Determination of Resistance to Aflatoxin Production in Maize Kernels and Detection of Fungal Colonization Using an *Aspergillus flavus* Transformant Expressing *Escherichia coli* β-Glucuronidase

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We thank S. Anderson, R. Reddix, D. Whittington, and H. Holen for technical assistance and B. Vinyard for statistical assistance.
Accepted for publication 9 June 1995.

ABSTRACT

Brown, R. L., Cleveland, T. E., Payne, G. A., Woloshuk, C. P., Campbell, K. W., and 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 85:983-989.

Thirty-one maize inbreds and the highly resistant GT-MAS:gk maize population were screened for resistance to aflatoxin production by *Aspergillus flavus* using a kernel-screening laboratory assay. Intact kernels from each line were evaluated in three trials. Significant differences among genotypes for resistance to aflatoxin production were found. Certain genotypes, previously shown to be resistant in field trials, demonstrated resistance in the kernel-screening assay. Twenty-two genotypes had intact-kernel resistance that was comparable to GT-MAS:gk. A separate experiment was done to visualize fungal colonization of internal tissue in susceptible and resistant maize kernels and to further elucidate the relationship between fungal colonization and aflatoxin production. Five genotypes screened in the inbred evaluation, were inoculated with an *A. flavus* aflatoxin-producing strain containing the *Escherichia coli* β-D-glucuronidase (GUS) reporter gene linked to an *A. flavus* β-tubulin gene promoter. Histochemical staining of nonwounded and wounded kernels detected differences in GUS expression among genotypes, and there was a relationship between GUS expression and the amount of aflatoxin detected in kernels. Minimal GUS expression was related to low aflatoxin production in wounded kernels of two inbreds previously identified in field trials as having moderate-to-high levels of resistance to aflatoxin production. These results suggest that resistance to aflatoxin production is directly related to resistance to fungal colonization in certain genotypes.

Additional keywords: mycotoxin.

Aflatoxins, toxic metabolites of the fungi *Aspergillus flavus* Link:Fr. and *A. parasiticus*, are potent carcinogens. These compounds pose serious health hazards to humans and domestic animals, because they frequently contaminate agricultural commodities (9,16). Aflatoxin contamination of maize (*Zea mays* L.) is a serious pre- and postharvest problem, because *A. flavus* may infect the crop prior to harvest and remain throughout harvest and storage (22).

Much of the effort to control aflatoxin contamination has been directed toward identifying maize genotypes resistant to both *A. flavus* infection and aflatoxin contamination (22). These studies are laborious, time-consuming, expensive, and often unsuccessful (15). However, a maize breeding population, GT-MAS:gk, with resistance to aflatoxin production was identified recently during extensive field testing (32). This population was derived from visibly classified segregating kernels obtained from a single fungus-infected hybrid ear (32). When the GT-MAS:gk population was tested for resistance to aflatoxin production with a kernel-screening assay (KSA), it demonstrated resistance as well (4,5,6). Further studies with GT-MAS:gk using the KSA also demonstrated resistance in wounded kernels and demonstrated that a living embryo was required for the expression of this phenomenon (5).

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In Illinois field trials, 27 inbreds have been identified as potentially resistant to aflatoxin production by *A. flavus* (8). In the present study, these inbreds and 4 susceptible checks were tested for resistance using the KSA and compared with GT-MAS:gk. The ability to differentiate resistant from susceptible lines using laboratory screening procedures would be an invaluable asset, given the labor, cost, and time constraints associated with field screening procedures (24). Several lines, which differed in resistance in the KSA, were examined further using an *A. flavus* strain transformed with the *Escherichia coli* β-D-glucuronidase (GUS) reporter gene, linked to an *A. flavus* β-tubulin gene promoter (33). The GUS gene fusion system is a useful tool for histochemical detection of fungal invasion of plant tissues and for quantifying fungal biomass (14,23,26,31). The lack of any detectable GUS activity in most higher plants and the stability of the GUS enzyme are two factors that contribute to the usefulness of this reporter construct (18,19,20). Also, the expression of the GUS gene has been shown, in previous studies, to have no effect on the pathogenicity of fungi to plants (7,23,26). We hypothesized that this technique would allow us to visualize and compare fungal colonization of aflatoxin-resistant and -susceptible maize kernels and to relate fungal colonization to aflatoxin production. Preliminary presentations of data from this study have been made (2).

