Identifying Aflatoxin Resistance-related Proteins/Genes through Proteomics and RNAi Gene Silencing[1]

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ABSTRACT

Aflatoxins are carcinogenic secondary metabolites produced mainly by *Aspergillus flavus* Link ex. Fries, and *A. prarasiticus* Speare during infection of susceptible crops, such as maize, cottonseed, peanuts and tree nuts. This paper will review research efforts in identifying aflatoxin resistance-related proteins/genes in maize. Similar strategies may be useful in peanut. For maize, although genotypes resistant to *A. flavus* infection or aflatoxin production have been identified, the incorporation of resistance into commercial lines has been slow due to the lack of selectable markers and poor understanding of host resistance mechanisms. Recently, resistance-associated proteins (RAPs) were identified through proteomic comparison of constitutive protein profiles between resistant and susceptible maize genotypes. These proteins belong to three major groups based on their peptide sequence homologies: storage proteins, stress-related proteins, and antifungal proteins. Preliminary characterization of some of these RAPs suggest that they play a direct role in host resistance, such as pathogenesis-related protein 10 (PR10), or an indirect role, such as glyoxalase I (GLX I), through enhancing the host stress tolerance. To verify whether these RAPs play a role in host resistance, RNA interference (RNAi) gene silencing technique was used to silence the expression of these genes in maize. RNAi vectors (*glx I* RNAi and *pr10* RNAi) were constructed using Gateway technology, and then transformed into immature maize embryos using both bombardment and *Agrobacterium* infection. The extent of gene silencing in transgenic callus tissues ranged from 20% to over 99%. The RNAi silenced transgenic maize seeds have also been obtained from plants regenerated from *Agrobacterium* transformed callus lines. Kernel screen assay of the transgenic maize kernels demonstrated a significant increase in susceptibility to *A. flavus* colonization and aflatoxin production in some of the silenced transgenic lines compared with non-silenced control kernels, suggesting the direct involvement of these two proteins in aflatoxin resistance in maize.

Key Words: maize, kernel protein, resistance, aflatoxin.

Aflatoxins are secondary metabolites produced mainly by *Aspergillus flavus* Link ex. Fries, and *A. parasiticus* Speare during infection of susceptible crops, such as maize, cottonseed, peanuts and tree nuts (Diener *et al.*, 1987, Payne, 1998). The dominant aflatoxin produced by these fungi is aflatoxin B_1, which is the most potent naturally occurring carcinogenic substance known (Squire, 1981). Aflatoxin contamination not only reduces the value of grain as an animal feed and as an export commodity (Nichols, 1983), but has also been linked to increased mortality in farm animals (Smith and Moss, 1985), and increased incidence of liver cancer in humans (Hsieh, 1989). In 2004, a severe outbreak of aflatoxicosis was reported in Kenya due to consumption of highly contaminated maize (as high as 8000 ppb), and 125 people died as a result (Azziz-Baumgartner *et al.*, 2005). Currently, over 50 countries have established regulations regarding the permissible level of aflatoxins in food and feed. In the U. S., the Food and Drug Administration prohibits interstate commerce of food and feed contaminated with levels of aflatoxin higher than 20 parts per billion (ppb, equivalent to 20 ng/g).

Infection of susceptible crops both pre-harvest and post-harvest by *A. flavus* and subsequent contamination with aflatoxins is a recurrent problem in the southern United States. Drought and hot weather conditions have been associated with increased aflatoxin contamination in the field (Payne, 1998). In support of this role for drought and high temperatures, lower soil temperature was found to reduce aflatoxin contamination in peanut (Hill *et al.*, 1983), while increased aflatoxin contamination has been observed in drought-treated peanuts with increased soil temperatures (Cole *et al.*, 1985). Dorner *et al.* (1989) also

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concluded that a higher soil temperature favors *A. flavus* growth and aflatoxin production, and a study on the effect of drought on peanut resistance to *A. flavus* by Wotton and Strange (1987) found that fungal colonization was inversely related to water supply, as was aflatoxin production. Holbrook *et al.* (2000) evaluated resistance to preharvest aflatoxin contamination in a set of peanut genotypes that had been documented as having varying levels of drought tolerance, and concluded that tolerant genotypes also had greatly reduced aflatoxin contamination. In spite of these findings, measures such as good cultural practices, harvesting at the optimum stage of maturity, rapid drying after harvesting, etc. (Lisker and Lillehoj, 1991), for controlling aflatoxin contamination in the field are not always available or cost-effective for growers (Payne *et al.*, 1986).

