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5

Identification of Gene Markers in Aflatoxin-Resistant Maize Germplasm for Marker-Assisted Breeding

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1. Introduction

Aflatoxins, the toxic and highly carcinogenic secondary metabolites of *Aspergillus flavus*, *A. parasiticus* are the most widely investigated of all mycotoxins due to their role in establishing the significance of mycotoxins in animal diseases, and to the regulation of their presence in food (Brown et al., 1998; Dorner et al., 1999). Aflatoxins pose serious health hazards to humans and domestic animals, because they frequently contaminate agricultural commodities (CAST, 1979; Diener et al., 1987). Presently, numerous countries have established or proposed regulations for controlling aflatoxins in food and feeds (Haumann, 1995); 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 M_1 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 demonstrated 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 (Gong et al., 2002). Also, a 2004 outbreak of acute aflatoxicosis in Kenya, due to ingestion of contaminated maize, resulted in 125 deaths (Probst et al., 2007).

Recognition of the need to control aflatoxin contamination of food and feed grains has elicited various approaches from researchers to eliminate this toxin from maize and other susceptible crops. The approach to enhance host resistance through conventional or molecular breeding gained renewed attention following the discovery of natural resistance to *A. flavus* infection and aflatoxin production in maize (Gardner et al., 1987; King & Scott, 1982; Widstrom et al., 1987; Scott & Zummo, 1988; Campbell & White, 1995; Brown et al., 1995, 1999). During the past two decades, maize genotypes with natural preharvest resistance to aflatoxin production have been identified through field screening (Scott & Zummo, 1988; Campbell & White, 1995; Warner et al., 1992). However, 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 (Brown et al., 1993, 1995]. 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 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 [Brown et al., 1993, 1995). This assay has several advantages, as compared to traditional field screening techniques (Brown et al., 1995): 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.

One drawback to using the known resistant maize lines to develop commercial lines is their poor agronomic quality (Brown et al., 1999). To overcome this, markers need to be identified to facilitate the incorporation of aflatoxin-resistance into lines with commercially-acceptable genetic backgrounds. The expression of maize kernel proteins has been implicated in kernel resistance to *A. flavus* infection/aflatoxin production (Cordero et al., 1992, 1994; Guo, et al., 1996; Huang et al., 1997). Using reverse genetics to identify genes that are associated with aflatoxin-resistance may lead to the discovery of breeding markers. These protein/gene markers could be used to transfer resistance to good genetic backgrounds while excluding undesirable traits. The purpose of this review is to highlight the discovery of resistance-associated proteins (RAPs) and their potential as breeding markers.

2. Discovery of Resistance-Associated Proteins (RAPs)

The development of the KSA by Brown *et al.* (Brown et al., 1995) facilitated the verification of maize kernel resistance under laboratory conditions in a short time. This accelerated the discovery of knowledge surrounding host resistance mechanisms. Using this assay, Brown *et al.* (Brown et al., 1993) discovered the existence of subpericarp resistance in maize kernels and that the expression of this resistance requires a live embryo, the latter indicating a potential role for kernel proteins in resistance. Guo *et al.* (1996) found that imbibition of kernels, before inoculation with *A. flavus*, significantly increased aflatoxin-resistance of susceptible maize genotypes. Further investigation revealed that susceptible genotypes were able to induce antifungal proteins upon fungal infection (Guo et al., 1996), suggesting that susceptible lines have the ability to induce an active defense mechanism after fungal infection. The usefulness of the KSA as an investigative tool is aided by the fact that KSA results correlate well with field results (Brown et al., 1995) and that aflatoxin buildup occurs after kernel maturity, a developmental phase where constitutive factors required for kernel resistance are highlighted by the KSA (Brown et al., 1995). Agronomic factors contributing to resistance have to be evaluated during field trials.

Examination of kernel proteins of several maize genotypes revealed differences between genotypes resistant or susceptible to aflatoxin contamination (Guo et al., 1997, 1998). Imbibed susceptible kernels contained increased levels of germination-induced ribosome inactivating protein (RIP) and zeamatin; both proteins have demonstrated growth-inhibitory activity *in vitro* against *A. flavus* (Guo et al., 1998). In another study, two kernel proteins were identified from a resistant maize inbred line (Tex6), which may contribute to resistance to aflatoxin contamination (Huang et al., 1997). When a commercial maize hybrid was inoculated with toxgenic 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). Lozovaya et al., 1998) reported that the presence of *A. flavus* caused an increase in β-1,3-glucanase activity in callus tissues from a resistant genotype, but not from a susceptible one. A more rapid and stronger induction of the PR-1 and PR-5 genes in maize leaves has also been observed in an incompatible interaction when compared to a compatible interaction upon pathogen infection (Morris et al., 1998). A 14 kDa trypsin inhibitor protein (TI) was found to express at high levels in resistant lines but at low levels or is missing in susceptible ones (Chen et al., 1998). This protein demonstrated antifungal activity against *A. flavus* and several other pathogenic fungi (Chen et al., 1999a), possibly through inhibition of fungal α-amylase activity and production (Chen et al., 1999b). This could limit the availability of simple sugars needed for fungal growth and aflatoxin production (Woloshuk et al., 1997).