MATERIALS AND METHODS

Maize entries. Thirty-one inbreds were obtained from the Department of Plant Pathology of the University of Illinois, Urbana (8). Twenty-seven inbreds were identified as potentially resistant, both

as inbreds and as F_1 crosses with the susceptible inbreds B73 and Mo17. Inbreds 33-16, NC232, B73, and Mo17 were included as susceptible checks. The GT-MAS:gk population was used as a resistant check. GT-MAS:gk seed was obtained from the USDA-ARS-Insect Biology and Population Management Laboratory in Tifton, GA (32). All seeds were dried in a forced-air oven at 45°C for 2 days and kept at room temperature in sealed plastic buckets containing silica gel desiccant until used in tests. Seeds were visually examined for cracks in the pericarp prior to selection for use in experiments.

Fungal strains and growth conditions. The *A. flavus* isolate (AF13) used in the inbred evaluation was isolated from agricultural soil in Arizona (11,13). The isolate produces large quantities of aflatoxins in developing cottonseed and corn and in culture (3,11). 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 vials containing 5 ml of distilled water (11,12). Conidia from 7- to 10-day-old cultures suspended in deionized water served as inocula (13).

An *A. flavus* isolate (GAP 2-4) transformed with the *E. coli* GUS gene linked to a β-tubulin gene promoter was used to detect GUS activity in maize kernels. Strain 656-2 (*w, leu, pyr, afl*R) was cotransformed with two plasmids. One plasmid (B9X2) contained the *pyr*4 gene from *Neurospora crassa* and the *afl*R+ gene from *A. flavus* (25). The *pyr*4 gene was utilized as a selectable marker for transformation. The *afl*R+ gene complemented the strain to an aflatoxin-producing phenotype. The second plasmid (GAP2) contained a reporter gene construct consisting of the *ben*A gene from *A. flavus* and the GUS gene (*uid*A) from *E. coli* (33). 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 from the 31 inbreds and from the GT-MAS:gk population were dipped into a suspension of *A. flavus* (AF13) conidia (4.0 × 10 conidia per ml) and evaluated using a KSA (5) designed as follows: kernels were placed individually in plastic vial caps (20 mm diameter, 6 mm height) that were placed side by side in an open tissue-culture dish (60 × 15 mm style; Becton Dickinson Co., Oxnard, CA). Individual culture dishes containing four kernels of each genotype were the experimental units. Culture dishes were placed side by side in a clear tray (243 × 243 × 18 mm; Nunc bioassay dish, Thomas Scientific, Swedesboro, NJ) lined wall to wall with 3-mm chromatography paper (Whatman International, Maidstone, England). The lid was placed on top of the tray but was not sealed. The experiment was replicated four times and performed three times. High humidity was maintained by adding 30 ml of sterile deionized water to the chromatography paper initially and 30 ml 3 days later. Kernels were incubated at 31°C for 7 days, after which they 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 (3,5).

Inoculations with GAP 2-4 and detection of GUS activity. Based on results from the inbred evaluation, resistant (CI2, T115, MI82, and GT-MAS:gk) and susceptible (NC232) genotypes were evaluated further for both GUS activity and aflatoxin contamination. Kernels from each genotype were wounded by puncturing them with a 26-gauge, 13-mm hypodermic needle (Becton Dickinson & Co., Rutherford, NJ) through the pericarp into the endosperm to a depth of 1 mm (5). Wounded and nonwounded kernels of these genotypes were surface-sterilized with a 4-min wash in 70% ethanol (with stirring) followed by a quick deionized-H_2O rinse and three 3-min washes (with stirring) in deionized H_2O (30). Kernels were inoculated by dipping them into a spore suspension of GAP 2-4 (4.0×10^6 conidia per ml), and evaluated using the KSA (5). Experimental units were individual culture dishes containing three wounded or nonwounded kernels of one genotype. The experiment was replicated seven times and performed twice. Kernels were incubated at 37°C for 1 day followed by 6 days at 31°C (5). Using the same experimental procedure, other kernels of each genotype were evaluated 1 and 3 days after inoculation. Noninoculated kernels of each genotype served as controls in all experiments.

After incubation, kernels were sliced longitudinally into halves to expose the embryo-endosperm interface. Each half was placed, cut-surface down, into a solution formulated as follows: 5 mg X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide; Sigma Chemical Co., St. Louis) dissolved in 50 ml of dimethyl formamide and brought up to 10 ml with 50 mM $NaPO_4$, pH 7.0 (19). Each halved kernel was stained in 1 ml of this solution for 16 h at 37°C. Blue color in kernel tissues indicated GUS activity. Kernels were examined visually for blue color in internal and external tissues of the kernel. Also, kernel internal tissues (embryo and endosperm) were rated as follows: 0 = no visible blue; 1 = <25% of tissue stained blue; 2 = 25% to 50% of tissue stained blue; 3 = >50% of tissue stained blue. After the rating, kernels were prepared for aflatoxin analyses as described above. The aflatoxin B_1 content of kernels from replicates from all tests was determined as in an earlier study (5).

