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A β-Glucuronidase Reporter Gene Construct for Monitoring Aflatoxin Biosynthesis in *Aspergillus flavus*†

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Aflatoxins are toxic and carcinogenic secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus*. Current research is directed at the elimination of these compounds in important food sources. The objective of this research was to develop a method to study the induction and regulation of aflatoxin biosynthesis by examining the expression of one aflatoxin pathway gene, *ver1*. The promoter region of *ver1* was fused to the β-glucuronidase (GUS) gene (*uidA*) from *Escherichia coli* to form the reporter construct, GAP13. *A. flavus* 656-2 was transformed with this construct. Aflatoxin production, GUS activity, and transcript accumulation were determined in transformants after shifting the cultures from a nonconducive medium to a medium conducive to aflatoxin biosynthesis. Transformants harboring GAP13 displayed GUS expression only when aflatoxin was detected in culture. Further, the transcription of the *uidA* gene driven by the *ver1* promoter followed the same profile as for the *ver1* genes. The results show that the GAP13 construct may be useful as a genetic tool to study the induction of aflatoxin in situ and to identify substances that affect the expression of genes involved in aflatoxin biosynthesis. The utility of this construct to detect inducers of aflatoxin biosynthesis in maize kernels was tested in a bioassay. A heat-stable inducer of aflatoxin with a molecular size of less than 10 kDa was detected in extracts from maize kernels colonized by *A. flavus*.

Aflatoxins are toxic and carcinogenic secondary metabolites produced by the fungi *Aspergillus flavus* Link: Fries and *A. parasiticus* Speare. These fungi readily colonize several important crops such as corn, cottonseed peanuts, and tree nuts (9). Currently, there are no commercial crops with resistance to infection by these fungi or to aflatoxin production. However, there is evidence that resistance and susceptibility are heritable genetic traits (5, 6, 13, 18, 22). The development of crops with resistance has been slowed by the complex nature of the host-pathogen interaction (18). *A. flavus* and *A. parasiticus* are saprophytes, but both can be pathogenic if the health of the host plant is stressed. Stress from drought conditions and high temperatures are important factors that lead to high levels of infection and aflatoxin contamination. Insects that damage host kernels also increase the likelihood of high aflatoxin contamination because they provide an infection site for fungi. A major difficulty in selecting resistant genotypes has been the lack of uniform environmental conditions in fields from year to year. All to often, there is one year of severe natural infection by *A. flavus* followed by little or no infection in the next year.

Genetic markers that follow the inheritance of resistance to aflatoxin contamination are needed. A potential source of markers may come from studies examining the host-fungus interaction to identify host metabolites that are important factors influencing aflatoxin biosynthesis. Factors affecting growth of *A. flavus* have the greatest impact on aflatoxin production. These factors include carbon, nitrogen, lipids, and minerals, and these nutrients as components of seeds may have profound effects on *A. flavus* (17). Studies on maize kernels have indicated that the sugary kernel genotypes support higher growth and more aflatoxin accumulation than starchy genotypes (26). Lipids from seeds were found to enhance the growth of *A. flavus*, but the stimulation of aflatoxin production by seed lipids was correlated with the potential to form lipoperoxides (10, 12, 20, 23). There is also a positive correlation between aflatoxin production and the zinc content in maize kernels (11). Despite these findings, we need a better understanding of which seed components influence aflatoxin production and how the environmental stresses that predispose a host plant to *A. flavus* infection and aflatoxin contamination also influence the quality and quantity of these seed components.

Measuring growth, sporulation, and aflatoxin production constitutes the traditional method used for studying resistance to *A. flavus* (26). However, such measurements are not conducive to the identification of specific seed metabolites that directly affect aflatoxin biosynthesis. Specifically, growth and sporulation are not always correlated with aflatoxin concentrations, and procedures that measure aflatoxin production are expensive and have variable results. Recently, several genes involved in aflatoxin biosynthesis have been characterized, and expression of these genes has been shown to correlate with aflatoxin production (7, 19, 24, 25, 28, 31). In this study, we have used one of these aflatoxin biosynthesis genes (*ver1*) to develop a reporter gene assay that can readily measure the activation of a gene for aflatoxin biosynthesis. The product of the *ver1* gene is involved in the conversion of versicolorin to sterigmatocystin in the aflatoxin biosynthetic pathway (25). We show here that measurements derived from this assay reflect aflatoxin accumulation in culture and the transcription of the aflatoxin gene in *A. flavus*. We also have used this assay to detect and partially characterize an aflatoxin inducer from maize kernels.