The above-studies indicate an important role for kernel proteins in disease resistance. Further investigation, supporting earlier work by Guo (1996), found that both constitutive and inducible proteins are required for kernel resistance to *A. flavus* infection and aflatoxin production (Chen et al., 2001). In fact, one major difference between resistant and susceptible genotypes is that resistant lines constitutively produce higher levels of antifungal proteins compared to susceptibles. Therefore, research on resistance genes/proteins has focused heavily on the identification of constitutively-produced kernel resistance-associated proteins or RAPs.

2.1 Using comparative proteomics for RAP discovery

To assist in the further identification of RAPs, proteomics approaches have been employed. This increased protein resolution and detection sensitivity by 10 to 20 fold over conventional approaches and, thus, enhanced ability to identify more constitutively-expressed RAPs. Kernel proteins from several resistant and susceptible genotypes were compared using large format 2-D gel electrophoresis. A number of protein spots, either unique or 5-fold up-regulated in resistant lines, were detected, isolated from preparative 2-D gels and identified using ESI-MS/MS after in-gel digestion with trypsin (Chen et al., 2002, 2007a). 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 (GLX I) and heat shock proteins, and (3) antifungal proteins, including TI. In total, 21 proteins upregulated in resistant versus susceptible lines have been identified using comparative proteomics (Table 1).

Antifungals	**Stress-related**	**Storage**
Zeamatin	Aldose reductase (ALD)	Globulin I
Trypsin Inhibitor 14 kD	Cold-regulated (ZmCORp)	Globulin II
Trypsin inhibitor 10 kD	Water stress inducible (WSI)	Cupin domain (Zmcup)
Ribosome inactivating (RIP)	Anionic peroxidase	Late embryogenesis (LEAIII)
B-1,3,-glucanase	Small heat shock protein	LEA 14
PR 10	Glyoxalase (GLX I)	
PR 10.1	Peroxiredoxin (PER1)	

Table 1. Resistance-associated proteins (RAPs) identified by proteomics[1,2]

No investigation has been conducted 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 maize kernels (Payne, 1998). Unique or higher levels of hydrophilic storage or stress-related proteins, such as the aforementioned, may put resistant lines at an advantage for the ability to synthesize proteins and defend against pathogens while under stress. Further studies including physiological and biochemical characterization, genetic mapping, plant transformation using RAP genes, RNAi gene silencing experiments and marker-assisted breeding should clarify the roles of stress-related RAPs in kernel resistance (Brown et al., 2003).

To conduct the above-described comparative proteomics studies, composite profiles for resistance and for susceptibility were developed from 2 D gels of several resistant or susceptible maize lines. This was done to homogenize nonresistance-related differences among lines within each group, and, therefore, facilitate the identification of resistance-related proteins. In using the composite gel approach, only those proteins that were five-fold upregulated in resistant versus susceptible lines were studied to minimize the chance of identifying proteins unrelated to host resistance.

An advancement in the aforementioned 2-D approach is the use of difference gel electrophoresis or DIGE in RAP discovery (Figure 1) (Luo et al., 2010). This represents an advancement because it eliminates side-by-side gel comparisons and therefore, gel-to-gel variability. Protein samples extracted from frozen embryo or endosperm are run on the same gel with an internal reference using CyDye DIGE fluorescent dyes for labeling (Minden et al., 2009). Treated samples are labeled by Cy5, control by Cy3 and the internal standard by Cy2. The internal standard consists of pooled protein from controls and treated samples. For IEF, Cy2-, Cy3-, and Cy5- labeled samples are mixed with an appropriate buffer and applied to an Immobiline DryStrip (pH 3-10). After the second dimension is completed, the CyDye-labeled gels are visualized using a fluorescent Image Reader. Other advantages of DIGE are that gel staining is unnecessary, quantifying protein spots using DIGE is easier and more accurate than the standard 2 D approach, and identified proteins of interest can be recovered from the same 2-D gel and used directly for downstream MS/MS analysis for mass finger printing or *de novo* peptide sequencing.

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[1] Table is adaptation and updated version of table from Cary et al., 2009 reference.

[2] Each protein was up-regulated in aflatoxin-resistant versus –susceptible lines.

