Novel Procedure for Identification of Compounds Inhibitory to Transcription of Genes Involved in Mycotoxin Biosynthesis

Seanna L. Annis,[†,‡] Luis Velasquez,[†] Haixin Xu,[†] Ray Hammerschmidt,[†] John Linz,[§] and Frances Trail*[,†]

Department of Botany and Plant Pathology and Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824

A novel assay is described for the identification and isolation of compounds that inhibit the transcription of genes involved in mycotoxin biosynthesis. The thin-layer chromatography-based assay was used to screen plant extracts for compounds that would inhibit the expression of the *β*-glucuronidase reporter gene under the control of an aflatoxin biosynthesis gene promoter in *Aspergillus parasiticus*. The assay was used to track purification of an inhibitory compound, cp2, from extracts of black pepper (*Piper nigrum*). Cp2 did not inhibit mycelial growth or the expression of the *β*-tubulin gene but did inhibit aflatoxin biosynthesis at the transcriptional level. Applications of cp2 to the control of mycotoxins are discussed.

Keywords: *Aspergillus; aflatoxin; β-glucuronidase; mycotoxin; TLC; pepper; Piper nigrum*

INTRODUCTION

Mycotoxins are fungal secondary metabolites that pose a significant public health problem worldwide. In many cases, mycotoxins are produced by fungi that invade crops before harvest and proliferate during storage. Fungicides have not been effective in the control of these fungi, and germplasm in affected crops has yielded few lines, with the exception of a recent line of corn (Huang et al., 1997), that consistently show resistance. Biological control using atoxigenic isolates of *Aspergillus flavus* has shown promise in controlling contamination (Bock and Cotty, 1999). Crop losses caused by many of these fungi are due to mycotoxin contamination, not the presence of the mycelia. In focusing control efforts on eliminating the mycotoxin contamination, we have developed a simple assay to detect compounds that inhibit the expression of aflatoxin biosynthetic genes.

Aflatoxin is a well-studied mycotoxin, and much of the molecular biology of the biosynthetic pathway has been elucidated in *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nidulans* (Brown et al., 1999). The *nor-1* and *ver-1* genes are two of the most studied genes of the pathway. The *nor-1* (Trail et al., 1994) encodes an enzyme that catalyzes the ketoreduction of norsolorinic acid to averantin, an early step in the pathway. The *ver-1* encodes a ketoreductase that catalyzes versicolorin A to demethylsterigmatocystin, near the end of the pathway (Skory et al., 1992). Coordinated induction of transcription of the biosynthetic genes is regulated in part by the action of *afl*R (Chang et al., 1993; Woloshuk and Payne, 1994; Yu et al., 1996). In culture, the aflatoxigenic *Aspergillus* species produce aflatoxins during idiophase, when logarithmic growth has slowed. The appearance of gene transcripts precedes the appearance of aflatoxin by 3−12 h (Burow et al., 1997; Skory et al., 1993).

There are many reports of substances that inhibit aflatoxin production, particularly in plant extracts (Hitokoto et al., 1978; Llewellyn et al., 1981; Madhyastha and Bhat, 1984). Hitokoto et al. (1978) examined the effects of chloroform extracts of herbal drugs and dry condiments, including black pepper (*Piper nigrum*), on the growth and toxin production of *A. parasiticus*, *A. flavus*, *Aspergillus ochraceus*, and *Aspergillus versicolor*. Their results showed that black pepper extracts reduced secretion of ochratoxin A (by *Aspergillus ochraceus*), sterigmatocystin (by *A. versicolor*), and aflatoxin (by *A. flavus* and *A. parasiticus*). Awuah and Kpodo (1996) used a norsolorinic acid-accumulating isolate of *A. parasiticus* to determine that extracts of black pepper inhibited the production of this aflatoxin precursor, but not mycelial growth. Recently, 13*S*-hydroxyperoxy fatty acids generated by a plant lipoxygenase were shown to repress transcription of aflatoxin pathway genes in *A. parasiticus* (Brown et al., 1999).

The objectives of this study were to develop a simple assay to screen for compounds that inhibit expression of aflatoxin genes. These compounds would be overlooked by assays designed to screen for fungitoxic or fungistatic activity. The assay was successfully used to track the purification of an inhibitory compound identified in black pepper extracts during the development of the assay.

