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A USA–Africa collaborative strategy for identifying, characterizing, and developing maize germplasm with resistance to aflatoxin contamination

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Abstract

Aflatoxin contamination of maize by *Aspergillus flavus* poses serious potential economic losses in the US and health hazards to humans, particularly in West Africa. The Southern Regional Research Center of the United States Department of Agriculture, Agricultural Research Service (USDA-ARS-SRRC) and the International Institute of Tropical Agriculture (IITA) initiated a collaborative breeding project to develop maize germplasm with resistance to aflatoxin accumulation. Resistant genotypes from the US and selected inbred lines from IITA were used to generate backcrosses with 75% US germplasm and F_1 crosses with 50% IITA and 50% US germplasm. A total of 65 S_4 lines were developed from the backcross populations and 144 S_4 lines were derived from the F_1 crosses. These lines were separated into groups and screened in SRRC laboratory using a kernel-screening assay. Significant differences in aflatoxin production were detected among the lines within each group. Several promising S_4 lines with aflatoxin values significantly lower than their respective US resistant recurrent parent or their elite tropical inbred parent were selected for resistance-confirmation tests. We found pairs of S_4 lines with 75–94% common genetic backgrounds differing significantly in aflatoxin accumulation. These pairs of lines are currently being used for proteome analysis to identify resistance-associated proteins and the corresponding genes underlying resistance to aflatoxin accumulation. Following confirmation tests in the laboratory, lines with consistently low aflatoxin levels will be inoculated with *A. flavus* in the field in Nigeria to identify lines resistant to strains specific to both US and West Africa. Maize inbred lines with desirable agronomic traits and low levels of aflatoxin in the field would be released as sources of genes for resistance to aflatoxin production.

Key words: *A. flavus*, aflatoxin resistance, breeding, maize inbred lines, proteome analysis

Introduction

Ear and kernel rots of maize are caused by a variety of fungi, most notably *Aspergillus*, *Botrydiplodia*, *Diplodia*, *Fusarium*, *Gibberella*, and *Macropomina*. Some of these fungi are widespread in temperate and tropical maize growing environments and can cause considerable economic losses by reducing grain quality in maize. When *Aspergillus flavus* infects maize plants, the developing kernel can be contaminated with aflatoxin, which renders the harvested grain unsuitable for food and animal feed. Aflatoxin accumulation in maize grain is a worldwide problem. In the southern United States, it is a chronic problem, but occasionally it affects the Midwestern Corn Belt [1]. Aflatoxin is a natural carcinogen, and its presence in maize grain poses serious health hazards to humans and domestic animals [2]. Contamination of maize with aflatoxin causes losses of millions of

dollars to growers, elevators, feed manufacturers and livestock feeders [3, 4].

Contamination with aflatoxin is also severe in warm, humid, tropical and subtropical maize growing environments that are conducive for growth and development of the different species of *Aspergillus* [5]. The effect of maize contaminated with aflatoxin can be more serious in developing countries than that in the United States because the largest proportion of the maize grain in many other countries is used for human consumption and there is limited capacity to monitor the level of aflatoxin contamination in the grain [5]. The widespread exposure to aflatoxin in Africa [5–7] had been implicated in increased incidence of acute toxicosis, liver cancer, and morbidity in children suffering from kwashiorkor [6, 8]. A recent study in Benin and Togo found a strong link between aflatoxin levels in children's blood and stunted growth and being underweight [9]. Some reports suggest that different methods of processing, including roasting, boiling, frying, baking and fermenting, may not be effective in eliminating aflatoxin from contaminated maze food products [5, 10]

Several approaches have been proposed for minimizing the severity of *Aspergillus* ear rot and the subsequent aflatoxin accumulation, but the most promising control strategy is the development of resistant maize genotypes [5, 11]. Pre-harvest host resistance to *A flavus* is economical to growers, leaves no harmful residue in food or the environment, and is compatible with other control measures. This compatibility makes host plant resistance valuable even when the level of resistance is less than desired. Also, pre-harvest resistance to infection by *A. flavus* can eliminate the need to decontaminate large quantities of aflatoxin-contaminated grain and avoid the uncertainties associated with receiving approval for detoxification from regulatory agencies [11]. Although several maize genotypes with resistance to aflatoxin accumulation have been identified, the majority of these sources of resistance lack desirable agronomic backgrounds and adaptation and their level of resistance is not adequate to satisfy commercial needs [11–13]. There is, therefore, a need for a breeding strategy to develop maize germplasm with desirable agronomic traits and enhanced levels of resistance to aflatoxin contamination.