To verify that GUS visual ratings compared well with quantitative measurement of GUS activity, three genotypes, MI82, 33-16, and B73, were tested using each approach. Kernels of each genotype were inoculated and incubated with GAP 2-4 for 7 days (KSA) and were either prepared for GUS visual examination as described above or assayed for GUS activity using the fluorogenic method of Jefferson (19). To apply the fluorogenic method, kernels (seven repetitions; three kernels per repetition) were first homogenized in a GUS extraction buffer (19) using a mortar and pestle. Homogenates were centrifuged, and the supernatants were used for quantitation of GUS activity. Values were recorded as specific activity—nanomoles of methyl-umbelliferone produced per minute per milligram of protein (27).

Statistical analyses. Aflatoxin values were transformed to $l_n[ng/(g + 1)]$ to stabilize the variance. Antilogs of the logarithmic means produced the geometric means. Analyses of variance were computed for tests of significance for the transformed aflatoxin values between genotypes, treatments, and their respective interactions. There were significant interactions ($P = 0.05$) between treatment and genotype in both GAP2-4 inoculation tests; therefore, an independent variable was created that has a level for each combination of genotype (entry) and treatment variable (10). A least significant ratio, which is the antilog of the least significant difference, was computed for comparisons between geometric means (29).

GUS expression rating data were analyzed using PROC CATMOD (Statistical Analysis Software System, SAS Institute, Inc., Cary, NC) on the response means to contrast all line/treatment pairs. This procedure compared mean ratings without the assumption of statistical normality.

RESULTS

Production of aflatoxin B_1 in 32 entries. The test and genotype variables did not interact in the three inbred screening tests ($P = 0.05$), allowing data from three tests to be pooled (Table 1). Aflatoxin levels in the 32 entries for combined tests ranged from 2 to 5,089 ng/g (Table 1). Among the five genotypes in this evaluation that were selected for further study using the GUS-transformed *A. flavus* isolate, NC232 was clearly the most susceptible, supporting significantly more aflatoxin production than 29 other entries. T115 supported less aflatoxin production than 20 other entries, whereas CI2 supported less than 17 entries, MI82 less than 11 entries, and GT-MAS:gk less than 9 entries. Inbred 33-16, included in the evaluation as a susceptible check, was the most susceptible. Mo17 and B73, also included as susceptible checks, were intermediate in susceptibility.

Production of aflatoxin in selected genotypes inoculated with GAP 2-4. There was a significant interaction ($P = 0.05$) between test and treatment variables; therefore, tests 1 and 2 are reported separately. Using the GUS transformant, maximum aflatoxin levels in nonwounded kernels ranged from 42 ng/g in test 1 to 3,229 ng/g in test 2 and ranged in wounded kernels from 5,796 ng/g in test 1 to 29,466 ng/g in test 2 (Table 2). For nonwounded kernels (Table 2) in test 1, CI2 was the only genotype to support significantly less aflatoxin production than NC232, whereas in test 2, MI82 supported less than all other entries. Among wounded kernels (Table 2), MI82 supported less aflatoxin production than did GT-MAS:gk, CI2, and NC232 in test 1 and less than all other entries in test 2. Also, among wounded kernels, T115 and GT-MAS:gk aflatoxin B_1 levels were lower than those of CI2 and NC232 in test 1, whereas T115 supported less aflatoxin production than CI2 in test 2.

Visible detection of GUS expression. Visual ratings of GUS activity (0 to 3 rating system) closely corresponded to quantitative measurements of GUS activity in the verification experiment. Genotypes with low visual ratings (0.85 ± 0.71 for MI82 and B73 combined) yielded low specific activity (137 ± 52 for MI82 and B73 combined) in the quantitative assay. Entry 33-16 with a very high visual rating (2.76 ± 0.43) yielded very high specific activity (4,756 ± 1,199) when quantitatively assayed.

