Chapter 11

Identification of Maize Breeding Markers through Investigations of Proteins Associated with Aflatoxin-Resistance

Robert L. Brown[1], Zhi-Yuan Chen[2], Thomas E. Cleveland[1], Abebe Menkir[3], Ahmad Fakhoury[4]

[1]USDA, ARS, SRRC, New Orleans, LA 70124
[2]Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803
[3]International Institute of Tropical Agriculture, Ibadan, Nigeria
[4]Southern Illinois University, Carbondale, IL 62901

The goal of a collaborative research project between International Institute of Tropical Agriculture (IITA) in Nigeria and ARS-Southern Regional Research Center (SRRC) in New Orleans is to develop maize inbred lines with resistance against aflatoxin contamination by *Aspergillus flavus*. A second goal is to identify gene markers in these lines to facilitate their use in U.S. breeding and African national programs. To accomplish this, comparative proteomics of near-isogenic lines varying in aflatoxin accumulation is being employed to identify kernel resistance associated proteins with (RAPs). A number of RAPs have been identified and several further characterized through physiological and biochemical investigations conducted to determine a potential role in resistance and, therefore, fitness as breeding markers. Three RAPs, a trypsin inhibitor, pathogenesis-related protein and glyoxalase I were investigated as well, using RNAi gene silencing and plant transformation. Results of proteome and characterization studies are discussed.

Screening for Resistance to Aflatoxin Accumulation

A number of approaches to eliminating aflatoxins from susceptible crops such as corn, cottonseed, peanut, and treenuts have been advanced, however, the best and most widely explored strategy is the development of preharvest host resistance. This is because *A. flavus* infects affected crops prior to harvest (*1*). Effective, reliable, and rapid screening techniques are indispensable prerequisites to breeding for resistance to aflatoxin accumulation in maize (*2, 3*). Brown et al. (*4*) developed a rapid laboratory-based kernel-screening assay (KSA) that creates higher and more uniform levels of infection and aflatoxin production and allows differentiation of resistant and susceptible maize genotypes. This assay provides consistent ranking of maize genotypes in different tests and the results seem to be correlated with resistance levels expressed by maize genotypes in field trials (*4*).

This rapid assay employs a very simple and inexpensive procedure (*4*). Kernels screened by the KSA are usually incubated for seven days in 100% humidity, at a temperature of 31°C that favors *A. flavus* growth and aflatoxin production. Aflatoxin amounts in kernels from KSA experiments can be obtained two weeks after experiments are initiated. KSA experiments confirmed GT-MAS:gk resistance to aflatoxin production and demonstrated that the resistance in otherwise viable kernels is maintained, when the pericarp barrier is breached (*5*). Penetration through the pericarp barrier was achieved by wounding the kernel with a hypodermic needle down to the endosperm, prior to inoculation. Wounding allows the fungus to freely access the seed without physical barriers, thereby facilitating differentiation between stable resistance mechanisms in operation, and physical attributes of the seed imparting temporary resistance. The results of this study indicated the presence of two levels of resistance: at the pericarp and at the subpericarp level. The former was supported by KSA studies which demonstrated a role for pericarp waxes in kernel resistance (*6, 7, 8*) and highlighted quantitative and qualitative differences in pericarp wax between GT-MAS:gk and susceptible genotypes (*9*). The KSA also confirmed sources of resistance among 31 inbreds tested in Illinois field trials (*4, 10*).

The KSA has several advantages as compared to traditional breeding techniques (*4*): 1) it can be performed and repeated several times throughout the year and outside of the growing season; 2) it requires few kernels; 3) it can detect/identify different kernel resistance mechanisms expressed; 4) it can dispute or confirm field evaluations (e.g. identify escapes); and, 5) relationships between laboratory findings and inoculations in the field have been demonstrated. The KSA has proven be a valuable complement to standard breeding practices in the evaluation of germplasm for aflatoxin-resistance. This was most recently demonstrated through the registration and release of six aflatoxin-resistant maize inbreds developed through a collaboration between IITA-Nigeria (International Institute of Tropical Agriculture) and the Southern Regional Research Center (SRRC) of USDA-ARS in New Orleans (*11*). Field trials, however, are irreplaceable for confirmation of resistance.

