin-synthesis cluster in isolates of *A. flavus* and *A. parasiticus* from the peanut growing areas involved in the project.

At the NPRL, Dr. Horn maintains a collection of aflatoxigenic and non aflatoxigenic strains of *Aspergillus* spp., including *Aspergillus flavus* NRRL3357 from which the genome has been sequenced (http://www.aspergillus flavus.org/genomics/). Dr. Horn has kindly made cultures available for our work. Gene fragments within the aflatoxin synthesis pathway will be used to make the constructs based on the sequencing information of *A. flavus* NRRL3357, either by amplification or synthetic genes. *Argonaute* binding will be taken into account during the design of the constructs (Karlowski et al. 2010). Fragments will be combined in the vector pENTR1A (Invitrogen) for their transfer into RNAi vectors by recombination using LR clonase of the Gateway system (Invitrogen). We have three vectors suitable for RNAi silencing in plants, two for constitutive silencing pB7GWIWG2(II),0 (Karimi et al. 2002) and pCAPD (Filichkin et al. 2007), and one for RNAi inducible silencing under the control of an ethanol inducible promoter, p4RHA52 (Arias, unpublished).

Genetic transformation of peanut will be done at the NPRL. Genetically-transformed plants will be used in challenge experiments with toxigenic strains of *A. flavus* and *A. parasiticus*. Preliminary screening for aflatoxins will be performed by TLC, whereas concentrations of these mycotoxins will be analyzed according to standard techniques at the NPRL (Sobolev et al. 2007), using UHPLC. Transformed plants will be grown in greenhouse for seed production. A method for accurate quantification of mycotoxins in individual peanut kernels will be developed. In general, UHPLC can process large number of samples using half the time and half the amount of solvents than HPLC, and protocols have been developed to effectively detect and quantify aflatoxins B_1, B_2, G_1, G_2 (López Grío et al. 2010).

Gene constructs that show promising results on these preliminary screenings will be used in combined transformations, making new constructs, or by stacking genes with different selectable markers. RNAi silencing of *Aspergillus* genes by translocation from the plant-host to the pathogen has not been reported, and nothing is known about the timing and extent of silencing achievable. Therefore, monitoring of aflatoxin synthesis genes and their gene expression will be done by Real-Time PCR to determine if silencing is taking place. Sample size, number of samples, and time for sampling after challenging the plant tissues with the aflatoxigenic *Aspergillus* will be empirically determined.

Should effective silencing be achieved, *A. flavus* transcriptome analysis will be performed for RNAi-harboring and control (non-RNAi) plants. Plant lines showing the highest suppression of the targeted aflatoxins will be used for further testing and crosses with a commercial cultivar. Effective silencing of targeted genes will be confirmed by Real-Time PCR. In addition to their support on the computational infrastructure for bioinformatics at GBRU, we will use the CLC Bio platform at NPRL to facilitate the workflow and create a custom computational pipeline at the NPRL for the large amounts of data generated by this project.

Short periods of training, 4-6 weeks at the NPRL, have been highly effective to accelerate the research project in ICRISAT, Malawi, so we plan to do each year one training for specific topics within the PMIL project. We are also in contact with the National Agriculture Research Organization (NARO) in Kampala, Uganda, one of the leading African countries in adopting genetically modified crops (*i.e.* banana), and that has developed its own method of peanut transformation (Dr. Kiggundu, personal communication). NARO also has the proper enclosure facilities to test transgenic peanuts, which we foresee as crucial in the future of RNAi research. Given the sequence specificity of the RNAi mechanism, in order to develop RNAi technology to be used in African countries, we need to know the genetic diversity of the aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus* in their peanut growing areas. For this, MS Sserumaga, from NARO, will collect samples from Uganda and Kenya, and bring them for processing to Malawi. Dr. Njoroge at ICRISAT Malawi will collect samples from Zambia, Mozambique and Malawi, will isolate DNA from *A. flavus* and *A. parasiticus* and will train Sserumaga in these processes. The DNA samples will be sent to the NPRL for analysis of their aflatoxin synthesis pathways; collaborators from the participating countries will be trained at the NPRL on the molecular characterization of isolates, on genetic transformation of peanut, and on

chemical analyses as needed. The information will be incorporated in making new RNAi constructs suitable for those Sub-Saharan African countries.