The approach to enhance host resistance through conventional breeding and/or genetic engineering has gained renewed attention following the discovery of natural resistance to *A. flavus* infection and aflatoxin production in maize (Gardner *et al.*, 1987; King and Scott, 1982; Widstrom *et al.*, 1987; Scott and Zummo, 1988; Campbell and White, 1995, Brown *et al.*, 1995; Brown *et al.*, 1999). Promising sources of resistant peanut germplasm have also been identified from a core germplasm collection, although resistance screening has proven to be a difficult task with this crop (Holbrook *et al.*, 2008). These peanut lines, however, have less than acceptable agronomic characteristics, and are thus being hybridized with commercially acceptable lines. One approach to enhance host resistance is to pyramid insect and fungal resistance genes into commercial germplasm to reduce fungal infection caused by insect damages (Guo *et al.*, 2000). Unfortunately, the progress toward developing resistant genotypes has been slow, mainly due to the lack of precise physical or chemical factors known to be associated with resistance (Widstrom *et al.*, 1984; Widstrom and Zuber, 1983, Brown *et al.*, 1999) and poor understanding of host resistance mechanisms. Without this information, breeders instead, have to rely on artificial inoculation in the field and to screen a large number of crosses in order to identify a resistant variety, which is very expensive, labor intensive, and time consuming.

For this review, we will mainly summarize research efforts in the following three areas: (1) the preliminary studies on host and fungus interaction, from which it was found that maize constitutive kernel proteins (antifungal and some stress related proteins) play as important a role as inducible kernel proteins in host resistance; (2) research efforts on identifying constitutive differences in the domestic maize lines differing in aflatoxin resistance; and (3) the characterization of these proteins to verify their importance to host resistance. At the end of this review, we will briefly describe a most recent effort where near isogenic lines that differ in aflatoxin resistance were used to enhance the identification of resistance related proteins/genes, especially regulatory proteins produced in low amounts. Since most of the molecular studies on host resistance to aflatoxin were conducted in maize, this review will mainly use studies on maize as examples. Similar strategies are applicable to identification of aflatoxin-resistance related genes in peanut.

Kernel Proteins and Host Resistance.

The development of a laboratory kernel screening assay (KSA) by Brown *et al.* (1995) enabled us to verify maize kernel resistance under laboratory (controlled) conditions in a short time, which accelerated our understanding of host resistance mechanisms. Using this assay, Brown *et al.* (1993) demonstrated the existence of a subpericarp resistance in maize kernels and that the expression of this resistance requires a viable embryo, indicating that biochemical factors may play a major role in resistance. Guo *et al.* (1997) found preimbibition significantly increased aflatoxin resistance of susceptible maize genotypes. Further investigation revealed that susceptible genotypes were able to induce the same antifungal proteins as resistant lines upon fungal infection, but at a slower pace or at lower levels compared to resistant maize lines (Guo *et al.*, 1997, Chen *et al.*, 2001). These studies also suggested that susceptible lines have the ability to induce an active defense mechanism if they were given enough time to imbibe water and induce antifungal proteins. Huang *et al.* (1997) identified two kernel proteins from a resistant corn inbred line (Tex6), which may contribute to resistance to aflatoxin contamination. When a commercial maize hybrid was inoculated with toxigenic and atoxigenic strains of *A. flavus* at milk stage, one chitinase and one β-1,3-glucanase isoform were detected in maturing infected kernels, while another isoform was detected in maturing uninfected kernels (Ji *et al.*, 2000). A study by Lozovaya *et al.* (1998) reported that the presence of *A. flavus* caused an increase in β-1,3-glucanase activity in callus tissues of a resistant genotype, but not in a susceptible one. A more rapid and stronger induction of the pathogenesis-related (*pr1* and *pr5*) genes in maize leaves has also been observed in a resistant reaction when compared to a susceptible reaction upon pathogen infection (Morris *et al.*, 1998). In another investigation, a 14 kDa trypsin inhibitor (TI) protein was found constitutively

produced at high levels in resistant lines but at low levels or was missing in susceptible ones (Chen *et al*., 1998). This protein demonstrated antifungal activity against *A. flavus* and several other pathogenic fungi (Chen *et al*., 1999), possibly through inhibition of fungal α-amylase activity and production. This could limit the availability of simple sugars needed for fungal growth and aflatoxin production (Woloshuk *et al*., 1997). All of these earlier studies indicated an important role for kernel proteins in disease resistance. Further investigation found that both constitutive and inducible proteins are required for kernel resistance to *A. flavus* infection and aflatoxin production (Chen *et al*., 2001). It also showed that one major difference between resistant and susceptible genotypes is that resistant lines have higher constitutive levels of antifungal proteins, stress-related proteins, and highly-hydrophilic storage proteins compared to susceptible lines. Therefore, constitutively the produced proteins have been the focus of a number of important investigations.

Identifying Host Resistance-Associated Proteins in Maize Using Proteomics.