History of IITA-ARS collaboration

Both the International Institute of Tropical Agriculture (IITA) and the Southern Regional Research Center (SRRC) of the USDA-ARS have invested considerable amount of resources and effort to combat the problem of *A. flavus* ear rot and the associated aflatoxin contamination in maize. IITA has hot spot locations for evaluating its breeding materials under naturally occurring severe disease pressure and continually selects promising materials based on visual assessment for reduced levels of ear rot infection caused by one or more fungal pathogens. Over the years, IITA had developed an array of maize inbred lines with desirable agronomic traits, good husk cover, reduced ear rots, and stress tolerance from diverse sources of germplasm. These lines may possess alleles for resistance to aflatoxin contamination and other mycotoxins that can be assessed by researchers in the US. USDA-ARS-SRRC has developed an efficient maize kernel-screening assay for evaluating resistance to aflatoxin production in a large number of genotypes. This assay has been used for evaluating the diverse elite inbred lines from IITA and also for carrying out rapid assessment of resistance in breeding lines derived from segregating populations. SRRC also has the state-of-the-art proteomics technology that has been employed to identify and characterize kernel resistance-associated proteins (RAPs). Previous studies identified a constitutively expressed maize kernel trypsin inhibitor protein (TI) associated with resistance in US germplasm [14]. In 1998, these mutually beneficial strengths of the two institutions prompted the formal research collaboration between IITA and SRRC. The collaborative research initiative has, therefore, brought together research efforts on elimination of aflatoxin contamination in maize at the two institutions that are complementary but have been undertaken independently.

Screening of IITA' maize inbred lines using KSA for selecting parental lines.

Effective, reliable, and rapid screening techniques are indispensable prerequisites to breeding for resistance to aflatoxin contamination in maize [11, 13, 15]. SRRC developed a rapid laboratory-based kernel-screening assay (KSA) that results in higher

and more uniform levels of infection and aflatoxin production and allows differentiation of resistant and susceptible maize genotypes [16, 17]. This assay provides consistent ranking of maize genotypes in different tests and the results seem to be correlated with resistance levels expressed by maize genotypes in field trials [16]. In a recent study conducted in Nigeria to evaluate five maize inbred lines selected for resistance to aflatoxin production using KSA under artificial inoculation in the field, three of the five inbred lines had low levels of aflatoxin in 2003 (Table 1). Two of the three inbred lines also had low aflatoxin levels in 2004. These results indicate that the laboratory-based KSA can be used as a pre-screening tool in a resistance-breeding program [11].

IITA supplied seeds of 76 inbred lines selected for moderate to high levels of resistance to ear rot caused by *Aspergillus*, *Botrydiplodia*, *Diplodia*, *Fusarium*, and/or *Macropomina* in the forest zone and mid-altitudes of West and Central Africa to the SRRC laboratory for screening. SRRC employed KSA [18] to evaluate the inbred lines and identified 18 lines with aflatoxin levels, which were as low as or lower than those of the best resistant lines from the USA [18]. Further studies involving some of these lines showed that the protein profiles in IITA lines were different from those of the lines from the USA, suggesting that a potential exists to identify traits in IITA lines not present in domestic lines. Also, assessment of fungal growth on selected lines using an *A. flavus* tester strain with a β-glucuronidase [GUS] gene linked to an *A. flavus* β-tubulin gene promoter [19] identified a unique line with low aflatoxin accumulation, but exhibiting a high level of fungal growth [18]. This observation suggests the possibility of identifying a kernel resistance trait directly inhibitory to aflatoxin biosynthesis rather than to fungal infection. The identification of several types of resistance traits could significantly enhance the development of resistant commercial lines by facilitating a strategy of pyramiding different resistance genes into good agronomic backgrounds. The resistant US genotypes and inbred lines from IITA, which were screened using KSA provide the basis for selecting parental lines in an on-going collaborative resistance-breeding project [14].

