Application of Differential Display RT-PCR and EST/Microarray Technologies to the Analysis of Gene Expression in Response to Drought Stress and Elimination of Aflatoxin Contamination in Corn and Peanut

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ABSTRACT

Aflatoxin contamination in the field is known to be influenced by numerous factors. Drought and high temperatures are conducive to Aspergillus flavus infection and aflatoxin contamination. This article will review the application of new molecular tools to study host resistance to aflatoxin contamination.
biotic and abiotic factors affecting preharvest aflatoxin contamination in corn and peanut. We will also summarize recent studies conducted in our laboratories to evaluate the relationship of drought tolerance and aflatoxin contamination, and discuss the progress in using genetic engineering approaches to control preharvest aflatoxin contamination. The application of molecular tools, such as proteomics, DD-RT-PCR (differential display reverse transcription-polymerase chain reaction), expressed sequence tag (EST) and gene chip technology (macro/microarray) to study gene expression in response to drought stress, and genetic transformation, will be reviewed. We have used DD-RT-PCR to display genes expressed in peanut and corn grown under drought stress vs irrigation condition. A new program has been initiated to use EST/microarray technology to study the whole genome as influenced by drought stress in corn and peanut. We are also studying A. flavus ESTs to better understand the genetic control and regulation of toxin biosynthesis. Because of the complexity of the Aspergillus-plant (corn and peanut) interactions, better understanding of the genetic mechanisms of resistance will be needed using both conventional and molecular breeding for crop improvement and control of preharvest aflatoxin contamination. Genetic improvement of crop resistance to drought stress is one component and will provide a good perspective on the efficacy of control strategy through genetic improvement.

Key Words: Aflatoxin; Corn; Peanut; Molecular tools; Genomics.

INTRODUCTION

A major milestone in biological science was the sequencing of the human genome which provided fundamentally new ways of studying the human body (Lander et al., 2001; Subramanian et al., 2001; Venter et al., 2001). Likewise, to the complexity of factors involved in preharvest aflatoxin contamination of corn and peanut crops, genomics could tremendously impact our understanding of host resistance mechanisms, genetic improvement of resistance to insects, invasion by Aspergillus spp., and improvement in drought tolerance for corn and peanut. The complete decoding of the 3 billion-letter human genetic codes marked an important milestone in biomedical research, suggesting that the human genome may contain fewer than the expected 50-100,000 genes. No matter how many genes are encoded in the human genome, only a fraction of them are expressed at a given time and a cell within the human body. This is likewise true in the plant genome. Further information is needed on the dynamics of gene expression in plants and how their expression is controlled in the context of a cell as a function of time and space.
Corn and peanut become contaminated with aflatoxins when subjected to prolonged periods of heat and drought stress. To meet the challenge of prevention of preharvest aflatoxin contamination, it will be necessary to have a more detailed understanding of the expression and function of the genetic material of corn and peanut in response to biotic/abiotic stresses. Moreover, the genes that control functions leading to plant reactions to environmental stress and fungal infection must be identified. In this paper, we will discuss drought stress, aflatoxin contamination, molecular tools used to study the genetic response to drought stress, and genetic engineering approaches to control aflatoxin contamination. Research objectives include “prospecting” for useful plant genes that can be characterized and transferred into plants. Genomic research will help identify and understand the function and control of genes to improve the desired traits. Identifying and characterizing those genes that control significant biological processes and agronomic performance are crucial in the development of genetic approaches for control of preharvest aflatoxin contamination.

**AFLATOXIN CONTAMINATION AND ENVIRONMENTAL FACTORS**

*A. flavus* and *A. parasiticus* can colonize seed of several agricultural crops including corn and peanut. This can result in the contamination of the seed with toxic fungal metabolite aflatoxins. These fungi are ubiquitous, being found virtually everywhere in the world. They are soil-borne, but prefer to grow on high nutrient media (e.g. seeds). *A. flavus* appears to be the primary aflatoxin-producing fungus on these commodities, although *A. parasiticus* also occurs frequently on peanut. Both fungi produce a family of related aflatoxins; the one most commonly produced by *A. flavus* are B1 and B2, while *A. parasiticus* produces two additional aflatoxins, G1 and G2. Damage due to insects or environmental stress (drought) can enable the fungi to invade seeds where they thrive at high temperatures and extremely dry conditions, such as those frequently experienced in the Southern U.S. during the summer. With the implications to human and animal health worldwide, the development of crop lines with reduced aflatoxin contamination, when grown under heat-and drought-stressed conditions, would be a valuable development in alleviating this problem.

