Strategies in Prevention of Preharvest Aflatoxin Contamination in Peanuts: Aflatoxin Biosynthesis, Genetics and Genomics

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ABSTRACT

Peanut (*Arachis hypogaea* L.), or groundnut, is an important crop economically and nutritionally in many tropical and subtropical areas of the world. It is also one of the most susceptible host crops to *Aspergillus flavus* resulting in aflatoxin contamination. The prevention or elimination of aflatoxin contamination in preharvest and postharvest crops is a serious challenge facing scientists. The recent International Conference on Groundnut Aflatoxin Management and Genomics held in Guangzhou, China, provided an international forum for discussions on the latest accomplishments, the development of strategies, and the initiation of cooperative research for the prevention of aflatoxin contamination. This review summarizes the progress in genetic and genomic research of peanuts and the toxin-producing fungus *A. flavus.* In particular, the pathway for production and the genetic regulation of afaltoxin, and the peanut-*Aspergillus* interaction are discussed. The use of a peanut-*Aspergillus* microarray will help scientists to study the crop-pathogen interaction; aids in the identification of genes involved in both fungal invasion and crop resistance, and ultimately enhance research to find solutions that prevent aflatoxin contamination in agricultural commodities.

Key Words: Aflatoxin contamination, genetics, genomics.

Throughout the world, aflatoxin contamination is considered one of the most serious food and feed safety issues. Chronic problems with aflatoxin contamination occur in the southern U.S., and are particularly troublesome in peanut, corn, cottonseed, and tree nuts. The impact of aflatoxin contamination on the agricultural economy is especially devastating during drought years when aflatoxin affects the more northern areas including the Midwestern corn belt. Economic losses in the years of major aflatoxin outbreaks have been estimated in the hundreds of millions of dollars. The realization of the unique nature of the aflatoxin problem and the need for novel technologies to ameliorate the impact of this problem became a focal point of discussion in 1988 at the first U.S. Aflatoxin Elimination Workshop held in New Orleans, LA. The Annual Aflatoxin Workshops (Table 1) have served as a forum to assemble USDA-ARS scientists, university faculty, representatives of the different commodities and industries, and international participants in a unique cooperative effort to develop aflatoxin control strategies through research and development.

Peanut (*Arachis hypogaea* L.), also called groundnut, is an important staple crop in many areas of the world and also is one of the most susceptible host species for *Aspergillus flavus* Link:Fr., which is the causal organism producing aflatoxin. The International Conference on Groundnut Aflatoxin Management and Genomics was held 5-9 November, 2006, in Guangzhou, China, and provided an international forum for scientific presentations and discussions about the latest accomplishments, strategies and cooperation in the prevention of aflatoxin contamination through genetics and genomics. Our presentation at this conference focused on searching for strategies in prevention of preharvest aflatoxin contamination through understanding the mechanisms of aflatoxin formation, pathogenicity of the fungus, and host crop resistance along with recent research on crop-fungus interaction using microarray by gene expression profilings. The joint effort between USDA-ARS at Tifton, GA and New Orleans, LA, and the J. Craig Venter Institute (JCVI) to produce a microarray to study the interaction between *Aspergillus* and peanut is also reviewed in this paper.

Aflatoxins and Importance of Aflatoxin Contamination

Aflatoxins. Mycotoxins are low molecular weight secondary metabolites produced by filamentous fungi and induce various degrees of toxicity to vertebrates, invertebrates, plants, and microorganisms. Aflatoxin was first associated with an outbreak of ''Turkey X disease'', which

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Table 1. USDA Annual Multicrop Aflatoxin Elimination Workshop.

Year	Location	Year	Location
1988	New Orleans, LA	1998	St. Louis, MO
1989	Peoria, IL	1999	Atlanta, GA
1990	St. Louis, MO	2000	Yosemite, CA
1991	Atlanta, GA	2001	Phoenix, AZ
1992	Fresno, CA	2002	San Antonio, TX
1993	Little Rock, AR	2003	Savannah, GA
1994	St. Louis, MO	2004	Sacramento, CA
1995	Atlanta, GA	2005	Raleigh, NC
1996	Fresno, CA	2006	Ft. Worth, TX
1997	Memphis, TN	2007	Atlanta, GA

occurred in 1960 near London, England, and killed approximately 100,000 poults (Blount, 1961; Forgacs and Carll, 1962). The cause of the disease was later associated with the feeding of peanut (groundnut) meal infested by *A. flavus*, and thus the toxins were named "aflatoxin" for the *A. flavus* toxin.