TABLE 1. Production of aflatoxin in maize inbreds inoculated with *Aspergillus flavus* strain AF13[z]

Entry	Aflatoxin B_1 (ng/g)	Entry	Aflatoxin B_1 (ng/g)
33-16	5,089 a	SP292	32 e–j
NC232	3,702 a	TR213	26 e–k
Tex6	1,213 a,b	Oh516	21 e–k
LB31	291 b,c	CO158	21 e–k
KYS	260 b–d	SDp262	16 f–l
MS214	151 b–e	75-R012	15 f–l
Mo17	99 c–f	B9	15 f–l
Ky58	82 c–g	75-R001	12 g–l
SDp031	75 c–g	B40	9 h–l
H103	63 c–h	N6	9 h–l
FR809	60 c–h	CH66-17	8 h–l
Y7	53 c–i	GT-MAS:gk	7 h–l
F486	41 c–i	MI82	7 i–l
N8	41 c–i	SD18	5 j–l
Oh513	41 c–i	CI2	4 k,l
B73	36 d–j	T115	2 l

[z] Values are averages of 12 observations made over three separate tests. Genotype and test variables did not interact ($P = 0.05$), allowing data from the three tests to be combined. Aflatoxin values were transformed to $l_n[ng/(g + 1)]$ to stabilize the variance, and antilogs of the logarithmic means produced geometric means. A least significant ratio (LSR) which is the antilog of the LSD, was computed for comparisons between geometric means. Means whose ratios do not exceed the LSR values are not significantly different ($P = 0.05$), and are followed by the same letter.

Average ratings of GUS expression in nonwounded kernels (Table 2) were significantly lower for MI82 (Figs. 1E and 2B) than for CI2 (Figs. 1B and 2A) and NC232 (Figs. 1A and 2E) in test 1 and lower for MI82 than for all other entries in test 2. Also, in test 2 ratings for CI2 were lower than ratings for T115, GT-MAS:gk, and NC232. T115 and GT-MAS:gk ratings also were lower than those for NC232 nonwounded kernels.

Average ratings of GUS expression in wounded kernels (Table 2) of entries MI82, T115, and GT-MAS:gk (Fig. 1E, D, and C, respectively) were significantly lower than ratings for CI2 and NC232 (Fig. 1B and A, respectively) in test 1. Also in test 1, CI2 ratings were lower than ratings for NC232. In test 2, average ratings of GUS expression in wounded kernels were lower for MI82 than for all other entries tested.

Growth of *A. flavus* in infected kernel tissue was easily detected by the blue color associated with GUS activity (Figs. 1 through 3). Mycelia exposed on the cut face of the kernel as well as mycelia growing under the pericarp surrounding kernels were stained blue in the GUS assay (Fig. 3). The blue indicative of GUS activity was more apparent in wounded kernels than in intact kernels for most genotypes in tests 1 and 2 (Fig. 1). No blue was detected in noninoculated kernels of any entry tested. When wounded kernels were assayed for GUS expression 7 days after inoculation, blue was observed in varying amounts of endosperm and/or embryo tissues in all entries. GUS activity was seen primarily in the embryo of all nonwounded kernels assayed for GUS expression 7 days after inoculation and of both nonwounded and wounded kernels assayed 1 or 3 days after inoculation (Fig. 2). Blue staining of tissues was observed through the kernel pericarp in all kernels assayed for GUS expression 7 days after inoculation (Fig. 3). Generally, the greater the visible expression of GUS activity in internal tissue, the greater the blue observed under pericarps.

DISCUSSION

Developing maize genotypes resistant to aflatoxin contamination has been both challenging and frustrating to researchers (24). There has been limited success in identifying resistant germ plasm under repeated testing and in different environments (15).

Table 2. Aflatoxin production and *Escherichia coli* β-D-glucuronidase (GUS) reporter gene expression in maize kernels inoculated with an *Aspergillus flavus* GUS transformant

Entry	Treatment	Test 1		Test 2	
		Aflatoxin B_1 (ng/g)[y]	GUS rating[z]	Aflatoxin B_1 (ng/g)	GUS rating
NC232	Nonwounded	42 b,c	0.69 c	3,229 b–d	2.24 a
	Wounded	5,796 a	2.55 a	11,986 a,b	2.29 a
CI2	Nonwounded	4 d	0.24 c,d	140 ef	0.67 c
	Wounded	1,887 a	1.29 b	29,466 a	2.43 a
GT-MAS:gk	Nonwounded	18 c,d	0.38 c,d	742 d,e	1.10 b
	Wounded	198 b	0.86 c	6,330 a–c	2.17 a
T115	Nonwounded	23 b–d	0.36 c,d	1,255 c,d	1.26 b
	Wounded	28 b–d	0.66 c	3,956 b–d	2.26 a
M182	Nonwounded	5 c,d	0.17 d	15 g	0.21 d
	Wounded	20 c,d	0.38 c,d	101 f	0.43 c,d

[y] Values are averages of seven replicates. There was an interaction between treatment and test variables ($P = 0.05$), so data from the two tests were kept separate. Aflatoxin values were transformed to $l_n[ng/(g + 1)]$ to stabilize the variance, and antilogs of the logarithmic means produced geometric means. Analyses of variance revealed significant interactions in both tests between treatment and genotype ($P = 0.05$); therefore, an independent variable was created that has a level for each combination of genotype and treatment variables. A least significant ratio (LSR), which is the antilog of the LSD, was computed for comparisons between geometric means. Means whose ratios do not exceed the LSR values are not significantly different ($P = 0.05$) and are followed by the same letter.

