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Development of Maize Host Resistance to Aflatoxigenic Fungi

Robert L. Brown, Deepak Bhatnagar,
Thomas E. Cleveland, Zhi-Yuan Chen and
Abebe Menkir

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http://dx.doi.org/10.5772/54654

1. Introduction

Aflatoxins, the toxic and highly carcinogenic secondary metabolites of *Aspergillus flavus* and *A.parasiticus* are the most widely investigated of all mycotoxins because of their central role in establishing the significance of mycotoxins in animal diseases, and the regulation of their presence in food [1, 2]. Aflatoxins pose serious health hazards to humans and domestic animals, because they frequently contaminate agricultural commodities [3, 4]. Presently, numerous countries have established or proposed regulations for controlling aflatoxins in food and feeds [5]; the US Food and Drug Administration (FDA) has limits of 20 ppb, total aflatoxins, on interstate commerce of food and feed, and 0.5 ppb of aflatoxin M1 on the sale of milk. However, many countries, especially in the developing world, experience contamination of domestic-grown commodities at alarmingly greater levels than does the U.S. Evidence of this was shown in a study that revealed a strong association between exposure to aflatoxin and both stunting (a reflection of chronic malnutrition) and being underweight (a reflection of acute malnutrition) in West African children [6]. Also, a 2004 outbreak of acute aflatoxicosis in Kenya, due to the ingestion of contaminated maize, resulted in 125 deaths [7].

Recognition of the need to control aflatoxin contamination of food and feed grains has elicited responses outlining various approaches from researchers to eliminate aflatoxins from maize and other susceptible crops. The approach to enhance host resistance through breeding gained renewed attention following the discovery of natural resistance to *A. flavus* infection and aflatoxin production in Maize [8-12]. While several resistant maize genotypes have

been identified through field screening, there is always a need to continually identify and utilize additional sources of maize genotypes with aflatoxin-resistance.

An important contribution to the identification/investigation of kernel aflatoxin-resistance has been the development of a rapid laboratory screening assay. The kernel screening assay (KSA), was developed and used to study resistance to aflatoxin production in GT-MAS:gk kernels [13, 14]. The KSA is designed to address the fact that aflatoxin buildup occurs in mature and not developing kernels. Although, other agronomic factors (e.g. husk tightness) are known to affect genetic resistance to aflatoxin accumulation in the field, the KSA measures seed-based genetic resistance. The seed, of course, is the primary target of aflatoxigenic fungi, and is the edible portion of the crop. Therefore, seed-based resistance represents the core objective of maize host resistance. Towards this aim, the KSA has demonstrated proficiency in separating susceptible from resistant seed [13, 14]. This assay has several advantages, as compared to traditional field screening techniques [14]: 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; 4) it can dispute or confirm field evaluations (identify escapes); and 5) correlations between laboratory findings and inoculations in the field have been demonstrated. The KSA can, therefore, be a valuable complement to standard breeding practices for preliminary evaluation of germplasm. However, field trials are necessary for the final confirmation of resistance.

2. Discovery of aflatoxin-resistance

2.1. Traditional screening techniques

Screening maize for resistance to kernel infection by *Aspergillus flavus* or for resistance to aflatoxin production is a more difficult task than most disease screening. Successful screening in the past had been hindered [15] by the lack of 1) a resistant control; 2) inoculation methods that yield infection/aflatoxin levels high enough to differentiate among genotypes (natural infection is undependable); 3) repeatability across different locations and years; and, 4) rapid and inexpensive methods for assessment of fungal infection and aflatoxin levels. Several inoculation methods, including the pinbar inoculation technique (for inoculating kernels through husks), the silk inoculation technique, and infesting corn ears with insect larvae infected with *A. flavus* conidia have been tried with varying degrees of success [9, 16]. These methods can each be useful, however, clarity must exist as to the actual resistance trait to be measured (e.g. husk tightness; silk traits; the kernel pericarp barrier; wounded kernel resistance), before an appropriate technique can be employed. Silk inoculation, however, (possibly more dependent upon the plant's physiological stage and/or environmental conditions) has proven to be the most inconsistent of the inoculation methods [17].

Plating kernels to determine the frequency of kernel infection and examining kernels for emission of a bright greenish-yellow fluorescence (BGYF) are methods that have been used for assessing *A. flavus* infection [15]. While both methods can indicate the presence of *A. flavus* in seed, neither can provide the kind of accurate quantitative or tissue-localization data

useful for effective resistance breeding. Several protocols have been developed and used for separation and relatively accurate quantification of aflatoxins [18].

2.2. Early identification of resistant maize lines

Two resistant inbreds (Mp420 and Mp313E) were discovered and tested in field trials at different locations and released as sources of resistant germplasm [11, 19]. The pinbar inoculation technique was one of the methods employed in the initial trials, and contributed towards the separation of resistant from susceptible lines [11]. Several other inbreds, demonstrating resistance to aflatoxin contamination in Illinois field trials (employing a modified pinbar technique) also were discovered [12]. Another source of resistance discovered was the maize breeding population, GT-MAS:gk. This population was derived from visibly classified segregating kernels, obtained from a single fungus-infected hybrid ear [10]. It tested resistant in trials conducted over a five year period, where a kernel knife inoculation technique was employed.

These discoveries of resistant germplasm may have been facilitated by the use of inoculation techniques capable of repeatedly providing high infection/aflatoxin levels for genotype separation to occur. While these maize lines do not generally possess commercially acceptable agronomic traits, they may be invaluable sources of resistance genes, and as such, provide a basis for the rapid development of host resistance strategies to eliminate aflatoxin contamination.

3. Investigations of resistance mechanisms/traits in maize lines

3.1. Molecular genetic investigations of aflatoxin-resistant lines

Chromosome regions associated with resistance to *A. flavus* and inhibition of aflatoxin production in maize have been identified through Restriction Fragment Length Polymorphism (RFLP) analysis in three "resistant" lines (R001, LB31, and Tex6) in an Illinois breeding program, after mapping populations were developed using B73 and/or Mo17 elite inbreds as the "susceptible" parents [20, 21]. Chromosome regions associated with inhibition of aflatoxin in studies considering all 3 resistant lines demonstrated that there are some regions in common. Regions on chromosome arms 2L, 3L, 4S, and 8S may prove promising for improving resistance through marker assisted breeding into commercial lines [21]. In some cases, chromosomal regions were associated with resistance to *Aspergillus* ear rot and not aflatoxin inhibition, and vice versa, whereas others were found to be associated with both traits. This suggests that these two traits may be at least partially under separate genetic control. QTL studies involving other populations have identified chromosome regions associated with low aflatoxin accumulation.