A proteomic approach was employed to increase protein resolution and detection sensitivity and, thus, enhance the ability to identify additional resistance-associated proteins (RAPs) (Chen *et al*., 2002). Endosperm and embryo proteins from several resistant and susceptible genotypes have been compared using large format 2-D gel electrophoresis. Due to genetic background differences among the genotypes, a comparative composite gel approach was used to identify proteins that are either unique (qualitative) or upregulated (quantitative) in resistant lines compared to susceptible ones for the purpose of homogenizing background differences. A high threshold (5 fold differences for embryo proteins) was used to identify upregulated proteins that are more likely to be involved in host resistance. Over a dozen protein spots, either unique or upregulated in resistant lines, have been identified, recovered from preparative 2-D gels and sequenced using ESI-MS/MS (Chen *et al*., 2002). These proteins can be grouped into three categories based on their peptide sequence homology: (1) storage proteins, such as globulin proteins (GLB1, GLB2), and late embryogenesis abundant proteins (LEA3, LEA14); (2) stress-responsive proteins, such as aldose reductase (ALD), a glyoxalase I protein (GLX I), and a 16.9 kDa heat shock protein; and (3) antifungal proteins (TI and PR10). These data are in agreement with the evidence from genetic studies that aflatoxin resistance is a quantitative multigene-controlled trait, which is also regulated by environmental factors (Davis and Williams, 1999; Paul *et al*., 2003; Brooks *et al*., 2005). These findings are also supported by field observations that aflatoxin production by *A. flavus* is associated with water and heat stresses (Payne, 1998). The purpose of the proteomic comparison was to identify proteins that related to host resistance. The repeated identification of storage proteins, stress-related proteins in addition to antifungal proteins indicated that kernel resistance may not only require the presence of high levels of antifungal proteins, but also requires the presence of high levels of stress-related proteins and highly hydrophilic storage proteins. Therefore, possession of unique or higher levels of hydrophilic storage or stress-related proteins, such as the aforementioned, may put resistant lines in an advantageous position over susceptible genotypes under stress, in the ability to delay fungal invasion, and induce an active defense response immediately upon fungal infection before kernels are overtaken by the fungus.

Functions of Resistance-Associated Proteins.

A literature review of the RAPs identified above indicates that storage and stress-related proteins may play important roles in enhancing stress tolerance of host plants. The expression of storage protein GLB1 and LEA3 has been reported to be stress-responsive and ABA-dependant (Thomann *et al*., 1992). Transgenic rice overexpressing a barley LEA3 protein HVA1 showed significantly increased tolerance to water deficit and salinity (Xu *et al*., 1996). The role of GLX I in stress-tolerance was first highlighted in an earlier study using transgenic tobacco plants overexpressing a *Brassica juncea* glyoxalase I (Veena *et al*., 1999).

The above proteome investigation indicated that GLX I may play an important role in maize resistance to aflatoxin accumulation by reducing the levels of methylglyoxal, which was shown to induce aflatoxin production *in vitro* (Chen *et al*., 2004b). PER1, a 1-cys peroxiredoxin antioxidant identified in our comparison (Chen *et al*. 2007), was demonstrated to be an abundant peroxidase, and may play a role in the removal of reactive oxygen species. The PER1 protein overexpressed in *Escherichia coli* demonstrated peroxidase activity *in vitro*. It is possibly involved in removing reactive oxygen species produced when maize is under stress conditions (Chen *et al*., 2007).

Another RAP that has been characterized further is the PR10. It showed high homology to pathogenesis-related protein 10 from rice (85.6% identical) and sorghum (81.4% identical), It also shares 51.9% identity to intracellular pathogenesis-related proteins from lily (AAF21625) and asparagus (CAA10720), and low homology to a RNase from ginseng. (Chen *et al*., 2006). The PR10

overexpressed in *E. coli* exhibited ribonucleolytic and antifungal activities. In addition, an increase in the antifungal activity against *A. flavus* growth was observed in the leaf extracts of transgenic tobacco plants expressing maize *pr10* gene compared to the control leaf extract (Chen *et al*., 2006). This evidence suggests that PR10 plays a role in kernel resistance by inhibiting fungal growth of *A. flavus*. Further, its expression during kernel development was induced in the resistant line GT-MAS:gk, but not in susceptible Mo17 in response to fungal inoculation (Chen *et al*., 2006). Evidence supporting a role for *pr10* in host resistance is also accumulating in other plants. A barley *pr10* gene was found to be specifically induced in the resistant cultivars upon infection by *Rhynchosporium secalis*, but not in near-isogenic susceptible plants (Steiner-Lange *et al*., 2003). In cowpea, a *pr10* homolog was specifically up-regulated in resistant epidermal cells inoculated with the rust fungus *Uromyces vignae* Barclay (Mould *et al*., 2003). A *pr10* transcript was also induced in rice during infection by *Magnaporthe grisea* (McGee *et al*., 2001).