MATERIALS AND METHODS

Fungal Strains and Medium. *A. parasiticus* SU-1 (NRRL5862) is a wild-type aflatoxin-producing strain and is the distant parent of all other strains used in this study. *A. parasiticus* G5 is a strain whose *nor-1* gene was replaced with a *nor-1* promoter fused to the *uidA* gene (GUS) from *Escherichia coli* encoding *β*-glucuronidase (Xu et al., 2000) and

* Author to whom correspondence should be addressed [e-mail trail@msu.edu; telephone (517) 432-2939; fax (517) 353-1929].

† Department of Botany and Plant Pathology.

‡ Present address: Department of Biological Sciences, 5735 Hitchner Hall, University of Maine, Orono, ME 04469-5735.

§ Department of Food Science and Human Nutrition.

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produces reduced levels of aflatoxin. *A. parasiticus* GAPN2 (Wilson, 1998) is an aflatoxin-producing transformant containing a β-tubulin (*benA*) promoter from *A. flavus* fused to the GUS reporter gene (Woloshuk and Payne, 1994). Strains were maintained as conidial stocks ($\sim 1 \times 10^8$ spores/mL) in 35% glycerol at −80 °C.

Peptone mineral salts (PMS) and glucose mineral salts (GMS) media (Buchanan and Lewis, 1984) were used to perform the nutritional shift assays. YES medium (2% yeast extract, 6% sucrose), an aflatoxin-inducing medium, was used to determine the effect of purified inhibitory compounds on growth. YES with 1.5% agar was used for sporulation assays.

Thin-Layer Chromatography (TLC) Bioassay and initial Screening of Black Pepper Extracts. Initial development of the TLC bioassay was performed while crude pepper extracts were screened for inhibitory compounds. Commercial ground black pepper (*Piper nigrum*) was extracted overnight with chloroform (1:1 w/v). The mixture was filtered through Whatman No. 1 paper, and the filtrates were concentrated by evaporation. Extracts were resuspended in chloroform (2:1, w/v, pepper/chloroform).

A bioassay to identify compounds with aflatoxin inhibitory properties was developed by modifying a screening method for fungitoxic substances (Homans and Fuchs, 1970). Compounds in the crude pepper extract (20 μL) were separated on TLC plates (silica gel on glass or polyester with fluorescent indicator, Sigma-Aldrich, St. Louis, MO) developed in a solvent system of cyclohexane/ethyl acetate (1:1 or 1:3). The plates were air-dried and then placed silica-side down on a UV transilluminator (302 nm), and UV absorbent spots were traced on an acetate sheet. A 10^6/mL spore suspension of *A. parasiticus* (G5 or GAPN2) was prepared in half-strength YES medium containing 0.3% agarose at 50 °C (20 mL was sufficient to cover a 10 × 20 cm TLC plate). The spore suspension was applied evenly across the surface of the TLC plate with a glass TLC plate spray apparatus. A low concentration of agarose was essential, as higher levels permitted sufficient hyphal growth to mask GUS expression. The sprayed TLC plate was placed in a moist chamber and incubated for 40−48 h at 28 °C in the dark for the GUS assays and for up to 3 days to detect effects on sporulation. Plates were removed from the incubation chamber when covered with an even layer of mycelia and some sporulation was visible. Areas of inhibition of fungal growth were traced on acetate sheets for comparison with UV absorbent spots. Plates were wrapped in plastic film and frozen for 1 h at − 80 °C and then thawed at room temperature for at least 30 min. The freeze/thaw cycle released the GUS enzyme from the mycelia and promoted even staining. Plates were then sprayed with a mixture of equal parts of freshly prepared X-gluc buffer [100 mM potassium phosphate buffer, pH 7.0, 0.03% K ferricyanide (Sigma), 0.1% X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt, Gold Biotechnology, Inc, St. Louis, MO)] and 0.6% agarose (at 50 °C). Plates were wrapped in plastic film, placed in a moist chamber, and incubated at 37 °C overnight. Areas that lacked GUS activity were traced onto acetate sheets and compared to the location of UV absorbent spots and areas of fungal growth inhibition.