In a study involving 2 populations from Tex6 x B73, conducted in 1996 and 1997, promising QTLs for low aflatoxin were detected in bins 3.05-6, 4.07-8, 5.01-2, 5.05-5, and 10.05-10.07 [22]. Environment strongly influenced detection of QTLs for lower toxin in different years;

QTLs for lower aflatoxin were attributed to both parental sources. In a study involving a cross between B73 and resistant inbred Oh516, QTL associated with reduced aflatoxin were identified on chromosomes 2, 3 and 7 (bins 2.01 to 2.03, 2.08, 3.08, and 7.06) [23]. QTLs contributing resistance to aflatoxin accumulation were also identified using a population created by B73 and resistant inbred Mp313E, on chromosome 4 of Mp313E [24]. This confirmed the findings of an earlier study involving Mp313E and susceptible Va35 [25]. Another QTL in this study, which has similar effects to that on chromosome 4, was identified on chromosome 2 [24]. A recent study to identify aflatoxin-resistance QTL and linked markers for marker-assisted breeding was conducted using a population developed from Mp717, an aflatoxin-resistant maize inbred, and NC300, a susceptible inbred adapted to the southern U.S. QTL were identified on all chromosomes, except 4, 6, and 9; individual QTL accounted for up to 11% of phenotypic variance in aflatoxin accumulation [26]. Lastly, in a study of population of F2:3 families developed from resistant Mp715 and a southern-adapted susceptible, T173, QTL with phenotypic effects up to 18.5% were identified in multiple years on chromosomes 1, 3, 5, and 10 [27].

A number of genes corresponding to resistance-associated proteins (RAPs), that were identified in proteomics studies (see section 3.5.1 below) have been mapped to chromosomal location using the genetic sequence of B73 now available online (http://archive.maizesequence.org/index.html) [28]. Using the DNA sequence of the RAPs and blasting them against the B73 sequence allowed us to place each gene into a virtual bin, allowing us to pinpoint the chromosomal location to which each gene maps. The chromosomes involved include the above-mentioned chromosomes 1, 2, 3, 7, 8 and 10, some in bins closely located to those described above. Another study also mapped RAPs to bins on the above-chromosomes as well as chromosomes 4 and 9 [29].

3.2. Kernel pericarp wax

Kernel pericarp wax of maize breeding population GT-MAS:gk has been associated with resistance to *Aspergillus flavus* infection /aflatoxin production. Previously, kernel wax of GT-MAS:gk was compared to that of 3 susceptible genotypes. Thin layer chromatography (TLC) of wax from these genotypes showed a band unique to GT-MAS:gk and a band unique to the three susceptible lines [30]. GT-MAS:gk kernel wax also was shown to inhibit *A. flavus* growth. A later investigation compared GT-MAS:gk wax resistance-associated traits to that of twelve susceptible maize genotypes [31]. TLC results of wax from these lines confirmed findings of the previous investigation, demonstrating both the unique GT-MAS:gk TLC band and the unique 'susceptible' band. Gas chromatography/mass spectroscopy (GC/MS) analysis of the whole wax component showed a higher percentage of phenol-like compounds in the resistant genotype than in the susceptibles. Alkylresorcinol content was dramatically higher in GT-MAS:gk wax than in susceptible lines. An alkylresorcinol, 5-methylresorcinol, also inhibited *in vitro* growth of *A. flavus*. Further research is needed for a clear identification of the component(s) responsible for kernel wax resistance and to determine its expression level in other maize lines.

3.3. Two levels of resistance

The KSA employs a very simple and inexpensive apparatus involving bioassay trays, petri dishes, vial caps as seed containers, and chromatography paper for holding moisture [14]. Kernels screened by the KSA are maintained in 100% humidity, at a temperature favoring *A. flavus* (31° C) growth and aflatoxin production, and are usually incubated for seven days. Aflatoxin data from KSA experiments can be obtained two to three weeks after experiments are initiated. KSA experiments confirmed GT-MAS:gk resistance to aflatoxin production and demonstrated that it is maintained even when the pericarp barrier, in otherwise viable kernels, is breached [13]. Penetration through the pericarp barrier was achieved by wounding the kernel with a hypodermic needle down to the endosperm, prior to inoculation. Wounding facilitates differentiation between different resistance mechanisms in operation, and the manipulation of aflatoxin levels in kernels for comparison with other traits (e.g. fungal growth; protein induction). The results of this study indicate the presence of two levels of resistance: at the pericarp and at the subpericarp level. The former was supported by the above-studies which demonstrated a role for pericarp waxes in kernel resistance [30], and highlighted quantitative and qualitative differences in pericarp wax between GT-MAS:gk and susceptible genotypes [31, 32].

3.4. Comparing fungal growth to toxin production

When selected resistant Illinois maize inbreds (MI82, CI2, and T115) were examined by the KSA, modified to include an *A. flavus* GUS transformant (a strain genetically engineered with a gene construct consisting of a β-glucuronidase reporter gene linked to an *A. flavus* beta-tubulin gene promoter for monitoring fungal growth) [14], kernel resistance to fungal infection in nonwounded and wounded kernels was demonstrated both visually and quantitatively, as was a positive relationship between the degree of fungal infection and aflatoxin levels [14, 33]. This made it possible assess fungal infection levels and to determine if a correlation exists between infection and aflatoxin levels in the same kernels. *A. flavus* GUS transformants with the reporter gene linked to an aflatoxin biosynthetic pathway gene could also provide a way to indirectly measure aflatoxin levels [34-36], based on the extent of the expression of the pathway gene.