Plant stresses, depending on the type of stress and the type of plant, may include factors affecting plant survival, growth, and development of seed for harvest. Tremendous efforts have been made by scientists worldwide to study the mechanisms of the environmental factors that affect crop yield (Cochard, 2002; Horn et al., 1995; Kolesnichenko et al., 2002; Norton et al., 2002;
Aflatoxin contamination in preharvest corn is affected by many factors including drought, temperature, humidity, planting date, irrigation, tillage, insect damage, resistance or susceptibility (see Widstrom et al.’s review in this issue). Among these factors, resistance to insects, fungal infection/aflatoxin formation, and drought stress are genetic properties of the crop variety. We have spent years trying to decipher the genetic mechanisms of resistance or susceptibility to insect damage, fungal invasion, and tolerance to adverse environmental conditions in relation to the level of aflatoxin contamination in the preharvest corn (Butrón et al., 2001; Guo et al., 1997; 2000; 2001; 2002a; Lynch et al., 2002). Likewise, the action of aflatoxin biosynthesis has also been investigated extensively (Bhatnagar et al., 2001; Yu, 2002). Our goal is to reduce or eliminate aflatoxin contamination by screening for genetic traits that contribute to a greater resistance to insect damage and to A. flavus infection as well as tolerance to environmental stresses, such as drought. Although significant progress has been made (Guo et al., 2002b,c; Li et al., 2000), the problem is far from solved.

DROUGHT STRESS/TOLERANCE AND AFLATOXIN CONTAMINATION

Several agronomic practices can reduce preharvest aflatoxin contamination in corn and peanut. These include the use of pesticides, altered cultural practices (such as irrigation), and the use of resistant varieties. Prevention of preharvest aflatoxin contamination is long-term and the best approach. Holbrook et al. (1994) developed a large-scale field screening technique to directly measure field resistance to preharvest aflatoxin contamination in peanut. This technique uses subsurface irrigation in a desert environment to allow an extended period of drought stress in the pod zone while keeping the plant alive. In initial field tests conducted in the desert environment without subsurface irrigation, peanut plants died and their seeds rapidly dehydrated in the soil before contamination could occur. The use of a small amount of subsurface irrigation, to prolong plant viability during the drought stress, resulted in higher and more consistent contamination. Sanders et al. (1993) also observed high levels of aflatoxin contamination when peanuts in the pod zone were artificially stressed with heat and drought while keeping plants nonstressed by providing root zone irrigation.

Drought tolerance is a characteristic that has the potential to serve as an indirect selection tool for resistance to preharvest aflatoxin contamination. Holbrook et al. (2000) evaluated the resistance to preharvest aflatoxin contamination in a set of genotypes that had been documented as having...
varying levels of drought tolerance (Rucker et al., 1995) and determined the correlation of drought tolerance characteristics with aflatoxin contamination. Drought tolerance was very effective in reducing aflatoxin contamination in Tifton, GA and significant positive correlations were observed between aflatoxin contamination and leaf temperature, and between aflatoxin contamination and visual stress ratings. A significant negative correlation was also observed between aflatoxin contamination and yield under drought stressed conditions. Leaf temperature, visual stress ratings and yield are all less variable and cheaper to measure than aflatoxin contamination. These characteristics may be useful as indirect selection tools for reduced aflatoxin contamination.

A similar relationship between drought tolerance and reduced aflatoxin contamination has been observed in a drought tolerant peanut cultivar in Australia (Cruickshank et al., 2000). The cultivar, ‘Streeton’ has up to 40% lower aflatoxin contamination during years of high aflatoxin incidence in comparison to other commercial cultivars. Physiological studies have shown that the lower aflatoxin incidence is associated with better root water uptake resulting in better maintenance of plant during severe end-of-season drought.

In corn, research has been conducted to evaluate drought tolerance of corn germplasm in rain-out shields for three years. In rain-out shield screening for drought tolerant germplasm, we identified and selected several corn lines with excellent drought tolerance based on a ‘stay-green’ character (Li et al., 2000). Further, we evaluated lines selected from the GT-MAS:gk population which have drought tolerance (Guo et al., 2001, 2002a). Multi-location field evaluation of commercial hybrids for drought tolerance and aflatoxin production demonstrated that drought tolerant commercial lines, in general, had lower aflatoxin contamination under drought condition. This positive association of drought tolerance with lower aflatoxin production is encouraging and hybrids made from drought-tolerant lines may be tested further to evaluate for drought tolerance, yield, and aflatoxin contamination.