[z] Expression of GUS on kernel halves was rated as follows: 0 = no visible activity; 1 = <25% of tissue stained blue; 2 = 25 to 50% of tissue stained blue; 3 = >50% of tissue stained blue. Values are averages of 42 observations. Letters were assigned to values after analysis of contrasts revealed differences and similarities ($P = 0.05$). Values followed by the same letter are not significantly different.

The recent identification of heritable resistance in the breeding population GT-MAS:gk, however, indicates that it may be possible to develop commercial maize lines with moderate-to-high resistance to aflatoxin production (4,5,6,32). Two types of resistance appear to function in kernels of this population: i) pericarp resistance, which may be due to the wax and cutin content (17), and ii) resistance associated with internal pericarp tissue (5,17). Currently, the resistance mechanisms present in this population are not well characterized. Consequently, the complete characterization of these mechanisms is important because this information could lead to the development of selectable markers for plant breeding as well as to the isolation of genes for use in the development of resistant transgenic plants.

One of the objectives of our study was to determine if other highly resistant maize genotypes could be identified. We used the same KSA used earlier to both confirm resistance and to detect the expression of different resistance mechanisms in GT-MAS:gk (5) to screen 27 inbreds for resistance to aflatoxin accumulation. This assay gave consistent rankings of the genotypes in three tests and allowed us to identify 22 genotypes with resistance comparable to GT-MAS:gk.

The KSA has several major advantages: i) it can be done at any time and repeated several times throughout the winter; ii) it requires very few kernels; iii) it can detect/identify different kernel resistance mechanisms expressed; iv) it can dispute or confirm field evaluations (i.e., genotypes rated as having high levels of re-

