Identification of Chemical Components of Corn Kernel Pericarp Wax Associated with Resistance to *Aspergillus flavus* Infection and Aflatoxin Production

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Kernel pericarp wax of the corn breeding population GT-MAS:gk has been associated with resistance to *Aspergillus flavus* infection and aflatoxin production. GT-MAS:gk wax, previously compared to waxes of three susceptible genotypes, was presently compared to wax of a different, and more numerous, group of susceptible lines. Wax separation by TLC confirmed previous findings, demonstrating a unique GT-MAS:gk band and a unique "susceptible" band. Only GT-MAS:gk wax inhibited the growth of *A. flavus;* however, no association was established, as before, between kernel wax abundance and resistance. Gas chromatography−mass spectroscopy (GC−MS) analysis of kernel whole wax showed a higher percentage of phenol-like compounds in wax from GT-MAS:gk than in waxes from the susceptible lines. The GT-MAS:gk unique band contained phenol-like compounds and ethyl-hexadecanoate; butyl-hexadecanoate was preeminent in most of the "susceptible bands". Alkylresorcinol (phenolic compounds) content was dramatically higher in GT-MAS:gk wax than in the wax of susceptible lines. An alkylresorcinol, 5-methylresorcinol, also inhibited in vitro growth of *A. flavus*. These and other phenolic compounds may contribute to kernel wax inhibition of *A. flavus* infection/aflatoxin production. Further investigation is needed to confirm a role for them in GT-MAS:gk resistance.

Keywords: *mycotoxin; host resistance; Zea mays; wax*

INTRODUCTION

Aflatoxins are secondary metabolites produced by the fungi *Aspergillus flavus* link: Fr. and *Aspergillus parasiticus* Speare (*1*). Aflatoxin contamination of corn (*Zea mays* L.) is a problem not only in the United States, but throughout the world (*2*). Aflatoxins cause mortality and reduce productivity in farm animals (*3*), and also are detrimental to humans: high concentrations have been associated with liver cancer (*4*). Infection by the fungus barely affects crop yield; however, it causes severe loss in crop value (*5*) because contamination with aflatoxins reduces the value of grain for domestic feed and for export. Aflatoxin levels in food and feed are regulated by the FDA (20 ppb, interstate commerce). Limits in several other countries, however, are even lower than those in the U.S. This creates a situation that could lead to trade disputes.

Since host resistance was determined to be a viable strategy for controlling preharvest aflatoxin contamination of corn, several genotypes have been identified as resistant to aflatoxin production (*6*−*10*). These resistant lines, however, have poor agronomic qualities, making them unsuitable for commercial use. Research efforts, therefore, focus on identifying kernel resistance traits in these lines that limit aflatoxin accumulation (*11*). Resistance traits that are inhibitory to either fungal growth or aflatoxin production include subpericarp components such as preformed and induced proteins, and kernel pericarp characteristics (*11*, *12*). In resistant genotypes evaluated for both fungal infection levels and aflatoxin accumulation, resistance to aflatoxin production appears to be primarily a function of fungal growth inhibition (*13*, *14*).

Corn kernel pericarp wax of the resistant corn population GT-MAS:gk was investigated, and an association between wax and resistance to aflatoxin production was demonstrated (*12*). When GT-MAS:gk wax was quantified and compared to that of three susceptible lines (*15*), it was shown to be more abundant on kernels than wax found on kernels of the susceptible genotypes. Also, growth media incorporating GT-MAS:gk wax (but not media incorporating wax of either susceptible line) was inhibitory to *A. flavus* growth. When pericarp wax of the above genotypes was subjected to thin-layer chromatography (TLC), a unique band present only in GT-MAS:gk was identified, as was a band present in the susceptible lines, but missing from GT-MAS:gk (*15*). These results raise the possibility of identifying kernel pericarp resistance factors that could eventually be used as markers when breeding to develop agronomically useful, aflatoxin-resistant corn lines.

The purpose of the present study was to identify the chemical composition of the wax components unique to GT-MAS:gk and of those unique to the susceptible genotypes. To achieve these objectives, the authors first confirmed the uniqueness of these resistance factors (kernel wax amount, inhibitory properties, and unique TLC bands) by investigating a group of susceptible lines different from and more numerous than the group previously studied (*15*). Then, the chemical compositions of the unique bands were determined. A preliminary presentation of these data has been made (*16*).