Attention has been given to the occurrence, biosynthesis, and toxicity of aflatoxins since these compounds are both carcinogenic and the most toxic of the known mycotoxins (Cole and Cole, 1987; Van Egmond, 1989; Yu *et al.*, 2004a, b,c; Yu *et al.*, 2006). Aflatoxins belong to a family of compounds with difuranocoumarins. Aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) are the four major aflatoxins based on their blue (B) or green (G) fluorescence under ultraviolet light and their relative mobility by thin-layer chromatography on silica gel. Aflatoxin M_1 is a hydroxylated derivative metabolized from aflatoxin B_1 by cows and secreted in milk (Van Egmond, 1989). In addition to aflatoxins B_1 and B_2, *A. flavus* produces cyclopiazonic acid, kojic acid, beta-nitropropionic acid, aspertoxin, aflatrem and aspergillic acid (Goto *et al.*, 1996). *Aspergillus parasiticus* Speare produces G_1 and G_2 in addition to aflatoxins B_1 and B_2.

Food safety and economic impact. *Aspergillus flavus* is a weak opportunistic plant pathogen and secretes aflatoxins into many agricultural crops. Preharvest aflatoxin contamination can occur when *A. flavus* infects peanut pods, corn ears, and cotton bolls with insect or mechanical damage (or if tissues are not amaged). Postharvest aflatoxin contamination can be problematic if grain storage is poorly managed. Outbreaks of acute aflatoxicosis from contaminated food in humans have been documented in Kenya, India, Malaysia, and Thailand as reported by the Council for Agriculture Science and Technology (CAST, 2003). For example, an outbreak of severe aflatoxicosis in humans occurred in more than 150 villages in western India in 1974 where 397 persons were affected and 108 persons died (Krishnamachari *et al.*, 1975). The largest and most severe outbreak of acute aflatoxicosis documented worldwide occurred in Kenya during 2004 and involved 317 cases and 125 deaths, mainly among children, due to consumption of aflatoxin contaminated corn (Lewis *et al.*, 2005; CDC, 2004).

Low dose consumption of aflatoxin contaminated food stuff causes chronic aflatoxicosis resulting in cancer, suppression of immunological reponses, and other "slow" pathological conditions. The liver is the primary target organ by toxic and carcinogenic aflatoxins. Cytochrome P450 enzymes in the liver convert aflatoxins to the reactive 8,9-epoxide form, which is capable of binding to both DNA and proteins (Eaton and Groopman, 1994). Aflatoxin B_1-DNA adducts can result in the GC to TA transversions in the p53, a DNA-repair, tumor-suppressor gene, at codon 249. Inactivation of the p53 tumor suppressor gene leads to the development of primary liver cancer (Bressac *et al.*, 1991; Hsu *et al.*, 1991).

Aflatoxin contamination also has a significant economic impact on worldwide agriculture. In the developing countries, food safety is the major problem where detection and decontamination policies are impractical. Due to food shortage in those countries, routine consumption of aflatoxin-contaminated food is widespread. The liver cancer incidence rates are 2 to 10× higher in developing countries than in developed countries (Henry *et al.*, 1999). In developed countries, the maximum allowable amount of aflatoxin in food and feed for human consumption and for livestock has been mandated by law. A guideline of 20 ppb (parts per billion) aflatoxin in food or feed substrate is the maximum allowable limit imposed by the U.S. Food and Drug Administration. The European Union has a maximum level of 2 ppb for aflatoxin B_1 and 4 ppb for total aflatoxins. The aflatoxin contaminated commodities are often destroyed if the aflatoxin content is higher than the mandated levels. This results in billions of dollars in yearly losses worldwide. In some parts of the southern U.S. such as in the southeast peanut, southern cotton belt, and mid-south corn farming regions, severe outbreaks of aflatoxin contamination occur frequently and resulted in enormous economic losses (CAST, 1989; Robens and Cardwell, 2005).

Genetics and Biochemistry of Aflatoxin Biosynthesis

Aflatoxin biosynthetic pathway, genes and enzymes. The elucidation of the aflatoxin biosynthetic pathway has been one area of research focus during the last decade. The major biochemical

pathway steps have been determined and the chemical structures of aflatoxin intermediates have been characterized (Minto and Townsend, 1997; Payne and Brown, 1998; Yu, 2004; Yu *et al.*, 2004a, c; Yu *et al.*, 2005). At least 23 enzymatic reactions are involved in aflatoxin formation. No less than 15 structurally-defined aflatoxin intermediates have been identified in the aflatoxin/ST (sterigmatocystin) biosynthetic pathway.