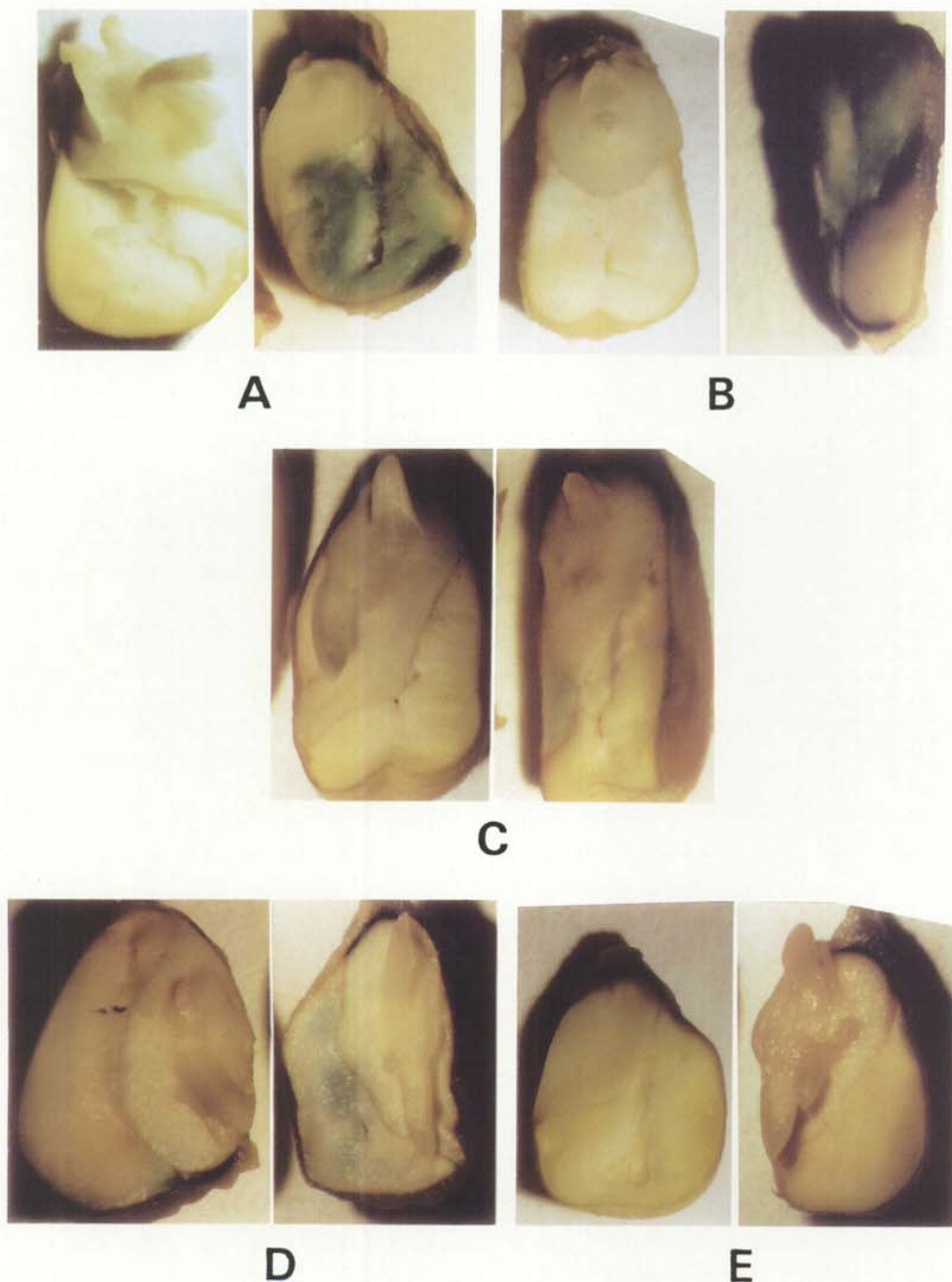

Fig. 1. Visualization of *Escherichia coli* β-D-glucuronidase reporter gene expression (blue) in typical nonwounded and wounded kernels for five maize genotypes. **A,** NC232; **B,** CI2; **C,** GT-MAS-gk; **D,** T115; and **E,** MI82. For each genotype, the nonwounded kernel is on the left, and the kernel pin-wounded in the endosperm is on the right.

sistance in the field may be escapes); and, v) there appears to be a relationship between laboratory findings and inoculations in the field. Besides the relationship between GT-MAS:gk field and laboratory findings (5,32), inbreds CI2 and T115, which demonstrated resistance to Aspergillus ear rot and aflatoxin accumulation in Illinois field trials (8), were resistant in the KSA. Inbred 33-16, which was extremely susceptible in the Illinois study, proved to be the same in the KSA, whereas B73 and Mo17 demonstrated moderate susceptibility in both field and laboratory studies (8).

The only major difference between the Illinois field study and the KSA was in the performance of inbred Tex6, which was extremely susceptible in the laboratory but very resistant in the field. Further testing with the KSA, of a different Tex6 seed lot, confirmed its resistance and detected wounded-kernel resistance as well (R. Brown, *personal communication*). Conflicting results by the KSA with Tex6 may point to a weakness of this screening procedure: its possible dependence on seed quality.

Further examination of three resistant genotypes along with GT-MAS:gk and a susceptible genotype, NC232, for colonization by *A. flavus* was done to learn more about the nature of resistance. Our objectives were to visualize fungal colonization in resistant and susceptible kernels and to relate fungal colonization to aflatoxin production. Colonization of kernels infected by a strain of *A. flavus* expressing the GUS gene was followed by visuali-