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MATERIALS AND METHODS

Corn Entries. Eleven commercial maize hybrids, Pioneer Hybrid Brands 3162, 3260, and 3223 (P3162, P3260, P3223), Dk 668, Cargill 688 (Car688), Mycogen 288 IMI (Myc 288 IMI), Dk 689, Garst 8325 (Gar8325), Cargill 7731 IMI (Car7731), Garst 8513 IT (Gar8513), 8412, and Deltapine G-4666 (G-4666), were obtained from the Louisiana State University Department of Plant Pathology and Crop Physiology (Baton Rouge, LA). The resistant maize genotype, GT-MAS:gk (*10*), was obtained from the USDA-ARS-IBPMRL in Tifton, GA. All kernels were dried and stored at 4 °C in airtight containers until used. Intact kernels of uniform size and shape were used in all experiments.

Fungal Cultures. *A. flavus*, strain AF13, which produces large quantities of aflatoxins in cottonseed and in corn kernels (*17*, *18*), was provided by P. J. Cotty of the USDA/ARS/SRRC (New Orleans, LA). The fungus was grown on V8 vegetable juice agar plates (5% V8 juice and 2% agar) at 31 °C in darkness. Conidia from 7-day old cultures served as inocula. All manipulations involving *A. flavus* were performed using a biological cabinet or a fume hood. Glassware in contact with this fungus or with aflatoxins was soaked overnight in a sodium hypochlorite solution prior to being washed.

Aflatoxin Determination in Corn Kernels. Kernels of each genotype were surface-sterilized (*19*) and then subjected to the kernel screening assay (KSA) for determining aflatoxin accumulation in corn kernels (*13*). The KSA is a rapid and relatively low-cost laboratory assay for screening corn kernels for resistance to aflatoxin accumulation (*11*). It is designed to provide optimal conditions for both fungal growth and aflatoxin production, and KSA results correlate well with field results (*11*). The inoculum concentration was 4×10^6 conidia/mL, and kernels were incubated for 7 days at 31°C. Each treatment was replicated 10 times (each replicate contained 4 seeds), and the experiment was performed twice. Aflatoxin B_1 content of kernels of each genotype was determined using the method of a previous study (*20*) that involves methylene chloride extraction, TLC separation, and quantification of aflatoxins with a scanning densitometer.

Effect of Whole Wax on *A. flavus* Growth. To remove kernel pericarp wax, corn kernels (50 g) from each genotype were surface-sterilized by washing the seeds with 10% sodium hypochlorite at pH 10.6 for 1 min, followed by three water rinses (3 min each), and drying (*21*). To each beaker of 50 g of kernels, 200 mL of chloroform was added with stirring for 60 s at room temperature (*15*). The kernels were then removed, and the chloroform was evaporated to dryness. Residual wax was redissolved in 3−5 mL of chloroform, and the contents of the beakers were transferred to small vials to dry. The dried content was re-suspended in 1.5 mL of chloroform. Then, 250 μL of the wax extract was spread with an inoculating loop onto Adye and Mateles (A&M) agar medium (*22*) and left to dry for approximately 2 h (to evaporate chloroform). Afterward, plates were inoculated with 10 μL of *A. flavus* suspension (1×10^6 conidia/mL) and incubated at 31 °C. Colony diameter was measured at 24, 72, 120, and 168 h. Each treatment was replicated four times and the experiment was performed twice.

Thin-Layer Chromatography of Pericarp Wax. Kernel pericarp wax was removed as described above. Contents of the small vials were redissolved in 0.5 mL of hexane. A 15-μL portion of each sample were applied to a TLC plate that was subsequently developed at 25 °C in a solvent system of benzene/chloroform (7:3, v/v). After development, the TLC plates were sprayed with concentrated sulfuric acid and charred until bands appeared. To isolate the unique bands, TLC plates were developed as mentioned above, and afterward visualized by putting the plates in a covered glass tank with iodine crystals until bands could be seen. Bands of interest were circled and the iodine was allowed to evaporate. The bands were then scraped and re-suspended in hexane for further analysis with gas chromatography−mass spectroscopy (GC−MS). The experiment was performed three times.

