

## Research Note

# Resistance to Aflatoxin Accumulation in Kernels of Maize Inbreds Selected for Ear Rot Resistance in West and Central Africa

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### ABSTRACT

Thirty-six inbred lines selected in West and Central Africa for moderate to high resistance to maize ear rot under conditions of severe natural infection were screened for resistance to aflatoxin contamination using the previously established kernel screening assay. Results showed that more than half the inbreds accumulated aflatoxins at levels as low as or lower than the resistant U.S. lines GT-MAS:GK or MI82. In 10 selected aflatoxin-resistant or aflatoxin-susceptible inbreds, *Aspergillus flavus* growth, which was quantified using an *A. flavus* transformant containing a GUS- $\beta$ -tubulin reporter gene construct, was, in general, positively related to aflatoxin accumulation. However, one aflatoxin-resistant inbred supported a relatively high level of fungal infection, whereas two susceptibles supported relatively low fungal infection. When kernels of the 10 tested lines were profiled for proteins using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, significant variations from protein profiles of U.S. lines were observed. Confirmation of resistance in promising African lines in field trials may significantly broaden the resistant germplasm base available for managing aflatoxin contamination through breeding approaches. Biochemical resistance markers different from those being identified and characterized in U.S. genotypes, such as ones inhibitory to aflatoxin biosynthesis rather than to fungal infection, may also be identified in African lines. These discoveries could significantly enhance the host resistance strategy of pyramiding different traits into agronomically useful maize germplasm to control aflatoxin contamination.

Aflatoxins, toxic secondary metabolites of the fungi *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare, are potent carcinogens. They pose serious health hazards to humans and domestic animals because of their frequent contamination of agricultural commodities, such as cottonseed, peanuts, tree nuts, or maize (8, 15). Aflatoxin contamination of maize (*Zea mays* L.) is a preharvest and a postharvest problem; *A. flavus* may infect the crop before harvest and remain throughout harvest, storage, and use (23).

Progress made in identifying maize genotypes that resist aflatoxin contamination has enhanced host resistance strategies for eliminating or controlling aflatoxin contamination (2, 3, 6, 7, 25, 28). These strategies also have benefited from the discovery of both maize kernel pericarp and subpericarp resistance to aflatoxin contamination (3, 6, 19). Regarding the former, kernel pericarp wax of a resistant genotype, GT-MAS:GK, has been implicated as potentially a physical barrier to fungal ingress and as chemically inhibitory to *A. flavus* growth and subsequent aflatoxin production (19, 24). Subpericarp resistance has been investigated through side-by-side comparisons of maize kernel

protein profiles of susceptible and resistant germplasm (11, 16, 17). Both constitutive and inducible proteins associated with resistance have been identified and characterized (10, 11, 16, 17, 20). It is hoped that these markers will play a role in the development of a strategy of pyramiding different resistance mechanisms against *A. flavus* infection and aflatoxin accumulation into agronomically useful maize germplasm through either breeding or genetic engineering. However, for this strategy to succeed, germplasm with higher levels of resistance and that use different mechanisms than those already identified will have to be discovered.

The purpose of the present study was to investigate the potential of 36 maize inbreds, adapted to the savanna and mid-altitude ecological zones of West and Central Africa, to resist aflatoxin accumulation. These lines were selected for resistance to ear rot under conditions of severe natural infection in their respective areas of adaptation and have moderate to high levels of resistance to ear rot. The major ear rot-causing fungi in these environments include *Aspergillus*, *Botrydiplodia*, *Diplodia*, *Fusarium*, and *Macropomina*. To determine the potential of these inbreds to resist aflatoxin production by *A. flavus*, the kernel screening laboratory assay (KSA) was used (3, 6). It also was used in

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conjunction with an *A. flavus* transformant expressing *Escherichia coli*  $\beta$ -glucuronidase (GUS) to assess fungal growth in selected resistant and susceptible maize genotypes (3, 6). The KSA has been used successfully in previous studies to identify maize genotypes resistant to aflatoxin production and, along with the above *A. flavus* GUS tester strain, to study resistance mechanisms and to assess fungal growth (3, 4, 6). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used in the present study to preliminarily assess these selected lines for protein profile diversity and, thus, potential to contain resistance markers varying from those observed in U.S. resistant lines. A preliminary report of aflatoxin accumulation assessment has been made (1).