Aflatoxins are synthesized from malonyl CoA, first with the formation of hexanoyl CoA, followed by formation of a decaketide anthraquinone (Bhatnagar *et al.*, 1992; Minto and Townsend, 1997). There are two fatty acid synthases (FAS) and a polyketide synthase (PKS) involved in the synthesis of the polyketide from acetyl CoA (Watanabe and Townsend, 2002). Norsolorinic acid (NOR) is the first stable aflatoxin intermediate identified in the pathway (Bennett, 1981). Aflatoxins are formed after a series of oxidation-reduction reactions. The generally accepted aflatoxin biosynthetic pathway scheme is: a hexanoyl CoA precursor → norsolorinic acid, NOR → averantin, AVN → hydroxyaverantin, HAVN → Oxoaverantin, OAVN → averufin, AVF → hydroxyversicolorone, HVN → versiconal hemiacetal acetate, VHA → versiconal, VAL → versicolorin B, VERB → versicolorin A, VERA → demethyl-sterigmatocystin, DMST → sterigmatocystin, ST → O-methylsterigmatocystin, OMST → aflatoxin B_1 and aflatoxin G_1 (Fig. 1). After the VHA step, there is a branch point in the pathway that leads to aflatoxins B_2 and G_2 formation (Yabe *et al.*, 2003; Yu *et al.*, 1998; Yu *et al.*, 2004c).

A total of 29 genes or open reading frame (ORF) are involved in aflatoxin formation. The first aflatoxin pathway gene identified was *aflD* (*nor-1*) that encodes a ketoreductase in *A. parasiticus* (Chang *et al.*, 1992) for the conversion of norsolorinic acid (NOR) to averantin (AVN). Disruption or deletion of the *aflD* (*nor-1*) gene leads to the accumulation of a brick-red pigment in the hyphae and blocks the synthesis of all aflatoxins and their intermediates beyond NOR (Bennett, 1981). A gene named *aflR* present in *A. parasiticus* and *A. flavus*, as well as in *A. nidulans* Eidam (originally named *afl-2* and *apa-2*), has been cloned by Chang *et al.*, (1993) and Payne *et al.* (1993). This is a positive regulatory gene involved in both the aflatoxin pathway gene expression in *A. flavus* and *A. parasiticus* and sterigmatocystin (ST) pathway gene expression in *A. nidulans*. These four genes for aflatoxin or ST biosynthesis are milestone discoveries.

There are two separate pathways leading to B-Group (AFB_1 and AFB_2) and G-Group (AFG_1 and AFG_2) aflatoxins (Yabe *et al.*, 1988). A gene, named *aflQ* (*ordA*), encoding a cytochrome P-450 monooxygenase, was demonstrated to be responsible for the conversion of *O*-methylsterigmatocystin (OMST) to AFB_1 and AFG_1, and demethyldihydrosterigmatocystin (DMDHST) to AFB_2 and AFG_2 (Prieto and Woloshuk, 1997; Yu *et al.*, 1998) in *A. parasiticus* and *A. flavus*. Expression and substrate feeding, using a yeast system, demonstrated that an additional enzyme was required for formation of G-group aflatoxins (AFG_1 and AFG_2) (Yu *et al.*, 1998). Functional studies demonstrated that the *aflU* (*cypA*) gene in *A. parasiticus*, encoding a cytochrome P450 monooxygenase, is responsible for the conversion of OMST to AFG_1 and DHOMST to AFG_2 (Ehrlich *et al.*, 2004). A partial deletion of this gene results in loss of G-group aflatoxin production in *A. flavus* (Ehrlich *et al.*, 2004).

The observation that the *aflD* (*nor-1*) and *aflM* (*ver-1*) genes were linked with the regulatory gene *aflR* in a common cosmid clone was the initial evidence indicating that aflatoxin pathway genes were clustered (Skory *et al.*, 1993; Trail *et al.*, 1995b). The completed aflatoxin pathway gene cluster was established when an 82 kb DNA sequence harboring a total of 29 aflatoxin biosynthetic pathway genes (or ORFs) and four sugar utilization genes were reported (Yu *et al.*, 2004c). The primary advantage of gene clustering may be for the purpose of coordinated gene expression.

