ABSTRACT

ROWE, CHRISTINA ELLA MARIE. DNA Markers for Resistance to Post-Harvest Aflatoxin Accumulation in *Arachis hypogaea* L. (Under the direction of Drs. THOMAS G. ISLEIB and SUSANA R. MILLA-LEWIS.)

Aflatoxin contamination causes economic loss for the global peanut (*Arachis hypogaea* L.) industry and raises human and animal health concerns. Peanut genotypes with resistance to aflatoxin accumulation should be an important part of an integrated aflatoxin management program. This study was conducted to identify AFLP markers tightly linked to genetic factors controlling reduced aflatoxin accumulation after infection with *Aspergillus flavus*. A segregating F_2 population was generated by crossing high-aflatoxin accumulating cultivar Gregory with low-aflatoxin accumulating interspecific tetraploid line GP-NC WS 2, phenotyped for aflatoxin accumulation using an *in vitro* assay, and screened with AFLP markers previously identified to be associated with reduced aflatoxin accumulation. An F-test was used to determine whether markers were associated with the trait, a genetic linkage map was generated, and interval mapping was used to identify regions of the genome that influence aflatoxin accumulation. Gregory supported significantly more aflatoxin production by *A. flavus* than GP-NC WS 2, and the F_2 population exhibited high-parent heterosis. Thirty-five of 38 AFLP markers used to screen the F_2 population had segregation distortion favoring the *A. hypogaea* cultivar. Six markers were significantly associated with reduced aflatoxin accumulation at the 5% significance level. Thirty-three markers were included in a genetic linkage map covering 60 cM and a putative QTL was identified at map position 9 cM. Linked markers could be utilized in a marker-assisted selection program to identify individuals that support low levels of aflatoxin accumulation.

DNA Markers for Resistance to Post-Harvest Aflatoxin Accumulation in *Arachis hypogaea* L.

by
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BIOGRAPHY

Christina Rowe grew up in Clayton, North Carolina, USA. She attended Clayton High School where she developed an interest in agriculture through coursework and participation in the National FFA Organization. After high school, she attended North Carolina State University (NCSU) where she obtained Bachelor of Science degrees in Horticultural Science and Agricultural Education. During her undergraduate career, Christina worked for the NCSU Sweetpotato Breeding and Genetics Program where she became interested in plant breeding. Following graduation in 2006, Christina started her Master of Science degree under the direction of Drs. Thomas G. Isleib and. Susana R. Milla-Lewis in the Department of Crop Science at NCSU. Christina will work as a research associate for Monsanto after graduation.

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LITERATURE REVIEW

PEANUT (*Arachis hypogaea* L.)

Uses, production, and economic importance

Arachis hypogaea is a native South American legume. The species is an important food crop and is grown in the tropical, subtropical, and warm temperate zones (Hammons, 1982). Peanut crops are grown for their seeds, oil, meal, and vegetative residue (Bunting, *et al.*, 1985). The two cotyledons comprise 96% of the seed by weight and contribute most to the economic importance of the plant (Moss and Rao, 1995). Peanuts are used mostly as an oilseed crop around the world, but are used for direct human consumption in the United States (Holbrook and Stalker, 2003), where peanut butter, salted peanuts, and confectionary products are most frequently consumed (Bunting *et al*., 1985).

In 2006/07, global peanut production reached 30.53 million metric tons, with the top three producers being China, India, and the United States (USDA PS&D, 2008). The value of production of peanuts for nuts in the United States in 2007 was $762.6 million (USDA QuickStats, 2008). There are three regions of production in the United States. Production in the Southeast states (Alabama, Florida, Georgia, Mississippi, and South Carolina) totaled 2.6 billion pounds in 2007, while Southwest production (New Mexico, Oklahoma, and Texas) totaled 785 million pounds, and Virginia-North Carolina production totaled 314 million pounds (USDA Crop Production, 2008).

Botany

Peanut is an annual herb and one of the few plant species that forms underground fruits (Moss and Rao, 1995). Peanut inflorescences are borne in the axils of leaves. Each bears up to five modified sessile papillionaceous flowers (Norden, 1980) that self-pollinate (Norden, 1973). After fertilization of the ovule, an intercalary meristem located adjacent to the basal ovule becomes active and a "peg" (botanically a carpophore or gynophore) is formed. Elongating pegs exhibit positive geotropism and grow into the soil (Smith, 1956). The peg becomes diageotropic after penetrating the soil, ceases to elongate, and a pod develops at the distal end (Ziv, 1981). The pod contains seeds consisting of two cotyledons, a hypocotyl, epicotyl, and radicle (Gregory *et al*, 1951).

Taxonomy

The genus *Arachis* belongs to the family Leguminosae, the tribe Aeschynomenaea, and the subtribe Stylosanthenae. *Arachis* is characterized by having a peg and geocarpic reproductive growth (Stalker and Simpson, 1995). The genus contains 80 species and has been divided into nine sections: *Arachis, Caulorrhizae, Erectoides, Extranervosae, Heteranthae, Procumbentes, Rhizomatosae, Trierectoides,* and *Triseminatae* (Valls and Simpson, 2005). Several species of *Arachis* are cultivated, but *A. hypogaea* is the only species that has been domesticated and widely distributed for commercial production of seeds and oil (Holbrook and Stalker, 2003; Stalker and Moss, 1987).

Arachis hypogaea is divided into two subspecies, subsp. *hypogaea* and subsp. *fastigiata* Waldron. Each subspecies is further divided into botanical varieties; subsp. *hypogaea* into var. *hypogaea* and var. *hirsuta* Köhler, subsp. *fastigiata* Waldron into var. *fastigiata*, var.

vulgaris Harz., var. *peruviana* Krapov. & W.C. Greg., and var. *aequatoriana* Krapov. & W.C. Greg. Subspecific and varietal classifications are based on morphological characteristics including growth habit, branching patterns, pubescence, stem color, and pod and seed size and shape (Krapovickas and Gregory, 1994). Forms intermediate to subspecies and varieties exist and are sometimes difficult to classify (Isleib and Wynne, 1983).

Market classes

In addition to taxonomic classification, the U.S. market divides *A. hypogaea* into four market types: virginia, runner, spanish, and valencia. Market types correspond loosely to botanical varieties, but are based primarily upon pod and seed characteristics. Virginia and runner market-type peanuts trace most of their ancestry to var. *hypogaea*, but U.S. cultivars of virginia and runner market-types have at least some ancestral contribution from subsp. *fastigiata*, mostly from var. *vulgaris*. In contrast, spanish cultivars trace almost exclusively to ancestors of var. *vulgaris* and valencia cultivars to var. *fastigiata* (Isleib *et al.*, 2001). Virginia market-types have larger pods than the other market types. Virginia market-type peanuts are used for salted peanuts and cocktail peanuts when shelled, or they are roasted and sold as in-shell peanuts (Knauft *et al*., 1987). Most of the production of virginia market-type peanuts is in Virginia, North Carolina, and South Carolina (Sholar *et al.*, 1995). Runner and spanish market-type peanuts are generally used to make peanut butter, candy, and oil (Knauft *et al.*, 1987). Runner market-type peanuts are grown in Georgia, Alabama, Florida, Texas, and Oklahoma, while spanish peanuts are grown in Oklahoma and Texas (Sholar *et al.*, 1995). Valencia peanuts are sold either roasted in the shell or boiled (Knauft *et al*., 1987), and are grown primarily in New Mexico and west Texas (Sholar *et al*., 1995). The

composition of the commercial peanut crop in the United States is approximately 83% runner types, 14% virginia types, 2.5% spanish types, and less than 1% valencia types (USDA Peanut Stocks, 2008).

Genetics

Species within the genus *Arachis* are predominantly diploid (2n=2x=20), though tetraploids (2n=4x=40) exist in sections *Arachis* and *Rhizomatosae* (Stalker and Simpson, 1995). *A. hypogaea* is an allotetraploid that likely evolved from two diploids in section *Arachis*, each diploid progenitor species contributing one genome to *A. hypogaea*, which possesses an A and a B genome. Random fragment length polymorphism (RFLP) analysis indicated that *A. duranensis* Krapov. & W.C. Greg. is the most likely donor of the A genome and *A. ipaensis* Krapov. & W.C. Greg. is most closely related to the B genome of domesticated peanut. Analysis of chloroplast DNA indicated that *A. duranensis* was the female parent of the hybridization event that produced domesticated peanut (Kochert *et al*., 1996).

A. hypogaea exhibits normal bivalent pairing and a low frequency of multivalent configurations, indicating that cultivated peanut is a highly diploidized tetraploid. This leads to disomic inheritance and normal Mendelian segregation. Multivalent association can be due to homoelogous pairing (including the formation of quadrivalents) between chromosomes of the two genomes (Smartt and Stalker, 1982). Various types of gene action govern the inheritance of quantitative traits in cultivated peanut. Additive genetic variance is the main component of genotypic variance for economically important traits. However, several instances of non-additive gene action have been reported. Heterosis is present in

peanut and usually indicates that non-additive gene action is important (Wynne and Coffelt, 1982). Heterosis exists for traits such as yield (Higgins, 1940), plant characteristics (Hassan and Srivastava, 1966; Wynne *et al.*, 1970), and maturity (Isleib and Wynne, 1983; Parker *et al.*, 1970). Additionally, the presence of epistatic variance has been detected in peanut by several investigators. Sandhu and Khehra (1976) detected important non-additive effects for yield characters and concluded that epistasis can be observed in peanut crosses. Significant levels of additive epistatic variance exist for traits such as yield and fruit characteristics in populations derived from diverse peanut lines (Isleib *et al.*, 1978). Other traits, such as early maturity, also exhibit segregation patterns indicative of epistatic effects (Upadhyaya and Nigam, 1994).

Utilization of wild species

Wild *Arachis* species have higher levels of resistance to many diseases and insects than do cultivated accessions (Stalker and Moss, 1987). Interspecific hybridization is difficult but remains the most promising method of introgressing disease and insect resistance from wild *Arachis* species when developing improved *A. hypogaea* cultivars (Holbrook and Stalker, 2003; Stalker and Simpson, 1995). Even when cross-compatible species are used, progeny are often sterile because of different ploidy levels, genomic incompatibilities, and cryptic genetic differences (Holbrook and Stalker, 2003). Species in section *Arachis* are most easily utilized in breeding programs. Species with multiple resistances are most important for crop improvement because fertility and sterility complications over generations of hybridization make 40-chromosome hybrid derivatives challenging to recover. Wild diploids *A. diogoi* Hoehne, *A. cardenasii* Krapov. & W.C.