Developing resistance to fungal infection in wounded as well as intact kernels would go a long way toward solving the aflatoxin problem (*12*). Studies

demonstrating subpericarp (wounded-kernel) resistance in corn kernels have led to research to identify subpericarp resistance mechanisms. When kernels of susceptible genotypes were allowed to imbibe water at 100% humidity at 31°C for 3 days prior to being inoculated in the KSA protocol, certain kernel proteins became elevated, and aflatoxin levels were drastically and significantly reduced compared to unimbibed controls (*13*). These observations suggest that even susceptible kernels contain proteins capable of being induced during imbibition that can inhibit growth and/or fungal elaboration of aflatoxins. A recent investigation into maize kernel resistance (*14*), however, determined that both constitutive and induced proteins are required for resistance to aflatoxin production. It demonstrated that one major difference between resistant and susceptible genotypes is the relatively high level of constitutively expressed antifungal proteins in resistant lines compared to susceptible lines. A function of these high levels of constitutive proteins may be to delay fungal invasion, and subsequent aflatoxin formation, until other antifungal proteins can be synthesized to form an active defense system.

Identification of Resistance-Associated Proteins (RAPs)

In a previous study, when imbibed susceptible kernels, for example, showed decreased aflatoxin levels, this was paralleled by increases in ribosome inactivating protein (RIP) and zeamatin (*15*). Both zeamatin and RIP have been shown to inhibit *A. flavus* growth *in vitro* (*15*). In another study, two kernel proteins were identified from a resistant corn inbred (Tex6) which may contribute to resistance to aflatoxin contamination (*16*). One protein, 28 kDa in size, inhibited *A. flavus* growth, while a second, over 100 kDa in size, primarily inhibited toxin formation. Recently, the antifungal protein was identified as an endochitinase and was shown to be a major contributor to Tex6 resistance (*17*). When a commercial maize hybrid was inoculated with aflatoxin and nonaflatoxin-producing strains of *A. flavus* at milk stage, one induced chitinase and one ß-1,3-glucanase isoform were detected in maturing infected kernels, while another isoform was detected in maturing uninfected kernels (*18*).

In another investigation, an examination of kernel protein profiles of 13 maize genotypes revealed that a constitutively-produced 14 kDa trypsin inhibitor protein (TI) is present at relatively high concentrations in seven resistant maize lines, but at low concentrations or is absent in six susceptible lines (*19*). The mode of action of TI against fungal growth may be partially due to its inhibition of fungal -amylase, limiting *A. flavus* access to simple sugars (*20*) required not only for fungal growth, but also for toxin production (*21*). TI also demonstrated antifungal activity against other mycotoxigenic species (*22*). The identification of these proteins may provide markers for plant breeders, and may facilitate the cloning and introduction of antifungal genes through genetic engineering into other aflatoxin-susceptible crops.

Using Comparative Proteomics to Identify RAPs

To increase protein reproducibility, spot resolution and detection sensitivity by 10 to 20 fold and, thus, enhance ability to identify more constitutively-expressed RAPs, a proteomics approach was employed. Kernel proteins from several resistant and susceptible genotypes were compared using large format 2-D gel electrophoresis, and over a dozen such protein spots, either unique or 5-fold up-regulated in resistant lines, were identified, isolated from preparative 2-D gels and analyzed using ESI-MS/MS after in-gel digestion with trypsin (*23, 24*). These proteins can be grouped into three categories based on their peptide sequence homology: (1) storage proteins, such as globulins (GLB1, GLB2), and late embryogenesis abundant proteins (LEA3, LEA14); (2) stress-responsive proteins, such as aldose reductase (ALD), glyoxalase I (GLX1) and heat shock proteins, and (3) antifungal proteins, including TI. In total, approximately 17 proteins upregulated in resistant versus susceptible lines have been identified using comparative proteomics (Table I).

Table I. Resistance-associated Proteins Identified in Maize Kernels using Proteomics

Antifungal	Stress-responsive	Storage
Zeamatin	Peroxiredoxin 1	Globulin I
TI (14 kDa)	Aldose reductase	Globulin II
PR-10	Glyoxalase I	LEA III
TI (10 kDa)	Anionic Peroxidase	LEA 14
β-1,3-glucanase	Cold Regulated Protein	
RIP	Water stress inducible	
	Heat shock	

SOURCE: Table adapted from Reference 25.