**GENETIC ENGINEERING AND PREVENTION OF AFLATOXIN FORMATION**

Genetic engineering approaches to control aflatoxin contamination in corn and peanut have focused on three main areas, resistance to the fungus, inhibition of aflatoxin production, and resistance to insects. The focus on resistance to insects is a result of the intimate relation between insect damage and aflatoxin contamination (Bowen and Mack, 1993; Cole et al., 1995; Lynch and Wilson, 1991; Widstrom, 1996). The _A. flavus_ and _A. parasiticus_ are able to survive and out-compete other fungi under hot, dry conditions.
These conditions are also conducive to the development of outbreak populations of certain insects such as the lesser cornstalk borer, *Elasmopalpus lignosellus* (Zeller), in the U.S. and termites (*Odontotermes* spp. and *Microtermes* spp.) in Africa that feed on peanut pods/kernels. Other insects such as the corn earworm, *Helicoverpa zea* (Boddie), and the fall armyworm, *Spodoptera frugiperda* (Smith) damage kernels as they feed in an ear of corn, providing a direct avenue for infection by *A. flavus* and *A. parasiticus*, and exacerbating the infection and contamination of ears with *A. flavus* and aflatoxin (McMillian et al., 1985). The highest levels of aflatoxin contamination in both corn and peanut are usually associated with insect damage (Lynch and Wilson, 1991; McMillian et al., 1985). Indeed, aflatoxin contamination in peanuts from insect-damaged pods is 30-60 times greater than that in undamaged pods. Thus, one approach to reduce aflatoxin contamination is to reduce insects damage.

### Insect Resistance in Transgenic Crops

The bacterium *Bacillus thuringiensis* (Bt) is ubiquitous and is unique in that it produces a protein (termed Cry proteins because of their crystalline nature) during sporulation that is toxic to certain insects. Over 240 insecticidal Cry proteins have been identified and sequenced (Crickmore et al., 2002). Each of these proteins is encoded by a single gene. Corn, cotton, potato and other crops have been genetically engineered to express one of these proteins for insect control. Transgenic Bt crops have been commercially available since the mid-1990's. In 2001, genetically engineered crops were grown on 130 million acres worldwide, up 19%, or almost 20 million acres from 2000. Of this total, 88.2 million acres were planted to transgenic crops in the U.S. in 2001 and included soybean, cotton, corn, and potato. Herbicide resistance accounted for 77% of the total acreage planted to transgenic crops, Bt crops for 15%, and stacked genes for herbicide and insect resistance accounted for 8%. Registration of Bt crop varieties was recently renewed for another seven years (EPA, 2001).

The primary target of Bt transgenic corn is the European corn borer, *Ostrinia nubilalis* (Hübner). This insect not only reduces the yield of corn grown in the Midwest by an estimated $1 billion annually (Ostlie et al., 1997), it is also associated with ear infections with *Fusarium* spp. and *A. flavus* (Dowd, 1998). Field studies in the Midwest with transgenic corn have consistently shown that hybrids that express the BT11 and MON810 events have a significantly lower incidence and severity of *Fusarium* ear rot and significantly lower concentrations of fumonisins than isogenic corn lines without the Bt gene (Dowd and Munkvold, 1999; Munkvold and Hellmich, 1999a; Munkvold et al., 1997). These events produce the Cry1Ab toxin in all
parts of the corn plant including silks and kernels. Events which do not express the Bt toxin in the kernels are less effective in reducing European corn borer damage and Fusarium ear rot (Munkvold and Hellmich, 1999a,b). Although present, the European corn borer is not the major pest of corn in the South and Southeast and the relationships between reduced insect damage in transgenic corn and aflatoxin in southern grown corn is not as clear as that for fumonisins in midwestern grown corn. There are two reasons for this difference. Commercially available Bt corn hybrids are not as effective against the corn earworm and fall armyworm as they are against the European corn borer. The environmental conditions conducive to the infection of corn with A. flavus and formation of aflatoxin are also much more severe, on average, in the South-Southeast than they are in the Midwest. In the South-Southeast, the corn earworm, fall armyworm and southwestern corn borer, Diatraea grandiosella Dyar, are the major lepidopterous pests of corn. Ear damage by the corn earworm and fall armyworm can be quite extensive and lead to increased levels of aflatoxin contamination under appropriate environmental conditions. In the Midwest, Bt corn reduced kernel infection by A. flavus and lowered aflatoxin concentrations in BT11 and MOB810 hybrids (Munkvold and Hellmich, 1999a). However, in the Southeast, no such relationship between insect resistance in Bt corn (YieldGard, BT11, MON810) and aflatoxin concentration could be established (Buntin et al., 2001). Although YieldGard corn did reduce the percentage of infested ears and the number of larvae in the ears, slower larval development did occur in ears of the resistant plants. Under heavy fall armyworm infestations, YieldGard corn did not reduce the percentage of infested ears, but did reduce the rate of larval development and the amount of kernel damage. Sims et al. (1996) reported reduced corn earworm feeding damage and reduced larval development on eight of 12 independently transformed lines of corn containing a Cry1Ab gene. Williams et al. (1997) reported significantly less fall armyworm leaf feeding damage, reduced survival and slower larval growth on Bt corn (BT11 event) and near immunity to feeding by the southwestern corn borer. These differences in the effect of Bt transgenic plants on corn insects is directly related to the susceptibility of the insects to the Cry1Ab protein; LC50 values ranged from 2.22 to 7.89 ng/cm² for the European corn borer (Marcón et al., 1999), considerably lower than the 70.3 to 221.3 ng/cm² for the corn earworm (Siegfried et al., 2000), and lower than the 0.36 to 10.22 μg/cm² for the fall armyworm (Lynch et al., 2002). Windham et al. (1999) reported that corn hybrid N6800Bt had a lower southwestern corn borer damage rating and about a 50% reduction in aflatoxin concentration than N6800 when they were artificially infested with A. flavus spores and southwestern corn borer larvae. Research conducted in South Texas with Cry2Ab, Cry1Ab, and
non-Bt isolines showed a positive correlation between the number of fall armyworm larvae per ear with ear insect injury rating at harvest and with aflatoxin content.