Factors affecting aflatoxin biosynthesis. Biotic and abiotic factors, either nutritional or environmental, are known to affect aflatoxin production in toxigenic *Aspergillus* species, although the molecular mechanisms for these effects are still unclear (Payne and Brown, 1998; Guo *et al.*, 2005a, b). Nutritional factors such as carbon, nitrogen, amino acid, lipid, and trace elements affect aflatoxin production (Feng and Leonard, 1998; Payne and Brown, 1998; Cuero *et al.*, 2003). Simple sugars such as glucose, sucrose, and maltose support aflatoxin formation, but peptone, sorbose, or lactose do not promote the toxin (Payne and Brown, 1998). However, the mechanism by which a carbon source is involved in the regulation of aflatoxin pathway gene expression is poorly understood. Nitrogen source affects aflatoxin formation in varying ways and production levels are different when *Aspergillus* spp. is on a nitrate versus a nitrite medium (Payne and Brown, 1998). Certain amino acids also can have opposing effects on aflatoxin production (Payne and Hagler, 1983). Recent studies using *A. flavus* show that tryptophan inhibits aflatoxin formation while tyrosine spikes aflatoxin production (Wilkinson *et al.*, 2007). Micronutrients (metal ions) were also reported to

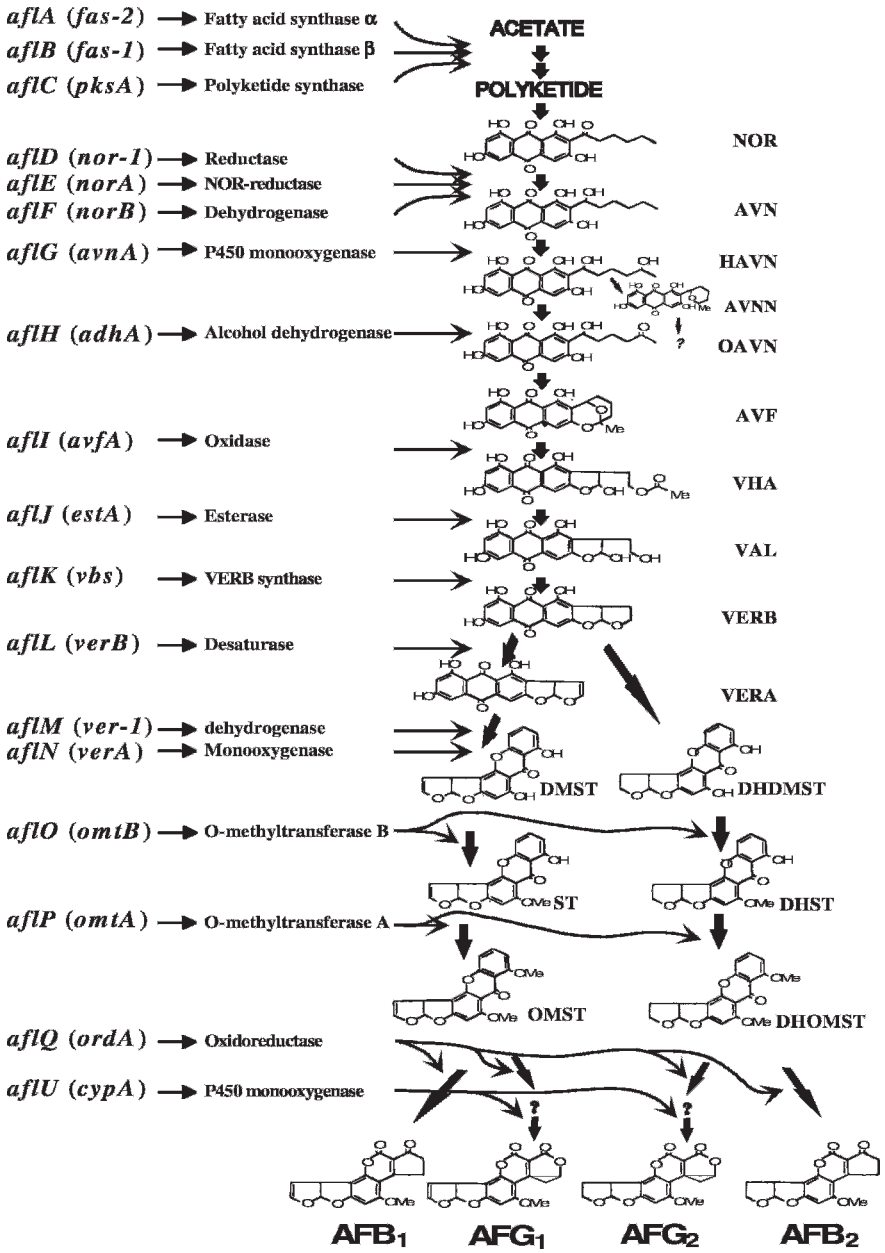

Fig. 1. Aflatoxin biosynthetic pathway (adopted from Yu et al., 2004c). Arrows indicate the connections from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and from the intermediates to the products in the aflatoxin bioconversion steps. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5-hydroxyaverantin; AVNN, averufanin; OAVN, oxoaverantin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2.