GC−MS Analysis of Whole Wax and of TLC Bands. Corn kernel pericarp wax from the surface of corn kernels (100 g) was removed as described above. The residual whole wax was redissolved in 0.2 mL of hexane and analyzed using GC−MS. Samples (1 μL) of whole wax or wax of TLC bands were injected into a splitless injector on an HP 5973 GC/MS system (Agilent Technologies, Palo Alto, CA). The injector temperature was held constant at 300 °C. The GC oven temperature was held for 1 min at 100 °C and then ramped 5 °C/min to 150 °C, and held there for 10 min. The temperature was then raised 10 °C/min to 300 °C, where it was held for 7 min (total time was 33 min). A 30 m × 0.25 μm i.d., DB-5 capillary column was used with helium as the carrier gas, under a constant flow of 40 cm/s. The total GC cycle time consisted of a 35 min run and a 5 min cool-down period.

The mass spectrophotometer was operated in the scan mode from *m*/*z* 50 to 550, and peak areas were determined for each compound by integrating a selected ion for that compound. All fatty acid peak areas were normalized on *m*/*z* 127 and the molecular ion was used as the qualifying ion. Steroids were integrated on their molecular ions. MSD chemstation software (from Agilent Technologies, Palo Alto, CA) was used to develop a spreadsheet containing samples and selected compounds. The experiment was performed twice.

Determination of Alkylresorcinols in Whole Wax. To further characterize the phenolic composition of kernel pericarp waxes in the corn genotypes, a colorimetric method for determining the amount of alkylresorcinols present in wax extracts using diazonium salt fast blue B was employed (*23*). Wax from the corn kernel surfaces (100 g of kernels) of nine genotypes, including GT-MAS:gk, was removed as described above. 5-Methylresorcinol (Aldrich, Milwaukee, WI), an alkylresorcinol compound, was used as a standard (the standard calibration curve was 0.00−80 μg/μL), and a Shimadzu UV-1601 spectrophotometer determined the amount of alkylresorcinols at 560 nm. Each treatment was replicated twice and the experiment was performed three times.

Effect of 5-Methylresorcinol on *A. flavus* Growth. A&M agar plates were poured incorporating different concentrations (0, 5, 10, 15, and 20 μg/μL) of 5-methylresocinol (Aldrich, Milwaukee, WI). Plates were inoculated with an *A. flavus* conidia suspension (10^6 conidia/mL) prepared from 7-day old cultures, and growth was measured at 24, 48, 72, 96, and 120 h. Each treatment was replicated four times and the experiment was performed twice.

Statistical Analysis. Analyses of data obtained from the aflatoxin determination and from the wax vs fungal growth experiments were performed with the Statistical Analysis Software System (SAS Institute, Inc., Cary, NC). Treatment replicates from each test in each experiment were first subjected to analysis of variance followed by mean comparisons of colony diameter values or square-root transformations of aflatoxin values. Transformations were performed to equalize treatment variances. Differences among treatment means were determined by the least significant difference test ($P = 0.05$).

Data obtained from the alkylresorcinol determination and the alkylresorcinol vs fungal growth experiments were analyzed with Microsoft Excel data analysis Add-In for Windows 95 and Sigma plot 2000 (SPSS Inc., Chicago, IL). Treatment means ± standard error were calculated.

Safety. Special precautions in handling the toxigenic fungus *A. flavus* were used, including fume hoods and biological cabinets during toxin extractions and handling of fungal spores. All glassware in contact with the fungus was soaked in sodium hypochlorite prior to washing and re-using.

RESULTS

Aflatoxin Accumulation in Corn Kernels. KSA results showed differences in aflatoxin B_1 accumulation among the genotypes tested (Figure 1). With the exception of Mycogen 288 IMI, all other corn genotypes accumulated more aflatoxin B_1 than did GT-MAS:gk. Experimental problems and a lack of seed prevented the acquisition of aflatoxin data for Garst 8325.