## MATERIALS AND METHODS

**Maize entries.** Thirty-six inbred lines were obtained from the International Institute of Tropical Agriculture in Ibadan, Nigeria. Genotype MI82, a resistant control, was obtained from the Department of Crop Sciences of the University of Illinois. GT-MAS:gk, another resistant control was obtained from the U.S. Department of Agriculture, Agricultural Research Service, Insect Biology Population Management Research Laboratory, in Tifton, Ga. Delta Pine G-4666 (DP), the susceptible control, was obtained from the Department of Plant Pathology and Crop Physiology at Louisiana State University. MI82 was identified as resistant to aflatoxin production as an inbred and as an F1 cross with susceptible inbreds B73 and/or Mo17 (3, 7). GT-MAS:gk was identified as resistant in numerous field trials and has been studied extensively in laboratory investigations (2, 28). DP is susceptible to the elaboration of aflatoxins (18). All kernels were kept in sealed plastic containers at 4°C until used.

**Fungal strains and growth conditions.** The *A. flavus* isolate (AF13) used in the evaluation of the 36 inbreds was isolated from agricultural soils in Arizona (12, 14). It produces large quantities of aflatoxins in developing cottonseed and maize and in culture (5, 12). Cultures were grown at 30°C in the dark on a 5% V-8 juice and 2% agar medium. Plugs (3 mm in diameter) of sporulating cultures were stored at 8°C on a long-term basis in 4-dram (14.788-ml) vials containing 5 ml of deionized water (12, 13). Conidia from 4- to 7-day-old cultures suspended in deionized water served as inocula.

An *A. flavus* isolate (GAP2-4) transformed with the *E. coli* GUS gene linked to an *A. flavus*  $\beta$ -tubulin gene promoter (3) was used to quantify fungal growth in maize kernels of selected inbreds. Cultures were grown at 37°C in the dark on potato dextrose agar. Conidia from 4- to 7-day-old cultures suspended in deionized water served as inocula.

**Inbred evaluation.** Kernels of 36 inbreds (divided and tested in four separate series, each containing nine inbreds plus two controls) and of genotypes MI82 or GT-MAS:gk and DP were surface sterilized (27) and then dipped into a suspension of AF13 conidia ( $4.0 \times 10^6$  conidia/ml) and evaluated using the KSA (3, 6). Each experimental unit contained 3 kernels that were replicated 10 times. Kernels were incubated with AF13 using the KSA protocol (31°C, 7 days) and afterward were removed and dried in a forced-air oven at 60°C for 2 days to stop fungal activity and prepare samples for aflatoxin analyses (3). The experiment was performed twice.

**Inoculations with GAP2-4 and quantification of GUS activity.** The 10 inbreds, four susceptible and six resistant, selected

for further evaluation using the GAP2-4 strain were, respectively, 1188, 603, 15, and KU and 1368, 502, 305, 102, 28, and 1823. Kernels of these 10 genotypes and of MI82 and DP were surface sterilized as above. They were inoculated by dipping them into a spore suspension of *A. flavus* GAP2-4 ( $4.0 \times 10^6$  conidia/ml) and were incubated at 31°C for 6 days using the KSA. Each treatment was replicated eight times, with each replicate containing three kernels. Tests were performed twice. After incubation, kernels were subjected to a protocol for fluorogenic GUS quantification (4, 21). For each enzyme reaction, 50  $\mu$ l of crude extract was added to the assay buffer containing the substrate, and reactions were stopped after 20 min. Preliminary kinetic studies were performed to identify the amount of crude extract to use in each test and a time point that fell within the linear portion of the enzyme reaction curve. GUS activity in samples was determined with a Gilford Fluoro IV spectrofluorometer (Corning Laboratory Sciences Co., Oberlin, Ohio); excitation was at 360 nm and emission at 455 nm. GUS activity was normalized through protein determinations in crude extracts using the method of Sedmak and Grossberg (26).