Purification of Active Inhibitory Compound. Commercial ground black pepper was suspended in cyclohexane (1:2 w/v) with constant stirring overnight. The mixture was filtered and then re-extracted with cyclohexane for another 4 h. The filtrates were combined and concentrated by rotoevaporation under vacuum at 40 °C. The concentrated cyclohexane extract had two phases: one soluble in cyclohexane and the other soluble in ethanol. The ethanol-soluble fraction contained cp2. Cp2 was recovered from the ethanol-soluble fraction by flash chromatography (FC) purification through silica gel (grade 9385, 230−400 mesh, 60A; Aldrich, Milwaukee, WI) as described by Still et al. (1978). The solvent system for FC was chloroform/toluene/acetone (25:40:6 v/v/v) and gave an R_f value of 0.35 for cp2. Fractions containing cp2 were pooled, dried by rotoevaporation, weighed, resuspended in the minimal amount of absolute ethanol required to dissolve the crystals, and stored at 4 °C.

To assess the concentration and purity of FC-purified cp2, ~10 mg of the concentrated fractions was loaded onto a preparatory TLC plate (20 × 20 cm, 1000 μm silica gel 60A; Whatman, Clinton, NY) and developed three times in hexanes/ethyl acetate (3:1). Areas covered by five bands visible under UV light were determined by SigmaScan (Sigma) from a digital image of the plate under UV light and expressed as a proportion of the total area covered by all five bands. The band corresponding to cp2 was detected by the TLC bioassay. The corresponding silica was removed from the plate and cp2 eluted with chloroform/ethanol (4:1). After evaporation of the solvent, crystals of cp2 were analyzed by GC-MS (Achenbach et al., 1986; Parmar et al., 1997).

Nutritional Shift Assays and Quantification of Sporulation. A nutritional shift protocol (Skory et al., 1993) was modified and used to determine the effect of cp2 on the expression of *nor-1*, *ver-1*, *benA*, and aflatoxin production. Conidia (1×10^6) of *A. parasiticus* SU-1 were inoculated into 100 mL of PMS (aflatoxin nonsupporting) in 250 mL flasks and incubated for 48 h at 250 rpm at 28 °C. Mycelia were harvested by vacuum filtration through Miracloth (Calbiochem, La Jolla, CA), weighed, and divided equally among all treatments. Mycelium was transferred to 100 mL of GMS medium (aflatoxin supporting) accompanied by the addition of FC-purified cp2 dissolved in absolute ethanol to produce final concentrations of 2.6−78 μg/mL of medium. Ethanol alone was added to control treatments. Mycelia and culture medium were harvested at 0, 10, 24, 36, and 48 h postmedium shift. Half of the mycelial mat (wet weight) was frozen at −80 °C for RNA extraction, and the other half was rinsed in dH_2O and dried at 60 °C for at least 2 days for dry weight determination. Culture medium was frozen at −80 °C for ELISA analysis. There were two flasks per treatment, and the experiment was repeated once. A time course experiment to compare the effect of cp2 (52 μg/mL of medium) on mycelial growth was performed using the nutritional shift method described above. Three repetitions of cultures at each time point and treatment were performed. Mycelial dry weights were compared using Proc GLM in SAS (Cary, NC) at a 95% confidence level.

The effect of cp2 on sporulation was examined using spores of *A. parasiticus* SU-1. A 500 μL aliquot of a suspension of 4.6×10^6 conidia/mL (from freezer stocks) was spread on the surface of YES agar medium. Filter disks, 6 mm in diameter (Whatman No. 1), were saturated with cp2 at concentrations of 2.6, 26, 52, or 260 μg/mL in absolute ethanol and placed on the surface of the plate. After 48 h, when the cultures were starting to sporulate, the disks were removed, placed in 200 μL of H_2O, and vortexed briefly to suspend conidia. Conidia were counted using a hemacytometer, and data were analyzed by Student's *t* test.

Isolation and Analysis of RNA. Total RNA was extracted from hyphae using the Trizol RNA extraction kit (Gibco BRL, Grand Island, NY) according to the manufacturer's directions. Northern analysis was performed as previously described (Trail et al., 1995). DNA probes were labeled with ^{32}P using a Random Primed DNA labeling kit (Boehringer Mannheim Biochemicals). The probes used were specific to *nor-1*, *ver-1*, or *benA* (Skory et al., 1993; Trail et al., 1994; Wu et al., 1996).