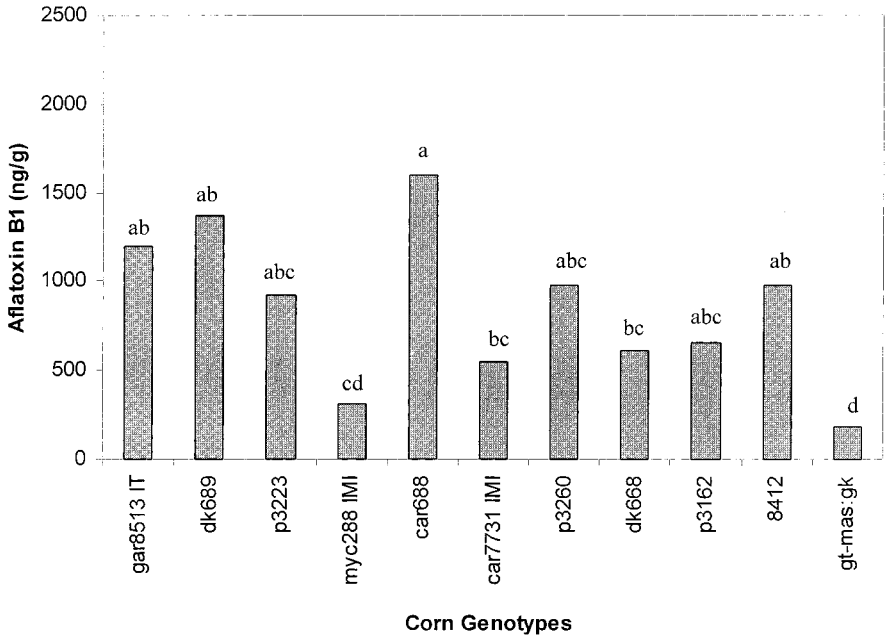

Figure 1. Aflatoxin accumulation (in ng/g) in eleven corn genotypes, including resistant population GT-MAS:gk. Bars represent means of aflatoxin values of twenty reps measured over two experiments. Values (bars) with the same letter are not significantly different by the least significant difference test ($P = 0.05$). Data were square-root transformed prior to analyses to equalize variances.

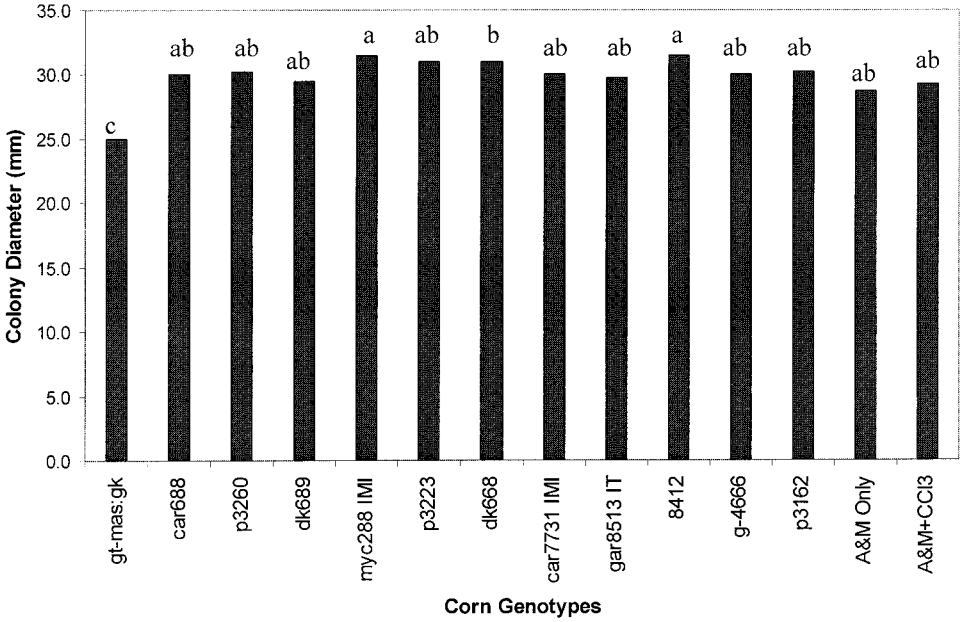

Figure 2. *A. flavus* colony diameter (mm) in A&M media amended with kernel pericarp wax of each genotype. Bars represent means of *A. flavus* colony diameter values of eight reps over two experiments after 120 h. Values (bars) with the same letter are not significantly different by the least significant difference test ($P = 0.05$). P3223, P3260, and P3162 are Pioneer Hybrid Brands. A&M media and A&M with chloroform (allowed to evaporate) served as controls.