No investigation has been conducted, thus far, to determine the possible direct involvement of stress-related proteins in host fungal resistance. However, increased temperatures and drought, which often occur together, are major factors associated with aflatoxin contamination of corn kernels (*26*). 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 in the ability to synthesize proteins and defend against pathogens under stress conditions. Further studies including physiological and biochemical characterization, genetic mapping, plant transformation using RAP genes, RNAi gene silencing experiments (*27*) and marker-assisted breeding should clarify the roles of stress-related RAPs in kernel resistance.

The screening of progeny generated through the collaborative project between IITA-Nigeria and USDA-ARS-SRRC facilitated the identification of near-isogenic lines from the same backcross differing significantly in aflatoxin accumulation (Table II), and proteome analysis of these lines is being conducted

Figure 9.1. Amino acids in lytic peptides. The symbols represent a molecular alphabet

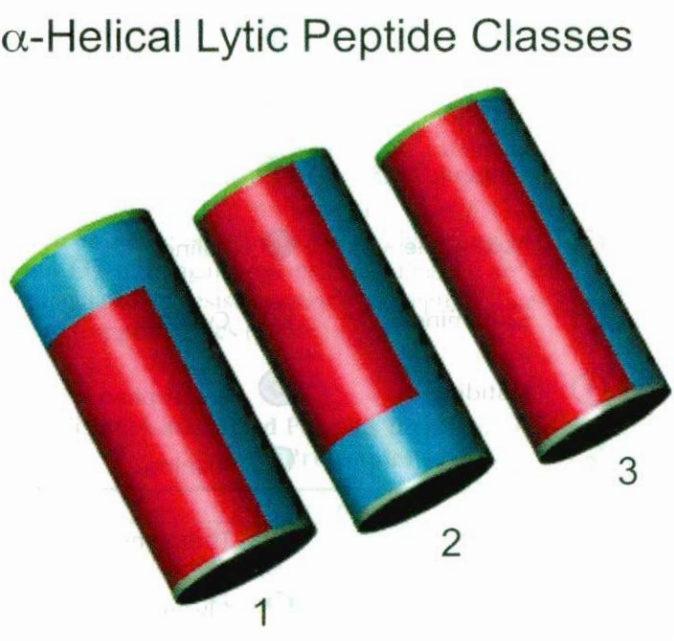

Figure 9.2. α-helical lytic peptide classes

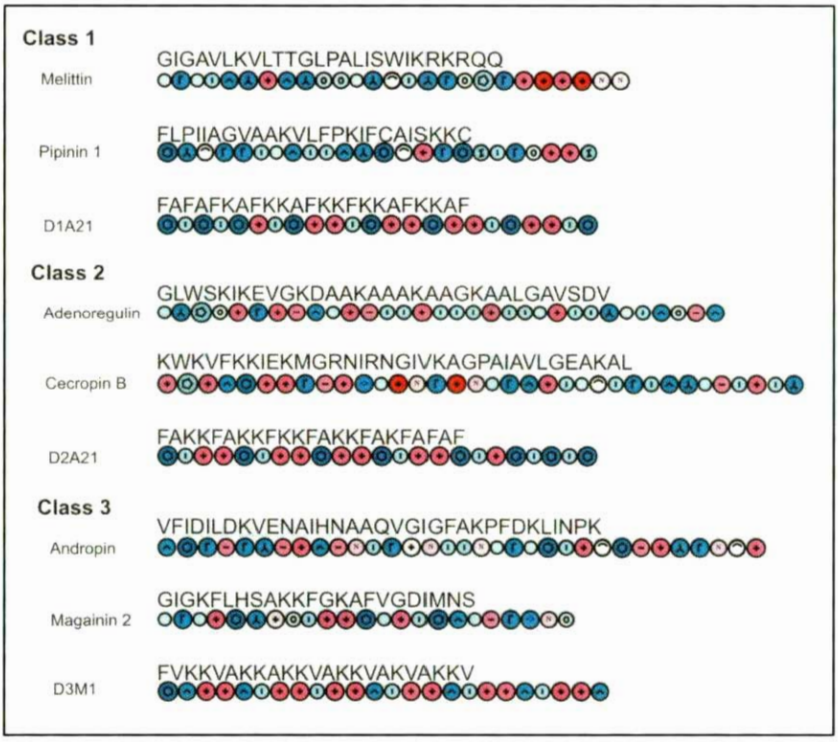

Figure 9.3. Natural lytic peptides.