Recently, It was demonstrated, using the KSA and an *F. moniliforme* strain, genetically transformed with a GUS reporter gene linked to an *A. flavus* β-tubulin gene promoter, that the aflatoxin-resistant genotype, GT-MAS:gk, inhibits growth of *F. moniliforme* as well [37]. This indicates that some resistance mechanisms may be generic for ear rotting/ mycotoxigenic fungi.

A more recent use of reporter genes was performed on cotton using a green fluorescent protein reporter; a GFP-expressing *A. flavus* strain to successfully monitor fungal growth, mode of entry, colonization of cottonseeds, and production of aflatoxins [38]. This strain provides for an easy, potentially non-destructive, rapid and economical assay which can be done in real time, and may constitute an advance over GUS transformants.

3.5. Resistance-associated proteins

Developing resistance to fungal infection in wounded as well as intact kernels would go a long way toward solving the aflatoxin problem [17]. Studies demonstrating subpericarp (wounded-kernel) resistance in maize kernels have led to research for identification of subpericarp resistance mechanisms. Examinations of kernel proteins of several genotypes revealed differences between genotypes resistant and susceptible to aflatoxin contamination [39]. Imbibed susceptible kernels, for example, showed decreased aflatoxin levels and contained germination-induced ribosome inactivating protein (RIP) and zeamatin [40]. Both zeamatin and RIP have been shown to inhibit *A. flavus* growth *in vitro* [40]. In another study, two kernel proteins were identified from a resistant corn inbred (Tex6) which may contribute to resistance to aflatoxin contamination [41]. One protein, 28 kDa in size, inhibited *A. flavus* growth, while a second, over 100 kDa in size, primarily inhibited toxin formation. When a commercial corn 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 was detected in maturing infected kernels, while another isoform was detected in maturing uninfected kernels [42].

In another investigation, an examination of kernel protein profiles of 13 maize genotypes revealed that a 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 [43]. 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 [44] required not only for fungal growth, but also for toxin production [45]. TI also demonstrated antifungal activity against other mycotoxigenic species [46]. 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.

An investigation into maize kernel resistance [47] determined that both constitutive and induced proteins are required for resistance to aflatoxin production. It also showed that one major difference between resistant and susceptible genotypes is that resistant lines constitutively express higher levels of antifungal proteins compared to susceptible lines. The real function of these high levels of constitutive antifungal proteins may be to delay fungal invasion, and consequent aflatoxin formation, until other antifungal proteins can be synthesized to form an active defense system.

3.5.1. Proteomic analysis

Two-dimensional (2-D) gel electrophoresis, which sorts proteins according to two independent properties, isoelectric points and then molecular weights, has been recognized for a number of years as a powerful biochemical separation technique. Improvements in map resolution and reproducibility [48, 49], rapid analysis of proteins, analytical soft ware and computers, and the acquisition of genomic data for a number of organisms has given rise to another application of 2-D electrophoresis: proteome analysis. Proteome analysis or "proteomics" is the analysis of the protein complement of a genome [50, 51]. This involves the systematic separation, identification, and quantification of many proteins simultaneously. 2-D

electrophoresis is also unique in its ability to detect post- and cotranslational modifications, which cannot be predicted from the genome sequence.

Through proteome analysis and the subtractive approach, it may be possible to identify important protein markers associated with resistance, as well as genes encoding these proteins. This could facilitate marker-assisted breeding and/or genetic engineering efforts. Endosperm and embryo proteins from several resistant and susceptible genotypes have been compared using large format 2-D gel electrophoresis, and over a dozen such protein spots, either unique or 5-fold upregulated in resistant maize lines (Mp420 and Mp313E), have been identified, isolated from preparative 2-D gels and analyzed using ESI-MS/MS after in-gel digestion with trypsin [52, 53]. These proteins, all constitutively expressed, can be grouped into three categories based on their peptide sequence homology: (1) storage proteins, such as globulins and late embryogenesis abundant proteins; (2) stress-responsive proteins, such as aldose reductase, a glyoxalase I protein and a 16.9 kDa heat shock protein, and (3) antifungal proteins, including the above-described TI.

During the screening of progeny developed through the IITA-USDA/ARS collaborative project, near-isogenic lines from the same backcross differing significantly in aflatoxin accumulation were identified, and proteome analysis of these lines is being conducted [54]. Investigating corn lines from the same cross with contrasting reaction to *A. flavus* should enhance the identification of RAPs clearly without the confounding effect of differences in the genetic backgrounds of the lines.

Heretofore, most RAPs identified have had antifungal activities. However, increased temperatures and drought, which often occur together, are major factors associated with aflatoxin contamination of maize kernels [55]. It has also been found that drought stress imposed during grain filling reduces dry matter accumulation in kernels [55]. This often leads to cracks in the seed and provides an easy entry site to fungi and insects. Possession of unique or of 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, and marker-assisted breeding should clarify the roles of stress-related RAPs in kernel resistance. RNAi gene silencing experiments involving RAPs may also contribute valuable information. [54].

3.5.2. Further characterization of RAPs

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 [56]. Transgenic rice overexpressing a barley LEA3 protein HVA1 showed significantly increased tolerance to water deficit and salinity [57]. 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 [58]. The substrate for glyoxalase I, methylglyoxal, is a potent cytotoxic compound produced spontaneously in all organisms under physiological

conditions from glycolysis and photosynthesis intermediates, glyceraldehydes-3-phosphate and dihydroxyacetone phosphate. Methylglyoxal is an aflatoxin inducer even at low concentrations; experimental evidence indicates that induction is through upregulation of aflatoxin biosynthetic pathway transcripts including the *AFLR* regulatory gene [59]. Therefore, glyoxalase I may be directly affecting resistance by removing its aflatoxin-inducing substrate, methylglyoxal. PER1, a 1-cys peroxiredoxin antioxidant identified in a proteomics investigation [60], 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 [60]. Another RAP that has been characterized further is the pathogenesis-related protein 10 (PR10). It showed high homology to PR10 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 [61]. 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 [61]. 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 [61]. Recently, a new *PR10* homologue was identified from maize (*PR10.1*) [62]. *PR10* was expressed at higher levels in all tissues compared to *PR10.1*, however, purified PR10.1 overexpressed in *E. coli* possessed 8-fold higher specific RNase activity than *PR10* [62]. This homologue may also play a role in resistance. 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 resistant cultivars upon infection by *Rhynchosporium secalis*, but not in near-isogenic susceptible plants [63]. In cowpea, a *PR10* homolog was specifically up-regulated in resistant epidermal cells inoculated with the rust fungus *Uromyces vignae* Barclay [64]. A *PR10* transcript was also induced in rice during infection by *Magnaporthe grisea* [65].