affect aflatoxin pathway gene expression (Bennett *et al.*, 1979; Cuero *et al.*, 2003). Lipids have tremendous effects on aflatoxin formation, not only as a nutritive source but also as substrates metabolized for acyl-CoA starter units (Maggio-Hall *et al.*, 2005) and as signaling molecules (Brodhagen and Keller, 2006).

Temperature, pH, water activity (drought stress) and other stresses are external environmental factors that can affect aflatoxin production (Cotty, 1988; Payne and Brown, 1998; Guo *et al.*, 2005b; Kim *et al.*, 2005, 2006; Sobolev *et al.*, 2007). Studies suggest that *aflR* transcription is responsive to a G-protein signaling cascade that is mediated by protein kinase A (Hicks *et al.*, 1997). This signaling pathway may respond to the environmental effects on aflatoxin biosynthesis. Optimal aflatoxin production is observed at temperatures near 30C (28 to 35C). Aflatoxin production is closely related to pH changes where biosynthesis in *A. flavus* occurs in acidic media, but is inhibited in alkaline media (Cotty, 1988).

Fungal developmental processes are associated with secondary metabolism such as sporulation and sclerotial formation (Bennett *et al.*, 1986; Calvo *et al.*, 2002; Chang *et al.*, 2002; Yu, 2006). Similar environmental conditions required for secondary metabolism and for sporulation are observed. It was also reported that the spore formation and secondary metabolite formation occur at about the same time (Hicks *et al.*, 1997; Trail *et al.*, 1995a). Mutants that have suppressed sporulation were unable to produce aflatoxins (Bennett and Papa, 1988). Some compounds in *A. parasiticus* that inhibit sporulation also have been shown to inhibit aflatoxin formation (Reib, 1982).

Oxidative stress induced aflatoxin biosynthesis in *A. parasiticus* has long been reported. Kim *et al.* (2006) showed that treatment of *A. flavus* with *tert*-butyl hydroperoxide induced significant increases in aflatoxin production. Similar treatment of *A. parasiticus* also induced aflatoxin production (Reverberi *et al.*, 2005; Reverberi *et al.*, 2006). However, hydrolysable tannins significantly inhibit aflatoxin biosynthesis with the main antiaflatoxigenic constituent being gallic acid (Mahoney and Molyneux, 2004). Gallic acid reduces expression of genes within the aflatoxin biosynthetic cluster. When certain phenolics or antioxidants such as ascorbic acid are added to oxidatively stressed *A. flavus*, aflatoxin production significantly declines with no effect on fungal growth (Kim et al. 2006).

Functional Genomics and Control Strategies of Aflatoxin Contamination

Althrough there is a fairly good understanding on the aflatoxin biosynthetic pathway and pathway cluster genes, many important questions still remain unanswered. Identification of all the genes responsible for aflatoxin formation and a better understanding of the regulatory mechanisms of aflatoxin formation of *Aspergillus* and the host crops will provide vital clues for devising strategies in solving aflatoxin contamination of food and feed.

Biological Control. Many natural isolates of *A. flavus* and *A. parasiticus* that do not produce aflatoxins. These non-aflatoxigenic strains have shown promise as a strategy to reduce preharvest aflatoxin contamination of crops (Dorner, 2005). Some of the non-aflatoxin-producing strains can out-compete the toxigenic strains in nature, and the strategy of competitive exclusion has been successfully practiced in cotton, peanut, and corn fields (Dorner, 2005). Cotty (1990) studied the competitiveness of seven non-toxigenic *A. flavus* strains in cotton in greenhouse experiments by co-inoculating them with representative toxigenic strains. Strain AF36 showed the highest survivability in greenhouse and field experiments and showed the largest reduction in aflatoxin levels. The EPA has approved AF36 as a biological control strain for field applications (Cotty, 1994). This strategy has also been shown to be effective in peanut and corn field studies (Dorner, 2005).