Peanut has also been genetically engineered to contain the Cry1Ac gene which confers resistance to feeding by the lesser cornstalk borer (LCB) (Singsit et al., 1996). This gene also confers resistance to the corn earworm and velvethenan caterpillar, but not to the fall armyworm (Lynch, unpublished data). The transgenic peanut is primarily aimed at control of the LCB since this insect is intimately associated with aflatoxin contamination. Only external scarification of peanut pods by LCB is needed to enhance infection of peanut kernels with *A. flavus* (Lynch and Wilson, 1991). Field test have been conducted to evaluate the efficacy of the Bt peanut in reducing lesser damage and aflatoxin contamination under drought-stress (Ozias-Akins et al., 2002). In 2000, there was no difference in the percentage of pods showing scarification due to LCB feeding on transgenic versus nontransgenic peanut pods. The aflatoxin concentration in scarified, transgenic peanut pods was significantly lower than in scarified, nontransgenic pods. The experiment was repeated in 2001, but aflatoxin analyses have not yet been reported.

**Fungal Resistance and Inhibition of Toxin Production in Transgenic Crops**

Progress has been made in the development of crop resistance to aflatoxin through genetic engineering. Research in Peggy Ozias-Akins’s lab, Tifton/Georgia, on aflatoxin reduction is using a three-tiered approach, i.e., resistance to insect damage using a Bt gene, resistance to fungal growth using the tomato anionic peroxidase gene (*tap1*) or an antifungal peptide D4E1, and inhibition of the aflatoxin biosynthetic pathway using the lipoxygenase gene *lox1* (Ozias-Akins et al., 2002).

Art Weissinger, North Carolina State University, is testing transgenic peanut containing synthetic Peptidyl Membrane Interactive Molecules (MIM) developed by Demegen, Inc. (Weissinger et al., 2002). His group developed transgenic peanut encoding D5C, an alpha-helical peptide that is highly active against *A. flavus*. In a test of 15 lines that carried the D5C transgene, none contained D5C mRNA and the peptide was not detectable using western blots. Furthermore, D5C transgenic peanut plants produced significantly fewer pods than control plants. Subsequent tests indicated that D5C was phytotoxic to peanut at levels required to kill *A. flavus*.

Demegen, Inc. has also developed other antimicrobial peptides that may warrant testing for *A. flavus* inhibition in transgenic plants (Zorner, 2002). D4E1 has emerged as one of the most active peptides against several species
of bacteria and fungi. Activity against *A. flavus* is also present, but at a lower level than that for other pathogens. Research is either planned or underway to integrate this gene in several crop species.