Table 1. Mean aflatoxin values of maize inbred lines selected for low aflatoxin production using the Kernel Screening Assay, which were evaluated under artificial inoculation in the field at Ibadan in Nigeria in 2003 and 2004

Inbred line	Field trials			KSA
	2003	2004	Combined	
	Aflatoxin (ppb)			
1368	1239	260	749	78
1823	370	96	233	39
TZMI102	1737	130	933	21
TZM104	314	310	312	268
TZMI502	237	93	165	70
Mean	779	178	436	95
SE	292	71	129	43

Breeding for resistance to aflatoxin contamination at IITA

Genetic variation for resistance to aflatoxin contamination is available in maize, making host plant resistance a feasible control option [5, 11, 13, 18]. Several studies have been conducted to determine the mode of inheritance of resistance to *Aspergillus* ear rot and aflatoxin accumulation in maize grain in diverse sources of inbred lines. Results of some studies show that resistance to ear rot and aflatoxin accumulation are quantitatively inherited [20–22], with additive gene effects playing a major role in conditioning the inheritance of resistance [21, 23–28]. However, other studies reported that dominance had a greater effect on resistance to aflatoxin accumulation than additive gene action [20, 29–33]. Broad-sense heritability estimates for both ear rot and aflatoxin levels were moderate to high [21, 33], suggesting that selection for resistance should be feasible. In spite of this considerable and significant progress in identifying sources of resistance and understanding their genetic basis, relatively little has been accomplished so far in utilizing the germplasm and information to breed maize for resistance to aflatoxin accumulation. The complex nature of inheritance of resistance, the erratic nature of field infection by *A. flavus* and the year-to-year variability in aflatoxin levels have limited transfer of resistance to elite maize inbred lines [12, 13]. The advent of new and efficient tools for screening maize genotypes in the field and laboratory has enhanced breeding strategies for developing resistant maize [14].

The pedigree and backcross breeding methods have been extensively used to develop lines with new combinations of agronomic traits and resistance to diseases. IITA has initiated a collaborative breeding project with SRRC to combine resistance of selected lines from IITA with resistance in the inbred lines from the US in order to develop improved inbred lines with desirable agronomic traits useful to breeding programs in the US. To achieve this objective, five elite tropical inbred lines from IITA (Babangoyo, KU1414-SR, 1368, 4001, and 9450) were crossed to eight genotypes from the USA ((B73×Tex6), C12, GT-MAS:gk, MI82, (MO 17×Tex6), MP420, OH516, and T115) with proven resistance to aflatoxin contamination [16, 30] to form 16 F_1 crosses. A backcross (BC1) was made to each F1 cross using the respective genotype from the US as a recurrent parent. The second objective of the breeding program is to combine resistance factors from the US germplasm with resistance in IITA inbred lines for use in tropical environments. To attain this objective, seven elite IITA inbred lines (Babangoyo, KU1414-SR, 1368, 4001, 5012, 9071, and 9450) were crossed to the resistant US genotypes listed above to develop 16 F_1 crosses.

As measurement of aflatoxin produced by *A. flavus* in maize is a relatively tedious and expensive procedure, it was not possible to assay aflatoxin production in a large number of single plants from the many segregating populations. Assessment of aflatoxin production was, thus, deferred until homozygous lines (S_4) were developed with selection for agronomic traits and resistance to diseases during the early stages of inbreeding. From 2000 to 2002 rainy seasons, ear-to-row selection was made to develop lines from each BC_1 or F_1 cross. At each stage of inbreeding, visual selection within and among lines was made on the basis of synchrony between pollen shed and silking, low ear placement, well-filled ears and resistance to lodging and diseases, including *Puccinia polysora* rust, *Bipolaris maydis* blight, and *Curvularia lunata* leaf spot, under naturally occurring disease pressure at Ibadan. Sixty-five S_4 lines were developed from the backcross populations and 144 S_4 lines were derived from F_1 crosses for screening using KSA. The number of lines derived from backcrosses was relatively fewer than that derived from the F_1 crosses because the majority of the lines from the former were susceptible to foliar diseases.