**Aflatoxin analyses.** The aflatoxin B<sub>1</sub> content of replicates from all tests was determined by a procedure used in an earlier study (6). This protocol involved methylene chloride extraction of aflatoxins from infected seed, thin layer chromatographic separation of compounds, and quantitation of aflatoxin B<sub>1</sub> using a scanning densitometer.

**Statistical analyses.** Analyses of aflatoxin data were performed with the Statistical Analysis Software System (SAS Institute, Inc., Cary, N.C.). Treatment replicates from each test were first subjected to analysis of variance followed by mean comparisons of log transformations of toxin values. Transformations were performed to equalize treatment variances. Differences among treatment means were determined by the least significant difference test.

**Protein extraction and gel electrophoresis.** Dry kernels (20 g) of each of the 10 selected genotypes were extracted for protein using the procedure of an earlier study (11). SDS-PAGE of protein extracts was performed using a 15% resolving gel with a 4% stacking gel according to Laemmli (22). Low-range protein markers (Sigma Chemical Co., St. Louis, Mo.) were used as molecular mass standards. The gels were electrophoresed (120 V, 1.5 h), stained with 0.125% Coomassie blue R-250 in 50% methanol and 10% acetic acid for 1 h at room temperature, and destained in 50% methanol and 10% acetic acid.

**Quantification of proteins.** The quantification of selected proteins (14 and 34 kDa) was performed using Bio-Rad's GS-700 Gel Densitometer (Bio-Rad Laboratories, Richmond, Calif.) and the associated Molecular Analyst software. The data presented in this report are the mean values of each genotype obtained in two experiments.

## RESULTS AND DISCUSSION

Aflatoxin B<sub>1</sub> levels in 19 of the 36 African lines tested, inbreds 1393, 5057, 1368, 5012, 103, 104, 502, 305, 102, 25, 20, 30, 34, 28, 2151, MmB90, 7271, 5052, and 1823, were approximately equivalent to levels measured in resistant controls, MI82 or GT-MAS:gk, in respective tests (Table 1). Toxin levels supported by inbreds 28 and 34 were lower than those supported by GT-MAS:gk. There was great variation in the overall amount of aflatoxin B<sub>1</sub> produced in test series A and B compared with series C and

TABLE 1. Aflatoxin accumulation in West and Central African maize genotypes

Line/toxin (ng/g) <sup>a</sup>			
Series A	Series B	Series C	Series D
1188/1839 A	603/1528 A	DP (S)/16.979 A	KU/9710 A
1394/1395 A	DP (S)/1231 A	15/15.633 A	4205/4885 B
DP (S)/1277 AB	604/1043 AB	38/8830 B	9484/3883 B
2097/1267 AB	301/1032 AB	39/7425 B	8071/2377 BC
1201/756 B	501/963 AB	35/6858 B	DP (S)/1604 CD
2096/557 BC	103/571 BC	25/5441 BC	2151/739 DE
1393/431 CD	MI82(R)/457 CD	20/4363 BC	MmB90/571 DE
5057/132 D	104/268 CD	MAS(R)/2212 CD	7271/151 E
1368/78 D	502/70 D	30/1781 DE	5052/129 E
MI82 (R)/41 D	305/34 D	34/720 E	MI82(R)/103 EF
5012/23 D	102/21 D	28/403 E	1823/39 F

<sup>a</sup> Values are averages of 20 replicates from two separate tests. Data were log transformed before analyses to equalize variances. Inbreds to be tested were divided into groups or "series" and tested at the same time. Each series contained a resistant and a susceptible control.

<sup>b</sup> Values in a column followed by the same letter are not significantly different ( $P = 0.05$ ) by the least significant difference test.

D; this variability phenomenon is routinely observed in aflatoxin studies. Confirmation of resistance in these lines through field evaluations could greatly increase the number of resistant inbred lines being investigated in aflatoxin-resistance breeding programs and could enhance the level of resistance accessible to breeders.

Fungal growth of the *A. flavus* GUS tester strain in kernels of 10 selected inbreds followed the typical pattern observed in U.S. lines, being generally high in susceptible and low in resistant inbreds. This may indicate the possible functioning of kernel antifungal resistance mechanisms.