Verification of RAP in Aflatoxin Resistance Using RNA Gene Silencing.

To directly demonstrate whether any RAP protein plays a key role in host resistance against *A. flavus* infection, an RNA interference (RNAi) vector to silence the expression of endogenous RAP genes (such as *pr10*, *glx I* and *ti*) in maize through genetic engineering was constructed (Chen *et al*., 2004a; Chen *et al*., unpubl. data, 2007). The degree of silencing using RNAi constructs is greater than that obtained using either co-suppression or antisense constructs, especially when an intron is included (Wesley *et al*., 2001). Interference of double-stranded RNA with expression of specific genes has been widely described (Fire *et al*., 1998; Gura, 2000). Although the mechanism is still not well understood, RNAi provides an extremely powerful tool to study functions of unknown genes in many organisms. This posttranscriptional gene silencing (PTGS) is a sequence-specific RNA degradation process triggered by a dsRNA, which propagates systemically throughout the plant, leading to the degradation of homologous RNA encoded by endogenous genes, and transgenes.

Both particle bombardment and *Agrobacterium*-mediated transformation methods were used to introduce the RNAi vectors into immature maize embryos. The former was used to provide a quick assessment of the efficacy of the RNAi vector in gene silencing. The latter, which can produce transgenic materials with fewer copies of foreign genes and is easier to regenerate, was chosen for generating transgenic kernels for evaluation of changes in aflatoxin resistance. It was demonstrated using callus clones from particle bombardment that *pr10* expression was reduced by an average of over 90% after the introduction of the RNAi vector (Chen *et al*., unpubl. data, 2007). The transgenic kernels also showed a significant increase in susceptibility to *A. flavus* infection and aflatoxin production. The data from this RNAi study clearly demonstrated a direct role of PR10 in maize host resistance to *A. flavus* infection and aflatoxin contamination.

RNAi vectors to silence other RAP genes, such as *glx I* and *ti*, have also been constructed, and introduced into immature maize embryos through both bombardment and *Agrobacterium* infection (Chen *et al*., unpubl. data, 2007). It will be very interesting to see the effect of silencing the expression of these genes in the transgenic kernels on host resistance to *A. flavus* infection and aflatoxin production.

Use of Near Isogenic Maize Lines to Identify Resistance Related Proteins

Genetic background differences among the lines used in proteomic comparisons increased our difficulty in identifying resistance-associated kernel proteins from either embryo or endosperm. To compensate for this, we had to use a composite gel approach and to focus on those proteins that are five-fold different in the level of expression between resistant and susceptible lines to minimize the chance of identifying proteins that are not related to host resistance. In addition, a lot of time is required to characterize each of the proteins through a series of studies to understand their functions and possible links to host disease resistance. To reduce the complications caused by diverse genetic background in the search for resistance related proteins, near isogenic lines from a resistant population GT-MAS:gk (PI 561859) (McMillian *et al*., 1993) and from the crosses between the African and the U.S. resistant inbred lines have been developed (Menkir *et al*., 2006; Guo *et al*., 2007).

Several sets of near isogenic maize lines were developed from the U.S. resistant maize population GT-MAS:gk as a result of repeated self-pollination by Guo *et al*. (2001, 2002, 2007). This population was derived from a commercial hybrid ear (a Pioneer hybrid) visibly segregating for fungal infection by *A. flavus* and selected for resistance to the fungal infection and reduction of aflatoxin contamination (Widstrom *et al*., 1987). McMillian *et al*. (1993) released the maize population GT-MAS:gk as a source of resistance to aflatoxin accumulation. To use the resistance traits from

GT-MAS:gk, such as physical pericarp wax (Guo *et al*., 1995, 1996; Russin *et al*., 1997) and antifungal proteins (Guo *et al*., 1997, 1998; Chen *et al*., 1998), efforts of self-pollination and selection have been made since 1996 for reduced aflatoxin contamination. By evaluating S1 families, Guo *et al*. (2001) demonstrated that considerable variation among the individual plants within the population GT-MAS:gk was detectable using random amplified polymorphic DNA (RAPD) markers and a laboratory aflatoxin bioassay. Guo *et al*. (2002) also evaluated the S5 generation using 113 restriction fragment length polymorphism (RFLP) probes for genetic variation and conducted 2-yr field tests for aflatoxin contamination. The aflatoxin concentrations and maturity among the S5 selfed lines were significantly different (Guo *et al*., 2002). Two inbred lines, GT601 (AM-1) (PI 644026) and GT602 (AM-2) (PI 644027), selected from GT-MAS:gk population have been released (Guo *et al*., 2007). GT601 (AM-1) flowers about one week earlier than GT602 (AM-2), with about 60 to 70 days from planting to flowering depending on the planting date. GT601 (AM-1) has a colorless pericarp, white cob, and browning silk, *P*-wwb; and GT602 (AM-2) has a colorless pericarp, red cob, and browning silk, *P*-wrb. GT601 (AM-1) had also been used in genetic quantitative trait locus (QTL) mapping studies for silk maysin production (Butrón *et al*., 2001) and *A. flavus* infection (Widstrom *et al*., 2003).