Effect of Whole Wax on *A. flavus* Growth. *A. flavus* colony diameter was significantly reduced on A&M media amended with GT-MAS:gk wax after 120 h of incubation compared to that of the controls (Figure 2). Significant reductions in colony diameter on media amended with GT-MAS:gk wax were not observed at any other time point. No growth reductions were seen at any time point on media amended with the wax of any of the eleven other genotypes. The above results were observed in each of the two experiments.

TLC Separation of Kernel Pericarp Wax. TLC of kernel wax showed differences between the resistant genotype, GT-MAS:gk, and the other eleven corn lines. The band unique to GT-MAS:gk and the band unique to aflatoxin-susceptible genotypes, previously identified (*15*), were confirmed in the present study. TLC bands other than these were similar in their R_f values and intensities among all entries tested.

GC−MS Analysis of Whole Wax and of Unique TLC Bands. Chemical analysis of the volatile fraction showed little differences in the fatty acid distribution (C-21 to C-35) among the genotypes (Figure 3). Steroid #1 (stigmasterol) constitituted 10% of the chemical composition of GT-MAS:gk, but higher levels of steroid #1 were found in Cargill 7731. Steroid #2 (campesterol) was detected only in Pioneer hybrid brand 3260. There were higher levels of phenolic compounds in GT-MAS:gk wax than in the other genotypes.

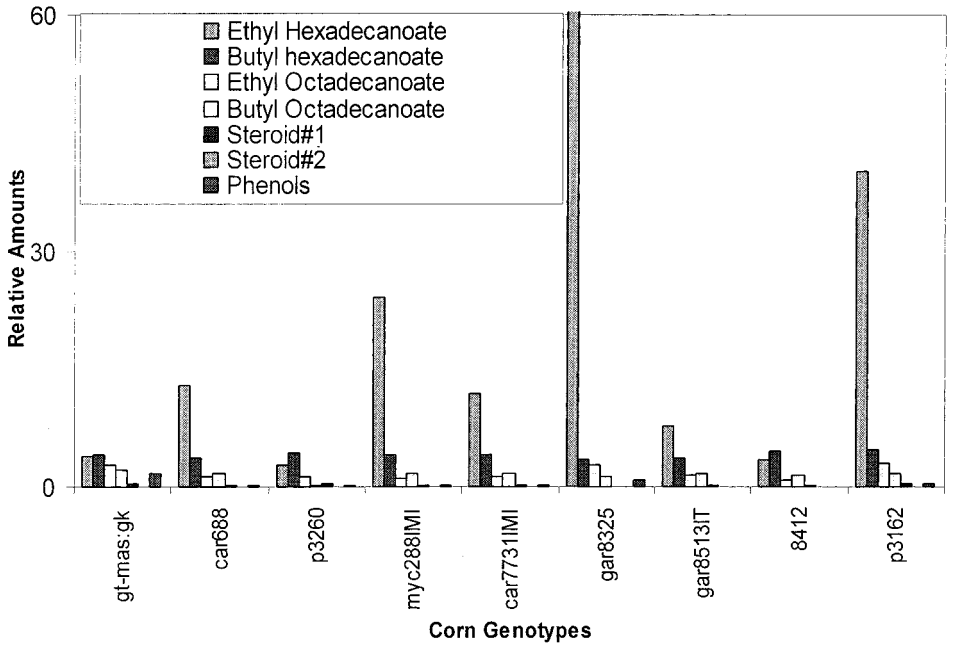

Figure 3. Chemical components of whole kernel pericarp wax of corn genotypes. Relative peak areas were normalized on C-25. Steriod # 1 = stigmasterol; steriod # 2 = campesterol.