Charles Woloshuk and colleagues (Woloshuk et al., 2002) at Purdue University are investigating the possibility that transgenic corn containing an α-amylase inhibitor will inhibit *A. flavus* infection. Their previous research had indicated that α-amylase produced by *A. flavus* may facilitate colonization and aflatoxin production in corn kernels. They also found that the α-amylase inhibitor from *Lablab purpureus* inhibits α-amylase production in several fungi, but not those from animals or plants. It also inhibits conidia germination and hypha growth of *A. flavus*.

**MOLECULAR TOOLS TO STUDY GENE EXPRESSION**

Plants tolerate environmental stress because of numerous physiological adaptations, which have been attributed to the function of various genes (Hasegawa et al., 2000). For example, in *Arabidopsis thaliana*, transcription of *RD* (Responsive to Dehydration) (Yamaguchi-Shinozaki et al., 1992) and *COR* (Cold Responsive) (Hajela et al., 1990) genes are activated by hyperosmotic or cold stress. The plant hormone abscisic acid (ABA) activates transcription of some *RD* and *COR* genes while *PLD* (phospholipase D) gene(s) transcription is activated by drought stress (Guo et al., 2002d; Maarouf EI et al., 1999; Sang et al., 2001; Xu et al., 2001). The genetic control of these traits for tolerance to abiotic stresses is complex. Drought tolerance, for example, may be determined by many genetic factors. Quite a number of plant features contribute to the drought tolerance and include both physiological and biological elements such as waxy skin layer on plant surfaces, size and number of stomata, extensiveness of root system, respiration rate, and nutritional status. These factors are genetically controlled. The isolation of one of these genes for a specific function is not easy, and the determination of all of these genes is almost impossible using traditional genetics and cloning techniques. The “one-gene-at-a-time” approach for analyzing gene expression is wholly inadequate. The development of Differential Display Reverse Transcription PCR (DD-RT-PRC) and Expressed Sequence Tag (EST) methods provided new tools for isolating more genes. It is now possible to locate multiple genes that enable plants to withstand biotic and abiotic stresses. There are several major tools used for gene expression analysis, and four of which will be discussed briefly herein. These include DD-RT-PCR, EST/microarray, proteomics, and transgenes/genetic transformation.
Expressed Gene Differential Display

DD-RT-PCR was first described by Liang and Pardee (Liang and Pardee, 1992), and is powerful and cost-effective method to detect variations in mRNA expression. DD-RT-PCR technology was developed to identify and isolate selectively those genes that are expressed in a temporally and spatially regulated manner in different tissues and organs. Differential display or DD-RT-PCR uses a limited number of short arbitrary primers in combination with the anchored oligo-dT primers to systematically amplify and visualize a certain proportion of the expressed genes (mRNA) in an organism or tissue. In cowpea, sunflower and tomato, cDNA libraries constructed from drought induced mRNA have been used to characterize genes associated with drought response. Differential display fragments can then be used as probes to screen the cDNA library to get the full-length drought-inducible genes. Using DD-RT-PCR, we have identified mRNA transcripts that are up-or down-regulated due to drought stress (Figure 1). In addition, differences in the composition of selected metabolite levels between the drought-tolerant and susceptible genotypes following drought stress may be determined.

![Image of cDNA fragments from DD-RT-PCR](image)

**Figure 1.** Verification of the cDNA fragments from DD-RT-PCR by reverse northern blot analysis, hybridized by probes of mRNA of no stress (A), mRNA of 2 days of drought stress (B), and mRNA of 4 days of drought stress (C) in the greenhouse. From panel A to C, signals that become stronger means up-regulated transcripts, and those that become weaker means down-regulated transcripts as induced by drought stress in corn.
To investigate gene expression pattern in response to induced drought stress in plant we have used differential display RT-PCR to differentiate gene expression in drought-susceptible and drought-tolerant corn and peanut (Cao et al., 2002; Xu, et al., 2002). Polymorphic mRNA transcripts have been identified. Some cDNA fragments that were up-or down-regulated by induced drought stress (Figures 1 and 2) have been cloned and sequenced. Using this method, we identified a novel PLD gene (Guo et al., 2002d; Xu et al., 2001). This gene encodes a putative phospholipase D, a primary enzyme responsible for the drought-induced degradation of membrane phospholipids in plants. The PLD gene expression under drought stress has been studied in the greenhouse using two peanut lines, Tifton 8 (drought tolerant) and Georgia Green (drought sensitive). Northern analyses showed that the PLD gene expression was induced sooner by drought stress in

**Figure 2.** Differential display using one-base anchored oligo-dT primers. Three RNA samples from the peanut cultivar COAN under drought stress 0, 5, and 8 days (from left to right) were compared by differential display using H-T11G (AAGCT11G) in combinations with six arbitrary 13-mers: H-AP8, H-AP9, H-AP10, H-AP11, H-AP12, and H-AP13.
Georgia Green than in Tifton 8 (Guo et al., 2002d). After the PLD gene in peanut is completely characterized we will attempt gene silencing using genetic transformation to suppress PLD gene expression and induce drought tolerance. The limitation of DD-RT-PCR is that it can be used to identify only those genes that are amplified by a few arbitrary primers within hundreds and perhaps thousands of expressed genes. In order to identify and isolate all of the expressed genes, expressed sequence tag (EST) offers the best solution.