Near isogenic lines with combined resistance traits from both the U.S. resistant inbred lines and the Africa lines with resistance to ear rot diseases and aflatoxin accumulation have also been developed at the International Institute for Tropical Agriculture (IITA) (Menkir *et al*., 2006). Dr. Menkir crossed five elite tropical inbred lines from IITA adapted to the Savanna and mid-altitude ecological zones of West and Central Africa with four U.S. maize lines with proven resistance to aflatoxin accumulation in Ibadan, Nigeria. These five Africa lines were selected for their resistance to ear rot caused by *Aspergillus*, *Botrydiplodia*, *Diplodia*, *Fusarium*, and *Macropomina*, and their potential resistance to aflatoxin accumulation (Brown *et al*., 2001; Menkir *et al*., 2006). The F1 crosses were backcrossed to their respective U.S. inbred lines and self-pollinated thereafter. The resulting lines were selected for resistance to foliar diseases and desirable agronomic characteristics under conditions of severe natural infection in their respective areas of adaptation. Sixty-four of the resulting S4 lines were screened through kernel screening assay (KSA), five pairs of the near isogenic lines were found to be significantly different in aflatoxin resistance (Chen *et al*., 2005). They share as high as 97% genetic similarities, but differ significantly in resistance levels. Using these lines in proteomic comparison to identify host resistance-associated proteins has several advantages: (1) gel comparisons and analyses become much easier; and (2) protein differences between resistant and susceptible lines as low as two-fold can be identified with confidence. In addition, this will increase our chance of identifying proteins that are directly involved in host resistance.

In a preliminary comparison of constitutive protein differences between those African near isogenic lines using proteomics, we identified a new category of resistance-associated proteins (putative regulatory proteins), including a serine/threonine protein kinase and a translation initiation factor 5A (Chen *et al*., unpubl. data, 2007). The genes encoding these two resistance associated regulatory proteins are being cloned and their potential role in host resistance to *A. flavus* infection and aflatoxin production will be investigated through RNAi.

Summary

Research efforts to understand host resistance mechanisms to *A. flavus* infection and aflatoxin contamination in the past indicated that maize kernel proteins, especially stress-related proteins and antifungal proteins, play a role in host resistance as demonstrated using RNAi gene silencing. The use of near isogenic maize lines in the search for aflatoxin resistance-associated proteins using proteomics will enhance our chance in identifying key proteins involved in host aflatoxin resistance. Enhancing the expression of these proteins can be an effective approach to control aflatoxin contamination in susceptible crops, such as maize and peanuts.

Literature Cited

Azziz-Baumgartner, E., K. Lindblade, K. Gieseker, H.S. Rogers, S. Kieszak, H. Njapau, R. Schleicher, L.F. McCoy, A. Misore, K. DeCock, C. Rubin, L. Slutsker, and the Aflatoxin Investigative Group. 2005. Case–control study of an acute aflatoxicosis outbreak, Kenya, 2004. Environ. Health Perspectives 113: 1779-1783.

Brooks, T.D., W.P. Williams, G.L. Windham, M.C. Willcox, and H.K. Abbas. 2005. Quantitative trait loci contributing resistance to aflatoxin accumulation in the maize inbred Mp313E. Crop Sci. 45:171-174.

Brown, R.L., Z.Y. Chen, A. Menkir, T.E. Cleveland, K. Cardwell, J. Kling, and D.G. White. 2001. Resistance to aflatoxin accumulation in kernels of maize inbreds selected for ear rot resistance in West and Central Africa. J. Food Prot. 64:396-400.

Brown, R.L., Z.Y. Chen, T.E. Cleveland, and J.S. Russin. 1999. Advances in the development of host resistance to aflatoxin contamination by *Aspergillus flavus*. Phytopathology (review) 89:113-117.

Brown, R.L., T.E. Cleveland, G.A. Payne, C.P. Woloshuk, K.W. Campbell, and D.G. White. 1995. Determination of resistance to aflatoxin production in maize kernels and detection of fungal colonization using an *Aspergillus flavus* transformant expressing *Escherichia coli* β-glucuronidase. Phytopathology 85:983-989.

Brown, R.L., P.J. Cotty, T.E. Cleveland, and N.W. Widstrom. 1993. Living maize embryo influences accumulation of aflatoxin in maize kernels. J. Food Prot. 56:967-971.