MATERIALS AND METHODS

Fungal isolates and culture methods. *A. flavus* 656-2 (*w leu pyrG* aflR) was used as the recipient strain in fungal transformations. This strain was created by parasexual crosses of strains 656 (*w met alf9*) and 650-33 (*t leu pyrG aflR*) (19, 28,

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29). Because of a mutated regulatory *aflR* gene, strain 656-2 does not produce aflatoxin (19, 28). The fungus was grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) supplemented with 10 mM uracil for production of conidia.

For experiments to study aflatoxin biosynthesis, plastic petri dishes (100 by 15 mm) containing 9.5 ml of peptone mineral salts (PMS) medium (3) were inoculated with 10^4 conidia of *A. flavus* and incubated for 3 days at 28°C. Sucrose low salts (SLS) medium (3) (15 ml) was placed beneath the resulting mycelial mats and dishes were incubated at 28°C. At various time points after the addition of SLS medium, samples (0.3 ml) were collected from underneath the mycelial mats and assayed for aflatoxin. In experiments in which the mycelial mats were harvested, the tissue was frozen in liquid nitrogen and stored at −80°C until further use.

For detection of inducers of aflatoxin biosynthesis in maize kernels, a modification of the culture technique was made to accommodate convenient testing of numerous samples. One milliliter of PMS medium and 10^6 spores of *A. flavus* transformant 13-22 (strain described below) were placed in each well of a 24-well tissue culture plate. After 3 days of incubation, the PMS was removed and replaced with solutions to be tested for aflatoxin-inducing activity. Twenty-four hours after the medium replacement, the mycelia were removed, homogenized, and measured for β-glucuronidase (GUS) activity.

Aflatoxin analysis. Aflatoxin concentrations were determined by an enzyme-linked immunoassay with aflatoxin B_1 monoclonal antibodies (8). The antibody and an aflatoxin B_1 horseradish peroxidase conjugate were kindly provided by J. Pestka (Michigan State University).

Promoter-reporter gene construction. The *ver1* gene from *A. flavus* was obtained by probing a genomic DNA library with the *ver1* gene from *A. parasiticus* (13) (kindly provided by J. E. Linz). Because of the sequence similarity between the *ver1* genes of *A. flavus* and *A. parasiticus*, it was possible to determine the translational start site for the *A. flavus* gene. A region 0.7 kb upstream from the ATG of the *ver1* gene was used as the promoter in the subsequent construction. A *Bam*HI site was inserted at the predicted translational start codon (ATG) of the *ver1* gene by site-specific mutagenesis (16). The region 5′ to the start codon of the gene was then excised by digestion with *Bam*HI, and the fragment was cloned into the vector GAP4 (27). This vector has a promoterless *Escherichia coli* GUS gene. The resulting construct was named GAP13.

Transformation and screening. The GAP13 construct and cosmid B9X2 were used to cotransform *A. flavus* 656-2, as previously described (19, 28). The cosmid B9X2 contains $aflR^+$ to restore aflatoxin production in strain 656-2 and a marker ($pyrG^+$) for transformant selection (19, 29).

GUS extraction and quantification. The protocol used for measuring GUS activity in fungal tissues was similar to Jefferson's protocol for measuring GUS activity in plant tissues (14, 15). Frozen tissue was placed in a 1.5-ml microcentrifuge tube along with 20 mg of glass beads (size, 100 to 200 μm). After the addition of 300 μl of GUS extraction buffer (12.5 mM PO_4 [pH 7.0], 7 μM β-mercaptoethanol, 1 mM EDTA, 0.001% Triton X-100, 0.001% sodium lauryl sarcosine), tissue was ground with a homogenizer for 30 s. After centrifugation for 1 min at 16,000 × *g*, the supernatant was assayed for protein by the Bradford method (1). GUS activity was determined by adding 50 μl of the protein sample obtained in the extraction procedure to 500 μl of assay buffer (1.25 M 4-methylumbelliferyl β-D-glucuronide in extraction buffer) preincubated at 37°C. After 5, 10, and 15 min, 100 μl of buffer was removed from reaction tubes and transferred to microcentrifuge tubes containing 900 μl of stop buffer (0.2 M Na_2CO_3). GUS activity was measured with a Hoefer TKO-100 fluorometer.