Host resistance through crop breeding and genetic engineering. *Aspergillus flavus* and *A. parasiticus* fungi can be found virtually everywhere in the world and are especially common in agricultural crop fields. Aflatoxin contamination tends to be greater when crops are subjected to insect damage and/or heat and drought stress. Various approaches have been suggested for genetic control of preharvest aflatoxin contamination including the development and use of crops with resistance to insects, resistance to plant stress (especially for tolerance to drought and high temperatures). Several sources of resistant germplasm were identified and released for crop genetic improvement in corn (McMillian *et al.*, 1993; Williams and Windham, 2001; Naidoo *et al.*, 2002; Guo et al. 2007a) and in peanuts (Cole *et al.*, 1995; Holbrook *et al.*, 2000; Guo *et al.*, 2006; Holbrook *et al.*, 2008). Crop resistance to aflatoxin contamination may be achieved by the three strategies: a) resistance to fungal invasion, b) inhibition of aflatoxin formation, and 3) resistance to insects and abiotic stress such as drought. Genetic engineering has been used in the last decade to improve crop resistance to diseases and insects. Examples are the development of Bt (*Bacillus thuringiensis*) corn (Ostlie *et al.*, 1997; Singsit *et al.*, 1996) and Bt peanut (Ozias-Akins *et al.*, 2002). Transgenic Bt corn and Bt peanut have shown reduced levels of aflatoxin contamination (Windham *et al.*, 1999; Dowd, 2001; Weissinger *et al.*, 2002).

Functional genomics as a tool in reducing aflatoxin contamination. Peanut genomics research can provide new tools to study plant-microbe interactions and enhance crop genetic characteristics. However, genomics research in peanut is behind many other crops due to the shortage of essential genome infrastructure, tools, and resources. Peanut genomic work has involved the development of markers, maps, and the classification of species. However, improvement of methods for gene and genome sequencing and analysis of gene expression promise to allow rapid progress for identification and understanding of gene function related to phenotype. DNA sequencing is one of the most-important sources of genomic informaiton. The peanut genome (2,800 Mb/1C) is large in comparison to many model plants such as *Arabidopsis* (128 Mb), rice (420 Mb), and *Medicago truncatula*

Gaertn. (500 Mb); and is larger than soybean *Glycine max* (L.) Merr. (1,100 Mb) and maize *Zea mays* L. (2,500 Mb) (Arumuganathan and Earle, 1991). The large genome size makes it unlikely that the peanut genome will be completely sequenced in the near future.

Sequencing of large numbers of Expressed Sequence Tags (ESTs) can deliver substantial amounts of genetic information on protein-coding sequences for comparative and functional genomic studies. ESTs provide putative genes, markers, and microarray resources to study peanut-*Aspergillus* interactions and to devise more effective breeding strategies to mitigate aflatoxin contamination. As of 5 October, 2007, a large number of ESTs of the top six plant species including *Arapidopsis* (1,276,692), rice (1,214,083), maize (1,159,264), wheat (1,050,932), oilseed rape (*Brassica napus*) (567,177) and barley (437,713) had been deposited to the GenBank database (dbEST release 100507, http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). However, only 41,372 *Arachis* ESTs were deposited as of 5 October, 2007, in GenBank, including 6,264 from the wild species *A. stenosperma* (Krapov. and W.C. Gregory.). These ESTs were from different tissues, some of which had been subjected to different abiotic and biotic stresses (Luo *et al.*, 2005a; Yan *et al.*, 2005; Proite *et al.*, 2007). Two submissions were 7454 and 2184 sequences from seed libraries of the Chinese accessions Luhua 14 and Shanyou 523, respectively. The majority represented the 21,777 ESTs (accession numbers ES702769 to ES724546) from developing seeds (Guo *et al.*, 2008a) of the U.S. runner type cultivar Tifrunner (Holbrook and Culbreath, 2007) and the Spanish breeding line GT-C20 (Guo *et al.*, 2008b). The seed and leaf EST originated from 10 non-normalized peanut cDNA libraries (Guo *et al.*, 2007b; Guo et al., 2008a), resulting in over 13,824 unigene ESTs, which will be used for the production of a long-oligo microarray for peanut. This sequence data has been made available to the community in order to develop genomic tools and resources for deciphering the biological function of genes in the peanut genome, including a) construction of peanut 70-mer oligo microarray, and b) development of markers/genes associated with resistance to biotic and abiotic stress. A total of 8402 oligos of 60-80bp in length have been designed for the oligo microarray (Guo *et al.*, 2007b).