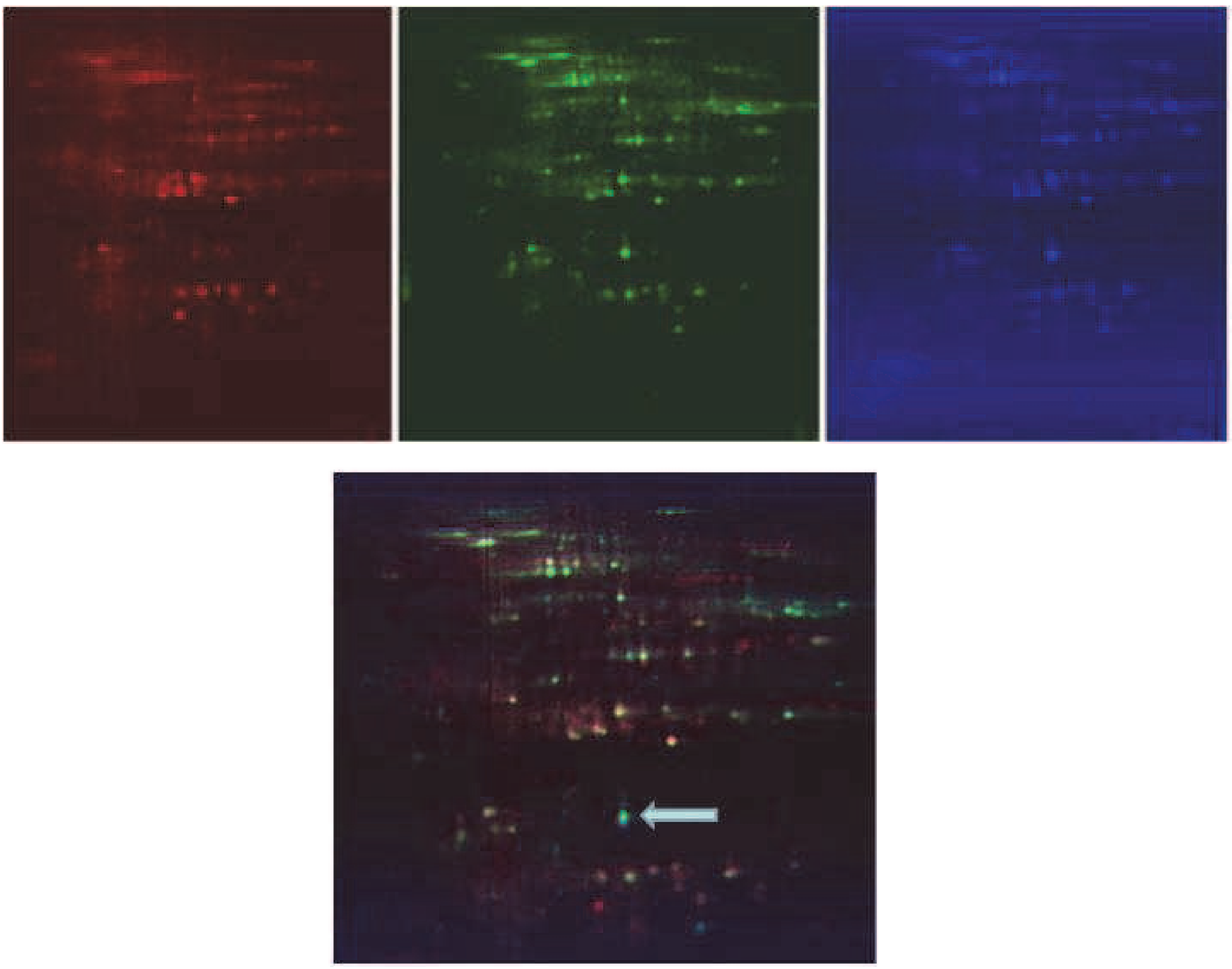

Fig. 1. DIGE experiment involving two closely-related maize lines varying in aflatoxin accumulation. **R**= resistant; **S**= susceptible. Red image: probe labeled by Cy3 = **S**. Green image: probe labeled by Cy5 = **R**; Blue image: probe labeled by Cy2 = reference. Bottom image: Images overlapped highlighting Heat Shock Protein 17.2 (arrow), 6.5 fold more abundant in **R** sample vs. **S** sample.