**Expressed Sequence Tag (EST) and Macro/Micro-array**

Expressed sequence tag (EST) is to sequence out cDNA (DNA copies of RNAs) clones in an expressed cDNA library and identify all of the unique sequences (genes) to study their functions. Generating sequences from cDNA fragments can be used to discover new genes and to assess their expression levels in the representative tissue (Ewing et al., 1999; Mekhedov et al., 2000). The level of an mRNA species in a specific tissue is reflected by the frequency of occurrence of its corresponding EST (expressed sequence tag) in a cDNA library. EST technologies are attractive because they do not rely on established sequence data from the organism under study, and they also fit well with labs already equipped to carry out high-throughput DNA sequencing (Adams et al., 1991). Auxiliary techniques, available to reduce the amount of sequencing, are subtraction hybridization (Sargent, 1987), representational difference analysis (RDA) (Hubank and Schatz, 1994) and suppression subtractive hybridization (SSH) (Diatchenko et al., 1996). The identified cDNA sequences, either fragments or homologous oligoes, can be used to fabricate a DNA microarray for functional study.

DNA microarrays or a gene chip typically consist of thousands of immobilized DNA sequences present on a miniaturized surface the size of a microscope slide. Arrays are used to analyze a sample for the presence of gene variations or mutations (genotyping), or for patterns of gene expression (Aharoni and Vorst, 2002). Microarrays are distinguished from macroarrays in that the DNA spot size is smaller, allowing for the presence of thousands of DNA sequences instead of the hundreds present on macroarrays. The samples of cDNAs are then prepared for expression analysis. The DNA samples are tagged with a radioactive or fluorescent label and applied to the array. Single stranded DNA will bind to a complementary strand of DNA. At positions on the array where the immobilized DNA recognizes a complementary DNA in the sample, binding or hybridization occurs. The labeled sample DNA marks the exact positions on the array where binding occurs, allowing automatic detection. The output consists of a list of hybridization events, indicating the
presence or the relative abundance of specific DNA sequences that are present in the sample, thus indicating how much a gene is turned on or off.

The mode of action, metabolism and biosynthesis of aflatoxins have been extensively studied in the last decade (Bhatnagar et al., 2001; Chang et al., 1993, 1995; Cleveland and Bhatnagar, 1991; Cleveland et al., 1997; Payne and Brown, 1998; Yu, 2002; Yu et al., 1995). For a better understanding of the genetic control and regulation of toxin production by A. flavus and the mechanism of toxin production in response to environmental conditions such as drought stress and temperature, fungal EST and Macro/Micro-array program are being carried out at the USDA/ARS Southern Regional Research Center in New Orleans, LA (Yu et al., 2002) and USDA-ARS Labs at Tifton, GA. Currently about 8,000 expressed unique genes have been identified from A. flavus EST program. A microarray containing these identified A. flavus genes will be produced to study gene expression and regulation and to identify factors involved in the plant-microbe interaction. The A. flavus EST program will help to identify genes that could be used to inhibit fungal growth or aflatoxin formation by the fungi. The EST and macro/micro-array projects in corn and peanut have been initiated in Tifton, GA, to study the gene expression profile of drought-response based on suppression subtractive hybridization (Luo et al., 2002). The preliminary ESTs show that some plant defense genes have been identified, such as some plant defense genes identified via EST as gene of small cysteine-rich antifungal protein, Ca2+/H+-exchanging protein, peroxidase, 14-3-3-like protein, glutathione S-transferase, and trypsin inhibitor.