Achievements and progress

Screening of advanced maize breeding lines using the Kernel Screening Assay

Seed samples of the first 65 S_4 lines derived from the backcross populations were sent to the USDA-ARS-SRRC laboratory in New Orleans. These lines were divided into 7 groups, each consisting of three to 12 S_4 lines along with the recurrent parent, a resistant and a susceptible inbred checks. Each group was screened for resistance to *A. flavus* infection/aflatoxin accumulation in a separate experiment using the laboratory-based kernel screening assay as described by Brown et al. [16, 17]. The maize inbred lines within each group exhibited a broad range in aflatoxin accumulation (Table 2). For example, the lines included in one of the groups differed significantly in their level of aflatoxin accumulation (Table 3). Seven S_4 lines (L08-L15) had significantly lower level of aflatoxin than the resistant US recurrent parent, MP420. L11, L12, L14, and L15 did not differ significantly in aflatoxin accumulation from the resistant US inbred check, MI82. Two pairs of S_4 lines (L02 with L10 and L05 with L12), which were advanced to the S_3 stage of inbreeding from the same single plant, differed significantly in aflatoxin accumulation (Table 3). Among the 65 S_4 lines evaluated in different groups, we found 23 lines with aflatoxin values significantly lower than their respective US resistant recurrent parent. Some of these lines also had aflatoxin levels similar to or lower than the resistant US inbred check, MI82.

The S_4 lines derived from tropical × temperate F_1 crosses were divided into three sets. The first two sets of inbred lines each were divided into groups and were screened for aflatoxin accumulation in the laboratory using the KSA. Significant differences in aflatoxin production were detected among the lines within each group (Table 2). As shown in Table 4, all the S_4 lines (TL03 to TL011) differed significantly in aflatoxin accumulation from the elite tropical parental line, 1368, but not from the resistant US inbred check, MI82. We found a pair of inbred lines (TL04 and TL09) that originated from the same single plant up to the S_3 stage of inbreeding but with contrasting aflatoxin levels. Of the 102 S_4 lines evaluated in different groups, 32 had significantly

Table 2. Minimum, maximum and mean aflatoxin values along with their standard deviation for each group of maize inbred lines evaluated in the laboratory using KSA in 2003 and 2004

Group[a]	Number of lines	Aflatoxin (ppb)			Standard error
		Minimum	Maximum	Mean	
Lines derived from backcrosses					
Group-I	14	6	5966	1651	415
Group-II	7	0	5214	1967	672
Group-III	10	358	5064	1538	525
Group-IV	13	76	10197	1499	740
Group-V	15	43	2109	930	174
Group-VI	12	147	7854	1559	660
Group-VII	10	136	8333	2677	740
	Lines derived from tropical × temperate crosses – Set-1				
Group-I	12	894	14787	6446	1298
Group-II	10	743	14154	6471	1391
Group-III	11	2992	9095	5969	533
Group-IV	9	0	4421	1914	452
Group-V	9	82	3668	2212	379
Group-VI	9	2187	9341	5288	882
Group-VII	9	388	2975	1473	312
Group-VIII	7	247	5590	3343	742
Group-IX	11	229	4644	1270	430
	Lines derived from tropical × temperate crosses – Set-2				
Group-I	11	990	17190	7904	1459
Group-II	11	126	7420	2108	788
Group-III	11	463	6628	2505	658
Group-IV	11	649	9524	5022	831
Group-V	13	701	18377	4234	1262

[a]The total number of maize inbred lines was organized into smaller groups to facilitate KSA analysis and appropriate comparisons.