2.1.1 Employing closely-related breeding lines to enhance RAP discovery

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 New Orleans (SRRC) facilitated the identification of closely-related lines from the same backcross differing significantly in aflatoxin accumulation, and proteome analysis of these lines has been conducted (Menkir, et al., 2006; Brown et al., 2001; Chen et al., 2005, 2011). Investigating maize 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 the U.S. resistant inbred lines with those of African lines, originally selected for resistance to ear rot diseases and for demonstrated potential aflatoxin-resistance (*via* KSA) (Menkir et al., 2006; Brown et al., 2001). 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 US resistant maize lines in Ibadan, Nigeria. The F1 crosses were backcrossed to their respective US inbred lines and self-pollinated thereafter. The resulting lines were selected through the S4 generation for resistance to foliar diseases and for desirable agronomic characteristics under conditions of severe natural infection in their respective areas of adaptation. Promising S5 lines were screened with the KSA and five pairs of closely-related lines were shown to be significantly different in aflatoxin resistance, while sharing as high as 97% genetic similarity (Chen et al., 2005, 2011). 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 proteins 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 (Chen et al., 2011). 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 recently registered and released six inbred lines with aflatoxin-resistance in good agronomic backgrounds, which also demonstrate good levels of resistance to southern maize blight and southern maize rust (Menkir et al., 2008). Resistance field trials for these lines on U.S. soil will be conducted; the ability to use resistance in these lines commercially will depend on having 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.

2.1.2 Proteomic investigation of maize rachis and silk tissues

A study was conducted to investigate the proteome of rachis tissue, maternal tissue that supplies nutrients to kernels (Pechanova, 2006). 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 support findings of Chen *et al*. (2001), who demonstrated that higher constitutive 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 [Peethambaran et al., 2010). Antifungal bioassays were performed using silk extracts from two aflatoxin-resistant and two–susceptible inbred lines. Silk extracts from resistant inbreds showed greater anti-fungal 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 between resistant and susceptible 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. Characterization of RAPs

A literature review of the RAPs that have been identified 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 (Table 2) 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 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 (Chen et al., 2004). 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 (Chen et al., 2007a), was demonstrated to be an abundant peroxidase (Table 2), 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 growing under stress (Chen et al., 2007a).

Another RAP that has been characterized further is the pathogenesis-related protein 10 (PR10) (Table 2). 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 [48]. 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). Recently, a new *PR10* homologue was identified from maize (*PR10.1*) (Xie et al., 2010). *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* (Xie et al., 2010). 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 (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).

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 (Chen et al., 2004b, 2010). The degree of silencing using RNAi constructs is greater than that obtained using either co-suppression or antisense constructs, especially when an intron is included (Wesley et al., 2001). Interference of double-stranded RNA with expression of specific genes has been widely described (Fire et al., 1998; Gura, 2000). Although the mechanism is still not well understood, RNAi provides an extremely powerful tool to study functions of unknown genes in many organisms. This posttranscriptional gene silencing (PTGS) is a sequence-specific RNA degradation process triggered by a dsRNA, which propagates systemically throughout the plant, leading to the degradation of homologous RNA encoded by endogenous genes, and transgenes.

Both particle bombardment and *Agrobacterium*-mediated transformation methods were used to introduce the RNAi vectors into immature maize embryos. The former was used to provide a quick assessment of the efficacy of the RNAi vector in gene silencing. The latter, which can produce transgenic materials with fewer copies of foreign genes and is easier to regenerate, was chosen for generating transgenic kernels for evaluation of changes in aflatoxin-resistance. It was demonstrated using callus clones from particle bombardment that *PR10* expression was reduced by an average of over 90% after the introduction of the RNAi vector Chen et al., 2010). 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 (Chen et al., 2010).

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., 2007b). 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 (Table 2) (Baker et al., 2009a). When tested against *A. flavus*, ZmCORp inhibited germination of conidia by 80% and decreased mycelial growth by 50%. 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 (Baker et al., 2009b).

3.1 Mapping genes

Chromosome regions associated with resistance to *A. flavus* infection 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 (White et al., 1995, 1998). Chromosome regions associated with inhibition of aflatoxin in studies considering all 3 resistant lines demonstrated that there are some chromosome regions in common. Regions on chromosome arms 2L, 3L, 4S, and 8S may prove promising for improving resistance in commercial lines through marker assisted breeding (White et al., 1998). In some cases, chromosomal regions were associated with resistance to *Aspergillus* ear rot and not aflatoxin inhibition, and vice versa, whereas other chromosomal regions 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 (Paul et al., 2003). 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) (Busboom & White, 2004). QTLs contributing resistance to aflatoxin accumulation were also identified using a population created by B73 and resistant inbred Mp313E, on chromosome 4 of Mp313E (Brooks et al., 2005). This confirmed the findings of an earlier study involving Mp313E and susceptible Va35 (Davis et al., 2000). Another QTL in this study, which has similar effects to that on chromosome 4, was identified on chromosome 2 (Brooks et al., 2005). 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 US. QTL were identified on all chromosomes, except 4, 6, and 9; individual QTL accounted for up to 11% of phenotypic variance in aflatoxin accumulation (Warburton et al., 2009).