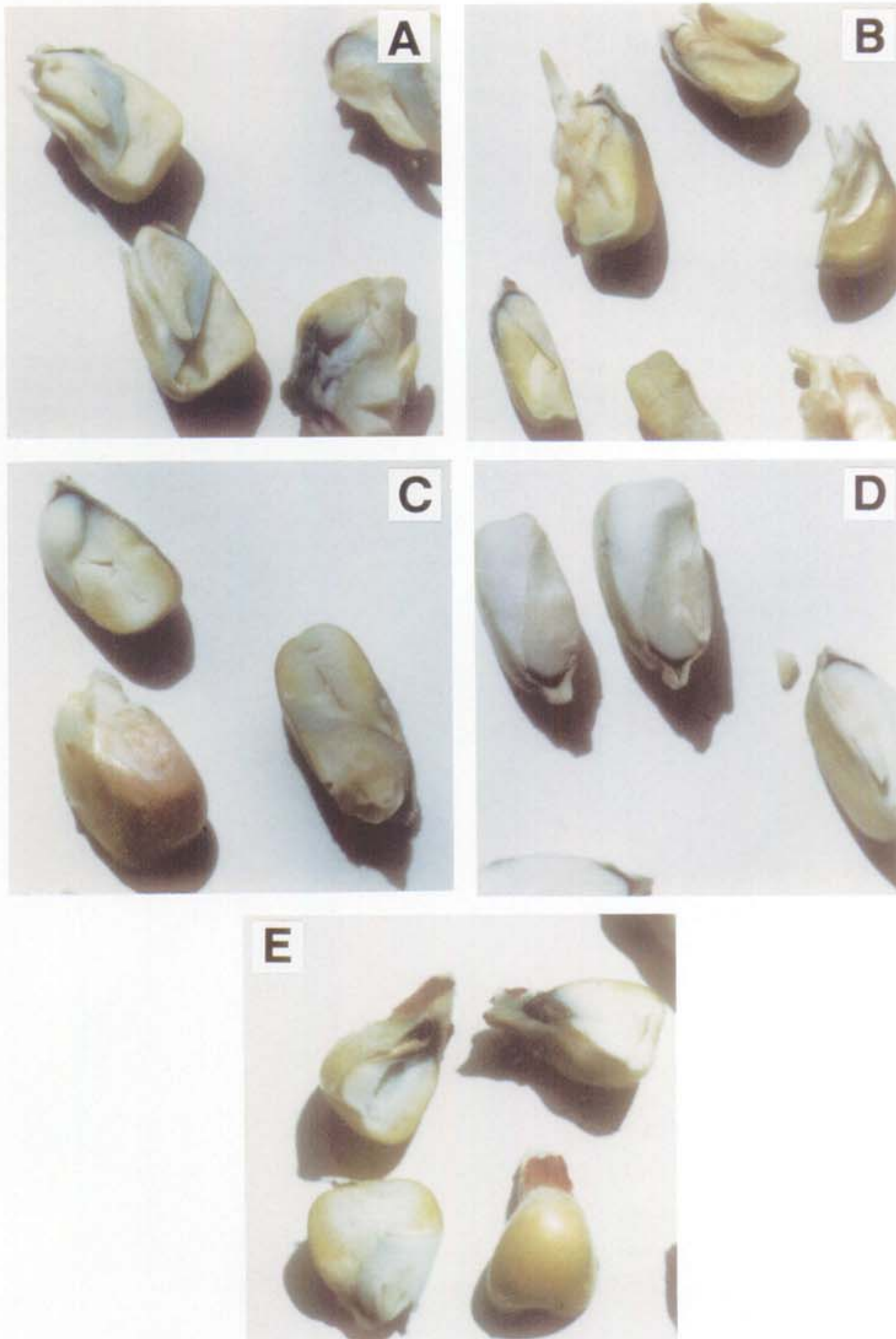

Fig. 2. Visualization of *Escherichia coli* β-D-glucuronidase (GUS) reporter gene expression (blue) in typical wounded kernels for five maize genotypes 3 days after inoculation (1 day after inoculation for entry NC232). **A,** CI2; **B,** MI82; **C,** GT-MAS:gk; **D,** T115; and **E,** NC232. GUS activity, where seen, is present only in the embryo for all entries. This is the same pattern seen in nonwounded kernels 1 or 3 days after inoculation and 7 days after inoculation (when GUS expression is seen).

zation of the blue product of the enzymatic reaction. This strain appeared to colonize tissue similarly to the wild-type strain AF13, as intact kernels of GT-MAS:gk, CI2, T115, NC232, and MI82 showed the same response with respect to aflatoxin production whether they were inoculated with AF13 or the strain with the reporter construct.

Results from these studies showed a strong relationship between fungal colonization and aflatoxin contamination. In the first test, kernels of maize entries GT-MAS:gk, MI82, and T115 supported relatively little fungal colonization and aflatoxin accumulation, even if they were wounded before inoculation. In the second test, there was greater overall fungal colonization and aflatoxin contamination in all kernels tested, with the exception of MI82, which continued to support both minimal fungal growth and toxin production. However, the pattern of aflatoxin accumulation among genotypes in test 2 was similar to that seen in test 1. Thus, genotypes GT-MAS:gk, MI82, and T115 appear to have a resistance mechanism independent of the kernel pericarp. In contrast, the extensive colonization and the high level of aflatoxin contamination of CI2 (a genotype rated as resistant in the intact-kernel evaluation) after wounding indicates that the pericarp contributes to the resistance of this genotype. These studies confirm earlier studies (16) that showed the pericarp to be important in some genotypes, whereas other mechanisms functioned in other lines.