Table 3. Mean aflatoxin values for a group of maize inbred lines derived from backcrosses evaluated in an experiment conducted at SRRC laboratory in New Orleans using KSA

Line	Pedigree	Aflatoxin (ppb)
L01	P3142 (Susceptible US check)	2110
L02	(MP420 × 4001 × MP420)-2-2-3-1-B	1675
L03	(MP420 × 4001 × MP420)-2-2-3-3-B	1594
L04	MP420 (Recurrent parent)	1464
L05	(MP420 × 9450 × MP420)-3-1-1-2-B	1449
L06	(MP420 × 9450 × MP420)-3-1-1-3-B	1370
L07	(MP420 × 4001 × MP420)-2-2-3-2-B	1249
L08	(MP420 × 4001 × MP420)-3-1-3-1-B	773
L09	(MP420 × 4001 × MP420)-3-1-3-2-B	793
L10	(MP420 × 4001 × MP420)-2-2-3-4-B	637
L11	(MP420 × 4001 × MP420)-3-1-2-1-B	320
L12	(MP420 × 9450 × MP420)-3-1-1-4-B	298
L13	MI82 (US resistant check)	110
L14	(MP420 × 4001 × MP420)-2-1-1-1-B	63
L15	(MP420 × 4001 × MP420)-3-1-2-2-B	43
Mean		930
SE		174

lower aflatoxin levels than the elite tropical inbred parent. About half of these lines did not differ significantly in aflatoxin accumulation from the resistant US inbred check, MI82. The third set of lines derived from tropical × temperate crosses will be screened using KSA in 2005.

Selection of genetically similar maize inbred lines for proteome analysis

In our collaborative breeding work, we used only one generation of backcrossing to the temperate inbred lines in order to recover segregates with superior agronomic traits. Also, we derived inbred lines from crosses between tropical and temperate parents to enhance the chances for developing inbred lines with desirable agronomic traits adapted to West and Central Africa. Inbred lines extracted from the same backcross or F1 cross, which are expected to share at least 75% common genetic background, were defined as genetically similar lines. Seven pairs of S5 lines derived from

Table 4. Mean aflatoxin values for a group of maize inbred lines derived from tropical × temperate crosses evaluated in an experiment conducted at SRRC laboratory in New Orleans using KSA

Line	Pedigree	Aflatoxin (ppb)
TL01	1368	7420
TL02	P3142 (Susceptible US check)	7146
TL03	1368 × MI82-13-1-1-1-B	2073
TL04	1368 × MI82-23-1-1-2-B	1481
TL05	1368 × MI82-19-4-1-1-B	1202
TL06	1368 × MI82-11-2-1-1-B	1161
TL07	1368 × MI82-23-1-1-1-B	770
TL08	MI82 (US resistant check)	673
TL09	1368 × MI82-23-1-1-3-B	578
TL10	1368 × MI82-11-1-1-1-B	561
TL11	1368 × MI82-17-1-1-1-B	126
Mean		2108
SE		788

backcrosses and 10 pairs of lines extracted from F1 crosses with 75–93.75% common genetic background differed significantly in aflatoxin accumulation. Use of such genetically similar inbred lines with contrasting aflatoxin accumulation in studies may allow identification of candidate genes underlying resistance to *A. flavus* infection/aflatoxin production without the confounding effects experienced with lines of diverse genetic backgrounds [14]. This in turn may facilitate identification of potential markers for rapid screening of genetic materials in a breeding program.