Proteomics

Proteomics is to identify and to study the proteins produced by a cell type and an organism (Wilkins et al., 1996). The term proteome refers to all the proteins expressed by a genome, and thus proteomics involves the identification of proteins in the organism and the determination of their role in physiological and biochemical functions. To study the proteins directly and to identify their genes is another effective method for gene expression analysis. There are tremendous advantage and practical applications in pharmaceutical industry for identifying drug receptors and inhibitory factors. The ca. 30,000 genes defined by the Human Genome Project translate into 300,000 to 1 million proteins when alternate splicing and post-translation modifications are considered. While a genome remains unchanged to a large extent, the proteins in any particular tissue change dramatically as genes are turned on and off in response to its environment. As sequencing of the entire genomes of many prokaryotes and eucaryotes has been completed, the technology is necessary to separate proteins from each other and to study proteins. The
main way this has been achieved is through 1-D or 2-D polyacrylamide gel electrophoresis (2-D PAGE) (Chen et al., 1998; Riccardi et al., 1998; Santoni et al., 1994). Using 2-D PAGE, separation of several thousands of different proteins can be achieved in one gel.

As a reflection of the dynamic nature of the proteome, some researchers prefer to use the term “functional proteome” to describe all the proteins produced by a specific cell in a single time frame (Gavin et al., 2002). Riccardi et al. (1998) reported that protein profiles change in response to water deficit in corn. The induced changes of protein profile in leaf tissue of three-week-old plants in response to drought or water deficit were studied by 2-D electrophoresis. Seventy-eight proteins out of a total of 413 showed a significant quantitative variation (increase or decrease), with 38 of them exhibiting a different expression in different genotypes. Eleven proteins increased by a factor of 1.3 to 5 in stressed plants and 8 proteins were detected only in stressed plants. Some proteins are already known to be involved in the response to water stress (responsive to ABA). Most cellular processes are carried out by multiprotein complexes. Through proteomics, new plant resistance genes could be identified and DNA markers could be derived from these proteins that could be used as markers for breeding selection or genetic transformation, such as antifungal protein(s) identified from corn kernels (Chen et al., 1999a,b; 2002).

Transgenes/Genetic Transformation

Molecular techniques allowed the identification, isolation, and characterization of genes that encode specific protein products controlling plant development (see above session of Molecular Tools). Genetic engineering is the next step to modify the genome of plants to contain and express foreign genes or modify native or endogenous genes to alter/enhance/suppress the traits of a plant in a specific manner (see above session of Genetic Engineering). Such foreign and modified genes are referred to as transgenes.

Expression Vector

Plant transformation involves the construction of an expression vector which will function in plant cells. Such a vector comprises DNA including a gene under control of or linked to a regulatory element, such as a promoter. Expression vectors include at least one genetic marker, linked to a regulatory element (a promoter) that allows transformed cells containing the marker to be either recovered by negative selection, such as inhibiting growth of cells that do not contain the selectable marker gene, or by positive selection by screening for the product encoded by the genetic marker.
One commonly used selectable marker gene for plant transformation is the neomycin phosphotransferase II (nptII) gene, which when placed under the control of plant regulatory signals confers resistance to kanamycin (Fraley et al., 1983). Another commonly used selectable marker gene is the hygromycin phosphotransferase gene which confers resistance to the antibiotic hygromycin (Elzen et al., 1985). Other selectable marker genes that confer resistance to antibiotics include gentamycin acetyl transferase (Hayford et al., 1988), streptomycin phosphotransferase, aminoglycoside-3’-adenyl transferase (Svab et al., 1990), and the bleomycin resistance (Hille et al., 1986). Selectable marker genes may also confer resistance to herbicides such as glyphosate, glufosinate or broxynil (Stalker et al., 1988). GUS (beta-glucuronidase) and luciferase represent another class of marker genes for plant transformation and require screening of presumptively transformed plant cells rather than direct genetic selection of transformed cells for resistance to an antibiotic (Jefferson, 1989; Koncz et al., 1987). More recently, a gene encoding Green Fluorescent Protein (GFP) has been utilized as a marker for gene expression (Sheen et al., 1995).

Promoter

Genes included in expression vectors must be driven by a nucleotide sequence comprising a regulatory element, a promoter. Several types of promoters are now well known in plant transformation. A plant promoter is capable of initiating transcription in plant cells. Promoters under developmental control include promoters that preferentially initiate transcription in certain tissues, such as leaves, roots, or seeds. Such promoters are referred to as tissue-preferred or tissue-specific, such as the phaseolin gene (Murai et al., 1983) and light-induced promoter (Timko et al., 1985). An inducible promoter is one that is under environmental control. Tissue-specific, tissue-preferred, and inducible promoters constitute the class of non-constitutive promoters. A constitutive promoter is one that is active under most environmental conditions, such as the 35S promoter from CaMV (Odell et al., 1988), rice actin (McElroy et al., 1990), or corn ubiquitin promoter (Christensen and Quail, 1989; Christensen et al., 1992). With an inducible promoter the rate of transcription increases in response to an inducing agent. A constitutive promoter is linked to a gene for expression or to a nucleotide sequence encoding a signal sequence which is linked to a gene for expression.