Greg., *A. stenosperma* Krapov. & W.C. Greg., and *A. batizocoi* Krapov. & W. C. Greg. are among the *Arachis* species most often utilized for interspecific hybridization in crossing programs. There are two primary methods for interspecific hybridization. In the first, triploid hybrids between *A. hypogaea* and diploid wild species are treated with colchicine to restore fertility at the hexaploid level. Cytologically unstable hexaploids spontaneously lose chromosomes during generations of selfing, and tetraploid progeny can be obtained (Stalker and Simpson, 1995). The second method for interspecific hybridization is development of a synthetic amphidiploid by crossing an A-genome diploid with a B-genome diploid. The F_1 diploid hybrid is treated with colchicine to produce a tetraploid that can be crossed with *A. hypogaea* (Simpson *et al.*, 1993).

One example of successful interspecific hybridization is a highly diverse *A. hypogaea* × *A. cardenasii* population originating from a hybrid made by Smartt and Gregory (1967). Tetraploid progeny were recovered by treating triploid hybrids with colchicine followed by generations of selfing. The progeny were found to be resistant to early leaf spot caused by *Cercospora arachidicola* S. Hori and late leaf spot caused by *Cercosporidium personatum* (Berk. & M.A. Curtis) Deighton (Stalker *et al.* 2002b; Stalker and Buete, 1993), rust caused by *Puccinia arachidis* Speg., bacterial wilt caused by *Ralstonia solanacearum* (Smith) Smith (Moss *et al.* 1997), peanut root-knot nematode *Meloidogyne arenaria* (Neal) Chitwood (Stalker *et al.* 2002a), and several insect pests (Moss *et al.* 1997; Stalker and Campbell, 1983; Stalker and Lynch, 2002). Xue *et al.* (2004) identified one interspecific tetraploid line from this population with resistance to aflatoxin accumulation. Progeny derived from an interspecific A×B genome cross were resistant to peanut root-knot nematode, as well as early

and late leaf spot (Simpson *et al.*, 1993). RFLP and random amplified polymorphic DNA (RAPD) marker analysis indicated that the mechanism for introgression from wild species into cultivated peanut is reciprocal chromosome recombination involving both genomes and not chromosome substitution (Garcia *et al.* 1995).

Transformation technologies are an alternative method for utilizing resistant species that will not hybridize with *A. hypogaea*, but single genes or gene complexes that confer high levels of resistance to insects and diseases have not been identified in peanut (Holbrook and Stalker, 2003). Thus, it would be difficult to identify suitable DNA sequences for transformation.

AFLATOXIN

Aflatoxins and Aspergillus

Aflatoxins are toxic and carcinogenic compounds produced by fungi in the genus *Aspergillus* P. Mich. (Payne, 1998). The genus *Aspergillus* is distributed worldwide and contains over 180 species. It is one of the most ubiquitous and abundant of all groups of fungi and is one of the most studied fungal groups (Dyer, 2007). *A. flavus* Link:Fr. and *A. parasiticus* Speare, the two *Aspergillus* species of most concern in agriculture, are predominant saprotrophs with limited parasitic ability (Payne, 1998). They are distributed worldwide and infect a number of crops (Jackson and Bell, 1969). Aflatoxin is recognized as one of the most important problems in the peanut industry, and a large effort has been made to eliminate the causal fungi and toxin from the seed chain (Stalker and Simpson, 1995). Both *A. flavus* and *A. parasiticus* colonize injured peanut seeds and sometimes invade intact pods; however, *A. flavus* is the more dominant species (Payne, 1998). *A. flavus* reproduces

by asexual conidia and has no known perfect state (Dyer, 2007). Damage to peanut pods increases susceptibility of seed to infection. Aflatoxin contamination of pods and seeds can occur in the soil prior to harvest, during harvest, or post-harvest during storage (Porter *et al.*, 1984). Conditions that support aflatoxin accumulation prior to harvest are high soil temperature and late season drought stress (Payne, 1998). Biological control of pre-harvest aflatoxin accumulation may be achieved by applying Afla-guard® (Circle One Global, Inc., Cuthbert, Ga.), a non-toxigenic strain of *A. flavus*, to the soil where it displaces toxigenic strains. Post-harvest aflatoxin accumulation usually occurs when peanuts are stored under conditions of high temperature and high humidity (Payne, 1998).

The four major aflatoxins that occur in crops are B1, B2, G1, and G2. *A. flavus* produces aflatoxins B1 and B2, while *A. parasiticus* produces all four aflatoxins (Payne, 1998). Aflatoxin B1 is the most toxic and best studied of the aflatoxins. The International Agency for Research on Cancer has designated aflatoxin as a human liver carcinogen (IARC, 1997). The U.S. Food and Drug Administration regulates maximum aflatoxin levels allowed in food and feed in the United States. Aflatoxin levels are not to exceed 20 parts per billion (ppb) in peanut products destined for human consumption or animal feed for immature animals. Levels are less stringent for animal feed intended for mature animals. Peanut products intended for breeding beef cattle, breeding swine, and mature poultry may contain up to 100 ppb aflatoxin. Peanut products intended for finishing beef cattle may have aflatoxin levels up to 300 ppb (US FDA, 2000). Other countries also set limits for aflatoxin contamination (Stoloff *et al*., 1991). The European Union (2006), a key importer of U.S.

peanuts, has set maximum total aflatoxin levels at 4 ppb and maximum aflatoxin B1 levels at 2 ppb for products intended for direct human consumption.

The U.S. peanut industry regulates the quality of domestically grown peanuts as they enter the market. Farmers stocks are divided into Segregations I, II, and III according to a visual assessment of damage and presence of *A. flavus*. Segregation I peanuts have little damage, no visible *A. flavus*, and are destined for human consumption. Lots containing kernels that are higher in damage but have no visible *A. flavus* are placed into Segregation II storage. Segregation II peanuts are generally crushed for oil (Sands, 1982), which is aflatoxin-free after refining (Dickens, 1977), but may be used for the edible market if a severe peanut shortage occurs. Segregation III lots have high amounts of damage and visible *A. flavus,* and must always be crushed for oil (Sands, 1982). Consequently, aflatoxin contamination has an unfavorable effect on the peanut industry. A survey of actual losses in peanuts during the 1993-1996 crop years was used to estimate the net cost of aflatoxin to the farmer, the buying point, and the sheller segment of the Southeast peanut industry to be about $25 million per year (Lamb and Sternitzke, 2001). Since the completion of Lamb and Sternitzke's study, the loan rate for peanuts has been reduced by approximately half (USDA Farm Bill, 2008), therefore the costs of aflatoxin contamination to the Southeast peanut industry are likewise reduced.

Resistance to Aspergillus flavus and aflatoxins in peanut

Four mechanisms of resistance to *Aspergillus* species in peanut have been studied. Resistance to *in vitro* seed colonization by *A. flavus* (IVSCAF) is related to characteristics of the testa (seed coat). Inoculations and incubation occur in a laboratory setting that provides

for optimum fungal growth. This type of resistance depends on the testa being intact and is consequently not always reliable (Mixon and Rogers, 1973; Mehan and McDonald, 1980). Resistance to field seed colonization by *A. flavus* (FSCAF) and preharvest aflatoxin contamination (PAC) is difficult to assess due to variability in *Aspergillus* growth under field conditions (Davidson *et al.*, 1983; Holbrook *et al.*, 1994). The final mechanism is resistance to aflatoxin production when colonized by a toxigenic strain of *Aspergillus* (Mehan and McDonald, 1980; Rao and Tulpule, 1967). Several sources of resistance to IVSCAF and FSCAF have been reported (Isleib *et al.*, 1994). Fewer sources of resistance to aflatoxin production have been reported, including wild species *A. cardenasii*, *A. duranensis* (Ghewande *et al.*, 1989), *A. pusilla* Benth., *A. chiquitana* Krapov. et al., and *A. triseminata* Krapov. & W.C. Greg. (Thakur *et al.*, 2000), as well as in *A. hypogaea* accessions U4-7-5 and VRR 245 (Mehan *et al.*, 1986). No germplasm is highly resistant to aflatoxin accumulation, but genotypes differ in the concentrations of aflatoxin they support. Adequate resistance to aflatoxin accumulation has not been incorporated into any agronomically desirable peanut cultivar (Xue *et al.*, 2004).

In vitro seed assay for screening for resistance

Xue *et al.* (2004) developed an *in vitro* assay to measure aflatoxin accumulation in peanut cotyledons. To perform the assay, the testa is removed from whole seeds and cotyledons are separated. Seed halves (cotyledons) are surface-sterilized then placed on moist filter paper in a plastic petri dish and inoculated with a suspension containing 1×10^6 conidia ml^{-1} of *A. flavus*. Petri dishes are placed on trays, which are then enclosed in plastic bags. Trays are stacked in an incubator at 28ºC for 8 d and kept moist during the incubation

period. Rigid spacers prevent the mass of the upper trays from compressing the petri dishes in the lower trays. At the end of 8 d, samples are rated for mycelial growth, color, and development of "fluffy" colonies. Samples are then dried for 1 d at 60ºC and then for 3 d at 50ºC. Once samples are dry, they are ground into a friable meal and stored in vials until they are analyzed for aflatoxin content by high-pressure liquid chromatography (HPLC). Aflatoxin is extracted from 2 g of ground sample with acetonitrile-water (24:1 vol:vol) in a 5:1 ratio of extractant volume to sample weight. The extract is purified using a Mycosep® 224 column (Romer Labs®, Washington, MO) and aflatoxin is measured by fluorescence HPLC as the post-column-generated bromide derivative. The assay is expensive, time consuming, and exhibits variation between runs (Xue *et al.*, 2004).

Resistance to aflatoxin accumulation in peanut wild species and interspecific lines

Xue *et al.* (2004) used the assay described above to evaluate resistance to aflatoxin production by *A. flavus* in *Arachis* species and interspecific tetraploid lines. Accessions of *A. cardenasii, A. duranensis, A. hypogaea*, and interspecific tetraploid lines were evaluated. The interspecific lines originated from a triploid interspecific hybrid between *A. hypogaea* PI 261942 and *A. cardenasii* accession PI 262141 (GKP 10017) (2n=2x=20) (Smartt and Gregory, 1967). The hybrid was treated with colchicine to restore fertility at the hexaploid (2n=6x=60) level. Hexaploids were allowed to self-pollinate for several generations until they reverted to the tetraploid level.

Arachis duranensis and *A. cardenasii* accumulated less aflatoxin than did *A. hypogaea* checks, but the difference between the two wild species was not significant. High amounts of variation were found among the interspecific lines for aflatoxin contamination. GP-NC

WS 2 was the only line that was not significantly different from the resistant *Arachis* species parent (*A. cardenasii* GKP 10017). Further work is needed to completely evaluate sources of resistance and move resistance genes into agronomically desirable accessions. Inoculation and analysis of aflatoxin content is time consuming and expensive, thus, the use of molecular markers tightly linked to aflatoxin resistance gene(s) would improve selection efficiency (Xue *et al.*, 2004).