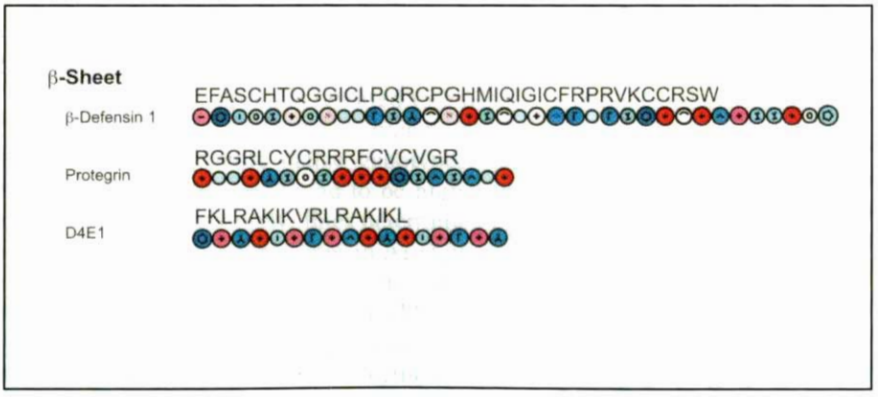

Figure 9.4. β-sheet peptides

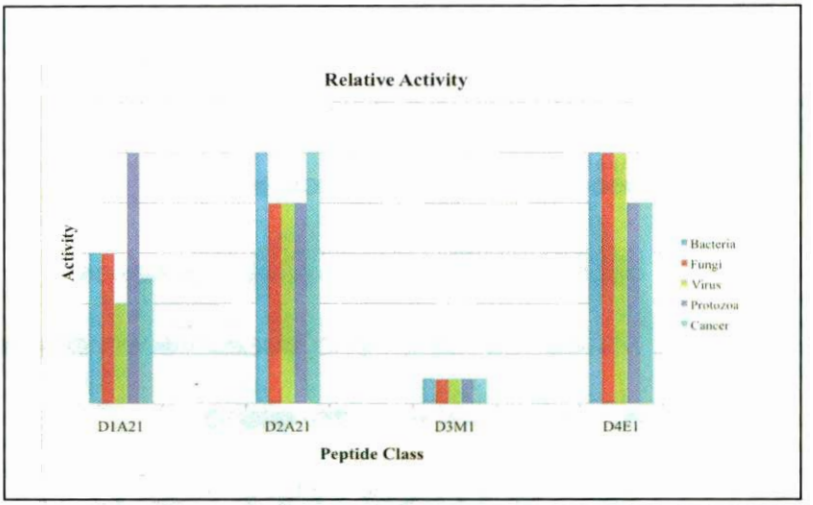

Figure 9.5. Relative Activity of selected synthetic lytic peptides (Jaynes, unpublished)

(*27, 28*). Investigating corn lines sharing close genetic backgrounds should enhance the identification of RAPs clearly without the confounding effects experienced with lines of diverse genetic backgrounds.

Table II. Near-Isogenic Maize Pairs Varying in Aflatoxin Levels via KSA

Line	Pedigree	AFB_1(ppb)
1	(GT-MAS:gk x KU1414SR x GT-MAS:gk)-8-1-2-3-B*6	1263 b
2	(GT-MAS:gk x KU1414SR x GT-MAS:gk)-8-1-2-4-B*6	11 e
25	1368 x GT-MAS:gk-8-1-1-4-B*4	5 h
31	1368 x GT-MAS:gk-8-1-1-3-B*3	1984 bc
86	1368 x MI82-23-2-1-3-B*4	156 f
87	1368 x MI82-23-2-1-5-B*4	1226 de
111	GT-MAS:gk x Babangoyo-1-1-3-B*6	3710 c
112	GT-MAS:gk x Babangoyo-1-1-1-B*2	276 fg

NOTE: Percentage of genetic similarity between pairs is as follows: Lines 1 and 2 =93.75%; Lines 25 and 31=87.5%; Lines 86 and 87=87.5%; Lines 111 and 112=75%.