Peanut ESTs developed from developing seeds and leaf tissues. Six different cDNA libraries were constructed from developing peanut seeds at three reproduction stages (R5, R6 and R7) from resistant and susceptible peanut genotypes. Tifrunner is susceptible to *Aspergillus* infection with higher aflatoxin contamination and resistant to Tomato Spotted Wilt Virus (TSWV) and GT-C20 is resistant to *Aspergillus* with reduced aflatoxin contamination and susceptible to TSWV. The developing peanut seed tissues were challenged by *A. parasiticus* and drought stress in the field. A total of 24,192 randomly selected cDNA clones from six libraries were sequenced. After removing vector sequences and quality trimming, 21,777 high-quality EST sequences were generated. Sequence clustering and assembling resulted in 8,689 unique EST sequences with 1,741 tentative consensus EST sequences (TCs) and 6,948 singleton ESTs. Functional classification was performed according to MIPS functional catalogue criteria. The unique EST sequences were divided into 22 categories. A similarity search against the non-redundant protein database available from NCBI (National Center for Biotechnology Information) indicated that 84.78% of the ESTs showed significant similarity to known proteins, of which 165 genes had been previously reported in peanuts. Furthermore, a comparison of these EST sequences with other plant sequences in The Institute for Genomic Research (TIGR) gene indices libraries showed that the percentage of peanut ESTs that matched to *A. thaliana*, maize (*Zea mays* L.), *M. truncatula*, rapeseed (*Brassica napus* L.), rice, soybean and wheat (*Triticum aestivum* (L.) Thell.) ESTs ranged from 33.84% to 79.46% with the sequence identity $\geq$ 80%. As expected, the peanut ESTs were more closely related to legume species than to cereal crops, and more homologous to dicot than to monocot plant species.

Two cDNA libraries were derived from peanut leaf tissues of the same peanut genotypes, Tifrunner and GT-C20, and 17,376 randomly picked clones were sequenced (Guo *et al.*, unpublished data). After removing vector sequences and discarding low quality sequences, a total of 14,432 high-quality EST sequences were generated. Sequence clustering and assembling resulted in 6,888 unique EST sequences with 1,703 tentative consensus EST sequences (TCs) and 5,185 singleton ESTs. Functional annotations were performed on the basis of MIPS functional catalogue criteria and Gene Ontology (GO). According to the MIPS, about 82.9% (5,717) of the unique sequences have no known putative functions; and the matched sequences can be sorted into 15 categories, including 1.2% with defense-related genes. The unique EST sequences from GT-C20 and Tifrunner were analyzed for their potential use for developing simple sequence repeat (SSR) markers. Among 3,976 GT-C20 and 2,912 Tifrunner unique se-

quences, 593 and 263 EST-SSRs were developed, respectively. The frequency of SSR-containing ESTs was 14.2% in GT-C20 and 8.41% in Tifrunner. On average, one SSR was found every 4.52 kb in GT-C20 and one every 5.89 kb in Tifrunner. Approximate 56.54% of the EST-derived SSRs were di-nucleotide, 35.59% were tri-nucleotide, and the remaining 7.87% was comprised of tetra-, penta- and hexa-nucleotide repeat motifs. Among the identified EST-SSRs, the two most abundant sequence motif types were AG and AT recovered both in GT-C20 and Tifrunner.

EST sequencing, the random sequencing of clones from cDNA libraries, is the most efficient approach for gaining an initial picture of genes in the peanut genome. Functional classification provides useful information about the different metabolic and regulatory pathways that may be associated with environmental stresses. The developed ESTs can be used to discover novel sequences or genes, to identify resistance-related genes and to detect the differences among alleles or markers between these resistant and susceptible peanut genotypes. Studies with ESTs have given rise to a new resource for the development of microsatellites in *A. hypogaea*. SSR-containing EST sequences (*He et al.*, 2005; Khanal *et al.*, 2008) have considerable potential for comparative mapping. Additionally, this large collection of cultivated peanut EST sequences will make it possible to construct microarrays for gene expression studies and for further characterization of host resistance mechanisms.