To directly demonstrate whether selected RAPs play 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 [59, 66]. 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 [67]. Interference of double-stranded RNA with expression of specific genes has been widely described [68, 69]. 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 [66]. 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 for PR10 in maize host resistance to *A. flavus* infection and aflatoxin contamination [66]. 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 [70]. 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.

ZmCORp, a protein with a sequence similar to cold-regulated protein and identified in the above-proteomic studies, was shown to exhibit lectin-like hemagglutination activity against fungal conidia and sheep erythrocytes [71]. When tested against *A. flavus*, ZmCORp inhibited germination of conidia by 80% and decreased 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 *A. flavus* growth, but not as great as the previously-mentioned 14 kDa TI [72].

3.5.3. Proteomic studies of rachis and silk tissue

A study was conducted to investigate the proteome of rachis tissue, maternal tissue that supplies nutrients to the kernels [75]. An interesting finding in this study is that after infection by *A. flavus*, rachis tissue of aflatoxin-resistant genotypes did not up-regulate PR proteins as these were already high in controls where they had strongly and constitutively accumulated during maturation. However, rachis tissue of aflatoxin-susceptible lines did not accumulate PR proteins to such an extent during maturation, but increased them in response to fungal infection. Given the relationship of the rachis to kernels, these results confirm findings of a previous investigation [47], which demonstrated levels of proteins in resistant *versus* susceptible kernels was a primary factor that determined kernel genetic resistance to aflatoxin contamination. Another study was conducted to identify proteins in maize silks that may be contributing to resistance against *A. flavus* infection/colonization [76]. Antifungal bioassays were performed using silk extracts from two aflatoxin-resistant and two–susceptible inbred lines. Silk extracts from resistant inbreds showed greater antifungal activity compared to susceptible inbreds. Comparative proteomic analysis of the two resistant and susceptible inbreds led to the identification of antifungal proteins including three chitinases that were differentially-expressed in resistant lines. When tested for chitinase activity, silk proteins from extracts of resistant lines also showed significantly higher chitinase activity than that from susceptible lines. Differential expression of chitinases in

maize resistant and susceptible inbred silks suggests that these proteins may contribute to resistance.

3.5.4. Transcriptomic analyses

To investigate gene expression in response to *A. flavus'* infection and to more thoroughly identify factors potentially involved in the regulation of RAP genes, a transcriptomic profile was conducted on maize kernels of two inbred lines that were genetically closely-related [73]. Similar work had previously been performed using Tex6 as the resistant line and B73 as the susceptible [74], however, in the study using closely-related lines, imbibed mature kernels were used (for the first time) and proved to be a quicker and easier approach than traditional approaches. The involvement of certain stress-related and antifungal genes previously shown to be associated with constitutive resistance was demonstrated here; a kinase-binding protein, Xa21 was highly up-regulated in the resistant line compared to the susceptible, both constitutively and in the inducible state.

4. Current efforts to develop resistant lines

4.1. Closely-related lines

Recently, the screening of progeny generated through a collaborative breeding program between IITA-Nigeria (International Institute of Tropical Agriculture) and the Southern Regional Research Center of USDA-ARS in Center (SRRC) of USDA-ARS in New Orleans facilitated the identification of closely-related lines from the same backcross differing significantly in aflatoxin accumulation, and proteome analysis of these lines is being conducted [77, 78]. Investigating corn lines sharing close genetic backgrounds should enhance the identification of RAPs without the confounding effects experienced with lines of diverse genetic backgrounds. The IITA-SRRC collaboration has attempted to combine resistance traits of U.S. resistant inbred lines with those of African lines, originally selected for resistance to ear rot diseases and for potential aflatoxin-resistance (*via* KSA) [77, 78]. Five elite tropical inbred lines from IITA adapted to the Savanna and mid-altitude ecological zones of West and Central Africa were crossed with four U.S. resistant maize lines in Ibadan, Nigeria. The five African lines were originally selected for their resistance to ear rot caused by *Aspergillus*, *Botrydiplodia*, *Diplodia*, *Fusarium*, and *Macropomina* [77, 78]. The F1 crosses were backcrossed to their respective U.S. inbred lines and self-pollinated thereafter. The resulting lines were selected through the S4 generation for resistance to foliar diseases and desirable agronomic characteristics under conditions of severe natural infection in their respective areas of adaptation. Promising S5 lines were screened with the KSA (Table 1). In total, five pairs of closely-related lines were shown to be significantly different in aflatoxin resistance, while sharing as high as 97% genetic similarity [79]. Using these lines in proteomic comparisons to identify RAPs has advantages: (1) gel comparisons and analyses become easier; and (2) protein differences between resistant and susceptible lines as low as twofold can be identified with confidence. In addition, the likelihood of identifying proteins that are directly involved in

host resistance is increased. In a preliminary proteomics comparison of constitutive protein differences between those African closely-related lines, a new category of resistance-associated proteins (putative regulatory proteins) was identified, including a serine/threonine protein kinase and a translation initiation factor 5A [29, 79]. 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 further investigated. Conducting proteomic analyses using lines from this program not only enhances chances of identifying genes important to resistance, but may have immediate practical value. The IITA-SRRC collaboration has registered and released six inbred lines with aflatoxin-resistance in good agronomic backgrounds, which also demonstrate good levels of resistance to southern corn blight and southern corn rust [80]. Resistance field trials for these lines on U.S. soil is being conducted; the ability to use resistance in these lines commercially will depend on having identified excellent markers, since seed companies desire insurance against the transfer of undesirable traits into their elite genetic backgrounds. The fact that this resistance is coming from good genetic backgrounds is also a safeguard against the transfer of undesirable traits.