MOLECULAR MARKERS

Molecular markers detect regions in a genome whose DNA base sequences differ from one individual to another. DNA markers are inherited with genes that are located nearby on a chromosome. Hence, they can be useful in detecting the presence or absence of a gene of interest (Paterson *et al.*, 1991). The use of markers to improve efficiency of selection is called "marker-assisted selection" (MAS). MAS is particularly useful when traits of interest are expensive or difficult to measure, or exhibit high environmental variation (Mohan *et al.*, 1997). MAS is most useful to plant breeders for tracing favorable allele(s) across generations, identifying pertinent individuals among segregating progeny, and identifying instances in which linkage of favorable alleles with unfavorable ones has been broken. Successful application of molecular markers depends upon a genetic linkage map with molecular markers tightly linked to major genes or quantitative trait loci (QTLs) controlling agronomic traits, recombination between markers associated with major genes or QTLs and the rest of the genome, and high through-put in a cost effective manner (Francia *et al.*, 2005).

QTLs associated with reduced aflatoxin accumulation in maize

QTLs associated with reduced aflatoxin accumulation have been identified in maize *Zea maize* L. mapping populations. Paul *et al.* (2003) examined two populations derived from maize inbred Tex6, which is associated with low aflatoxin accumulation, crossed with B73, a historically important inbred, for QTLs associated with aflatoxin accumulation. Two and three QTLs were detected, each explaining about 20% of the variation for the trait. QTLs for reduced aflatoxin accumulation were attributed to both parents, but environment strongly influenced detection of QTLs in different years, even at a very low significance threshold. The underlying genetic basis for the production of aflatoxin is probably several small effect genes that are influenced by the environment. Brooks *et al.* (2005) detected QTLs associated with reduced aflatoxin accumulation in a cross derived from Mp313E, a maize inbred with reduced aflatoxin accumulation, and B73. Two QTLs explaining 7% to 18% of the variation for the trait were significant in three out of four environments. Important QTL regions were rarely the same in different years.

Marker applications in peanut

Historically, cultivated peanut has shown limited DNA polymorphism, however, variation among species has been detected by RFLP analysis (Kochert *et al.*, 1991) and single-primer amplification (Halward, *et al.*, 1992). Amplified fragment length polymorphism (AFLP) analysis indicated that significant variation exists both among and within species, but interspecific variation is higher than intraspecific variation for most *Arachis* species (Milla *et al.*, 2005). Introgressing wild species DNA into *A. hypogaea* lines and evolving marker techniques have exposed DNA polymorphisms in peanut. Garcia *et al.*

(1995) was the first to analyze alien gene introgression in peanut. RFLP and RAPD markers were efficient in the detection of alien chromosome introgression.

The first successful case of using MAS in peanuts was the identification of RAPD markers linked to a root-knot nematode (RKN) resistance gene derived from wild peanut species, likely *A. cardenasii* (Burow *et al.*, 1996). RAPD markers "result from DNA sequence variation at primer binding sites and from DNA length differences between primer binding sites" (Powell *et al.*, 1996). Discovery of three markers linked to RKN resistance in peanut saved time and effort in assessing the subterranean phenotype. Additionally, such markers can contribute to comparative analysis by being a starting point for map-based cloning of resistance genes and by recognizing genes at corresponding positions in chromosomal segments derived from a common ancestor (Burow *et al.*, 1996).

The AFLP technique also has proved useful for peanut genetic studies. It is based on the selective polymerase chain reaction (PCR) amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: 1) digestion of the DNA with restriction enzymes and ligation of oligonucleotide adapters, 2) selective amplification of sets of restriction fragments, and 3) gel analysis of the amplified fragments. The technique is an effective tool to reveal restriction fragment polymorphisms (AFLP markers) (Vos *et al*., 1995). AFLP markers are reproducible, detect high levels of polymorphism, generate markers that are widely distributed throughout the genome, and do not require prior knowledge of the genome because primers of arbitrary sequence are used (Lu *et al.*, 1996; Prabhu and Gresshoff, 1994). The AFLP technique has been used in peanut to identify DNA markers linked to resistance to the aphid vector, *Aphis craccivora* Koch., that causes

groundnut rosette disease, and to construct a partial genetic linkage map for cultivated peanut (Herselman *et al.*, 2004).

Simple sequence repeats (SSR), also known as microsatellites, produce a higher level of polymorphism than other DNA markers in cultivated peanut (He *et al.*, 2003). SSRs consist of short, repeated sequences and have been found to be highly polymorphic in eukaryotic DNA. This technique requires prior sequence information to drive the PCR reaction (Akkaya *et al.*, 1992). A previous study demonstrated that the GA/CT repeat is the most frequently dispersed microsatellite in peanut and used five highly polymorphic markers to differentiate 24 cultivated genotypes (He *et al.*, 2003). The first SSR-based linkage map in *Arachis* was reported in 2005 (Moretzsohn *et al.*); the partial map contained 11 linkage groups in the diploid A genome. In 2007, the first comprehensive genetic map with SSR markers in peanut was reported. The map covered 20 linkage groups and 679 cM (Hong *et al.*, 2007).

Segregation distortion

Estimates of recombination frequencies may be biased by deviations of single-locus segregation ratios from the expected ratios. Markers tightly linked to genes that are affected by gametic or zygotic selection may show segregation distortion. Distortion may also be caused by structural rearrangements, such as translocations, that affect the viability of gametes. Dominant markers provide poor information in the case of segregation distortion and should be used with caution (Lorieux *et al.*, 1995). Segregation distortion has been documented in a wide array of plant species. Xu *et al.* (1997) compared segregation distortion across species and populations. An examination of 53 populations revealed the

highest frequency of segregation distortion (73%) in an interspecific recombinant inbred (RI) tomato (*Solanum* L.) population and the lowest frequency of distortion (5.4%) among an intraspecific *Cuphea* P. Browne population. The most extreme example of segregation distortion is a rice (*Oryza sativa* L.) intraspecific RI population that was reported to have 98.4% of marker loci skewed toward the *indica* parent allele (Wang *et al.*, 1994). Reports of segregation distortion in *Arachis* species range from 25% to 53%. When cultivated peanut was crossed with a synthetic amphidiploid, RFLP analysis revealed 25% of markers spread over 20 linkage groups with significant deviation from the expected 1:1 segregation pattern. Surprisingly, 68% of the distorted markers showed an excess of the synthetic parent allele (Burow, *et al.*, 2001). Additionally, 27% of RAPD and RFLP markers showed segregation distortion in an interspecific diploid backcross population (Garcia *et al.*, 2005). During construction of the first SSR-based genetic linkage map in *Arachis*, 53% of co-dominant SSR markers showed segregation distortion. Non-distorted markers were used to construct a framework and distorted markers were later added to the map (Moretzsohn *et al.*, 2005).

Markers associated with aflatoxin accumulation

As a first step in implementing a MAS program for reduced aflatoxin accumulation in peanut, 15 interspecific tetraploid lines phenotyped by Xue *et al.* (2004) for reduced aflatoxin accumulation and a set of plant introductions (PI 250906, PI 290626, PI 590299, and PI 590325) known to have resistance to aflatoxin accumulation were screened with 256 AFLP primer combinations in order to determine the extent of polymorphism in the interspecific lines and to identify associations between polymorphisms and reduced aflatoxin accumulation (Milla *et al.*, 2007). Low levels of polymorphism were detected among the

plant introductions, and higher levels were detected among the interspecific lines. Bands from 835 marker loci ranging in size from 56 to 690 base pairs were scored as present or absent, and the two genotypic classes were compared to determine whether they supported differing levels of aflatoxin accumulation. At $P \leq 0.05$, 69 markers were associated with total aflatoxin, 60 with aflatoxin B1, and 60 with aflatoxin B2. At $P \leq 0.01$, 36 markers were associated with total aflatoxin, 36 with aflatoxin B1, and 46 with aflatoxin B2. All 36 markers associated with total aflatoxin and aflatoxin B1 accumulation in the interspecific line GP-NC WS 2 were linked in repulsion with the trait. It is probable that reduced aflatoxin accumulation is conferred by *A. cardenasii*-derived genomic regions (Milla *et al.*, 2007).

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DNA Markers for Resistance to Post-Harvest Aflatoxin

Accumulation in *Arachis hypogaea* L.

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INTRODUCTION

Aflatoxins are toxic and carcinogenic secondary metabolites produced by several species of fungi in the genus *Aspergillus* P. Mich. *A. flavus* Link:Fr., which produces aflatoxins B1 and B2, and *A. parasiticus* Speare, which produces aflatoxins B1, B2, G1, and G2, are the primary species of concern in agriculture (Payne, 1998). Aflatoxin contamination raises human and animal health concerns (IARC, 1997), and causes economic loss for the farmer, the buying point, and the sheller segment of the U.S. peanut (*Arachis hypogaea* L.) industry (Lamb and Sternitzke, 2001). Pre- and post-harvest contamination of peanut by aflatoxin has been recognized as one of the most important problems in the peanut industry (Porter *et al*., 1984), and a large effort has been made to eliminate the causal fungi and the toxin from the seed chain (Stalker and Simpson, 1995). Aflatoxin contamination occurs pre-harvest under conditions of high soil temperature and late season drought stress or post-harvest under conditions of high temperature and high humidity in storage (Payne, 1998). The primary method to control aflatoxin contamination is management of the environmental conditions that support aflatoxin production and accumulation. Environmental management is difficult to achieve, therefore, peanut genotypes with resistance to colonization by *Aspergillus* species or to aflatoxin accumulation should be part of an integrated aflatoxin management program (Xue *et al.*, 2004).

Resistance to aflatoxin production when colonized by a toxigenic strain of *Aspergillus* has been evaluated (Mehan and McDonald, 1980; Rao and Tulpule, 1967) and a few sources of resistance identified, primarily among *Arachis* wild species (Ghewande *et al.*, 1989; Mehan *et al.*, 1986; Thakur *et al.*, 2000). No agronomically acceptable peanut cultivar has

high levels of resistance to aflatoxin accumulation (Xue *et al.*, 2004). This is expected, as *Arachis* wild species possess higher levels of resistance to many diseases and insects than do cultivated accessions (Stalker and Moss, 1987). Interspecific hybridization is an effective method for introgressing disease and insect resistance from wild species into *A. hypogaea* (Holbrook and Stalker, 2003).

Xue *et al.* (2004) developed an *in vitro* aflatoxin assay to measure aflatoxin accumulation in peanut cotyledons. Seven accessions of *Arachis* wild species *A. cardenasii* Krapov. & W.C. Greg. and 29 of *A. duranensis* Krapov. & W.C. Greg., along with 17 interspecific tetraploid lines from a highly diverse population derived from the cross *A. hypogaea* × *A. cardenasii* (Smartt and Gregory, 1967) were screened for aflatoxin accumulation when colonized by *A. flavus* (Xue *et al.*, 2004). *A. duranensis* and *A. cardenasii* accumulated less aflatoxin then did *A. hypogaea* checks, but the difference between the two wild species was not significant. High amounts of variation were found among the interspecific lines for aflatoxin accumulation. One interspecific tetraploid line, GP-NC WS 2 (Stalker and Beute, 1993), was identified as supporting very low levels of aflatoxin accumulation. GP-NC WS 2 was the only line tested that was not significantly different for aflatoxin accumulation from the resistant *Arachis* species parent (*A. cardenasii* GKP 10017).