RAP Gene	**Activity vs. A. flavus**	**Resistance-related enzyme rx**	**Mapping Bin**	**Other**
Heat Shock a	nda	nda	1.03	
PR – 10	+[1]	Rnase	1.03	knockout=Suscept
TI-14 kDa	+high	Inhib. trypsin	2.06	Inhib. amylase
WSI	nda	nda	3.07	
Zeamatin	+low	Inhib. Trypsin	7.04	
Heat Shock b	nda	nda	8.01	
ZmCorp	+	Lectin	8.04	
GLXI	nda	Forms D-lactate	10.3	knockout
RIP	+	lytic	nda	
PER 1	nda	peroxidase	nda	
B-1,3 glucanase	+	glucanase	nda	

Table 2. Evidence supporting the candidacy of selected RAPs as breeding markers[1]

A number of RAP genes identified in the proteomics studies have been mapped to chromosomal location (Table 2) using the genetic sequence of B73 now available online

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[1] + denotes presence of activity; nda denotes no data available.

(http://archive.maizesequence.org/index.html). 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 (Brown et al., 2010). The chromosomes involved include the above-mentioned chromosomes 1, 2, 3, 7, 8 and 10, some in bins closely located to those described above. This adds support to proteomic data and characterization results that suggest the involvement of 14 kDa TI, water stress inducible protein, zeamatin, one of the heat shock, cold-regulated, glyoxalase I and PR10 proteins in aflatoxin-resistance. From the above QTL investigations, it is observed that variation can exist in the chromosomal regions associated with *Aspergillus* ear rot and aflatoxin inhibition in different mapping populations. This suggests the presence of different genes for resistance in the different identified resistant germplasm. It will be important to map resistant lines investigated through proteomics or to obtain data from associative mapping panels regarding gene location.

4. Conclusion

Host resistance as a strategy for eliminating aflatoxin contamination of maize is closer to being a reality due to the identification of genotypes with natural resistance to aflatoxin accumulation and the development of new inbred lines through breeding. However, to exploit this resistance for the benefit of maize growers, markers have to be identified to facilitate the transfer of resistance to elite proprietary backgrounds that have commercial value. The identification of resistance-associated proteins goes a long way towards providing the novel markers that will be indispensible to any commercial breeding undertaking. Characterization studies including RNAi gene silencing and gene mapping are instrumental in building a case for the involvement of selected RAPs in kernel resistance to aflatoxin contamination.

Here, a listing of RAPs identified through comparative proteomics is presented along with evidence of the potential of selected RAPs as breeding markers. Investigations of RAPs, as discussed above, not only impact the development of commercially-useful resistant maize lines, but provide an expanding base of knowledge concerning nature’s requirements for creating a durable resistance against the opportunistic pathogen, *A. flavus*. It remains to be determined, how the different categories of proteins, antifungal, stress-related, storage and others contribute to the total picture of resistance. Future investigations (e.g., proteomics and microarray analysis) may also impact aflatoxin-resistance through the discovery of RAPs down-regulated in resistant lines, RAPs induced upon fungal infection and also factors involved in the regulation of RAPs. These discoveries will not only contribute to the development of aflatoxin-resistant maize lines, they may aid other susceptible crops and assist in meeting the challenges of other mycotoxin-producing fungi, while enhancing our understanding of host plant interactions with fungi.

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6. References

Baker, R., Brown, R.L., Cleveland, T.E., Chen, Z.-Y. & Fakhoury, A. (2009a). COR, a maize lectin-like protein with antifungal activity against *Aspergillus flavus*. *Journal of Food Protection,* Vol. 72, pp. 120–127

Baker, R., Brown, R.L., Cleveland, T.E., Chen, Z.-Y. & Fakhoury, A. (2009b). ZmTI, a maize trypsin inhibitor with limited activity against *Aspergillus flavus*. *Journal of Food Protection,* Vol. 72, pp. 85–188

Brooks, T.D., Williams, W.P., Windham, G.L., Wilcox, M.C. & Abbas, H. (2005). Quantitative trait loci contributing resistance to aflatoxin accumulation in maize inbred Mp313E. *Crop Science,* Vol. 45, 171–174

Brown, R.L., Cotty, P.J., Cleveland, T.E. & Widstrom, N.W. (1993). Living maize embryo influences accumulation of aflatoxin in maize kernels. *Journal of Food Protection,* Vol. 56, 967–971

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

Brown, R.L., Bhatnagar, D., Cleveland, T.E. & Cary, J.W. (1998). Recent advances in preventing mycotoxin contamination. In: *Mycotoxins in Agriculture and Food Safety*; K.K. Sinha, & D. Bhatnagar, (Eds), pp. 351-379, Marcel Dekker: New York, NY, USA

Brown, R.L., Chen, Z.-Y., Cleveland, T.E. & Russin, J.S. (1999). Advances in the development of host resistance to aflatoxin contamination by *Aspergillus flavus*. *Phytopathology,* Vol. 89, pp. 13–117