Analysis of Gene Expression. Macroarray (nylon-based) and microarray (glass slide-based) screening methods allow the simultaneous determination of expression levels of thousands of genes, making it possible to obtain a global view of the transcriptional state in a cell or tissue, and to associate genes with functions or specific physiological conditions. Recently, microarray technology has been demonstrated in a small scale pilot studies (Luo *et al.*, 2005b; Luo *et al.*, 2005c). EST-derived cDNA microarrays of 400 unigenes were probed under different conditions. Luo et al. (2005c) identified 25 ESTs that were potentially associated with drought stress or that responded to *A. parasiticus* challenge, and further study is warranted. Likewise, 56 up-regulated transcripts were identified and confirmed by real-time PCR upon infection with *Cercosporidium personatum* (Berk. et Curt.) Deighton. A long oligonucleotide microarray consisting of more gene-elements is under development (Guo *et al.*, 2007b). Luo *et al.* (2005c) reported differentially expressed genes using a cDNA microarray containing 768 spots of 384 cDNA unigenes. They compared two treatments (*A. parasiticus* infection and drought stress) in order to better understand the relationship of drought stress and aflatoxin contamination in peanut. This enabled them to screen for genes differentially expressed in different environments. The expression of some genes selected from the microarray analyses have been validated by using real-time PCR. Further evaluation for individual genes will be needed in more genotypes to confirm the possible association of the gene expression with the resistant phenotype.

Aspergillus flavus functional genomics. ESTs, whole genome sequencing, and microarray technologies are robust genomic tools that provide high throughput capabilities (Kim *et al.*, 2003; Yu *et al.*, 2004d). These should be available tools for identification of genes involved in aflatoxin production and for studying the regulatory mechanisms for their expression. An EST project has been completed using the wild type strain NRRL 3357 (ATCC# 20026). More than 26,110 cDNA clones from a normalized cDNA expression library were sequenced at TIGR. After assembly and annotation, 7,218 unique sequences were identified (Yu *et al.*, 2004d). These EST sequences have been released to the public at the NCBI GenBank Database. The *A. flavus* Gene Index was constructed at TIGR (http://www.tigr.org) which is currently maintained and curated by the Dana Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi). From this EST database, an additional four new transcripts (*hypB*, *hypC*, *hypD*, and *hypE*) were identified. Several categories of genes were identified that could potentially be involved, directly or indirectly, in aflatoxin production, such as in global regulation, signal transduction, pathogenicity, virulence, and fungal development. A genomic DNA amplicon microarray consisting of 5002 gene-elements was also constructed at TIGR. Profiling of genes involved in aflatoxin formation using microarrays performed at the Southern Regional Research Center of USDA-ARS and at TIGR identified hundreds of genes that are significantly up or down regulated (Kim *et al.*, 2003; OBrian *et al.*, 2003; Price *et al.*, 2006). Further study on their functional involvement in aflatoxin formation is underway.

Sequencing of the *A. flavus* whole genome has been completed. Primary assembly indicated that the *A. flavus* genome consists of eight chromosomes and the genome size is about 36.3 Mb. Annotation of the *A. flavus* genome sequence data, with the help of *A. flavus* EST database, demonstrated that there are over 12,000 functional genes in the *A. flavus* genome. Genes responsible for the biosynthesis of secondary metabolites such as aflatoxins

are those encoding polyketide synthases, nonribosomal peptide synthethases, cytochrome P450 monooxygenases, fatty acid synthases, carboxylases, dehydrogenases, reductases, oxidases, oxidoreductases, epoxide hydrolases, oxygenases, and methyltransferases (Yu *et al.*, 2004c). Primary annotation revealed that there exist more than two dozen PKSs, many NRPS, and numerous cytochrome P450 enzymes in the *A. flavus* genome. A whole genome microarray has been constructed at TIGR. Comparative genome hybridization (CGH) and functional genomics studies using these genomic resources are and will be performed for genome analysis. A proposed microarray is under construction in TIGR to include 8,402 peanut genes and more than 12,000 *A. flavus* genes and will be made available to collegeaues to study crop-pathogen interaction and for identifying genes involved in both fungal invasion and crop resistance.

Conclusions

To reduce or eliminate preharvest and postharvest aflatoxin contamination in crops is a serious challenge facing scientists today. In this presentation, we have reviewed the discovery of the aflatoxin pathway genes, gene clusters, and genetics and genomics progress made in peanut and *A. flavus*. The availability of a peanut-*Aspergillus* microarray will give scientists a valuable tool for studying crop-pathogen interaction and for identifying genes involved in both fungal invasion and crop resistance. The rapid progress in genomic research of both host plants and fungal pathogens should lead to a better understanding of the mechanisms of aflatoxin formation, pathogenicity of the fungus, and crop-fungus interactions. Developing novel strategies to control aflatoxin contamination is the ultimate goal for scientist. Efforts are also being made to understand fungus biology and the mechanism of aflatoxin biosynthesis using genomic tools. This will contribute greatly to achieve the goal of devising novel strategies to eliminate preharvest aflatoxin contamination resulting in a safer food and feed supply.

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