Entry	Aflatoxin B_1 (ppb)
Susceptible control	10197 a
22*	1693 b
19	1284 bc
28	1605 bcd
27	1025 bcd
21	1072 bcd
26	793 bcde
20	574 cde
24	399 cde
GT-MAS:gk	338 de
25*	228 e
23	197 e
Resistant control	76 e

Table 1. KSA screening of IITA-SRRC maize breeding materials which identified 2 closely related lines (87.5% genetic similarity), #22 and #25, from parental cross (GT-MASgk x Ku1414SR) x GT-MAS:gk; these contrast significantly in aflatoxin accumulation. Values followed by the same letter are not significantly different by the least significant difference test (P = 0.05).

4.2. Recent breeding efforts

Recent breeding efforts towards the development of aflatoxin-resistant maize lines has resulted in a number of germplasm releases including the above-mentioned IITA-SRRC inbreds. In 2008, TZAR 101-106, derived from a combination of African and southern-adapted U.S. lines are being field-tested in different parts of the Southern U.S. (Figure 1) [80]. These have also exhibited resistance to lodging and common foliar diseases. GT-603 was released in 2011, after having been derived from GT-MAS:gk [81], while Mp-718 and Mp-719 were released as southern adapted resistant lines which are both shorter and earlier than previous Mp lines [82, 83]. These lines are also being tested as inbreds and in hybrid combinations in the southern U.S. [83].

Figure 1. Inoculation of maize ears with *Aspergillus flavus* spores using a 'side needle' wound technique for field evaluations of TZAR lines developed through IITA-SRRC program.

5. Conclusion

The host resistance approach to eliminating aflatoxin contamination of maize has been advanced forward by the identification/development of maize lines with resistance to aflatoxin accumulation. However, to fully exploit the resistance discovered in these lines, markers must be identified to transfer resistance to commercially useful backgrounds. Towards this goal numerous investigations have been undertaken to discover the factors that contribute to resistance, laying the basis for exploiting these discoveries as well.

These investigations include QTL analyses to locate regions of chromosomes associated with the resistant phenotype, and the discovery of kernel resistance-related traits. We now know that there are two levels of resistance in kernels, pericarp and subpericarp. Also, there is a two-phased kernel resistance response to fungal attack: constitutive at the time of fungal attack and that which is induced by the attack. Thus far, it's been demonstrated that natural resistance mechanisms discovered are antifungal in nature as opposed to inhibiting the aflatoxin biosynthetic pathway.

One of the most important discoveries, thus far, has been that of resistance-associated proteins or RAPs. Due to the significance of the constitutive response, constitutive RAPs were investigated first, although induced proteins are being studied as well. Investigations of other tissues such as rachis and silks begin to provide a more complete picture of the maize resistance response to aflatoxigenic fungi. RAP characterization studies provide greater evidence that these proteins are important to resistance, although clearly, more investigations are needed. Looking at data collectively that's been obtained from different types of studies may enhance the identification of markers for breeding. A good example of this may be the supporting evidence provided by QTL data to proteomic and RAP characterization data suggesting the involvement of 14 kDa TI, water stress inducible protein, zeamatin, heat shock, cold-regulated, glyoxalase I, cupin-domain and PR10 proteins in aflatoxin-resistance. It will be interesting to determine if this marker discovery approach can lead to the successful transfer of a multigene-based and quantitative phenomenon such as aflatoxin-resistance to commercially-useful genetic backgrounds.

Acknowledgements

Author details

Robert L. Brown[1*], Deepak Bhatnagar[1], Thomas E. Cleveland[1], Zhi-Yuan Chen[2] and Abebe Menkir[3]

*Address all correspondence to: Robert.brown@ars.usda.gov

1 USDA-ARS, Southern Regional Research Center, New Orleans, LA, USA

2 Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA, USA

3 International Institute of Tropical Agriculture, Ibadan, Nigeria

References

[1] Brown RL., Bhatnagar D., Cleveland TE., Cary JW. Recent Advances in Preventing Mycotoxin Contamination. In: Sinha KK., Bhatnagar D. (eds) Mycotoxins in Agriculture and Food Safety, Marcel Dekker: New York, NY, USA; 1998. p351-379.

[2] Dorner JW., Cole RJ., Wicklow DT. Aflatoxin Reduction in Maize through Field Application of Competitive Fungi. Journal of Food Protection 1999; 62 650–656.

[3] CAST. Aflatoxins and Other Mycotoxins: An Agricultural Perspective CAST Report No. 80; Council for Agricultural Science and Technology: Ames, IA; 1979.

[4] Diener UL., Cole RJ., Sanders TH., Payne GA., Lee LS. Epidemiology of Aflatoxin Formation by *Aspergillus flavus*. Annual Review of Phytopathology 1987; 25 249–270.

[5] Haumann F. Eradicating Mycotoxins in Food and Feeds. Inform 1995; 6 248–256.

[6] Gong YY., Cardwell K., Hounsa A., Egal S., Turner PC., Hall AJ.,Wild CP. Dietary Aflatoxin Exposure and Impaired Growth in Young Children from Benin and Togo: cross sectional study. British Medical Journal 2002; 325 20–21.

[7] Probst C., Njapau H., Cotty PJ. Outbreak of an Acute Aflatoxicosis in Kenya in 2004: Identification of the Causal Agent. Applied and Environmental Microbiology 2007; 73 2762–2764.

[8] Gardner CAC., Darrah LL., Zuber MS., Wallin JR. Genetic Control of Aflatoxin Production in Maize. Plant Disease 1987;71 426–429.

[9] King SB., Scott GE. Screening Maize Single Crosses for Resistance to Preharvest Infection of Kernels by *Aspergillus flavus*. Phytopathology 1982;72 942.

[10] Widstrom NW., McMillian WW., Wilson DM. Segregation for Resistance to Aflatoxin Contamination Among Seeds on an Ear of Hybrid Maize. Crop Science 1987;27 961–963.

[11] Scott GE., Zummo N. Sources of Resistance in Maize to Kernel Infection by *Aspergillus flavus* in the Field. Crop Science 1988;28 505–507.

[12] Campbell KW., White DG. (1995). Evaluation of Maize Genotypes for Resistance to Aspergillus Ear Rot, Kernel Infection, and Aflatoxin Production. Plant Disease 1995;79 1039–1045.