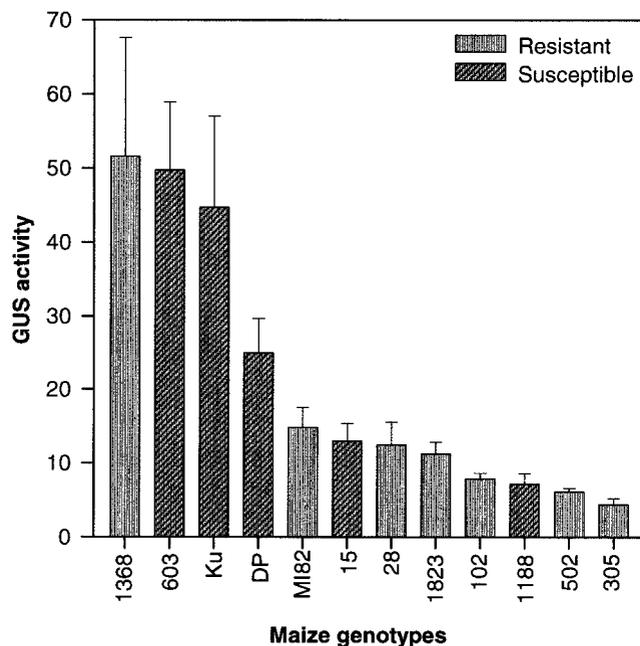


FIGURE 1. Fungal growth measured through specific GUS activity in nM of methylumbelliferone (MU) produced per min per mg of protein in 10 selected resistant and susceptible African inbreds. Bars represent GUS activity means  $\pm$  standard error of 16 repetitions measured during two experiments. MI82 is the resistant control; DP, the susceptible control.

Typical quantities (measured in nM of methylumbelliferone produced per min per mg of protein) of fungal growth (Fig. 1) were observed in susceptible inbreds 603 and KU and in resistant inbreds 305, 502, 102, 1823, and 28. However, growth of *A. flavus* in kernels of susceptible inbreds 1188 and 15 was atypical. It was lower than and equal to, respectively, growth in resistant control MI82. Fungal growth in resistant inbred 1368 varied from levels normally encountered in resistant inbreds. The levels of *A. flavus* measured in inbred 1368 were as high as those observed in susceptible inbreds 603 and KU. The discovery of high fungal growth in a maize line that accumulates low levels of aflatoxins could lead to the identification and characterization of a kernel resistance mechanism(s) directly inhibitory to aflatoxin biosynthesis rather than to fungal infection. This might enhance the development of maize germplasm through the pyramiding of diverse resistance traits and yield maize lines capable of sustaining resistance to aflatoxin formation over time and in different environments. The discovery that aflatoxin-susceptible inbreds 1188 and 15 support low levels of fungal growth may provide investigators with the opportunity to study in vivo substrate effects on aflatoxin production, an opportunity up to now not afforded by U.S. lines under investigation.

Genetic diversity may have been demonstrated in the electrophoretic profiles of the African inbreds compared with U.S. lines. Not only is there variation from the typical profiles as represented by MI82 and DP, but there is significant diversity among the African resistant and susceptible lines as well. On SDS-PAGE gels, a 14-kDa protein band corresponding to the size of a trypsin inhibitor protein, whose constitutive levels were previously correlated with resistance in U.S. lines (including MI82) (11), is easily observed as is a 34-kDa band corresponding to ribosome-inactivating protein (Fig. 2). Average percentage of protein content of the 14-kDa band for susceptible inbreds was relatively high compared with that found in a previous study involving U.S. genotypes (11) and as a group were not