Isolation and analysis of RNA and DNA. Total RNA was extracted from lyophilized mycelia of *A. flavus*, as previously described (19). For Northern (RNA) hybridization analysis, 10 μg of total RNA was electrophoresed through a 1.2% agarose gel containing 1.5% formaldehyde, transferred to a Zeta-Probe nylon membrane (Bio-Rad, Richmond, Calif.), and hybridized with ^{32}P-labeled DNA probes. Fungal genomic DNA isolations and Southern hybridization analysis were performed as previously described (27).

RESULTS

The promoter region of the *ver1* gene was fused 5′ to the *uidA* gene from *E. coli* that codes for GUS to generate the *ver1*-GUS construct, GAP13. The rationale for making such a construct to monitor aflatoxin biosynthesis is that growth conditions that induce aflatoxin biosynthesis activate transcription of the aflatoxin pathway gene, *ver1*. Therefore, measurement of the *ver1* promoter activity in GAP13 by the expression of GUS activity should reflect aflatoxin-related gene expression. The GAP13 construct was transferred along with vector B9X2 ($aflR^+$) into *A. flavus* 656-2. Two GAP13-B9X2 cotransformants (13-1 and 13-22) confirmed by Southern analysis to contain the GUS constructs were selected for further analysis. To verify the accuracy of the reporter in this system, aflatoxin production and GUS activity were determined in transformants 13-1 and 13-22 after shifting the cultures from a medium that does not support aflatoxin production (PMS) to a medium that does support aflatoxin production (SLS). Cultures of the fungi grown for 3 days on PMS medium were transferred to either SLS medium or fresh PMS medium and monitored at 6-h intervals for the accumulation of aflatoxin, the presence of *ver1* and *uidA* transcripts, and GUS activity. The two transformants containing the reporter construct were similar to each other with respect to aflatoxin accumulation, *ver1* transcript accumulation, and GUS expression. Aflatoxin accumulation and GUS expression for transformant 13-22 are shown in Fig. 1. In SLS medium, aflatoxin accumulation was detected at 18 h of incubation. No aflatoxin was detected in PMS medium. GUS activity paralleled the accumulation of aflatoxin in SLS medium (Fig. 1), whereas GUS did not accumulate in PMS medium (data not shown).

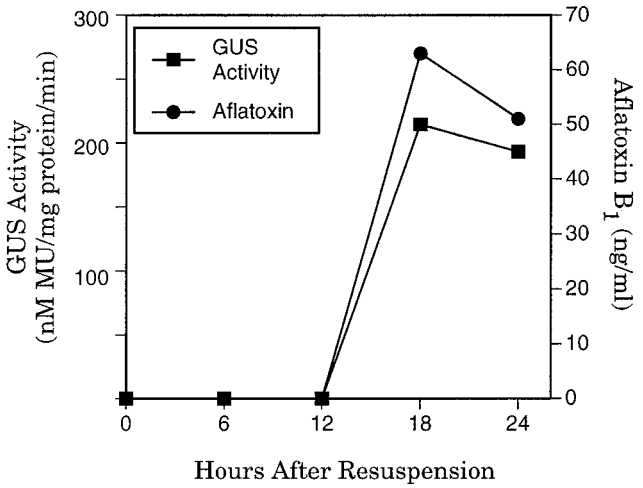

FIG. 1. Accumulation of GUS activity and aflatoxin B_1 in *A. flavus* transformant 13-22. Cultures were grown for 3 days at 28°C in PMS medium. At zero time, the culture medium was replaced with SLS medium. GUS activity and aflatoxin B_1 concentration were determined at 6-h intervals. MU, methylumbelliferone.