[13] Brown RL., Cotty PJ., Cleveland TE., Widstrom NW. (1993). Living Maize Embryo Influences Accumulation of Aflatoxin in Maize Kernels. Journal of Food Protection 1993; 56 967–971.

[14] Brown RL., Cleveland TE., Payne GA., Woloshuk CP., Campbell KW., White DG. 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 1995; 85 983–989.

[15] King SB., Wallin JR. Methods for screening corn for resistance to kernel infection and aflatoxin production by Aspergillus flavus. In:Diener, UL., Asquith RL., Dickens JW. (eds) Aflatoxin and *Aspergillus flavus* in Corn (Southern Cooperative Bulletin 279). Auburn University, Alabama, 1983; p77.

[16] Tucker DH Jr., Trevathan LE., King SB., Scott GE. Effect of Four Inoculation Techniques on Infection and Aflatoxin Concentration of Resistant and Susceptible Corn Hybrids Inoculated with *Aspergillus flavus*. Phytopathology 1986; 76 290-293.

[17] Payne GA. Aflatoxin in Maize. Critical Reviews in Plant Science 1992; 10 423-440.

[18] Shotwell O. Aflatoxin detection and determination in corn. In: Diener UL.,Asquith RL., Dickens JW. (eds) Aflatoxin and *Aspergillus flavus* in Corn (Southern Cooperative Bulletin 279)., Auburn University, Alabama, 1983; p38.

[19] Windham GL, Williams WP. *Aspergillus flavus* Infection and Aflatoxin Accumulation in Resistant and Susceptible Maize Hybrids. Plant Disease 1998; 82 281-284.

[20] White DG., Rocheford TR., Kaufman B., Hamblin AM. Chromosome regions associated with resistance to *Aspergillus flavus* and inhibition of aflatoxin production in maize. In: Proceedings of USDA-ARS Aflatoxin Elimination Workshop, Atlanta, GA, USA, 1995; p.8.

[21] White DG., Rocheford TR., Naidoo G., Paul C., Hamblin AM. Inheritance of molecular markers associated with, and breeding for resistance to Aspergillus Ear Rot and aflatoxin production in corn using Tex6. In: Proceedings of USDA-ARS Aflatoxin Elimination Workshop, St. Louis, MO, USA, 1998; pp. 4–6.

[22] Paul C., Naidoo G., Forbes A., Mikkilineni V., White D., Rocheford T. Quantitative Trait Loci for Low Aflatoxin Production in Two Related Maize Populations. Theoretical and Applied Genetics 2003; 7 263–270.

[23] Busboom KN. White DG. Inheritance of Resistance to Aflatoxin Production and Aspergillus Ear Rot of Corn from the Cross of Inbreds B73 and Oh516. Phytopathology 2004; 94 1107–1115.

[24] Brooks TD., Williams WP., Windham GL., Wilcox MC., Abbas H. Quantitative Trait Loci Contributing Resistance to Aflatoxin Accumulation in Maize Inbred Mp313E. Crop Science 2005; 45 171–174.

[25] Davis GL., Windham GL., Williams WP. QTL for Aflatoxin Reduction in Maize. Maize Genetics Conference Abstracts 2000; 41 139.

[26] Warburton ML., Brooks TD., Krakowsky MD., Shan X., Windham GL., Williams WP. Identification and Mapping of New Sources of Resistance to Aflatoxin Accumulation in Maize. Crop Science 2009; 49 1403–1408.

[27] Warburton ML., Brooks TD., Windham GL., Williams WP. Identification of Novel QTL Contributing Resistance to Aflatoxin Accumulation in Maize. Molecular Breeding 2011; 27 491-499.

[28] Brown RL., Chen Z-Y., Luo M., Menkir A., Fakhoury A., Bhatnagar D. Discovery and Characterization of Proteins Associated with Aflatoxin-Resistance: Building a Case for Their Potential as Breeding Markers. Toxins 2010 2(4) 919-933.

[29] Chen Z-Y., Brown R L., Menkir A., Cleveland TE. Identification of Resistance-Associated Proteins in Closely-Rrelated Maize Lines Varying in Aflatoxin Accumulation. Molecular Breeding 2012; 30 53-68.

[30] Guo BZ., Russin JS., Cleveland TE., Brown RL., Widstrom NW. Wax and Cutin Layers in Maize Kernels Associated with Resistance to Aflatoxin Production by *Aspergillus flavus*. Journal of Food Protection 1995; 58 296-300.

[31] Gembeh SV., Brown RL., Grimm C., Cleveland TE. Identification of Chemical Components of Corn Kernel Pericarp Wax Associated with Resistance to *Aspergillus flavus* Infection and Aflatoxin Production. Journal of Agricultural and Food Chemistry 2001; 49 4635-4641.

[32] Russin JS., Guo BZ., Tubajika KM., Brown RL., Cleveland TE., Widstrom NW. Comparison of Kernel Wax from Corn Genotypes Resistant or Susceptible to *Aspergillus flavus*. Phytopathology 1997; 87 529-533.

[33] Brown RL., Cleveland TE., Payne GA., Woloshuk CP., White DG. Growth of an *Aspergillus flavus* Transformant Expressing *Escherichia coli* ß- glucuronidase in Maize Kernels Resistant to Aflatoxin Production. Journal of Food Protection 1997; 60 84-87.

[34] Brown RL., Brown-Jenco CS., Bhatnagar D., Payne GA. Construction and preliminary evaluation of an *Aspergillus flavus* reporter gene construct as a potential tool for screening aflatoxin resistance. Journal of Food Protection 2003a; 66 1927-1931.

[35] Brown-Jenco C S., Brown R L., Bhatnagar D., Payne G A. 1998, Use of an omtA(p)::GUS reporter construct to evaluate corn for resistance to aflatoxin accumulation. Proceedings of the USDA-ARS Aflatoxin Elimination Workshop, St. Louis, 1998; pp. 23.

[36] Payne GA. 1997. Characterization of inhibitors from corn seeds and the use of a new reporter construct to select corn genotypes resistant to aflatoxin accumulation. Proceedings of the USDA-ARS Aflatoxin Elimination Workshop, Memphis, TN, 1997; pp 66-67.