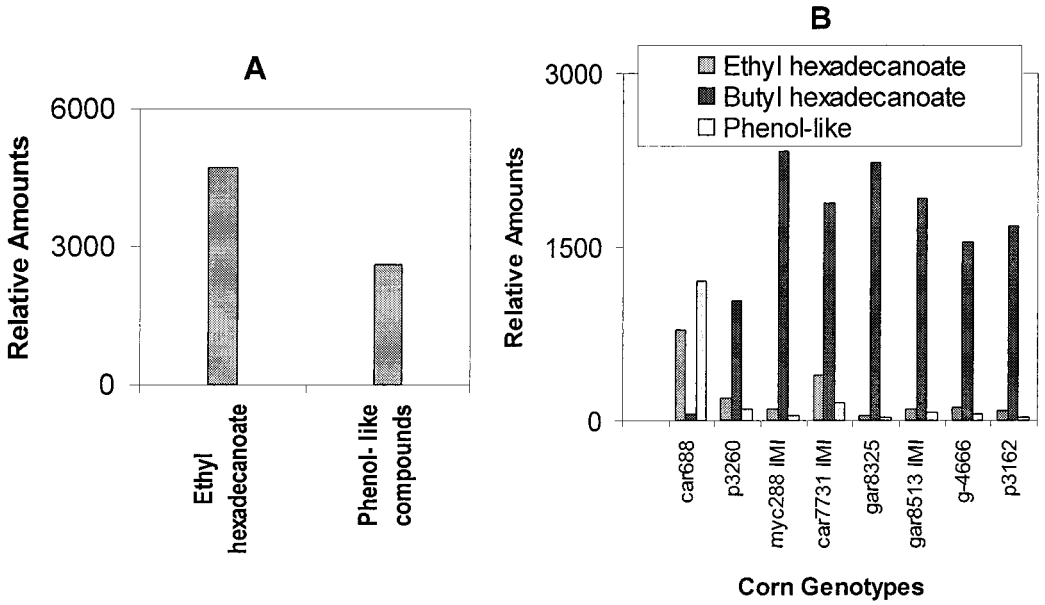

Figure 4. (A) Chemical components present in TLC band unique to GT-MAS:gk. Relative peak areas were normalized on C-25. (B) Chemical components present in TLC band unique to the susceptible genotypes. Relative peak areas were normalized on C-25.

Ethyl-hexadecanoate was present in each line tested, but was higher in amount in the whole wax of several susceptible lines than in GT-MAS:gk. Analysis of individual bands, however, revealed ethyl-hexadecanoate and phenol-like compounds present in the unique GT-MAS:gk band (Figure 4a), whereas butyl-hexadecanoate was the most prevalent compound in the unique "susceptible" band in most lines (Figure 4b). No data on the chemical composition of either whole wax or the unique band were obtained for P3223, Dk 688, or Dk 689 due to the inability to obtain more seed.

Determination of Alkylresorcinols in Whole Wax. Results showed GT-MAS:gk to have a significantly higher amount of alkylresorcinols than all other genotypes tested (Figure 5). Seven of the other eight genotypes contained alkylresorcinol levels not significantly different from each other.

Effect of 5-Methylresorcinol on *A. flavus* Growth. Growth of *A. flavus* in the presence of an alkylresorcinol compound, 5-methylresorcinol, is shown in Figure 6. The highest growth inhibition occurred on media amended with 15 or 20 μg/μL of 5-methylresorcinol at 96 h. Growth inhibition also occurred at 72 h at a 10, 15, or 20 μg/μL concentration. No fungal growth was observed at either 0 or 24 h, and no growth inhibition was observed at 48 or 120 h.

DISCUSSION

Formation of aflatoxins in agricultural commodities before harvest in hot and humid areas is difficult to