Butrón, A., R.G. Li, B.Z. Guo, N.W. Widstrom, M.E. Snook, T.E. Cleveland, and R.E. Lynch. 2001. Molecular markers to increase corn earworm resistance in a maize population. Maydica 46:117-124.

Campbell, K.W. and D.G. White. 1995. Evaluation of corn genotypes for resistance to aspergillus ear rot, kernel infection, and aflatoxin production. Plant Dis. 79:1039-1045.

Chen, Z.Y., R.L. Brown, T.E. Cleveland, and K.E. Damann. 2004a. Investigating the roles of an aflatoxin resistance-associated protein in maize using RNAi. Phytopathology 94:S18.

Chen, Z.Y., R.L. Brown, T.E. Cleveland, K.E. Damann, and J.S. Russin. 2001. Comparison of constitutive and inducible maize kernel proteins of genotypes resistant or susceptible to aflatoxin production. J. Food Prot. 64:1785-1792.

Chen, Z.Y., R.L. Brown, K.E. Damann, and T.E. Cleveland. 2002. Identification of unique or elevated levels of kernel proteins in aflatoxin-resistant maize genotypes through proteome analysis. Phytopathology 92:1084-1094.

Chen, Z.Y., R.L. Brown, K.E. Damann, and T.E. Cleveland. 2004b. Identification of a maize kernel stress-related protein and its effect on aflatoxin accumulation. Phytopathology 94:938-945.

Chen, Z.Y., R.L. Brown, K.E. Damann, and T.E. Cleveland. 2007. Identification of maize kernel endosperm proteins associated with resistance to aflatoxin contamination by *Aspergillus flavus*. Phytopathology 97:1094-1103.

Chen, Z.Y., R.L. Brown, A.R. Lax, T.E. Cleveland, and J.S. Russin. 1999. Inhibition of plant pathogenic fungi by a corn trypsin inhibitor over-expressed in *Escherichia coli*. Applied Environ. Microbiol. 65:1320-1324.

Chen, Z.Y., R.L. Brown, A.R. Lax, B.Z. Guo, T.E. Cleveland, and J.S. Russin. 1998. Resistance to *Aspergillus flavus* in corn kernels is associated with a 14 kDa protein. Phytopathology 88:276-281.

Chen, Z.Y., R.L. Brown, A. Menkir, K.E. Damann, and T.E. Cleveland. 2005. Proteome analysis of near isogenic maize lines differing in the level of resistance against *Aspergillus flavus* infection/aflatoxin production. Phytopathology 95:S19.

Chen, Z.Y., R.L. Brown, K. Rajasekaran, K.E. Damann, and T.E. Cleveland. 2006. Evidence for involvement of a pathogenesis-related protein in maize resistance to *Aspergillus flavus* infection / aflatoxin production. Phytopathology 96:87-95.

Cole, R.J., T.H. Sanders, R.A. Hill, and P.D. Blankenship. 1985. Mean geocarposphere temperatures that induce preharvest aflatoxin contamination of peanuts under drought stress. Mycopathologia 91:41-46.

Davis, G.L. and W.P. Williams. 1999. QTL for aflatoxin reduction in maize. Maize genetics conference 41:22.

Diener, U.L., R.J. Cole, T.H. Sanders, G.A. Payne, L.S. Lee, and M.A. Klich. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. Annu. Rev. Phytopathol. 25:249-270.

Dorner, J.W., R.J. Cole, T.H. Sanders, and P.D. Blankenship. 1989. Interrelationship of kernel water activity, soil temperature, maturity, and phytoalexin production in preharvest aflatoxin contamination of drought-stressed peanuts. Mycopathologia 105:117-128.

Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. Nature 391:806-811.

Gardner, C.A.C., L.L. Darrah, M.S. Zuber, and J.R. Wallin. 1987. Genetic control of aflatoxin production in maize. Plant Dis. 71:426-429.

Guo, B.Z., R.L. Brown, A.R. Lax, T.E. Cleveland, J.S. Russin, and N.W. Widstrom. 1998. Protein profiles and antifungal activities of kernel extracts from corn genotypes resistant and susceptible to *Aspergillus flavus*. J. Food Prot. 61:98-102.

Guo, B.Z., A. Butrón, H. Li, N.W. Widstrom, and R.E. Lynch. 2002. Restriction fragment length polymorphism assessment of the heterogeneous nature of maize population GT-MAS:gk and field evaluation of resistance to aflatoxin production by *Aspergillus flavus*. J. Food Prot. 65:167-171.

Guo, B.Z., Z.Y. Chen, R.L. Brown, A.R. Lax, T.E. Cleveland, J.S. Russin, A.D. Mehta, C.P. Selitrennikoff, and N.W. Widstrom. 1997. Germination induces accumulation of specific proteins and antifungal activities in corn kernels. Phytopathology 87:1174-1178.