Methods for Transformation

Numerous methods for plant transformation have been developed, including biological and physical. One method for introducing an expression
vector into plants is based on the natural transformation system of *Agrobacterium* (Horsch et al., 1986). *A. tumefaciens* and *A. rhizogenes* are plant pathogenic soil bacteria which genetically transform plant cells. The Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, carry genes responsible for genetic transformation of the plant (Kado, 1991). Another method is referred to as direct gene transfer, microprojectile-mediated transformation wherein DNA is carried on the surface of microprojectiles. The expression vector is introduced into plant tissues with a bolistic device (gene gun) that accelerates the microprojectiles to penetrate plant cell walls and membranes (Klein et al., 1987; Ye et al., 1990). In maize, several target tissues can be bombarded with DNA-coated microprojectiles in order to produce transgenic plants, including callus, immature embryos, and meristematic tissue.

**SUMMARY AND PROSPECTS**

In traditional genetics, a genetic trait of interest is targeted and then research to identify the gene that caused, or coded, for that trait is studied for several years. In the new paradigm of genomics, however, we take the opposite approach in that we map out all of the genes of an organism first, and then work to figure out their functions later. The first step is known as structural genomics, and the second step is called functional genomics (Fernandes et al., 2002; Kantety et al., 2002; Woo et al., 2001). The information obtained from genomics can be applied to the development of commercial crops for high yield with no or low aflatoxin contamination through genetic engineering (Estruch et al., 1997). A practical example of genetic engineering is the genetically improved (Bt) corn that protects against insects, and genetically engineered cotton that protects against the bollworm (Dowd, 2001). These innovative products not only increase crop yields but also dramatically reduce the cost for insecticides and environmental contamination.

Genes are key components in manipulating plants and animals with more desirable and economic/agronomic traits. To reduce yield losses and to study genetic factors involved with plant stresses, The National Science Foundation granted $8.4 million to the ‘‘Functional Genomics of Plant Stress Tolerance Project’’ that is being conducted by scientists at Purdue University, the University of Arizona and Oklahoma State University. The corn genomics project is expected to define and to discover the full suite of genes in corn as a route to new fundamental discoveries in plant biology, and to find immediate application in basic research for use in the commercial arena. Corn genomics promises development of new commercial corn varieties that are able to withstand environmental stresses such as drought and heat as well as
resistance to insects and plant pathogens. A peanut genomics project will be launched soon.

EST/microarray technology can be used to detect a whole set of genes transcribed under specific conditions and to study the biological functions of genes of interest. EST and microarray technology provides a tool for rapid identification of genes of interest expressed by plants under fungal challenge or environmental stress conditions. They can help in our understanding of the biological functions, coordination of gene expression in response to internal and external factors, mechanisms of plant-fungal interaction, plant-environmental interaction, fungal pathogenicity anti-fungal properties, and the mechanism of genetic regulation in relation to plant tolerance to biotic and abiotic stresses. This technology allows us to study a complete set of genes simultaneously for screening and identifying the most important host-resistance and stress-tolerance genes among hundreds or even thousands of relevant genes that could be used in genetic engineering for developing commercial crops. Our A. flavus/corn/peanut EST/Microarray programs are expected to provide valuable information on prevention and elimination of aflatoxin contamination in these crops.

In the effort to prevent preharvest aflatoxin contamination in corn and peanut, knowledge obtained from A. flavus EST/Microarray program can be integrated into the corn genomics program for identifying host-resistance and stress tolerance genes, and, at the same time, identify biological targets for anti-fungal growth or inhibition of toxin formation by fungi. Corn/peanut genomics combined with the A. flavus EST/Microarray project will give us more specific genetic information to target critical regulatory component(s) and gene(s) involved in aflatoxin biosynthesis. These gene(s) can be engineered into commercial crops to solve the problems of preharvest aflatoxin contamination of food and feed.

REFERENCES


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Mekhedov, S., Martínez de Ilárduya, O., Ohlrogge, J. (2000). Toward a


Siegfried, B. D., Spencer, T., Nearman, J. (2000). Baseline susceptibility of the corn earworm (Lepidoptera: Noctuidae) to the Cry1Ab toxin from *Bacillus thuringiensis*. *J. Econ. Entomol.* 93:1265–1268.


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