To determine if the induction of GUS activity correlated with increased RNA transcript accumulation from the *ver1-uidA* reporter gene, Northern analysis was conducted on RNA isolated from transformant 13-22. As shown in Fig. 2, *uidA* transcripts were detected only at 18 and 24 h in the SLS cultures, while no transcript was detected at any time in RNA from the PMS medium. Transcript accumulation of the native *ver1* gene was also analyzed and found to parallel the accumulation of the *uidA* reporter gene. These data indicate that

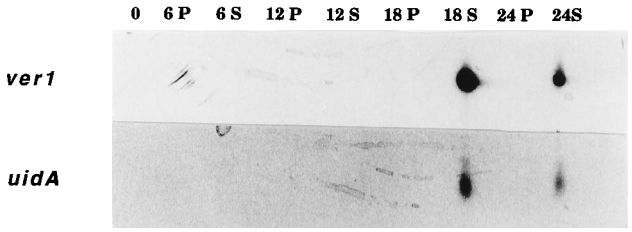

FIG. 2. Accumulation of *ver1* and *uidA* transcripts in transformant 13-22. Cultures were grown for 3 days at 28°C. At zero time, the culture medium was replaced with fresh PMS (P) or SLS (S) medium. Total RNA was isolated from cultures at 6-h intervals. Northern blots were hybridized with radiolabeled probes specific for *ver1* or *uidA*.

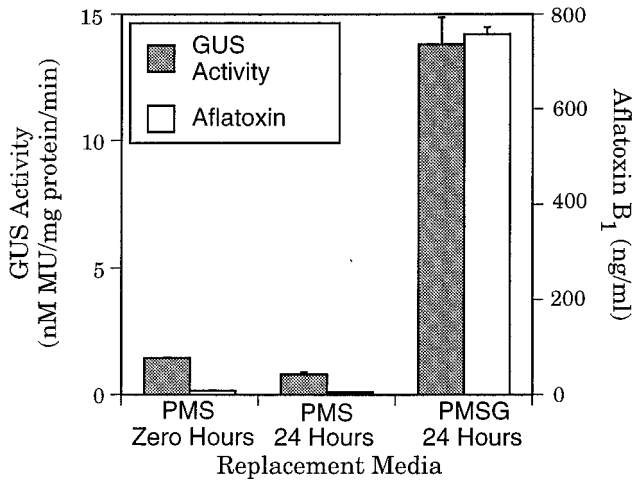

FIG. 3. Accumulation of GUS activity and aflatoxin B_1 in *A. flavus* transformant 13-22 after medium replacement with fresh PMS or PMSG medium. Cultures were grown for 3 days at 28°C. At zero time, the culture medium was replaced. GUS activity and aflatoxin B_1 concentration were determined at zero time and after 24 h of incubation. Values are the average of three replicates, and the bars represent the standard errors. MU, methylumbelliferone.

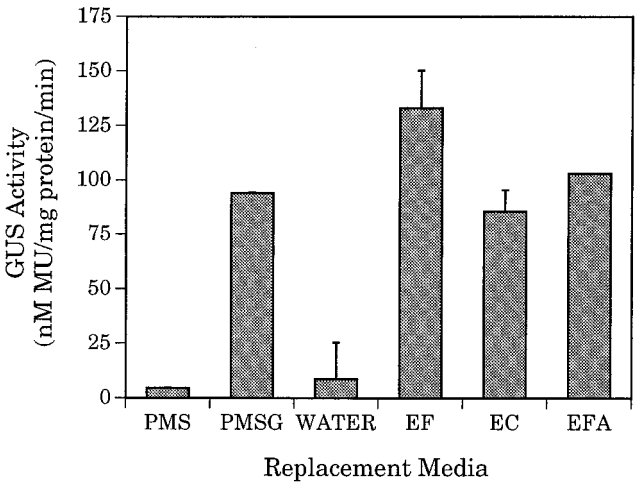

FIG. 4. Accumulation of GUS activity in *A. flavus* transformant 13-22 after medium replacement with fresh PMS, PMSG, water, extracts from fungus-treated kernels (EF), control extracts (EC), and autoclaved EF (EFA). Cultures were grown for 3 days at 28°C. At zero time, the culture medium in the bioassay was replaced. GUS activity was determined after 24 h of incubation. Values are the average of three replicates, and the bars represent the standard errors. MU, methylumbelliferone.

factors affecting the expression of the native *ver1* promoter are correctly perceived by the *ver1*-promoter construct.