Quantitative trait loci (QTLs) associated with reduced aflatoxin accumulation have been identified in maize *Zea maize* L. mapping populations. However, environment strongly influenced detection of QTLs, even at low significance thresholds (Paul *et al.*, 2003; Brooks *et al*., 2005). Historically, cultivated peanut has shown limited DNA polymorphism,

however, variation among species has been detected by random fragment length polymorphism (RFLP) analysis (Kochert *et al.*, 1991) and single-primer amplification (Halward *et al.*, 1992). Amplified fragment length polymorphism (AFLP) analysis indicated that significant variation exists both among and within species, but interspecific variation is higher than intraspecific variation for most *Arachis* species (Milla *et al.*, 2005). Marker-assisted selection (MAS) in peanut was first used in 1996 when random amplified polymorphic DNA (RAPD) markers linked to a root-knot nematode (RKN), *Meloidogyne arenaria* (Neal) Chitwood, resistance gene derived from wild peanut species saved considerable time and effort in assessing this subterranean phenotype (Burrow *et al.*, 1996). AFLP markers were used for MAS for resistance to the aphid vector, *Aphis craccivora* Koch., of groundnut rosette disease, and to construct a partial genetic linkage map (Herselman *et al.*, 2004). The use of molecular markers tightly linked to aflatoxin resistance genes would improve selection efficiency when developing improved peanut cultivars with resistance to aflatoxin accumulation, given that the aflatoxin assay is time-consuming, expensive, and exhibits variation between runs.

As a first step in implementing a MAS program for reduced aflatoxin accumulation in peanut, 15 interspecific tetraploid lines phenotyped by Xue *et al.* (2004) for reduced aflatoxin accumulation and a set of plant introductions (PI 250906, PI 290626, PI 590299, and PI 590325) known to have resistance to aflatoxin accumulation were screened with 256 AFLP primer combinations in order to determine the extent of polymorphism in the interspecific lines and to identify associations between polymorphisms and reduced aflatoxin accumulation (Milla *et al.*, 2007). Low levels of polymorphism were detected among the

plant introductions, and higher levels were detected among the interspecific lines. Bands from 835 marker loci ranging in size from 56 to 690 base pairs were scored as present or absent, and the two genotypic classes were compared to determine whether they supported differing levels of aflatoxin accumulation. At $P \leq 0.05$, 69 markers were associated with total aflatoxin accumulation, 60 with aflatoxin B1, and 60 with aflatoxin B2. At $P \leq 0.01$, 36 markers were associated with total aflatoxin accumulation, 36 with aflatoxin B1, and 46 with aflatoxin B2. All 36 markers associated with total aflatoxin and aflatoxin B1 accumulation in the interspecific line GP-NC WS 2 were linked in repulsion with the trait. It is probable that reduced aflatoxin accumulation is conferred by *A. cardenasii*-derived genomic regions (Milla *et al.*, 2007).

The primary objective of this work was to screen a segregating F_2 population derived from a cross between high-aflatoxin accumulating cultivar Gregory (Isleib *et al.*, 1999) and *A. cardenasii*-derived low-aflatoxin accumulating interspecific tetraploid line GP-NC WS 2 with candidate AFLP markers previously identified to be associated with reduced aflatoxin accumulation in order to identify markers closely linked to genetic factors controlling the trait. The ultimate goal is to utilize linked markers in a MAS program in order to expedite development of improved cultivars with reduced aflatoxin accumulation

MATERIALS AND METHODS

Population development

An F_2 population segregating for reduced post-harvest aflatoxin accumulation was generated by crossing Gregory, a Virginia market-type cultivar that supports high levels of aflatoxin and has superior agronomic characteristics, with GP-NC WS 2, an interspecific

tetraploid line originating from the *A. hypogaea* × *A. cardenasii* cross by Smartt and Gregory (1967) that supports low levels of aflatoxin, but is not suitable for use as a cultivar. F_1 plants from the cross Gregory × GP-NC WS 2 were grown in the field at a winter nursery in Juana Diaz, PR and allowed to self-pollinate to produce F_2 seed.

The F_2 seeds were central to multiple aspects of this research. The cotyledons were used in the *in vitro* assay to phenotype for aflatoxin accumulation. Furthermore, F_2 plants had to be cultivated for both DNA extraction for genotyping with AFLP markers and for generation advancement. Cotyledons are destroyed during the aflatoxin assay; therefore, the embryonic axes were removed from whole seeds prior to performing the assay and regenerated via tissue culture. Cotyledons were stored in envelopes at -20°C until the aflatoxin assay was performed. *In vitro* embryo regeneration allowed for the preservation of F_2 plants without expending the cotyledons.

Embryonic axes were excised from seeds and placed in petri dishes. Under sterile conditions, embryonic axes were surface sterilized in 70% ethanol for 1 min, 10% commercial bleach for 10 min, and rinsed three times in sterile distilled water. Embryos were allowed to air dry for 10 min, then transferred to Magenta jars containing approximately 30 ml tissue culture media consisting of Murashige and Skoog (1962) (MS) basal salt macronutrient and micronutrient solutions, MS vitamin solution, 3% sucrose, and 2% agar at pH 5.8. The tissue culture media was sterilized in an autoclave, and 50 μg ml^{-1} ampicillin plus 1 μg ml^{-1} amphotericin B were incorporated into the media. Magenta jars were sealed with Parafilm® (Pechiney Plastic Packaging Company, Chicago, IL) and placed in a growth chamber at 28°C on a 16 h light:8 h dark interval for 1 to 2 mo or until the plants

reached approximately 10 cm tall. Explants were then transferred to 7.6 cm clay pots, wrapped in plastic, and allowed to remain in the growth chamber for 3 to 4 d more to harden. Plants were subsequently transferred to the greenhouse and transplanted into flats filled with Metro-mix® medium (Sun Gro Horticulture, Bellevue, WA).

Aflatoxin assay

Segregating F_2 individuals plus susceptible check Gregory, and resistant checks GP-NC WS 2 and PI 262141 (*A. cardenasii* GKP 10017) were assayed to determine their ability to support aflatoxin accumulation when colonized by a toxigenic strain of *A. flavus* using the assay described by Xue *et al.* (2004) with some modifications. Each experimental unit was an inoculated cotyledon (seed half) in a 35 mm petri dish. Seed testas were removed to eliminate the potential barrier to fungal growth. Cotyledons were cut into four pieces (seed eighths), surface-sterilized by immersion in 10% commercial bleach for 3 min, then rinsed once in sterile distilled water. Cotyledon pieces were placed on two sheets of sterile filter paper moistened with sterile water. Each seed piece was inoculated with 12.5 µl of suspension containing 1×10^6 conidia ml^{-1} of *A. flavus* strain NRRL 3357 (Nat. Ctr. for Agric. Utilization Res., Peoria, IL).

A 7×7 modified Latin square design was used with one replicate on each of five trays to assay the F_2 population. Because it is not possible to replicate individuals in a segregating F_2 population, replicated checks were used to estimate position effects. Parents Gregory and GP-NC WS 2 were alternated along the diagonal. One *A. cardenasii* GKP 10017 sample was placed above and below the diagonal on each tray. Rows and columns were randomized for each tray and F_2 plants were filled in around the checks in numerical order. F_2 identities

were assigned arbitrarily, therefore they were already randomized and this pattern simplified record keeping. Each tray was wrapped in a clear plastic bag and sealed loosely with tape. Trays were stacked in an incubator at 28°C for 6 d. Short sections of PVC pipe were inserted between trays to prevent the weight of the stack from resting on the experimental units. Trays were rotated vertically each day of incubation by moving the top tray in the stack to the bottom. The moisture level of each petri dish was monitored daily and additional water was added to dishes that appeared dry. The optimum moisture regime was saturated filter paper with no free-standing water.

Due to profuse fungal development, the incubation period for the aflatoxin assay described by Xue *et al.* (2004) was curtailed by 2 d. After 6 d from the start of incubation, one unit each of Gregory and *A. cardenasii* GKP 10017 were removed from the tray, aflatoxin was extracted with chloroform, and spotted on a thin layer chromatography plate to qualitatively verify that aflatoxin had accumulated and that the two checks supported differing levels of aflatoxin. The remaining units were rated for mycelial growth, color, and development of fluffy colonies on the proportional scale of 0 (no growth, green color, or presence of fluffy colonies) to 10 (dense mycelium on all seed pieces, dark green color, or profuse fluffy colonies on all seed pieces) as previously described by Xue *et al.* (2004). Samples were weighed and transferred to scintillation vials. Ceramic grinding beads and 60:40 methanol:water solvent (volume in µls equal to four times mg fresh weight) were added to the scintillation vials. Samples were homogenized by grinding for 1 min and analyzed for aflatoxin B1 and B2 content by mass spectrometry at the N.C. State Univ. Genomic Sciences Laboratory.

Aflatoxin B1 and B2 values were log-transformed [Y'=ln(Y+0.5)] to stabilize error variance, and analysis of variance was conducted using the general linear model procedure (PROC GLM) of SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC). PROC GLM was used to adjust means to a common position effect within the growth chamber and to separate the means. Means of the transformed data were back-transformed ($Y=e^{Y'}-0.5$) to present values in parts per billion (ppb).

AFLP genotyping

DNA was extracted from regenerated plants once they were established in the greenhouse following the protocol described by Stein *et al.* (2001). DNA was quantified with a Nanodrop 1000™ spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at -20°C. One hundred eighty-three F_2 individuals were screened with 39 markers previously found to be associated with low aflatoxin accumulation (Milla *et al.*, 2007) (Table 1) using the protocol described by Myburg and Remington (2000). Each restriction digest was performed in a 30 µl volume with 500 ng genomic DNA, 5X restriction/ligation buffer (50 mM Tris HAc, 50 mM MgAc, 250 mM KAc, 25 mM DTT, and 250 ng/µl BSA), 12 units *Eco*RI, and 8 units *Mse*I for 1 to 3 h at 37°C.

Adapter ligations were performed in 25 µl volumes with 20 µl digested DNA plus 5 pMol *Eco*RI adapter, 50 pMol *Mse*I adapter, 10 mM ATP, 5X restriction/ligation buffer, and 0.5 Weiss units T4 DNA ligase for 3 to 15 h at 37°C. Reactions were diluted 1:10 with sdH_2O.