[37] Brown RL., Cleveland T E., Woloshuk CP., Payne GA., Bhatnagar D. Growth Inhibition of a *Fusarium verticillioides* GUS Strain in Corn Kernels of Aflatoxin -Resistant Genotypes. Applied Microbiology and Biotechnology 2001a; 57 708-711.

[38] Rajasekaran K., Cary JW., Cotty PJ., Cleveland TE. Development of a GFP-Expressing *Aspergillus flavus* Strain to Study Fungal Invasion, Colonization, and Resistance in Cottonseed. Mycopathologia 2008; 165 89-97.

[39] Guo BZ., Brown RL., Lax AR., Cleveland TE., Russin JS., Widstrom NW. Protein Profiles and Antifungal Activities of Kernel Extracts from Maize Genotypes Resistant and Susceptible to *Aspergillus flavus*. Journal of Food Protection 1998; 61 98–102.

[40] Guo BZ., Chen Z-Y., Brown RL., Lax AR., Cleveland TE., Russin JS., Mehta A.D., Seli-trennikoff CP., Widstrom NW. Germination Induces Accumulation of Specific Proteins and Antifungal Activities in Maize Kernels. Phytopathology 1997; 87 1174–1178.

[41] Huang Z., White DG., Payne GA. Maize Seed Proteins Inhibitory to *Aspergillus flavus* and Aflatoxin Biosynthesis. Phytopathology 1997; 87 622–627.

[42] Ji C., Norton RA., Wicklow DT., Dowd PE. Isoform Patterns of Chitinase and β-1,3-glucanase in Maturing Maize Kernels (*Zea mays* L.) Associated with *Aspergillus flavus* Milk Stage Infection. Journal of Agricultural and Food Chemistry 2000; 48 507–511.

[43] Chen Z-Y., Brown RL., Lax AR., Guo BZ., Cleveland TE., Russin JS. Resistance to *Aspergillus flavus* in Maize Kernels is Associated with a 14 kDa Protein. Phytopathology 1998; 88 276–281.

[44] Chen Z-Y., Brown RL., Russin JS., Lax AR., Cleveland TE (1999b). A Maize Trypsin Inhibitor with Antifungal Activity Inhibits *Aspergillus flavus* α-amylase. Phytopathology 1996b; 89 902–907.

[45] Woloshuk CP., Cavaletto JR., Cleveland TE. Inducers of Aflatoxin Biosynthesis from Colonized Maize Kernels are Generated by an Amylase Activity from *Aspergillus flavus*. Phytopathology 1997; 87 164–169.

[46] Chen Z-Y., Brown RL., Lax AR., Cleveland TE., Russin JS. Inhibition Plant Pathogenic Fungi by a Maize Trypsin Inhibitor Over-Expressed in *Escherichia coli*. Applied and Environmental Microbiology 1999a; 65 1320–1324.

[47] Chen ZY., Brown RL., Cleveland TE., Damann KE., Russin JS. Comparison of Constitutive and Inducible Maize Kernel Proteins of Genotypes Resistant or Susceptible to Aflatoxin Production. Journal of Food Protection 2001; 64 1785–1792.

[48] Gorg A., Postel W., Gunther S., Weser J., Improved Horizontal Two-Dimensional Electrophoresis with Hybrid Isoelectric Focusing in Immobilized pH Gradients in the First Dimension and Laying-On Transfer to the Second Dimension. Electrophoresis 1985; 6 599-604.

[49] Gorg A., Postel W., Gunther S.,The Current State of Two-Dimensional Electrophoresis with Immobilized pH Gradients. Electrophoresis 1988; 9 531-546.

[50] Pennington SR., Wilkins MR., Hochstrasser DF., Dunn MJ. Proteome Analysis: From Protein Characterization to Biological Function. Trends in Cell Biology 1997; 7 168-173.

[51] Wilkins MR., Pasquali C., Appel RD., Ou K., Golaz O., Sanchez JC., Yan JX., Gooley AA., Hughes G., Humphrey-Smith I., Williams KL., Hochstrasser DF. From Proteins to Proteomes: Large-Scale Protein Identification by Two-Dimensional Electrophoresis and Amino Acid Analysis. Biotechnology 1996; 14 61-65.

[52] Chen Z-Y., Brown RL., Damann KE., Cleveland TE. 2000. Proteomics analysis of kernel embryo and endosperm proteins of corn genotypes resistant or susceptible to *As-*

pergillus flavus infection. In: Proceedings of the USDA-ARS Aflatoxin Elimination Workshop, 2000; pp. 88.

[53] Chen Z-Y., Brown R.L., Damann K.E., Cleveland T.E. Identification of Unique or Elevated Levels of Kernel Proteins in Aflatoxin-Resistant Maize Genotypes through Proteome Analysis. Phytopathology 2002; 92 1084–1094.

[54] Brown RL., Chen Z-Y., Menkir A., Cleveland TE. Using biotechnology to enhance host resistance to aflatoxin contamination of corn. African Journal of Biotechnology 2003b; 2 557-562.

[55] Payne GA. Process of Contamination by Aflatoxin-Producing Fungi and Their Impact on Crops. In: Sinha KK., Bhatnagar D. (eds), Mycotoxins in Agriculture and Food Safety. Marcel Dekker, New York, NY, USA, 1998; pp. 279-306.

[56] Thomann E.B., Sollinger J., White C., Rivin C.J. Accumulation of Group 3 Late Embryogenesis Abundant Proteins in *Zea mays* Embryos. Plant Physiology 1992; 99 607–614.

[57] Xu D., Duan X., Wang B., Hong B., Ho THD., Wu R. Expression of a Late Embryogenesis Abundant Protein Gene HVA1, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice. Plant Physiology 1996; 110 249–257.

[58] Veena V., Reddy S., Sopory SK. Glyoxalase I from Brassica juncea: Molecular Cloning, Regulation and Its Over-expression Confer Tolerance in Transgenic Tobacco under Stress. Plant Journal 1999; 17 385–395.

[59] Chen ZY., Brown RL., Damann KE., Cleveland TE. Identification of a Maize Kernel Stress-Related Protein and Its Effect on Aflatoxin Accumulation. Phytopathology 2004; 94 938–945.