With transformant 13-22, an assay was developed to test for inducers of aflatoxin biosynthesis. Multiwell culture plates were used instead of petri plates because the 24-well plates allowed the testing of small samples. Growth was uniform between wells, and there was ample mycelial growth in a well for analysis. For the assay, conidia of *A. flavus* transformant 13-22 were inoculated into PMS medium in the wells. After growth for 3 days, the PMS medium was replaced with test solutions. After a 24-h induction period, aflatoxin and/or GUS activity was determined. Figure 3 shows that when PMS plus 6% glucose (PMSG), a medium conducive to aflatoxin production, was added to the assay both aflatoxin and GUS activity were induced, indicating a response similar to that observed in the petri dishes.

Pioneer maize hybrid 3369A is considered one of the hybrids more susceptible to *A. flavus* infection and aflatoxin contamination. When kernels of this hybrid were inoculated with conidia of strain GAP13-22 and incubated for 2 days, aflatoxin production and GUS activity were detected, suggesting the presence of a seed-associated aflatoxin inducer. However, when kernels were ground, resuspended in PMS medium, and added to the fungal assay, no induction of GUS activity or accumulation of aflatoxin was detected after 24 h of incubation (data not shown). To determine if a potential inducer of aflatoxin biosynthesis required solubilization, two types of extracts of ground maize were tested in the assay. One extract (EF) was from kernels treated by growing the aflatoxigenic *A. flavus* 3357 on a water suspension of ground kernels for 4 days at 37°C. A second extract (EC) was a suspension of the ground kernels without the fungus but incubated under the same conditions. When tested in the assay, both extracts induced GUS activity. Figure 4 shows the results of a representative experiment. Data from numerous experiments showed that the EF extracts always resulted in high GUS activity, whereas GUS activity induced by the EC extracts was variable. The EF extracts also consistently resulted in higher GUS activity than the EC extracts. There was also a notable difference in appearance between the two extracts. Extracts from the fungus-treated kernels (EF) were clear, and those of the EC extracts were opaque.

The inducing activity in EF extracts was not inactivated by autoclaving the extracts for 15 min prior to testing in the assay (Fig. 4). In six heat stability experiments, the amount of GUS activity induced by autoclaved extracts ranged from 77 to 95% of the nonautoclaved extracts. The molecular size of the inducing activity was also investigated. Passage through a glass microfiber filter (Whatman GF/A) clarified the EC extracts, but the filtrate was inactive. In contrast, the EF extracts remained active after filtration. The EF extracts also were passed through ultrafiltration membranes with 100- and 10-kDa exclusion (Centricon C-100 and C-10; Amicon Inc.). Analysis of the filtrate indicated that the GUS-inducing activity in the extracts passed through these filters (Fig. 5).

DISCUSSION

A number of genes are involved in aflatoxin biosynthesis. Several of these genes have been cloned, and their expression appears to be regulated by *aflR* (19, 28). Currently, little is known about the mechanism of gene regulation by *aflR* or the specific regions in the promoters of the pathway genes that interact with the regulatory element. The objective of our study was to develop a reporter gene-based assay to monitor promoter activity of the aflatoxin pathway genes. To do this, a construct was made with the promoter of one pathway gene (*ver1*) cloned immediately upstream of the *uidA* gene from *E. coli*. We chose *uidA* as a reporter gene because it has been used successfully in plant (14, 15, 30) and fungal (4, 21) systems to monitor gene expression.