Pre-amplifications were performed with primers corresponding to the adapter sequences plus an additional selective nucleotide in 15 µl volumes with 5 µl diluted

restriction/ligation product plus 10X PCR buffer (New England Biolabs, Ipswich, MA), 2.5 mM each dNTP, 8.3 µM each primer, and 0.8 units Taq. Reaction conditions were 28 cycles 94°C/15 s, 60°C/30 s, 72°C/60 s plus 1 s/cycle extension; 1 cycle 72°C/2 min. Products were diluted 1:20 with low TE pH 8.0.

Selective amplifications were performed with primers corresponding to the pre-amplification primer plus three additional selective nucleotides in 19 µl volumes with 4 µl diluted pre-amplification product plus 10X PCR buffer, 2.5 mM each dNTP, 1 µM IRD-labeled E-primer, 8.3 µM M-primer, and 1.2 units Taq. Reaction conditions were 13 cycles 94°C/10 s, 65°C/30 s minus 0.7°C/cycle, 72°C/60 s; 25 cycles 94°C/10 s, 56°C/30 s, 72°C/60 s plus 1 s/cycle extension; 1 cycle 72°C/2 min.

PCR products were separated on 8% polyacrylamide sequencing gels using a LI-COR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, Nebraska). Polymorphic bands were scored using the Quantar software package (Keygene Inc., Rockville, MD) as present (1) or absent (0). Markers that were not repeatable were discarded. Markers were deemed repeatable if the previously screened checks amplified with the same AFLP genotype as reported by Milla-Lewis *et al.* (2007). Markers were given eight-character names comprised of the last two nucleotides of the E-primer, followed by an underscore, the last two nucleotides of the M-primer, and the band size in base pairs. A chi-square test for goodness-of-fit was conducted to test whether each marker fit the expected 3:1 segregation ratio. PROC GLM of SAS statistical software was used to conduct an F-test for each marker to determine whether aflatoxin B1, aflatoxin B2, and total aflatoxin accumulation differed significantly for the two AFLP genotype classes. Significance at $P \leq 0.05$ was considered

evidence for linkage between the marker and a genetic factor(s) controlling aflatoxin accumulation. To select the best model, a full model that included tray, row, and column effects was fit. Design effects with P-values greater than 0.05 were eliminated iteratively starting with those with the highest P-values. Partial R^2 values were calculated for each marker adjusted for the presence of design effects, to determine the marker's contribution to the total variation for aflatoxin accumulation. A second R^2 value was computed to reflect the proportion of the genotypic variance for which the marker accounted.

Linkage analysis and interval mapping

A pair-wise chi-square test of independence was conducted using Microsoft® Office Excel version 12 (Microsoft® Corporation, Redmond, WA) to investigate linkage. JoinMap® 3.0 (Kyazma,® Wageningen, Netherlands) was also used to assign linkage groups based on a minimum logarithm of odds (LOD) score of 3.0. The LOD score calculated by JoinMap® for the recombination frequency is based on the G^2 statistic for independence in a two-way contingency table. The G^2 statistic has a chi-square distribution under the null hypothesis and is calculated by the formula $2\sum O\ln(O/E)$, where "O" is the observed and "E" the expected number of individuals in a cell, and "ln" the natural logarithm (van Ooijen and Voorrips, 2001). Results of the test of independence and JoinMap® were compared and linkage groups were determined.

JoinMap® was used to conduct regression mapping with recombination frequency threshold smaller than 0.4, independence LOD score larger than 1.0, and Kosambi's mapping function to translate recombination frequency into map distances. Three maps were assembled and compared to determine the most probable map. First, regression mapping was

conducted on all linked markers using JoinMap's® default mapping procedure of building the map by adding loci one at a time, starting with the most informative pair. The order was verified with a ripple within a moving window of three adjacent markers after each locus was added (van Ooijen and Voorrips, 2001). The next map was assembled by first mapping the most tightly linked markers (17 markers linked at LOD 10.0), setting the resulting map as a fixed order, and then adding the remaining markers to the map using JoinMap's® default mapping procedure. The final map was constructed by starting with the group of 17 markers linked at LOD 10.0 and adding a few markers to the map at a time in order of descending LOD linkage scores. One marker, TC_TC134, was discarded due to insufficient linkage to estimate a map location. A linked marker with an undetermined map position is possible when using JoinMap® software because the LOD grouping procedure uses linkage to any locus already in a group to determine whether another locus belongs in the group, but the mapping procedure requires any marker fitted onto the map to have at least two distinct links on the map. If there are not two links due to the stringent thresholds, the locus is deemed insufficiently linked and a map position cannot be estimated.

Mapmaker (Whitehead Institute, Cambridge, MA) was also used to construct a genetic linkage map of the most tightly linked markers. The Suggest Subset command was used to select six informative markers and the Compare command was used to determine a starting order based on the maximum likelihood map for all possible orders. The Try command was used to add remaining markers to the map. Map distances were calculated using the Kosambi mapping function. The final sequence was checked using the Ripple command with a window size of 5 and log-likelihood threshold of 2.0.

MapQTL® Version 5 (Kyazma®, Wageningen, Netherlands) was used to conduct interval mapping in order to calculate QTL positions along the best genetic linkage map for aflatoxin B1, B2, and total aflatoxin. A QTL likelihood map was calculated by determining the likelihood for the presence of a segregating QTL (LOD scores) at 1 cM intervals along the map. MapQTL® was used to conduct a permutation test as described by Churchill and Doerge (1994) using 1,000 iterations to determine the significance threshold of the LOD score at $P \leq 0.05$.

RESULTS AND DISCUSSION

Aflatoxin assay

Mycelial growth and green color did not vary across the experiment. Mycelial development was profuse and each petri dish had a dark green color. Both characters received maximum scores when rated on the scale described by Xue *et al.* (2004). Development of fluffy colonies varied; though most samples received low scores. Development of fluffy colonies was not associated with aflatoxin accumulation.

Position effects for tray and column were significant for aflatoxin accumulation. Significance groups based on adjusted means for log-transformed aflatoxin values revealed that *A. cardenasii* was not significantly different from Gregory or GP-NC WS 2 for the accumulation of aflatoxin B1 and B2. Gregory and GP-NC WS 2 were significantly different for aflatoxin B1 and B2 accumulation. Numerically, *A. cardenasii* produced less aflatoxin B1 than Gregory and GP-NC WS 2, and less aflatoxin B2 than Gregory but more than GP-NC WS2. It was not possible to significantly distinguish *A. cardenasii* from Gregory due to large standard errors associated with *A. cardenasii* values resulting from low levels of

replication of *A. cardenasii* in the experimental design. The F_2 group exhibited high-parent heterosis by accumulating more aflatoxin B1 and B2 on average than either parent (Table 2). Heterosis indicates non-additive gene action such as over-dominance or epistasis.

AFLP analysis

Thirty-eight of the 39 markers used to screen the segregating F_2 population were repeatable and utilized in AFLP analysis. Segregation distortion from the expected 3:1 ratio was present in 35 of the 38 markers at the $P \leq 0.05$ significance level. At $P \leq 0.01$, 28 markers had segregation distortion (Table 1). The distortion favored Gregory, the *A. hypogaea* cultivar, in all cases.

Segregation distortion, or deviation from the expected Mendelian proportion of individuals in a given genotypic class (Xu *et al.*, 1997), may be caused by gametic selection, zygotic selection, or chromosomal rearrangements (Lorieux *et al.*, 1995). Levels of segregation distortion in peanut reportedly range from 25% (Burow *et al.*, 2001) to 53% (Moretzsohn *et al.*, 2005) of markers showing significant deviation from the expected segregation ratio. Dominant markers provide poor linkage information in the case of segregation distortion and should be used with caution when mapping (Lorieux *et al.*, 1995). Because nearly all of the available markers had segregation distortion, there was no alternative to using distorted markers to generate a genetic linkage map in this study.

In addition to the possible genetic causes of segregation distortion, the embryo regeneration may have impacted allele frequencies in the F_2 population. The tissue culture protocol used for embryo regeneration was 81% effective in the F_2 population. Embryo regeneration was attempted on 227 individuals and successful on 183. It is possible that

alleles from the parent that was more recalcitrant in tissue culture were under-represented in the 183 individuals that were recovered via embryo regeneration. Regeneration frequencies for Gregory and GP-NC WS 2 were not compared due to an insufficient supply of GP-NC WS 2 seeds.

Six markers were significantly associated with aflatoxin accumulation in the F_2 population at $P \leq 0.05$ (Table 3). One additional marker had P-values near 0.05 but not reaching the significance threshold. R^2 values for the model that included both design effects and the markers were around 0.50, but R^2 values for markers alone were low. These significant markers could be utilized in a MAS program to identify individuals that support low levels of aflatoxin accumulation.

Linkage analysis and interval mapping

According to a pair-wise chi-square test of independence, all markers were in one linkage group at $P \leq 0.05$. At $P \leq 0.01$ and $P \leq 0.001$ all markers were in one linkage group except for one unlinked marker (GC_CC600). JoinMap® results indicated that 34 markers were in one linkage group with four unlinked markers (GC_CC600, GG_TC374, AC_TT247, and GG_CG202). The linkage group identified by JoinMap® was used to construct the linkage map.

The map created by JoinMap's® default mapping procedure was 83 cM long. The map resulting from setting 17 fixed loci and then adding all remaining loci to the map at once was 60 cM long. The map constructed by setting 17 fixed loci and adding remaining loci to the map in small groups in order of descending linkage LOD scores was 62 cM long. All three linkage maps were similar; particularly the maps generated using 17 fixed markers,

which have only a few inverted loci. When Mapmaker was used to map the 17 tightly linked loci, the resulting map had a much greater length of 203 cM and the map order was not similar to the maps produced by JoinMap.® Mapmaker's algorithms are not adept at handling distorted markers. The LOD scores used for linkage analysis by Mapmaker are based on the 10-log likelihood ratio comparing the estimated recombination frequency with 0.5 and are affected by segregation distortion. JoinMap's® use of the test for independence, which is not affected by segregation distortion, when generating LOD scores helps eliminate mapping errors introduced by distorted markers. JoinMap® expresses the goodness-of-fit measure as a chi-square value, where large chi-square values correspond to poor goodness-of-fit (van Ooijen and Voorrips, 2001). The shortest map had the smallest chi-square value, and was used for QTL analysis (Fig. 1).

Interval mapping indicated one probable QTL at 9 cM. LOD scores peaked at 2.06, 1.83, and 1.94 for aflatoxin B1, aflatoxin B2, and total aflatoxin, respectively (Fig. 2). The permutation test indicated that the 5% significance threshold is at LOD=2.7 for all three traits. A LOD score of 2.06 is at P=0.165, LOD 1.83 is at P=0.242, and 1.94 is at P=0.202. The QTL explained 5.8% of the variance for aflatoxin B1, 6.0 % for aflatoxin B2, and 5.8 % for total aflatoxin. The highest peak is directly above the marker AC_CT164, which was the most significant (lowest P-values) marker according to the F-tests performed using SAS statistical software to determine whether markers were associated with aflatoxin accumulation.