The use of the *A. flavus* strain with the reporter gene construct illustrated the colonization of maize kernels over time. The embryo, primarily in the scutellum region, was the first tissue internal to the pericarp to be colonized in kernels of all five genotypes, regardless of treatment. Initial colonization of the kernel appears to proceed through the pedicel and aleurone (a thin layer of tissue just under the pericarp) layers in unwounded kernels. Colonization of kernel endosperm tissue was not seen in kernels sampled earlier than 7 days after inoculation, and extensive colonization of this tissue occurred only in wounded kernels. The extent of fungal colonization of kernel embryonic tissues, and the subsequent spread of the fungus to endosperm tissue was related to the genotype. Those genotypes susceptible to aflatoxin accumulation (i.e., NC232) were more extensively colonized than the resistant genotypes.

In a previous study (21), embryonic and aleurone tissue were colonized by *A. flavus* preferentially over endosperm tissue, but endosperm tissue was colonized more extensively in germinating kernels. In the present study, fungal ingress into the endosperm appeared to proceed from the embryonic scutellum region and/or the wound in the pericarp. Thus, embryonic tissue appears to be the favored substrate for the fungus. This conclusion is supported by earlier studies (5) that showed that GT-MAS:gk kernels wounded in the embryo region prior to inoculation with *A. flavus* supported four to five times more aflatoxin production than did a similarly treated susceptible line. The high levels of aflatoxin produced on embryo tissue could be related to the high lipid content in this tissue (1,28).

This study shows the usefulness of the GUS gene fusion system in phytopathological studies. In this study, we were able to use this system to compare the colonization of resistant and susceptible lines and to follow the colonization of kernel tissue by the fungus. The results presented here provide new evidence for the existence of factors within the seed that confer resistance to aflatoxin accumulation. Also, these results suggest that resistance in the maize genotypes examined may be to the fungus itself and not to the synthesis of aflatoxin.

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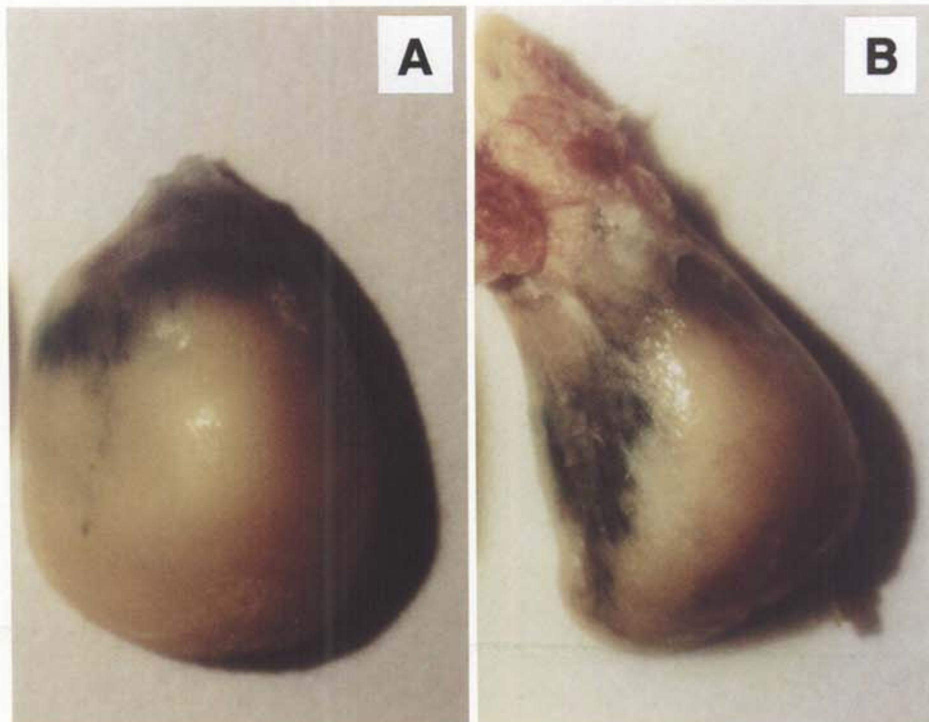

Fig. 3. Visualization of *Escherichia coli* β-D-glucuronidase (GUS) reporter gene expression (blue) under the pericarp in typical maize kernels from two genotypes. **A,** MI82, nonwounded; **B,** NC232, wounded. GUS is seen under the pericarp of nearly all kernels 7 days after inoculation, whether the kernels were inoculated nonwounded or wounded, even in kernels that showed no GUS expression in the internal tissues.

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