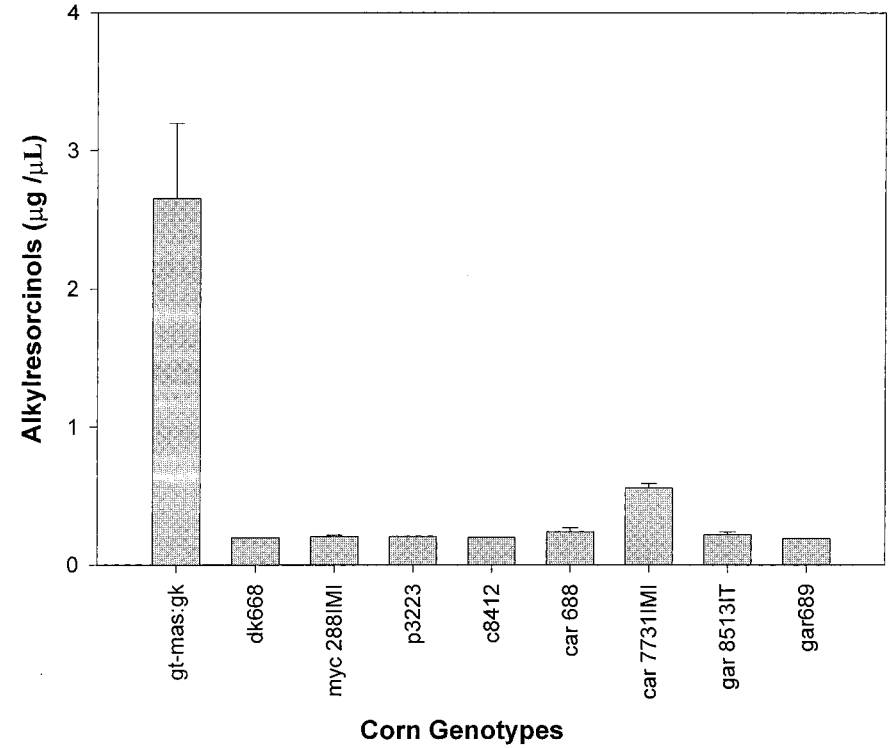

Figure 5. Alkylresorcinol content (μg/μL) of eight corn genotypes compared to that of GT-MAS:gk. Bars represent alkylresorcinol means ± standard error of six replicates over three experiments.

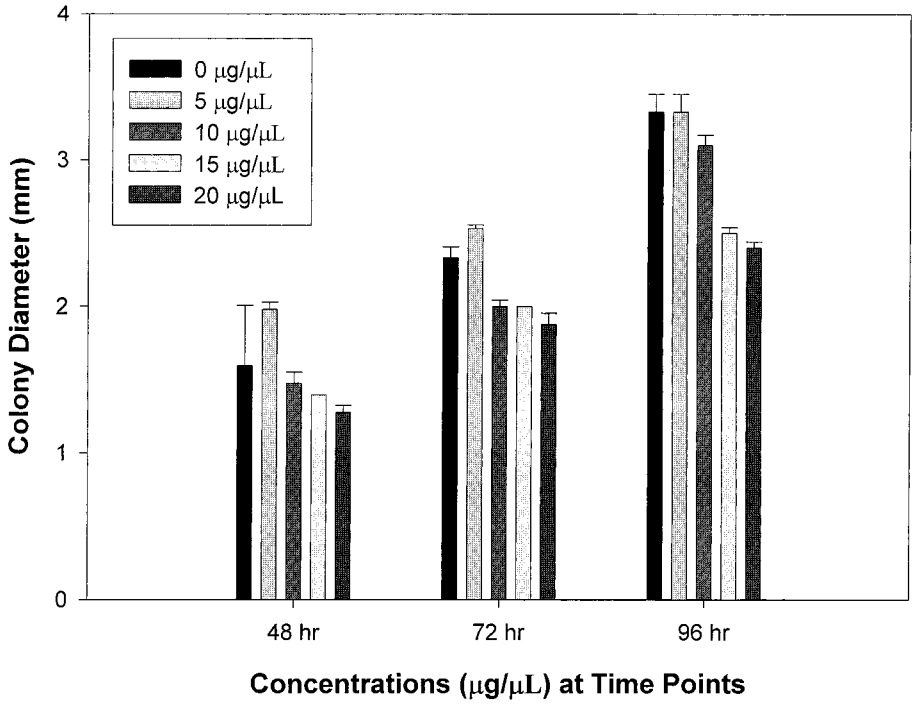

Figure 6. Growth of *A. flavus* in A&M media amended with 5-methylresorcinol. Bars represent colony diameter means ± standard error of eight replicates over two experiments.

control through conventional agronomic practices (*4*). Promising methods of control presently being investigated include biological control (replacing toxigenic strains of *A. flavus* with nontoxigenic strains in the field) (*17*) and developing host resistance against fungal growth and/or aflatoxin production (*24*). The latter method can involve marker-assisted breeding and/or genetic engineering. The present research was conducted to identify specific kernel pericarp wax traits of the resistant genotype GT-MAS:gk, and to investigate their potential to contribute to host resistance.