Though the LOD scores did not reach the 5% significance threshold, it is likely that genetic factors controlling aflatoxin accumulation are present at the highest peak.

Confounding effects from the experimental design and the aflatoxin trait itself may mask significant results when stringent significance thresholds are applied. Aflatoxin accumulation is a highly variable trait, even when measured under *in vitro* conditions. Furthermore, despite high replication for each marker, the F_2 individuals were not replicated in the aflatoxin assay. These factors cause noise in the data that interferes with significant signals.

Six markers have been identified that are associated with reduced aflatoxin accumulation in a segregating F_2 population derived from the cross Gregory × GP-NC WS 2. AC_CT164 is the most useful marker for MAS. It has the lowest P-values and is located at the highest LOD peak on the interval map. Flanking significant markers GC_AA339 and GA_GT310 are useful for assessing recombination between the putative QTL and the surrounding genome.

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Table 1. AFLP markers used to screen the F_2 population segregating for reduced aflatoxin accumulation and their χ^2 test statistic for goodness-of-fit to the expected 3:1 segregation ratio.

Marker Name[†]	Size in bps	Source of Band	χ^2 (1df) Test Statistic	χ^2 (1df) P-value	Signif. [‡]
AT_GG073	73	Gregory	7.2	0.007	**
CA_AC073	73	Gregory	.	.	.
TC_TC134	134	GP-NC WS 2	204.3	<0.001	***
GG_CC160	160	Gregory	7.2	0.007	**
GG_GT163	163	Gregory	36.3	<0.001	***
AC_CT164	164	Gregory	6.3	0.012	*
CC_CC115	115	GP-NC WS 2	131.8	<0.001	***
CC_CC174	174	Gregory	17.9	<0.001	***
TA_CA188	188	Gregory	9	0.003	**
TA_AC198	198	Gregory	9	0.003	**
GG_CG202	202	Gregory	8.7	0.003	**
CC_TC214	214	Gregory	20.2	<0.001	***
AG_TC226	226	Gregory	24.3	<0.001	***
AC_TT247	247	Gregory	30.4	<0.001	***
CT_CC278	278	Gregory	7.8	0.005	**
GT_AT289	289	Gregory	37.7	<0.001	***
TC_GG310	310	Gregory	5.2	0.022	*
GA_AA315	315	Gregory	8	0.005	**
TG_CC192	192	Gregory	14.4	<0.001	***
TG_CC212	212	Gregory	20.8	<0.001	***
TG_CC332	332	Gregory	1.5	0.218	NS
GC_AA339	339	Gregory	15.9	<0.001	***
CA_CA347	347	Gregory	7.6	0.006	**
GA_GT184	184	Gregory	9.6	0.002	**
GA_GT310	310	Gregory	21.8	<0.001	***
GA_GT347	347	Gregory	21.5	<0.001	***
GC_CT294	294	Gregory	4.7	0.03	*
GC_CT356	356	Gregory	0	0.853	NS
CG_TG319	319	Gregory	4.4	0.037	*
GG_TC374	374	Gregory	0.7	0.393	NS
CT_CA384	384	Gregory	6.6	0.01	**
TT_GC409	409	Gregory	5	0.025	*
TA_AT437	437	Gregory	14.2	<0.001	***

[†]Markers were assigned eight character names comprised of the last two nucleotides from the E-primer, followed by an underscore, the last two nucleotides of the M-primer, and the band size in base pairs.

[‡]The *, **, and *** denote statistical significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 1. Continued.

Marker Name	Size in bps	Source of Band	χ^2 (1df) Test Statistic	χ^2 (1df) P-value	Signif.
TT_CC460	460	Gregory	10.5	0.001	***
AC_AC509	509	Gregory	7.1	0.008	**
CC_TA331	331	Gregory	6.1	0.013	*
CC_TA515	515	Gregory	14.7	<0.001	***
TC_AT588	588	Gregory	20.6	<0.001	***
GC_CC600	600	GP-NC WS 2	22	<0.001	***

Table 2. Adjusted means with standard errors for production of aflatoxins by experimental groups.

Group	Obser-vations	Aflatoxin B1			Aflatoxin B2			Total Aflatoxin		
		Untrans-formed	Transformed[†]	Back-trans-formed	Untrans-formed	Transformed	Back-trans-formed	Untrans-formed	Transformed	Back-trans-formed
		ppb		*ppb*	*ppb*		*ppb*	*ppb*		*ppb*
A. cardenasii	9	32658±8303[a]	10.231±0.217[ab]	27754	1365±515[a]	6.993±0.397[ab]	1089	34023±8794[a]	17.224± 0.599[ab]	28843
F_2	181	93250±1758[b]	11.335±0.046[c]	83712	7785±109[b]	8.743±0.084[c]	6264	101035±1863[b]	20.078±0.127[c]	89976
Gregory	24	50321±5106[a]	10.708±0.134[a]	44730	2379±317[a]	7.640±0.244[a]	2080	52699±5408[a]	18.349±0.368[a]	46810
GP NC WS2	25	37847±4791[a]	10.258±0.125[b]	28503	1613±297[a]	6.765±0.229[b]	867	39459±5075[a]	17.023±0.346[b]	29370

[†]Values were log transformed to stabilize error variance, and then back-transformed to present values in ppb.
[a]Means followed by the same letter are not significantly different at $P \leq 0.05$.

Table 3. Significant AFLP markers in the segregating F_2 population and their respective R^2 values.

		Aflatoxin B1						Aflatoxin B2						Total aflatoxin					
		Untransformed			Log transformed			Untransformed			Log transformed			Untransformed			Log transformed		
Marker	Source of band	P-value	Model $R^{2\dagger}$	Marker $R^{2\ddagger}$	P-value	Model R^2	Marker R^2	P-value	Model R^2	Marker R^2	P-value	Model R^2	Marker R^2	P-value	Model R^2	Marker R^2	P-value	Model R^2	Marker R^2
			%	%		%	%		%	%		%	%		%	%		%	%
CC_CC115	GP-NC WS2	0.024	41.0	5.6	0.060	48.5	2.5	0.006	36.6	9.8	0.045	51.1	2.3	0.020	40.7	6.0	0.047	50.7	2.3
AG_TC226	Gregory	0.061	41.1	7.8	0.051	48.5	5.1	0.059	38.3	8.7	0.106	50.9	2.6	0.059	40.9	7.9	0.076	50.6	3.4
GG_CC160	Gregory	0.026	42.7	8.0	0.031	50.1	4.5	0.052	36.9	7.1	0.081	51.9	2.3	0.027	42.3	7.9	0.053	51.8	3.0
AC_CT164	Gregory	0.005	43.7	13.2	0.020	50.2	5.3	0.002	38.6	19.5	0.023	52.8	4.0	0.004	43.3	13.8	0.020	52.4	4.4
TT_GC409	Gregory	0.065	39.7	5.4	0.058	47.9	3.5	0.017	35.5	11.4	0.038	51.0	3.4	0.055	39.4	5.9	0.042	50.4	3.4
GC_AA339	Gregory	0.047	41.0	7.4	0.060	48.8	4.0	0.036	36.4	10.0	0.086	51.3	2.6	0.044	40.7	7.6	0.071	51.0	3.0
GA_GT310	Gregory	0.045	43.4	9.4	0.150	50.2	2.6	0.010	38.5	21.3	0.086	52.7	3.1	0.037	43.1	10.4	0.102	52.3	2.9

† Model R^2 includes sums of squares associated with experimental design effects, *e.g.*, trays and columns within the growth chamber, as well as marker effect.

‡ Marker R^2 estimates the percentage of genotypic variance explained by the marker.

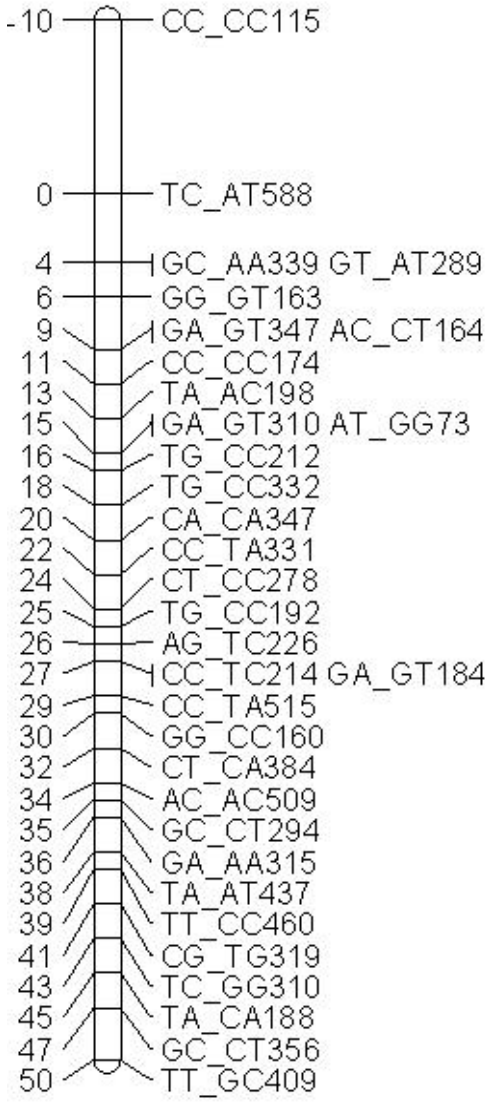
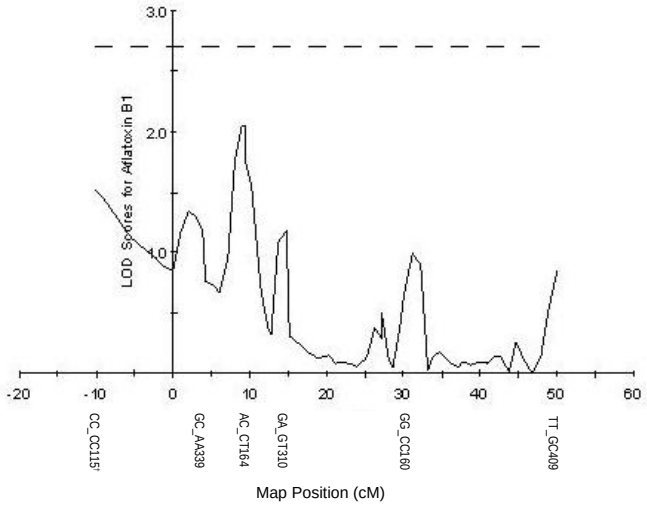
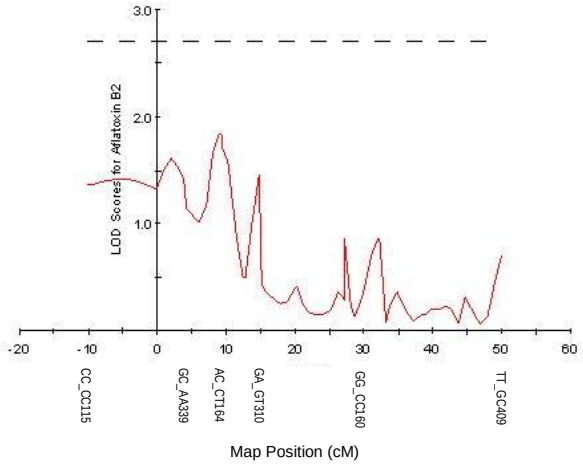
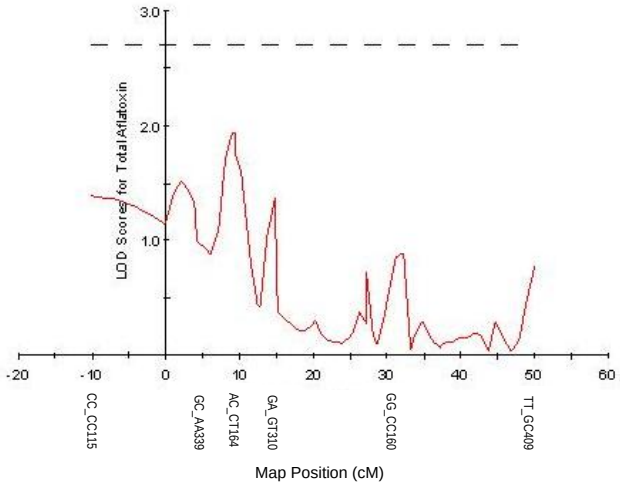


Fig. 1. Best genetic linkage map for AFLP loci generated using JoinMap® software. Map positions estimated using Kosambi mapping function and reported in cM.