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

Brown, R.L., Chen, Z.-Y., Menkir, A. & Cleveland, T.E. (2003). Using biotechnology to enhance host resistance to aflatoxin contamination of maize—A Mini-review. *African Journal of Biotechnology,* Vol. 2, pp. 557–562

Brown, R.L., Chen, Z.-Y., Warburton, M., Luo, M., Menkir, A., Fakhoury, A. & Bhatnagar, D. (2010). Discovery and characterization of proteins associated with aflatoxin-resistance: evaluating their potential as breeding markers. *Toxins*, Vol. 2, pp. 919-933

Busboom, K.N. & White, D.G. (2004). Inheritance of resistance to aflatoxin production and Aspergillus ear rot of maize from the cross of inbreds B73 and Oh516. *Phytopathology,* Vol. 94, pp. 1107–1115

Campbell, K.W. & White, D.G. (1995). Evaluation of maize genotypes for resistance to aspergillus ear rot, kernel infection, and aflatoxin production. *Plant Disease,* Vol. 79, pp. 1039–1045

Cary, J.W., Rajasekaran, K., Yu, J., Brown, R.L., Bhatnagar, D. & Cleveland, T.E. (2009). Transgenic approaches for pre-harvest control of mycotoxin contamination in crop plants. *World Mycotoxin Journal,* Vol. 2, pp. 203–214

CAST. (1979). Aflatoxins and other mycotoxins: An agricultural perspective; CAST Report No. 80; Council for Agricultural Science and Technology: Ames, IA

Chen, Z.-Y., Brown, R.L., Lax, A.R., Guo, B.Z., Cleveland, T.E. & Russin, J.S. (1998). Resistance to *Aspergillus flavus* in maize kernels is associated with a 14 kDa protein. *Phytopathology,* Vol. 88, pp. 276–281

Chen, Z.-Y., Brown, R.L., Lax, A.R., Cleveland, T.E. & Russin, J.S. (1999a). Inhibition plant pathogenic fungi by a maize trypsin inhibitor over-expressed in *Escherichia coli*. *Applied and Environmental Microbiology,* Vol. 65, pp. 1320–1324

Chen, Z.-Y., Brown, R.L., Russin, J.S., Lax, A.R. & Cleveland, T.E. (1999b). A maize trypsin inhibitor with antifungal activity inhibits *Aspergillus flavus* α-amylase. *Phytopathology,* Vol. 89, 902–907

Chen, Z.Y., Brown, R.L., Cleveland, T.E., Damann, K.E. & Russin, J.S. (2001). Comparison of constitutive and inducible maize kernel proteins of genotypes resistant or susceptible to aflatoxin production. *Journal of Food Protection,* Vol. 64, pp. 1785–1792

Chen, Z.-Y., Brown, R.L., Damann, K.E. & Cleveland, T.E.(2002). Identification of unique or elevated levels of kernel proteins in aflatoxin-resistant maize genotypes through proteome analysis. *Phytopathology,* Vol. 92, pp. 1084–1094

Chen, Z.Y., Brown, R.L., Damann, K.E. & Cleveland, T.E.(2004). Identification of a maize kernel stress-related protein and its effect on aflatoxin accumulation. *Phytopathology,* Vol. 94, pp. 938–945

Chen, Z.Y., Brown, R.L., Cleveland, T.E. & Damann, K.E. (2004b). Investigating the roles of an aflatoxin resistance-associated protein in maize using RNAi. *Phytopathology,* Vol. 94, pp. S18

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

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

Chen, Z.Y., Brown, R.L., Damann, K.E. & Cleveland, T.E. (2007a) Identification of maize kernel endosperm proteins associated with resistance to aflatoxin contamination by *Aspergillus flavus*. *Phytopathology,* Vol. 97, pp. 1094–1103

Chen, Z.-Y.[1], Brown, R.L.[2], Damann, K.E.[1] & Cleveland, T.E. [2] (2007b). [1]Louisiana State University, Baton Rouge, LA; [2]USDA-ARS-SRRC, New Orleans, LA. Unpublished work

Chen, Z.-Y., Brown, R.L., Damann, K.E. & Cleveland, T.E. (2010). PR10 expression in maize and its effect on host resistance against *Aspergillus flavus* infection/aflatoxin production. *Molecular Plant Pathology,* Vol. 11, pp. 69–81

Chen, Z.-Y., Brown, R. L., Menkir, A. & Cleveland, T.E. (2011) Identification of resistance-associated proteins in closely- related maize lines varying in aflatoxin accumulation. *Molecular Breeding*, Vol. 27, DOI 10. 1007/s11032-011-9597-3

Cordero, M.J., Raventós, D. & San Segundo, B. (1992). Induction of PR proteins in germinating maize seeds infected with the fungus *Fusarium moniliforme*. *Physiological. and Molecular Plant Pathology,* Vol. 41, pp. 189–200