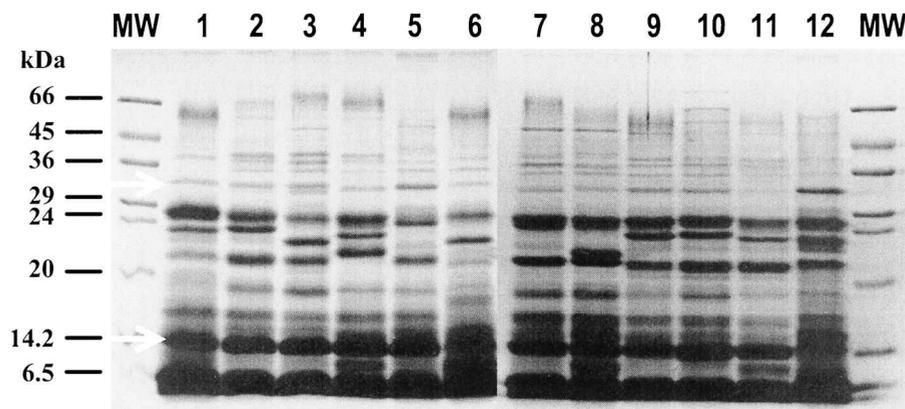


FIGURE 2. SDS-PAGE analysis of extracted kernel proteins of 10 selected African inbreds. Resistant inbred profiles are in lanes 1 through 6 and susceptible inbred profiles are in lanes 9 through 12. Lane 1, 1368; lane 2, 102; lane 3, 305; lane 4, 28; lane 5, 502; lane 6, 1823; lane 7, susceptible control, DP; lane 8, resistant control, MI82; lane 9, KU; lane 10, 603; lane 11, 15; and lane 12, 1188. White arrows point to 14- and 34-kDa protein bands.

different from the resistant African inbreds (Table 2). The possibility that susceptible African inbreds (unlike tested U.S. susceptible lines) could possess high levels of this trypsin inhibitor may indicate that traits or interactions between traits, different from those in U.S. lines, are responsible for the resistance demonstrated in the African inbreds. DP, uncharacteristically for U.S. susceptible genotypes, expressed a high level of the 14-kDa protein in these tests and did so in previous tests as well (9).

The highest levels of the 34-kDa protein band were seen in resistant inbred 502 and in susceptible inbred 1188 (Table 2), and as in U.S. lines (unpublished data), this band was not associated with resistance. Another band, approximately 23 kDa in size, was observed in profiles of all susceptible inbreds and in resistant inbreds 1368, 102, and 28 that was not present in either the resistant or the susceptible control (MI82 and DP, respectively). There also is variation among resistant and susceptible African inbreds in the pro-

TABLE 2. Quantification of the relative content of a 14-kDa protein band and a 34-kDa band in the total kernel protein extracts of the 10 selected African genotypes based on analysis of 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles after staining with Coomassie brilliant blue R-250

Entries	Resistance to <i>A. flavus</i> <sup>a</sup>	Protein band	
		14 kDa	34 kDa
1368	R	8.98	2.22
102	R	14.55	1.97
305	R	15.96	1.40
28	R	20.53	1.55
502	R	16.64	3.96
1823	R	4.43	1.71
MI82 (C)	R	11.81	1.46
DP (C)	S	13.53	1.35
1188	S	3.80	5.98
15	S	14.54	0.59
603	S	17.59	1.69
KU	S	13.37	2.17

<sup>a</sup> R, potentially resistant; S, susceptible; both determined by KSA.

tein bands above the 34-kDa region. Overall, there appears to be greater diversity in protein profiles among the African inbreds than between MI82 and DP, representatives of U.S. resistant and U.S. susceptible classes, respectively. There also appears to be quantitative differences among maize lines in many individual protein bands. Further investigation, however, is needed to quantify and compare the expression of specific proteins and determine associations with resistance.

In the last several years, it has been shown that maize germplasm as a whole possesses genes that increase resistance of the possessing genotype to aflatoxin accumulation by *A. flavus*. However, all identified U.S. resistant lines display less than desirable agronomic qualities (2). This fact highlights the need to identify and characterize specific resistance traits that then can be transferred to commercially desirable germplasm through marker-assisted breeding or through maize transformation. A few resistance traits have been characterized from resistant lines that appear to prevent aflatoxin buildup indirectly through fungal growth inhibition (3, 11, 19); the characterization of other traits is currently being pursued. The African inbreds evaluated in the present study provide the potential to broaden the present narrow base of aflatoxin-resistant maize germplasm and enhance the levels of resistance attainable. They also may provide resistance genes currently not expressed in U.S. tested lines and even “weapons” useful against other serious fungal diseases of crops.

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