Fig. 2. QTL map for linkage group associated with reduced accumulation of aflatoxin B1, aflatoxin B2, and total aflatoxin. The LOD threshold significance level, determined by a permutation test using 1,000 iterations, is indicated by horizontal dashes at LOD=2.7. †Markers significant at P≤0.05.


LOD Scores for Aflatoxin B1
3.0
2.0
1.0
-20
-10
0
10
20
30
40
50
60
CC_CC115'
GC_AA339
AC_CT164
GA_GT310
GG_CC160
TT_GC409
Map Position (cM)


LOD Scores for Aflatoxin B2
3.0
2.0
1.0
-20
-10
0
10
20
30
40
50
60
CC_CC115
GC_AA339
AC_CT164
GA_GT310
GG_CC160
TT_GC409
Map Position (cM)


LOD Scores for Total Aflatoxin
3.0
2.0
1.0
-20
-10
0
10
20
30
40
50
60
CC_CC115
GC_AA339
AC_CT164
GA_GT310
GG_CC160
TT_GC409
Map Position (cM)

APPENDICES

APPENDIX A: SAS program used to analyze aflatoxin and AFLP marker data.

```
*log transform data;
data logX06002;
      set X06002;
      log_ng_gAFB1=log(ng_gAFB1+0.5);
      log_ng_gAFB2=log(ng_gAFB2+0.5);
run;

proc print data=logX06002;
run;

proc glm data=logX06002;
      class tray row column group sample;
      model log_ng_gAFB1 log_ng_gAFB2=tray row column group sample(group);
      lsmeans group/stderr pdiff;
run;
*Row and sample(group) not significant;

proc glm data=logX06002;
      class tray row column group sample;
      model log_ng_gAFB1 log_ng_gAFB2=tray column group sample(group);
      lsmeans group/stderr pdiff;
run;
*This model is good;

*Gives LSMeans for each F2;
proc glm data=logX06002;
      class tray row column sample;
      model log_ng_gAFB1 log_ng_gAFB2=tray column sample;
      lsmeans sample/stderr pdiff;
run;

*Use a macro to determine whether genotype classes differ in aflatoxin
accumulation for individual markers;


*Example w/ one marker;
proc glm data=logX06002;
      class tray column group sample TC_TC134;
      model log_ng_gAFB1 log_ng_gAFB2=tray column group TC_TC134(group);
      lsmeans TC_TC134(group)/stderr pdiff;
run;

*The macro;
options mcompilenote=all;
%LET Marker1 = TC_TC134;
%LET Marker2 = TA_CA188;
%LET Marker3 = CC_CC174;
%LET Marker4 = CC_CC115;
%LET Marker5 = TA_AC198;
%LET Marker6 = CC_TC214;
```

```
%LET Marker7 = AG_TC226;
%LET Marker8 = GG_CC160;
%LET Marker9 = AC_CT164;
%LET Marker10 = CT_CC278;
%LET Marker11 = TG_CC332;
%LET Marker12 = TG_CC212;
%LET Marker13 = TG_CC192;
%LET Marker14 = GC_AA339;
%LET Marker15 = AT_GG073;
%LET Marker16 = GT_AT289;
%LET Marker17 = GG_GT163;
%LET Marker18 = GA_AA315;
%LET Marker19 = CG_TG319;
%LET Marker20 = GA_GT347;
%LET Marker21 = GA_GT310;
%LET Marker22 = GA_GT184;
%LET Marker23 = TC_GG310;
%LET Marker24 = CA_CA347;
%LET Marker25 = CT_CA384;
%LET Marker26 = AC_AC509;
%LET Marker27 = GG_TC374;
%LET Marker28 = TT_GC409;
%LET Marker29 = CC_TA515;
%LET Marker30 = CC_TA331;
%LET Marker31 = TC_AT588;
%LET Marker32 = GC_CC600;
%LET Marker33 = TT_CC460;
%LET Marker34 = TA_AT437;
%LET Marker35 = GG_CG202;
%LET Marker36 = GC_CT356;
%LET Marker37 = GC_CT294;

* %marker_model takes in the name of a marker variable and executes the
model for it;
%macro marker_model(arg);
proc glm data=logX06002;
      class tray column group sample &arg;
      model log_ng_gAFB1 log_ng_gAFB2=tray column group &arg(group);
      lsmeans &arg(group)/stderr pdiff;
      run;
%mend marker_model;

* %marker_iterator simply goes through each marker name one at a time and
calls the model macro;
%macro marker_iterator;
data=_null_;
set=logX06002;
run;
%do i = 1 %to 37;
      %marker_model(&&marker&i)
%end;
%mend marker_iterator;
```

```
%marker_iterator

*remember:  do not use a semicolon when calling the macro;


*Backtransform data for reporting in thesis;
data groupmeans;
      input Group$ log_ng_gAFB1 log_ng_gAFB2;
      datalines;
      Ac 10.2311534  6.99315767
      F2 11.3351398  8.74264385
      Gr 10.7084030  7.64018924
      WS 10.2577620  6.76497233
      ;
run;

proc print data=groupmeans;
run;

data ppb;
      set groupmeans;
      ppbAFB1=(EXP(log_ng_gAFB1)-0.05);
      ppbAFB2=(EXP(log_ng_gAFB2)-0.05);
run;

proc print data=ppb;
run;


*Compare backtransformed values to raw values;
proc glm data=X06002;
      class tray row column group sample;
      model ng_gAFB1 ng_gAFB2=tray column group sample(group);
      lsmeans group/stderr pdiff;
run;

*run macro on raw aflatoxin values;
options mcompilenote=all;
%LET Marker1 = TC_TC134;
%LET Marker2 = TA_CA188;
%LET Marker3 = CC_CC174;
%LET Marker4 = CC_CC115;
%LET Marker5 = TA_AC198;
%LET Marker6 = CC_TC214;
%LET Marker7 = AG_TC226;
%LET Marker8 = GG_CC160;
%LET Marker9 = AC_CT164;
%LET Marker10 = CT_CC278;
%LET Marker11 = TG_CC332;
%LET Marker12 = TG_CC212;
%LET Marker13 = TG_CC192;
%LET Marker14 = GC_AA339;
%LET Marker15 = AT_GG073;
```

```
%LET Marker16 = GT_AT289;
%LET Marker17 = GG_GT163;
%LET Marker18 = GA_AA315;
%LET Marker19 = CG_TG319;
%LET Marker20 = GA_GT347;
%LET Marker21 = GA_GT310;
%LET Marker22 = GA_GT184;
%LET Marker23 = TC_GG310;
%LET Marker24 = CA_CA347;
%LET Marker25 = CT_CA384;
%LET Marker26 = AC_AC509;
%LET Marker27 = GG_TC374;
%LET Marker28 = TT_GC409;
%LET Marker29 = CC_TA515;
%LET Marker30 = CC_TA331;
%LET Marker31 = TC_AT588;
%LET Marker32 = GC_CC600;
%LET Marker33 = TT_CC460;
%LET Marker34 = TA_AT437;
%LET Marker35 = GG_CG202;
%LET Marker36 = GC_CT356;
%LET Marker37 = GC_CT294;


* %marker_model takes in the name of a marker variable and executes the
model for it;
%macro marker_model(arg);
proc glm data=logX06002;
      class tray column group sample &arg;
      model ng_gAFB1 ng_gAFB2=tray column group &arg(group);
      lsmeans &arg(group)/stderr pdiff;
      run;
%mend marker_model;

* %marker_iterator simply goes through each marker name one at a time and
calls the model macro;
%macro marker_iterator;
data=_null_;
set=logX06002;
run;
%do i = 1 %to 37;
      %marker_model(&&marker&i)
%end;
%mend marker_iterator;

%marker_iterator

*remember:  do not use a semicolon when calling the macro;
```

```
*analyze total aflatoxin;
data totalAF;
      set logX06002;
      logB1B2=log_ng_gAFB1+log_ng_gAFB2;
      B1B2=ng_gAFB1+ng_gAFB2;
run;

proc print data=totalAF;
run;

options mcompilenote=all;
%LET Marker1 = TC_TC134;
%LET Marker2 = TA_CA188;
%LET Marker3 = CC_CC174;
%LET Marker4 = CC_CC115;
%LET Marker5 = TA_AC198;
%LET Marker6 = CC_TC214;
%LET Marker7 = AG_TC226;
%LET Marker8 = GG_CC160;
%LET Marker9 = AC_CT164;
%LET Marker10 = CT_CC278;
%LET Marker11 = TG_CC332;
%LET Marker12 = TG_CC212;
%LET Marker13 = TG_CC192;
%LET Marker14 = GC_AA339;
%LET Marker15 = AT_GG073;
%LET Marker16 = GT_AT289;
%LET Marker17 = GG_GT163;
%LET Marker18 = GA_AA315;
%LET Marker19 = CG_TG319;
%LET Marker20 = GA_GT347;
%LET Marker21 = GA_GT310;
%LET Marker22 = GA_GT184;
%LET Marker23 = TC_GG310;
%LET Marker24 = CA_CA347;
%LET Marker25 = CT_CA384;
%LET Marker26 = AC_AC509;
%LET Marker27 = GG_TC374;
%LET Marker28 = TT_GC409;
%LET Marker29 = CC_TA515;
%LET Marker30 = CC_TA331;
%LET Marker31 = TC_AT588;
%LET Marker32 = GC_CC600;
%LET Marker33 = TT_CC460;
%LET Marker34 = TA_AT437;
%LET Marker35 = GG_CG202;
%LET Marker36 = GC_CT356;
%LET Marker37 = GC_CT294;
```

```
* %marker_model takes in the name of a marker variable and executes the
model for it;
%macro marker_model(arg);
proc glm data=totalAF;
      class tray column group sample &arg;
      model B1B2=tray column group &arg(group);
      lsmeans &arg(group)/stderr pdiff;
      run;
%mend marker_model;

* %marker_iterator simply goes through each marker name one at a time and
calls the model macro;
%macro marker_iterator;
data=_null_;
set=totalAF;
run;
%do i = 1 %to 37;
      %marker_model(&&marker&i)
%end;
%mend marker_iterator;

%marker_iterator

*remember:  do not use a semicolon when calling the macro;
```

APPENDIX B: Supplementary figures.