Cordero, M.J., Raventos, D. & San Segundo, B. (1994). Differential expression and induction of chitinases and β-1,3-glucanases in response to fungal infection during germination of maize seeds. *Molecular and Plant Microbe Interactions,* Vol. 7, pp. 23–31

Davis, G.L., Windham, G.L. & Williams, W.P. (2000) QTL for aflatoxin reduction in maize. *Proceedings of the Maize Genetics Conference Abstracts,* Vol. 41, pp. P139, Coeur d'Alene, Idaho, March, 2000

Diener, U.L., Cole, R.J., Sanders, T.H., Payne, G.A. & Lee, L.S. (1987). Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annual Review of Phytopathology,* Vol. 25, pp. 249–270

Dorner, J.W., Cole, R.J. & Wicklow, D.T. (1999). Aflatoxin reduction in maize through field application of competitive fungi. *Journal of Food Protection,* Vol. 62, pp. 650–656

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

Gardner, C.A.C., Darrah, L.L., Zuber, M.S. & Wallin, J.R. (1987). Genetic control of aflatoxin production in maize. *Plant Disease,* Vol. 71, pp. 426–429

Gong, Y.Y., Cardwell, K., Hounsa, A., Egal, S., Turner, P.C., Hall, A.J. & Wild, C.P. (2002). Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study. *British Medical Journal,* Vol. 325, pp. 20–21

Guo, B.Z., Russin, J.S., Cleveland, T.E., Brown, R.L. & Widstrom, N.W. (1996). Resistance to aflatoxin contamination in maize as influenced by relative humidity and kernel germination. *Journal of Food Protection,* Vol. 59, pp. 276–281

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

Guo, B.Z., Brown, R.L., Lax, A.R., Cleveland, T.E., Russin, J.S. & Widstrom, N.W. (1998). Protein profiles and antifungal activities of kernel extracts from maize genotypes resistant and susceptible to *Aspergillus flavus. Journal of Food. Protection,* Vol. 61, pp. 98–102

Gura, T. (2000). A silence that speaks volumes. *Nature,* Vol. 404, pp. 804–808, ISSN

Haumann, F. (1995). Eradicating mycotoxins in food and feeds. *Inform,* Vol. 6,pp. 248–256

Huang, Z., White, D.G. & Payne, G.A. (1997). Maize seed proteins inhibitory to *Aspergillus flavus* and aflatoxin biosynthesis. *Phytopathology,* Vol. *87*,pp. 622–627

Ji, C., Norton, R.A., Wicklow, D.T. & Dowd, P.E. (2000). 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,* Vol. 48, pp. 507–511

King, S.B. & Scott, G.E. (1982). Screening maize single crosses for resistance to preharvest infection of kernels by *Aspergillus flavus*. *Phytopathology,* Vol. *72*, pp. 942

Lozovaya, V.V., Waranyuwat, A. & Widholm, J.M. (1998). β-1,3-glucanase and resistance to *Aspergillus flavus* infection in maize. *Crop Science,* Vol. *38*, pp. 1255–1260

Luo, M., Brown, R.L., Chen, Z.-Y., Menkir, A., and Bhatnagar, D. (2010). Identification of gene marker sets for screening maize lines for resistance to aflatoxin contamination. *Proceedings of the 3rd International Conference of Plant Molecular Breeding*, pp. 74, Beijing China, Sept. 5-9, 2010.

McGee, J.D., Hamer, J.E. & Hodges, T.K. (2001). Characterization of a PR-10 pathogenesis-related gene family induced in rice during infection with *Magnaporthe grisea*. *Molecular and Plant Microbe Interactions,* Vol. *14*, pp. 877–886

Menkir, A., Brown, R.L., Bandyopadhyay, R.., Chen, Z.-Y. & Cleveland, T.E. (2006). A USA-Africa collaborative strategy for identifying, characterizing, and developing maize germplasm with resistance to aflatoxin contamination. *Mycopathologia,* Vol. 162, pp. 225–232

Menkir, A., Brown, R.L., Bandyopadhyay, R. & Cleveland, T.E. (2008). Registration of six tropical maize germplasm lines with resistance to aflatoxin contamination. *Journal of Plant Registrations* Vol. 2, pp. 246–250

Minden, J.S., Dowd, S.R., Meyer, H.E., & Stuhler, K. (2009). Difference gel electrophoresis. *Electrophoresis,* Vol. 30, pp. S156-S161

Morris, S.W., Vernooij, B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C.C. Frederiksen, R.A.., Bhandhufalck, A. & Hulbert, S. (1998). Induced resistance response in maize. *Molecular and Plant Microbe Interactions,* Vol. *11*, pp. 643–658

Mould, M.J., Xu, T., Barbara, M., Iscove, N.N. & Heath. M.C. (2003). cDNAs generated from individual epidermal cells reveal that differential gene expression predicting subsequent resistance or susceptibility to rust fungal infection occurs prior to the fungus entering the cell lumen. *Molecular and. Plant Microbe Interactions,* Vol. *16*, pp. 835–845

Paul, C., Naidoo, G., Forbes, A., Mikkilineni, V., White, D. & Rocheford, T. (2003) Quantitative trait loci for low aflatoxin production in two related maize populations. *Theoretical and Applied Genetics*, Vol. 07, pp. 263–270

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

Pechanova, O. (2006). Proteomic analysis of maize rachis from inbred lines resistant and susceptible to *Aspergillus flavus*. Ph.D. Dissertation, Mississippi State University, USA, pp. 230.