Previous research in our laboratory (*12*, *15*) demonstrated the presence of an antifungal trait(s) in GT-MAS:gk kernel wax, which was absent in three susceptible lines tested. In the present study, we first demonstrated the higher accumulation of aflatoxins in nine of 10 genotypes tested against GT-MAS:gk. Although Mycogen 288 IMI aflatoxin levels were not

significantly different from GT-MAS:gk levels in data presented in Figure 1, further evaluation (data not shown) failed to confirm equally low accumulation of aflatoxins in Mycogen 288 IMI. Bioactivity assay results of Mycogen 288 IMI wax versus *A. flavus* growth, and wax chemical profiles of this genotype also are consistent with susceptibility and not with GT-MAS:gk profiles.

The authors demonstrated, again, the ability of GT-MAS:gk wax to uniquely inhibit *A. flavus* growth. In previous investigations, GT-MAS:gk kernels also were shown to be more abundant in pericarp wax than three susceptible lines. When wax amounts were compared among all lines tested in the present study, a relationship with resistance was not established (data not shown). However, TLC separation of pericarp wax of eleven susceptible lines and of GT-MAS:gk confirmed the association of resistance or susceptibility with unique TLC bands, as previously demonstrated in the study employing only three susceptible lines (*15*).

GC−MS analysis of the whole kernel wax of the tested genotypes revealed that kernel pericarp wax contains primarily long-chain hydrocarbons, esters, fatty acids, alcohols, and steroids. There is variation in the amounts of these compounds among genotypes, however, notable differences occur only in steroid and phenol content. In GT-MAS:gk, no evidence of ergosterol-like steroids was observed in the whole wax injection, nor in the isolated TLC band. Relatively higher amounts of ergosterol-like steroids were seen in Cargill 7731, Garst 8325, Garst 8513IT, and Pioneer Brand hybrid 3162, compared to those of the other susceptible lines. Although GC−MS data were not obtained for P3223, Dk 668, or Dk 689, all other data for these three lines in the present study fit the profile for aflatoxin-susceptibility.

GT-MAS:gk whole wax had a phenolic content that was higher than the phenolic contents of all susceptible lines tested. Analysis of the unique TLC band of GT-MAS:gk also indicated phenol-like compounds as significant components of this band. In a previous investigation, a mixture of 5-alkylresorcinol homologues isolated from etiolated rice seedlings inhibited the rice blast fungus, *Pyricularia oryzae* (*25*). In another study, a similar mixture of 5-alkylresorcinol homologues isolated from the peel of mango fruit inhibited *Alternaria alternata*, causal agent of black spot disease of mango fruits (*26*). Other studies also implicate phenolic compounds and in particular, alkylresorcinols, in antifungal resistance (*27*−*29*).

In an effort to further characterize the phenolic compounds present in kernel wax in the present study, the alkylresorcinol content was measured in the whole wax and compared among corn genotypes. Results showed GT-MAS:gk amounts to be significantly and dramatically higher than those of all susceptible lines tested. That an alkylresorcinol, 5-methylresorcinol, also inhibited *A. flavus* growth, suggests that alkylresorcinol abundance in GT-MAS:gk wax compared to wax of susceptible lines may contribute to kernel resistance to *A. flavus* infection and subsequent aflatoxin contamination demonstrated by this genotype.

Here we have identified the chemical composition of the whole kernel pericarp wax of GT-MAS:gk, of susceptible genotypes, and of specific wax TLC bands associated with resistance or susceptibility to aflatoxin elaboration. Efficacy against *A. flavus* growth also has been demonstrated in vitro by an alkylresorcinol; this class of phenolic compounds has been determined to be higher in amount in whole GT-MAS:gk wax than in wax of susceptible lines. Phenol-like compounds have been shown to be pre-eminent as a component in the GT-MAS:gk unique TLC band as well. Future investigations are needed to characterize the alkylresorcinols and other phenol-like compounds present in GT-MAS:gk pericarp wax and confirm their role in resistance. Studies also are needed to further characterize the identified chemical components of the unique TLC bands, and to clarify the significance of the TLC bands themselves to resistance. It also may be useful to determine the role of pericarp wax in the resistance of other corn genotypes, as well as the chemical composition of these waxes. These efforts may facilitate using resistance-associated compounds in kernel pericarp wax in marker-assisted breeding strategies aimed at developing commercially useful, resistant corn lines.

ABBREVIATIONS USED

FDA, Food and Drug Administration; TLC, thin-layer chromatography; GC−MS, gas chromatography−mass spectroscopy; A&M, Adye and Mateles; KSA, kernel screening assay.

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