Tray 1

G	1	3	5	6	C	7
8	9	10	11	12	13	W
14	G	15	16	17	G	18
21	W	W	22	23	24	25
26	28	29	30	W	31	32
33	34	36	W	37	38	39
C	G	40	41	42	G	43

37 F_2s
2 *A. cardenasii* (C)
5 GP-NC WS 2 (W)
5 Gregory (G)

Tray 2

44	45	49	50	51	52	W
53	54	W	58	59	60	61
C	62	64	65	G	66	67
G	68	69	G	C	70	71
72	73	74	75	76	W	77
G	78	83	G	84	85	86
87	W	88	W	89	91	92

37 F_2s
2 *A. cardenasii* (C)
5 GP-NC WS 2 (W)
5 Gregory (G)

Tray 3

94	95	G	96	G	C	97
98	99	100	W	101	102	103
104	105	106	107	108	109	W
111	W	112	113	114	116	117
118	120	G	122	G	123	124
125	126	128	130	C	G	131
W	132	W	133	134	135	136

37 F_2s
2 *A. cardenasii* (C)
5 GP-NC WS 2 (W)
5 Gregory (G)

Tray 4

138	141	142	143	144	146	W
148	W	150	151	153	154	156
157	158	G	160	162	G	162
164	165	166	W	167	168	169
G	170	C	171	172	173	174
C	175	G	176	178	G	179
181	182	183	184	W	W	186

37 F_2s
2 *A. cardenasii* (C)
5 GP-NC WS 2 (W)
5 Gregory (G)

Tray 5

W	187	189	190	W	191	192
193	194	G	C	195	196	197
198	W	200	201	W	202	204
205	G	C	G	206	207	208
209	210	212	213	214	W	216
217	218	219	223	224	225	W
226	G	227	G	228	229	230

36 F_2s
2 *A. cardenasii* (C)
6 GP-NC WS 2 (W)
5 Gregory (G)

Fig. 1. Modified Latin square experimental design used for aflatoxin assay.

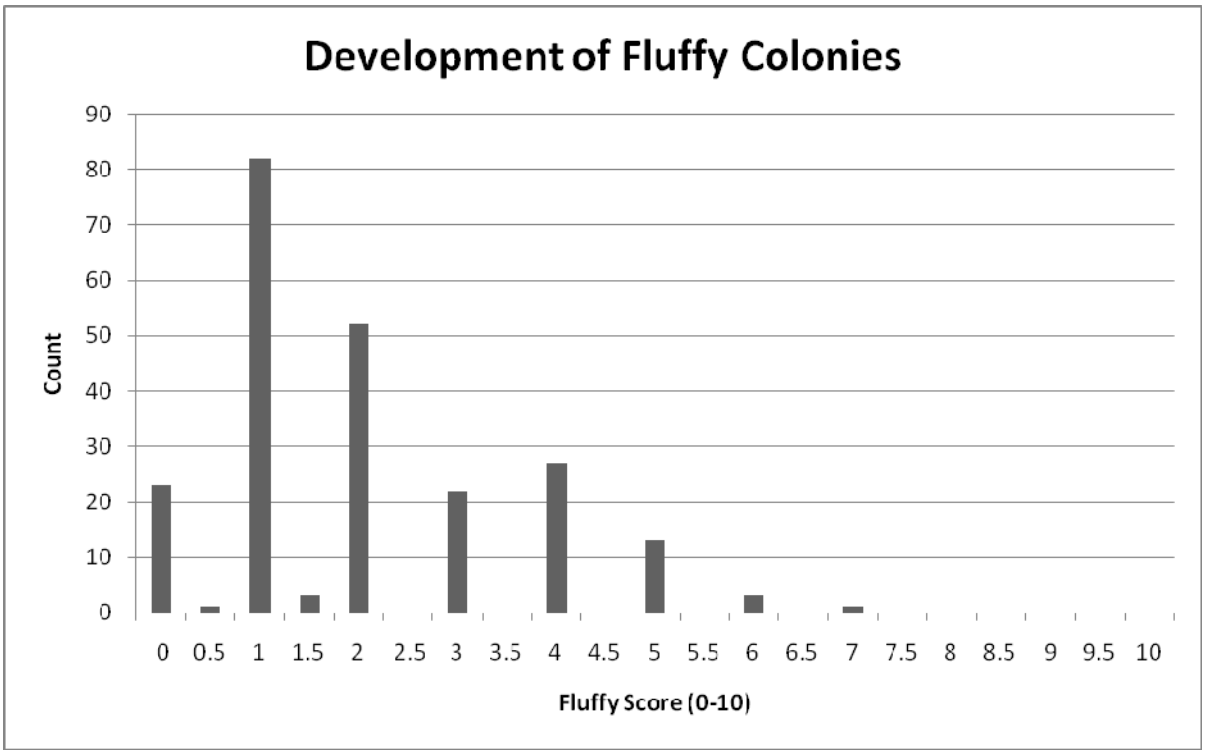


Fig. 2. Chart of fluffy score ratings for aflatoxin assay.

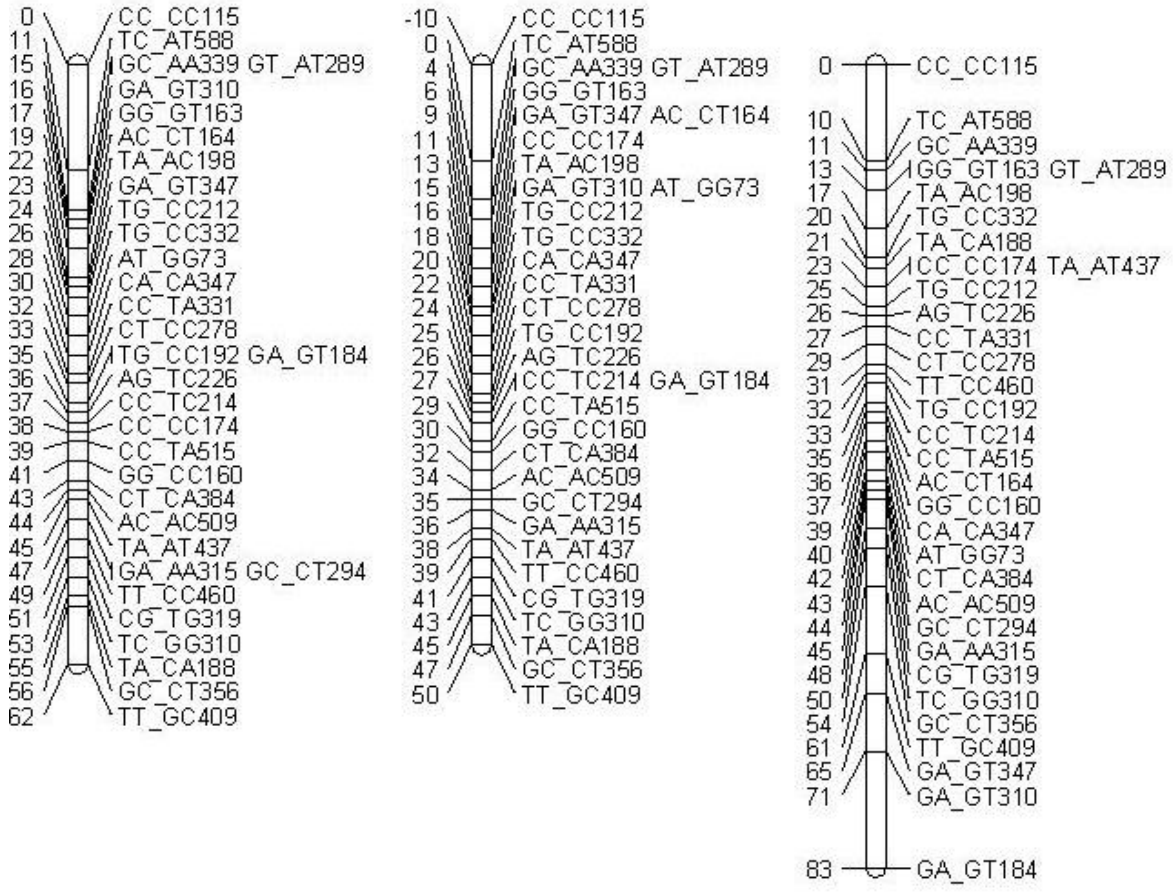


Fig. 3. Genetic linkage maps generated using JoinMap® software. Map positions estimated using Kosambi mapping function and reported in cM. Left: map based on 17 fixed loci with remaining loci added to the map in order of descending linkage LOD score. Middle: map generated by mapping 17 fixed loci and adding remaining loci using JoinMap® default procedures (best map). Right: map generated by JoinMap® default mapping procedures.

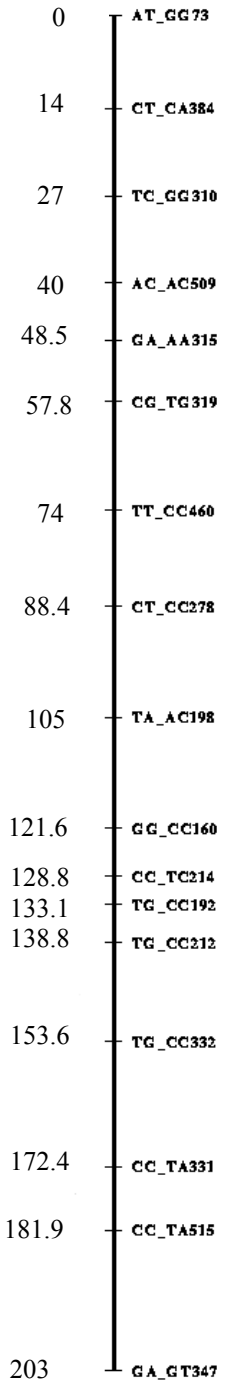


Fig. 4. Genetic linkage map generated using Mapmaker software. Map contains 17 tightly linked loci with positions estimated using Kosambi mapping function and reported in cM.