Identification of potential markers through comparative proteomics

SRRC uses proteomics technology to identify and characterize kernel RAPs. Previous protein biochemistry protocols identified a constitutively expressed corn kernel trypsin inhibitor protein (TI) quantitatively associated with resistance. TI demonstrated efficacy against growth of *A. flavus* and seven other corn pathogens. It also inhibited fungal alpha-amylase activity, which potentially inhibits both fungal growth and aflatoxin biosynthesis induction. TI is presently being employed in QTL studies, marker-assisted breeding, and transformation projects. A proteome analysis of US inbred lines with resistance and susceptibility to aflatoxin accumulation, identified several new kernel embryo and endosperm proteins either unique or 5-fold upregulated in resistant lines [34, 35]. While antifungal proteins were among these, storage proteins and water-stress proteins also were found to be associated with resistance. A recent investigation of GLX1 demonstrated its effect on aflatoxin accumulation through the reduction of its aflatoxin-inducing substrate, methylglyoxal [34, 35]. These investigations, thus far, have had to rely on side-by-side comparisons of resistant with susceptible genotypes with different genetic backgrounds. The identification of genetically similar lines differing significantly in aflatoxin accumulation from among the S_4 lines developed through our collaborative breeding project has enhanced proteome analysis. When genetically similar pairs of lines were subjected to proteome analysis, several RAPs, categorized as stress-related, and a putative regulatory protein were identified [14]. The discovery of an association between stress-related proteins and aflatoxin-resistance may be very important, since drought is known to drastically increase aflatoxin contamination of maize. Expression of these activities may enable a plant to defend against fungal invasion under stress conditions. Analysis of kernel endosperm proteins of IITA lines is currently under way.

Selection of resistant maize inbred lines for further testing and eventual release

The maize inbred lines selected for low aflatoxin accumulation using KSA will be tested further in the laboratory to confirm consistency of their resistance to aflatoxin accumulation. The best lines with consistently low aflatoxin levels will be tested in Nigeria under artificial field inoculation with *A. flavus* to assess the effectiveness of their resistance across the different strains of *A. flavus*. The selected inbred lines will also be tested in hybrid combinations to determine their combining ability. The best inbred lines that combine desirable agronomic traits with consistently low levels of aflatoxin in the laboratory and in the field would be selected for release as sources of genes for resistance to aflatoxin production.

Potential benefits of the collaborative research

The collaboration between SRRC and IITA has facilitated the sharing of resistant maize

germplasm to support the breeding of aflatoxin-resistant maize lines in backgrounds useful to the US and to the national programs in West and Central Africa. The progress in breeding for resistance over the last few years can be considered in relation to its current and potential future impact on commercial production of maize in the US and West and Central Africa. The inbred lines emanating from the collaborative breeding project are expected to have combined resistance factors coming from temperate and tropical germplasm. These resistance factors are likely to provide higher levels of resistance to aflatoxin accumulation in the future. Such lines can be exploited by maize breeders in the US as new sources of resistance for developing maize cultivars with strong intrinsic resistance to *A. flavus* infection/aflatoxin contamination. They can also serve as sources of resistance to other ear rot causing and mycotoxin-producing fungi as well as desirable agronomic traits to broaden and diversify the germplasm base of adapted US maize germplasm. The resistant lines with good agronomic traits would ultimately be used to accelerate breeding efforts of national programs in West and Central Africa aimed at combating mycotoxin contamination. Some of these lines could be used for developing synthetic varieties and hybrids that could be rapidly deployed to farmers' fields to combat aflatoxin contamination. A potential also exists to generate basic genetic information using some of these inbred lines to formulate an effective breeding strategy for resistance to mycotoxins.

Conclusion

The advances made in identifying genetically similar maize lines with contrasting aflatoxin levels has established a firm base for further studies to characterize the mechanisms of resistance to *A. flavus* infection and the subsequent aflatoxin production. The type of resistance mechanisms operating in kernels of resistant lines will be determined using *A. flavus* GUS or GFP reporter strains. Proteome analyses will also be performed on genetically similar lines differing significantly in aflatoxin accumulation to identify RAPs and their corresponding genes. Quantitative trait loci (QTL) studies and RNAi gene silencing experiments will follow to confirm the effect of these genes. Understanding specific mechanisms of resistance based on knowledge of RAPs and identification of genomic regions linked to resistance to aflatoxin accumulation can provide the impetus for germplasm development and efficient and deliberate introgression of genes from a diverse array of germplasm to adapted maize cultivars. Furthermore, identification of different resistance mechanisms will be useful for pyramiding multiple resistance factors for developing stronger and more durable resistance to aflatoxin accumulation.

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