Further Characterization of RAPs

Of the constitutively-expressed proteins identified through proteomics, several have been further investigated to understand their potential involvement in resistance. Among those investigated are: 1) aldose reductase (ALD), 2) glyoxalase I (GLX-I), 3) pathogenesis related protein 10 (PR-10), 4) peroxiredoxin antioxidant (PER1), 5) cold-regulated protein (ZmCORp), and 6) trypsin inhibitor-10kDa (ZmTIp).

Aldose reductase, which is reported to have a role in plant stress tolerance, is found constitutively-produced at higher levels in kernel embryo tissue of resistant versus susceptible maize genotypes (*24*). However, after *A. flavus'* infection, ALD activity was found to be higher in highly susceptible lines than in resistant lines. Interestingly, lines determined to be intermediately susceptible varied from highly susceptible lines in ALD production.

Glyoxalase I, also isolated from kernel embryo, is involved in the conversion of cytotoxic methylglyoxal (MG) into D-lactate, along with GLX II, and is suggested to be important to plant stress tolerance (*29*). Higher GLX-I activity was observed in maize kernels of resistant genotypes than in susceptibles both constitutively and after *A. flavus'* infection. However, infection significantly increased MG levels in two of three susceptible lines. MG was also shown to induce aflatoxin production in vitro; its mode of action may be to stimulate expression of the aflatoxin regulatory gene *aflR*.

During an investigation of PR-10, which was isolated from kernel endosperm (*30*), it was discovered that during kernel development, *pr-10* expression increased fivefold between 7 and 22 days after pollination, and was induced upon *A. flavus* infection in the resistant but not the susceptible genotype. It was also shown that PR-10 had ribonuclease and antifungal activities. Leaf extracts of transgenic tobacco plants expressing *pr-10* also demonstrated RNase activity and inhibited *A. flavus* growth.

PER1 protein, also produced in the endosperm, demonstrated peroxidase activity *in vitro*. Also, *per1* expression was significantly higher in a resistant genotype versus a susceptible one during the late stages of development, and was significantly induced upon *A. flavus* infection (31).

ZMCORp protein has a sequence similar to cold-regulated protein, however, it exhibited lectin-like hemagglutination activity against fungal conidia and sheep erythrocytes (*32*). When tested against *A.flavus*, it was shown to inhibit germination of conidia by 80% and to decrease mycelial growth by 50% when germinated conidia were incubated with the protein. Quantitative real-time RT-PCR revealed ZmCORp to be expressed 50% more in kernels of a resistant maize line versus a susceptible.

ZmTIp, a 10 kDa trypsin inhibitor, had an impact on fungal growth, but not as great as previously investigated TIs (*33*).

Of the six proteins discussed above, GLX I, PR-10 and also the 14 kDa TI are being investigated as to impact on aflatoxin formation through RNAi gene silencing (34). Data, while still being evaluated, indicate an important role for PR-10 in aflatoxin-resistance, as transgenic maize plants where *pr-10* expression is significantly decreased also accumulate significantly more aflatoxin compared to controls.

Conclusions

A great deal of progress has been made towards controlling aflatoxin contamination of maize through the development of germplasm resistant to the growth of aflatoxigenic species, and or biosynthesis of toxins by these species. Therefore, the identification of resistance traits in maize can, using marker-assisted breeding, facilitate a more rapid development of resistant, commercially-useful germplasm. Genetic engineering provides a tool especially useful for transferring resistance genes identified in maize into crops with little natural genetic diversity (e.g. cotton), and for testing the efficacy of the putative resistance genes.

Comparative proteome analysis of maize kernels has identified categories of proteins other than the expected antifungals that are associated with aflatoxin-resistance. The relationships among plant stresses such as drought, high temperature and fungal infection may indicate a connection between kernel stress tolerance and its ability to resist *A. flavus* colonization.

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