Identifying Aflatoxin Resistance-related Proteins/Genes through Proteomics and RNAi Gene Silencing¹

Z.-Y. Chen^{2*}, R.L. Brown³, B.Z. Guo⁴, A. Menkir⁵, and T.E. Cleveland³

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 B₁, 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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²Asst. Prof., Depart. of Plant Pathology and Crop Physiology, Louisiana State Univ. Agric. Center, Baton Rouge, LA 70803.

³Research Plant Pathologist, Southern Regional Research Center, USDA-ARS, New Orleans, LA 70179.

⁴Research Plant Pathologist, USDA-ARS, Crop Protection and Management Unit, Tifton, GA 31793.

⁵Plant Breeder, International Institute of Tropical Agriculture, Ibadan. Nigeria.

^{*}Corresponding author (e-mail: zchen@agcenter.lsu.edu)

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 differ-

ences 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 trans-

Both particle bombardment and Agrobacteriummediated 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 (OTL) 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.

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