We have shown by transcript analysis, aflatoxin accumulation, and GUS expression that the reporter construct reliably monitors the promoter activity of the *ver1* gene. In all cases, the expression of GUS by the reporter constructs was dependent on the culture conditions that induced aflatoxin formation. Also, the profile of aflatoxin accumulation in induced cultures was the same as that of GUS activity. The induction of aflatoxin biosynthesis involves the transcriptional induction of the

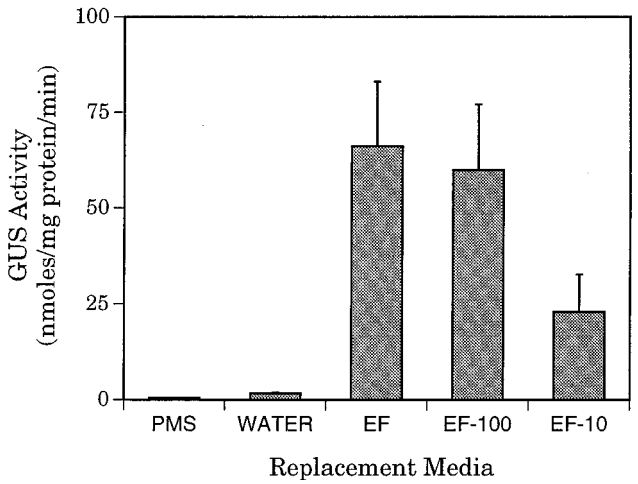

FIG. 5. Accumulation of GUS activity in *A. flavus* transformant 13-22 after medium replacement with fresh PMS, PMSG, water, or extracts from fungus-treated kernels. Cultures were grown for 3 days at 28°C. At zero time, the culture medium in the bioassay was replaced. Prior to testing, kernel extracts (EF) were passed through ultrafiltration membranes with 100 (EF-100)- and 10 (EF-10)-kDa exclusion. GUS activity was determined after 24 h of incubation. Values are the average of three replicates, and the bars represent the standard errors.

pathway genes. In all cases the induction of the *ver1* transcripts mirrored the induction of the *uidA* transcripts from the *ver1-uidA* reporter construct. Thus, the promoter-GUS construct developed in this study can be utilized effectively to examine the timing and magnitude of gene expression of at least one pathway gene.

The utility of the *ver1*-GUS construct is as a molecular probe to identify specific potential compounds that induce or inhibit aflatoxin biosynthesis. Aflatoxin biosynthesis is induced in vivo, and it has been observed that in some host genotypes there is fungal growth with little aflatoxin formation (9). This implies that there may be compounds in seeds that are stimulatory or inhibitory to aflatoxin biosynthesis. The reporter construct developed in this study offers a way to identify these compounds that is easier and more reliable than assaying for aflatoxin accumulation. The assay is also more sensitive than measuring aflatoxin accumulation because it measures a specific step early in the pathway rather than the accumulation of a final product.

In this study, we took the initial steps in utilizing the GUS reporter in an assay to detect an inducing activity in a susceptible maize hybrid. The identity of the inducer(s) was not determined, but it was shown to be heat stable and have a molecular size of less than 10 kDa. The aflatoxin-inducing component was also more readily extracted from the maize kernels if the kernels were colonized by the fungus during the extraction period. These data suggest that the inducer of aflatoxin biosynthesis is a complex seed molecule that is degraded by the action of fungal extracellular enzymes. This hypothesis is supported by the loss of inducing activity after the EC extract was passed through a glass fiber filter. Furthermore, the inducing activity in EC extracts was not consistent between experiments. An explanation for this observation is that during the 24-h induction period of the assay, the fungus may produce an extracellular enzyme that degrades the complex molecules to simpler, inducing molecules. The identification of the inducing molecule(s) is beyond the scope of this study. We speculate that the macromolecule may be amylose or amylopectin and that an α-amylase may be produced by the fungus to release the inducer. However, we also recognize that the data could be interpreted to suggest that *A. flavus* produces a metabolite capable of directly inducing aflatoxin production.

Further research with transformants harboring the *ver1*-GUS reporter gene will prove valuable in studying several other areas related to aflatoxin biosynthesis. *A. flavus* transformants expressing GUS are being used to study the effects of the regulatory gene *aflR* on the expression of pathway genes and to determine the promoter elements in *ver1* that are required for gene expression. Transformants expressing GUS are also useful in studies on fungal growth and aflatoxin gene expression in situ. Research to monitor fungal growth in kernels of different maize genotypes with a strain containing the *uidA* gene fused to a constitutive promoter is under way (2). The *ver1* construct described in this study should also allow us to address which tissue types induce aflatoxin formation. Such studies are important for devising control strategies involving transgenic plants as these will identify the seed tissue in which the resistance gene should be expressed.

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