[60] Chen ZY., Brown RL., Damann KE., Cleveland TE. Identification of Maize Kernel Endosperm Proteins Associated with Resistance to Aflatoxin Contamination by *Aspergillus flavus*. Phytopathology 2007; 97 1094–1103.

[61] Chen Z.Y., Brown RL., Rajasekaran K., Damann KE., Cleveland TE. Evidence for Involvement of a Pathogenesis-Related Protein in Maize Resistance to *Aspergillus flavus* Infection/Aflatoxin Production. Phytopathology 2006; 96 87–95.

[62] Xie YR., Chen Z-Y., Brown RL., Bhatnagar D. Expression and Functional Characterization of Two Pathogenesis-Related Protein10 Genes from *Zea mays*. Journal of Plant Physiology 2010; 167 121–130.

[63] Steiner-Lange S., Fischer A., Boettcher A., Rouhara I., Liedgens H., Schmelzer E., Knogge W. Differential Defense Reactions in Leaf Tissues of Barley in Response to infection by *Rhynchosporium secalis* and to Treatment with a Fungal Avirulence Gene Product. Molecular and Plant-Microbe Interactions 2003; 16 893–902.

[64] Mould M.J., Xu T., Barbara M., Iscove NN., Heath MC. cDNAs Generated from Individual Eepidermal Cells Reveal that Differential Gene Expression Predicting Subse-

quent Resistance or Susceptibility to Rust Fungal Infection Occurs Prior to the Fungus Entering the Cell Lumen. Molecular and Plant-Microbe Interactions 2003; 16 835–845.

[65] McGee JD., Hamer JE., Hodges TK. Characterization of a PR-10 Pathogenesis-Related Gene Family Induced in Rice during Infection with *Magnaporthe grisea*. Molecular and Plant-Microbe Interactions 2001; 14 877–886.

[66] Chen Z-Y., Brown RL., Damann KE., Cleveland TE. PR10 Expression in Maize and Its Effect on Host Resistance against *Aspergillus flavus*Iinfection/Aflatoxin Production. Molecular Plant Pathology 2010; 11 69–81.

[67] Wesley SV., Helliwell CA., Smith NA., Wang MB., Rouse DT., Liu Q., Gooding PS., Singh SP., Abbott D., Stoutjesdijk PA. Robinson SP., Gleave AP., Green AG., Waterhouse PM. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant Journal 2001; 27 581–590.

[68] Fire A. Xu S., Montgomery MK., Kostas SA., Driver SE., Mello CC. Potent and Specific Genetic Interference by Double Stranded RNA in *Caenorhabditis elegans*. Nature 1998; 391 806–811.

[69] Gura T. A silence that speaks volumes. Nature 2000; 404 804–808.

[70] Chen Z-Y., Damann KE. Louisiana State University, Baton Rouge, LA; Brown RL., Cleveland TE. USDA-ARS-SRRC New Orleans, LA., Unpublished work. 2007.

[71] Baker R., Brown RL., Cleveland TE., Chen Z-Y., Fakhoury A. COR, a Maize Lectin-like Protein with Antifungal Activity against *Aspergillus flavus*. Journal of Food Protection 2009a; 72 120–127.

[72] Baker R., Brown RL., Cleveland TE., Chen Z-Y., Fakhoury A. ZmTI, a Maize Trypsin Inhibitor with Limited Activity against *Aspergillus flavus*. Journal of Food Protection 2009b; 72 85–188.

[73] Luo M., Brown R L., Chen Z-Y., Menkir A., Yu J., Bhatnagar D. Transcriptional Profiles Uncover *Aspergillus flavus* Induced Resistance in Maize Kernels. Toxins 2011; 3 766-785.

[74] Luo M., Liu J., Lee RD., Scully BT., Guo B. Monitoring the Expression of Maize Genes in Developing Kernels under Drought Stress Using Oligo-Microarray. Journal of Integrative Plant Biology 2010; 52(12) 1059-1074.

[75] Pechanova O., Pechan T., Williams, WP., Luthe DS. Proteomic Analysis of Maize Rachis: Potential Roles of Constitutive and Induced Proteins in Resistance to Aspergillus flavus Infection and Aflatoxin Contamination. Proteomics 2011; 11 114-127.

[76] Peethambaran B., Hawkins L., Windham GL., Williams WP., Luthe DS. Antifungal Activity of Maize Silk Proteins and Role of Chitinases in *Aspergillus flavus* Resistance. Toxin Reviews 2010; 29 27–39.

[77] Brown RL., Chen Z-Y., Menkir A., Cleveland T.E., Cardwell K., Kling, J., White D.G. Resistance to Aflatoxin Accumulation in Kernels of Maize Inbreds Selected for Ear Rot Resistance in West and Central Africa. Journal of Food Protection 2001b; 64 396–400.

[78] Menkir A., Brown RL., Bandyopadhyay R.., Chen Z-Y., Cleveland TE. A USA-Africa Collaborative Strategy for Identifying, Characterizing, and Developing Maize Germplasm with Resistance to Aflatoxin Contamination. Mycopathologia 2006; 162 225–232.

[79] Chen ZY., Brown RL., Menkir A., Damann KE., Cleveland TE. Proteome Analysis of Near Isogenic Maize Lines Differing in the Level of Resistance against *Aspergillus flavus* Infection/Aflatoxin Production. Phytopathology 2005; 95 S19.

[80] Menkir A., Brown RL., Bandyopadhyay R., Cleveland TE. Registration of Six Tropical Maize Germplasm Lines with Resistance to Aflatoxin Contamination. Journal of Plant Registrations 2008; 2 246–250.

[81] Guo BZ., Krakowsky MD., Ni X., Scully BT., Lee RD., Coy AE., Widstrom NW. Registration of Maize Inbred Line GT603. Journal of Plant Registrations 2011; 5(2) 211-214.

[82] Williams WP.,Windham GL. Registration of Mp718 and Mp719 Germplasm Lines of Maize. Journal of Plant Registrations 2012; 6 200-202.

[83] Scully BT., Guo BZ., Ni X., Williams WP., Henry WB., Krakowsky MD., Brown RL. Development of Aflatoxin and Insect Resistant Corn Inbreds Adapted to the Southern U.S. Proceedings of the Corn Utilization and Technology Workshop, Abstract (in press).