Guo, B.Z., R. Li, N.W. Widstrom, R.E. Lynch, and T.E. Cleveland. 2001. Genetic variation in the maize population GT-MAS:gk and the relationship with resistance to *Aspergillus flavus*. Theor. Appl. Genet. 103:533-539.

Guo, B.Z., J.S. Russin, T.E. Cleveland, R.L. Brown, and K.E. Damann. 1996. Evidence for cutinase production by *Aspergillus flavus* and its possible role in infection of corn kernels. Phytopathology 86:824-829.

Guo, B.Z., J.S. Russin, T.E. Cleveland, R.L. Brown, and N.W. Widstrom. 1995. Wax and cutin layers in maize kernels associated with resistance to aflatoxin production by *Aspergillus flavus*. J. Food Prot. 58:296-300.

Guo, B.Z., N.W. Widstrom, T.E. Cleveland, and R.E. Lynch. 2000. Control of preharvest aflatoxin contamination in corn: Fungus-plant-insect interactions and control strategies. Recent Res. Devel. Agricultural and Food Chem. 4:165-176.

Guo, B.Z., N.W. Widstrom, R.D. Lee, A.E. Coy, and R.E. Lynch. 2007. Registration of maize germplasm GT601 (AM-1) and GT602 (AM-2). J. Plant Registrations 1:153-154.

Gura, T. 2000. A silence that speaks volumes. Nature 404:804-808.

Hill, R.A., P.D. Blankenship, R.J. Cole, and T.H. Sanders. 1983. Effects of soil moisture and temperature on preharvest invasion of peanuts by the *Aspergillus flavus* group and subsequent aflatoxin development. Appl. Environ. Microbiol. 45:628-633.

Holbrook, C.C., B.Z. Guo, D.M. Wilson, and P. Timper. 2008. The U.S. breeding program to develop peanut with drought tolerance and reduced aflatoxin contamination. Peanut Sci. (This Issue).

Holbrook, C.C., C.K. Kvien, K.S. Ruckers, D.M. Wilson, and J.E. Hook. 2000. Preharvest aflatoxin contamination in drought tolerant and intolerant peanut genotypes. Peanut Sci. 27:45-48.

Hsieh, D.P.H. 1989. Potential human health hazards of mycotoxins, pp. 69-80. *In* S. Natori, K. Hashimoto, and Y. Ueno (eds.). Mycotoxins and Phycotoxins. Elsevier, Amsterdam.

Huang, Z., D.G. White, and G.A. Payne. 1997. Corn seed proteins inhibitory to *Aspergillus flavus* and aflatoxin biosynthesis. Phytopathology 87:622-627.

Ji, C., R.A. Norton, D.T. Wicklow, and P.E. Dowd. 2000. Isoform patterns of chitinase and β-1,3-glucanase in maturing corn kernels (*Zea may* L.) associated with *Aspergillus flavus* milk stage infection. J. Agr. Food Chem. 48:507-511.

King, S.B. and G.E. Scott. 1982. Screening maize single crosses for resistance to preharvest infection of kernels by *Aspergillus flavus*. Phytopathology 72:942.

Lisker, N. and E.B. Lillehoj. 1991. Prevention of mycotoxin contamination (principally aflatoxins and *Fusarium* toxins) at the preharvest stage, pp. 689-719. *In* J.E. Smith and R.S. Henderson (eds.). Mycotoxins and Animal Foods. CRC Press, Inc., Boca Raton, FL.

Lozovaya, V.V., A. Waranyuwat, and J.M. Widholm. 1998. β-1,3-glucanase and resistance to *Aspergillus flavus* infection in maize. Crop Sci. 38:1255-1260.

McGee, J.D., J.E. Hamer, and T.K. Hodges. 2001. Characterization of a PR-10 pathogenesis-related gene family induced in rice during infection with *Magnaporthe grisea*. Mol. Plant-Microb. Interact. 14:877-886.

McMillian, W.W., N.W. Widstrom, and D.M. Wilson. 1993. Registration of GT-MAS:gk maize germplasm. Crop Sci. 33:882.

Menkir, A., R.L. Brown, R. Bandyopadhyay, Z.Y. Chen, and T.E. Cleveland. 2006. A U.S.A.-Africa collaborative strategy for identifying, characterizing, and developing maize germplasm with resistance to aflatoxin contamination. Mycopathologia 162:225-232.

Morris, S.W., B. Vernooij, S. Titatarn, M. Starrett, S. Thomas, C.C. Wiltse, R.A. Frederiksen, A. Bhandhufalck, and S. Hulbert. 1998. Induced resistance response in maize. Mol. Plant-Microb. Interact. 11:643-658.