Each phase of the project will be used for training of the involved personnel in different areas. During the entire project, undergraduate and graduate students, visitor scientists, technicians and postdocs will be given training on the various phases of the project. This will include fungal isolation and characterization, molecular cloning, tissue culture, genetic transformation, bioinformatics, aflatoxin analysis, and peanut breeding.

Milestones:

12 Months	1) Make at least 3 constructs with various combinations of aflatoxin-synthesis gene fragments; 2) Make a collection of about 200 isolates of *A. flavus* and *A. parasiticus* from peanut growing areas
24 Months	1) Transform at least 3 RNAi constructs into peanut, and screen putative transformants by PCR; 2) Complete most of the culture collection and perform DNA extractions, start genetic characterization of isolates
36 Months	1) Advance in siRNA signal understanding, mobility and half life; 2) Challenging of plant tissue with aflatoxigenic fungi, aflatoxin quantification under various treatments and conditions; 3) Genetic characterization of *Aspergillus* spp. to improve RNAi constructs
48 Months	1) Start crosses of peanut harboring RNAi that can reduce aflatoxin accumulation; 2) Make new RNAi constructs based on the *Aspergillus* spp. genetic diversity information; 3) Provide potentially useful constructs to the centers in Africa where genetic transformation of peanut will take place

Time Line:

Year 1		Year 2		Year 3		Year 4	
Sep/Mar	Mar/Aug	Sep/Mar	Mar/Aug	Sep/Mar	Mar/Aug	Sep/Mar	Mar/Aug
Design of molecular constructs, cloning of gene fragments, sequencing of clones for RNAi, making binary vector constructs for RNAi of aflatoxin biosynthesis genes, transform *Agrobacterium*. Get feedback from *Aspergillus flavus* and *Aspergillus parasiticus* sequencing of aflatoxin cluster from different peanut growing areas, and modify constructs when necessary.							
Transform peanut line Exp27-1516 with each of the RNAi constructs following the standard protocol developed at the NPRL.							
Provide training to undergraduate students on various techniques, including tissue culture, molecular tools, and chemical analyses, during the entire duration of the project. Provide training to one person each year (4-6 week periods), that is technical personnel and/or scientists from the countries participating in the project.							
Screen putative transformant plants by PCR, bring to maturity those identified as positive by PCR, perform Southern blots to determine copy number.							
Isolation of *Aspergillus flavus* and *Aspergillus parasiticus* from soil and seeds, DNA extraction, sequencing of multiple regions of the aflatoxin synthesis gene cluster							
	Develop a method to quantify aflatoxins in individual peanut kernels		Analyze aflatoxins in individual peanut kernels, and in *Aspergillus* exposed to transgenic plants				
		Analysis of distribution of small interfering RNA (siRNA) in plant tissues, regulation of gene silencing, environmental and physiological conditions required for production of siRNAs, effective doses, and stability of siRNAs.					
		Soil planting of transgenic lines for seed production in greenhouse					
			High-throughput sequencing of small RNAs from transgenic plants				
				Start making crosses between RNAi transformant peanuts and a commercial cultivar			
		Explore the feasibility of performing Real-Time PCR screening of aflatoxin synthesis genes in aflatoxigenic *Aspergillus* after being exposed to transgenic plant tissue. To determine what genes are being silenced by the RNAi constructs, we will pursue the development a method to expose the fungus to plant tissues and later analyze gene expression.					
		Experiments challenging various plant organs and developmental stages using aflatoxin producing strains of *Aspergillus flavus*					

Gender research strategy

Undergraduate students that participate in research projects at NPRL are mostly from the historically black institution Albany State University, and are normally selected by the school in equal number of males and females. We are currently working to bring to the Masters' program at UGA (funded by other grant), a female technician who performs aflatoxin analyses in ICRISAT Malawi. Also, a female postdoc from ICRISAT is scheduled to receive training at the NPRL within our PMIL proposal.

Outcomes and Impacts

The most important outcomes of this project will be: a) RNAi technology to mitigate aflatoxins in peanut and therefore safer food; b) trained personnel in the various aspects of the project to enable them to develop and/or accelerate their own research programs; c) knowledge of the genetic diversity of the aflatoxin synthesis pathway of aflatoxigenic fungi in the countries participating in this project, and a collection of fungal isolates that can be used to mine for information in future projects; d) a better understanding on the mechanism and efficiency of RNAi technology in the peanut-plant system.

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