Peethambaran, B., Hawkins, L., Windham, G.L., Williams, W.P. & Luthe, D.S. (2010). Antifungal activity of maize silk proteins and role of chitinases in *Aspergillus flavus* resistance. *Toxin Reviews,* Vol. 29, pp. 27–39

Probst, C., Njapau, H. & Cotty, P.J. (2007). Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. *Applied and Environmental Microbiology,* Vol. *73*, pp. 2762–2764

Scott, G.E. & Zummo, N. (1988). Sources of resistance in maize to kernel infection by *Aspergillus flavus* in the field. *Crop Science,* Vol. 28, pp. 505–507

Steiner-Lange, S., Fischer, A., Boettcher, A., Rouhara, I., Liedgens, H., Schmelzer, E. & Knogge, W. (2003). Differential defense reactions in leaf tissues of barley in response to infection by *Rhynchosporium secalis* and to treatment with a fungal avirulence gene product. *Molecular and Plant Microbe Interactions,* Vol. 16, pp. 893–902

Thomann, E.B., Sollinger, J., White, C. & Rivin, C.J. (1992). Accumulation of group 3 late embryogenesis abundant proteins in *Zea mays* embryos. *Plant Physiology,* Vol. 99, pp. 607–614

Veena, V., Reddy, S. & Sopory, S.K. (1999). Glyoxalase I from Brassica juncea: Molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. *Plant Journal,* Vol. 17, pp. 385–395

Warburton, M.L., Brooks, T.D., Krakowsky, M.D., Shan, X., Windham, G.L. & Williams, W.P. (2009). Identification and mapping of new sources of resistance to aflatoxin accumulation in maize. *Crop Science,* Vol. 49, pp. 1403–1408

Warner, S.A.J., Scott, R. & Draper, J. (1992). Characterization of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. *Plant Molecular Biology,* Vol. 19, pp. 555–561

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

White, D.G., Rocheford, T.R., Kaufman, B. & Hamblin, A.M. (1995). Chromosome regions associated with resistance to *Aspergillus flavus* and inhibition of aflatoxin production in maize. *Proceedings of the USDA-ARS Aflatoxin Elimination Workshop,* pp. 8, Atlanta, GA, USA, October, 1995

White, D.G., Rocheford, T.R., Naidoo, G., Paul, C. & Hamblin, A.M. (1998). Inheritance of molecular markers associated with, and breeding for resistance to Aspergillus Ear Rot and aflatoxin production in maize using Tex6. *Proceedings of the USDA-ARS Aflatoxin Elimination Workshop,* pp. 4-6, St. Louis, MO, USA, October, 1998

Widstrom, N.W., McMillian, W.W. & Wilson, D.M. (1987). Segregation for resistance to aflatoxin contamination among seeds on an ear of hybrid maize. *Crop Science,* Vol. 27, pp. 961–963

Woloshuk, C.P., Cavaletto, J.R. & Cleveland, T.E. (1997). Inducers of aflatoxin biosynthesis from colonized maize kernels are generated by an amylase activity from *Aspergillus flavus*. *Phytopathology,* Vol. 87, pp. 164–169

Xie, Y.R., Chen, Z.-Y., Brown, R.L. & Bhatnagar, D. (2010). Expression and functional characterization of two pathogenesis-related protein10 genes from *Zea mays*. *Journal of. Plant Physiology,* Vol. 167, pp. 121–130

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D. & Wu, R. (1996). Expression of a late embryogenesis abundant protein gene HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiology,* Vol. 110, pp. 249–257

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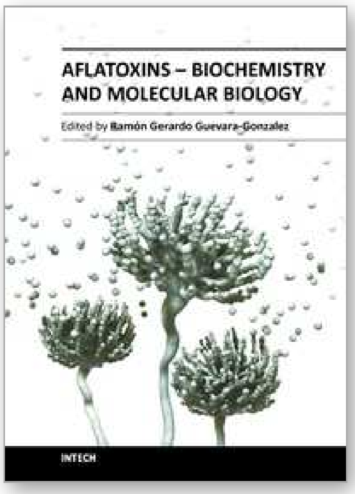

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Aflatoxins â€“ Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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