Mould, M.J., T. Xu, M. Barbara, N.N. Iscove, and M.C. Heath. 2003. cDNAs generated from individual epidermal cells reveal that differential gene expression predicting subsequent resistance or

susceptibility to rust fungal infection occurs prior to the fungus entering the cell lumen. Mol. Plant-Microb. Interact. 16:835-845.

Nichols, T.E. Jr. 1983. Economic impact of aflatoxin in corn. South. Coop. Ser. Bull. 279:67-71.

Paul, C., G. Naidoo, A. Forbes, V. Mikkilineni, D. White, and T. Rocheford. 2003. Quantitative trait loci for low aflatoxin production in two related maize populations. Theor. Appl. Genet. 107:263-270.

Payne, G.A. 1998. Process of contamination by aflatoxin-producing fungi and their impact on crops, pp. 279-306. *In* K.K. Sinha and D. Bhatnagar (eds.). Mycotoxins in Agriculture and Food Safety. Marcel Dekker, New York.

Payne, G.A., D.K. Cassel, and C.R. Adkins. 1986. Reduction of aflatoxin contamination in corn by irrigation and tillage. Phytopathology 76:679-684.

Russin, J.S., B.Z. Guo, K.M. Tubujika, R.L. Brown, T.E. Cleveland, and N.W. Widstrom. 1997. Comparison of kernel wax from corn genotypes resistance or susceptible to *Aspergillus flavus*. Phytopathology 87:529-533.

Scott, G.E. and N. Zummo. 1988. Sources of resistance in maize to kernel infection by *Aspergillus flavus* in the field. Crop Sci. 28:505-507.

Smith, J.E. and M.O. Moss. 1985. Mycotoxins: Formation Analyses and Significance. John Wiley and Sons, Chichester, NY.

Squire, R.A. 1981. Ranking animal carcinogens: A proposed regulatory approach. Science 214:877-880.

Steiner-Lange, S., A. Fischer, A. Boettcher, I. Rouhara, H. Liedgens, E. Schmelzer, and W. Knogge. 2003. Differential defense reactions in leaf tissues of barley in response to infection by *Rhynchosporium secalis* and to treatment with a fungal avirulence gene product. Mol. Plant-Microb. Interact. 16:893-902.

Thomann, E.B., J. Sollinger, C. White, and C.J. Rivin. 1992. Accumulation of group 3 late embryogenesis abundant proteins in *Zea mays* embryos. Plant Physiol. 99:607-614.

Veena, V.S. Reddy, and S.K. Sopory. 1999. Glyoxalase I from *Brassica juncea*: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. Plant J. 17:385-395.

Wesley, S.V., C.A. Helliwell, N.A. Smith, M.B. Wang, D.T. Rouse, Q. Liu, P.S. Gooding, S.P. Singh, D. Abbott, P.A. Stoutjesdijk, S.P. Robinson, A.P. Gleave, A.G. Green, and P.M. Waterhouse. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 27:581-590.

Widstrom, N.W., A. Butrón, B.Z. Guo, D.M. Wilson, M.E. Snook, T.E. Cleveland, and R.E. Lynch. 2003. Control of preharvest aflatoxin contamination in maize by pyramiding QTL involved in resistance to ear-feeding insects and invasion by *Aspergillus spp*. Eur. J. Agron. 19:563-572.

Widstrom, N.W., W.W. McMillian, and D.M. Wilson. 1987. Segregation for resistance to aflatoxin contamination among seeds on an ear of hybrid maize. Crop Sci. 27:961-963.

Widstrom, N.W., W.W. McMillian, D.M. Wilson, D.L. Garwood, and D.V. Glover. 1984. Growth characteristics of *Aspergillus flavus* on agar infused with maize kernel homogenates and aflatoxin contamination of whole kernel samples. Phytopathology 74:887-890.

Widstrom, N.W. and M.S. Zuber. 1983. Prevention and control of aflatoxin in corn: sources and mechanisms of genetic control in the plant, pp. 72-76. *In* U.L. Diener, R.L. Asquith, and J.W. Dickens (eds.). Aflatoxin and *Aspergillus flavus* in Corn. So. Coop Series Bull. Auburn, AL: Alabama Agric. Exp. Stn.

Woloshuk, C.P., J.R. Cavaletto, and T.E. Cleveland. 1997. Inducers of aflatoxin biosynthesis from colonized maize kernels are generated by an amylase activity from *Aspergillus flavus*. Phytopathology 87:164-169.

Wotton, H.R. and R.N. Strange. 1987. Increased susceptibility and reduced phytoalexin accumulation in drought-stressed peanut kernels challenged with *Aspergillus flavus*. Appl. Environ. Microbiol. 53:270-273.

Xu, D., X. Duan, B. Wang, B. Hong, T.H.D. Ho, and R. Wu. 1996. Expression of a late embryogenesis abundant protein gene HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol. 110:249-257.