ELISA Analysis of Aflatoxin production. Direct competitive ELISA analyses to determine concentrations of aflatoxin B_1 (Pestka, 1988) were performed on samples of the culture medium from the nutrient shift assays using aflatoxin B_1 monoclonal antibodies and aflatoxin B_1−horseradish peroxidase conjugate.

RESULTS

TLC Bioassay. The TLC bioassay was developed during screening of crude pepper extracts for compounds that inhibited expression of aflatoxin biosynthetic genes. Compounds in chloroform extracts of black pepper were resolved on TLC plates using different solvent systems. The developed plates were coated with either *A. para-*

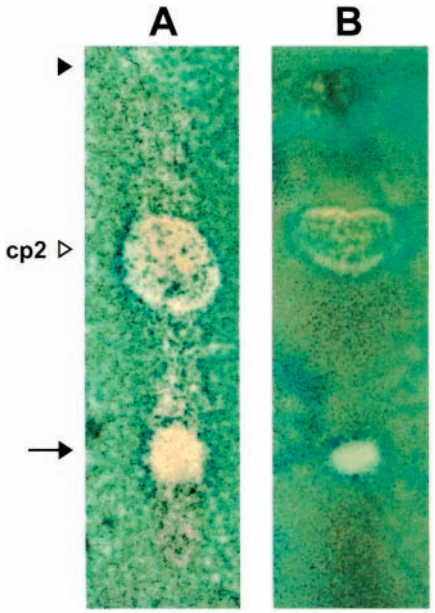

Figure 1. Identification of inhibitors of aflatoxin biosynthetic gene transcription by a TLC-based bioassay. TLC plates with resolved crude black pepper extract were coated with *A. parasiticus* strains G5 (*nor-1*−GUS fusion) (A) or GAPN2 (*benA*−GUS fusion) (B). Plates were subsequently coated with the GUS substrate X-gluc. Blue areas indicate the presence of GUS activity. Fungal growth was seen in the area corresponding to the compound cp2. An unidentified fungitoxic compound inhibited fungal growth in both strains (arrow). The front is indicated by an arrowhead.

siticus G5 harboring the *nor-1*−GUS fusion or *A. parasiticus* GAPN2 harboring the *benA*−GUS fusion. Plates were examined for fungal growth and for GUS activity as indicated by the presence of a blue pigment. Areas with mycelia that did not turn blue contained compounds that inhibited expression of GUS (Figure 1). Cp2 was isolated from an area of the plate that inhibited expression of the *nor-1*−GUS fusion but not the *benA*−GUS fusion (Figure 1). An unidentified fungitoxic compound that inhibited fungal growth in both strains was also found (Figure 1). TLC plates coated with the fungus that were allowed to grow for 2−3 days revealed areas corresponding to cp2-inhibited sporulation (not shown), although mycelial growth was not affected.

Purification of the Bioactive Compound, Cp2, Using the TLC Bioassay. The TLC bioassay was used to track the bioactive compound, cp2, through its purification from crude pepper extracts. Following flash chromatography of the extracts, three of five bands visible under UV light following TLC (29% of the total area of all bands) were not bioactive. The other two bands were both bioactive. One of these two bands comprised 45% of the area and contained 98% pure cp2 as determined by GC-MS. The other band, comprising 26% of the area, contained a piperine, a well-described antimicrobial compound produced by black pepper (Madhyastha and Bhat, 1984), as determined by GC-MS and TLC analysis. The cp2 and piperine bands partially overlapped; the overlapping region was visible under UV and avoided when cp2 was recovered from the plate.

Large amounts of cp2 could be easily obtained by FC, so the bioactive FC fraction was used as a source for cp2 in growth and aflatoxin bioassays. There was the possibility of contamination of this fraction by small amounts of piperine. In the initial TLC bioassay with crude pepper extracts, there was a slight inhibition of growth at the site of cp2, which was determined to be due to the comigration of cp2 with piperine (Figure 1B). Nutritional shift assays to determine the effect of piperine on aflatoxin biosynthesis and mycelial growth indicated piperine is highly fungistatic but did not consistently inhibit aflatoxin biosynthesis (data not shown). The FC-purified cp2 fraction did not inhibit mycelial growth (Tables 1 and 2); therefore, the effects of piperine in the FC fraction on treated cultures were considered to be minimal.

Table 1. Effect of Cp2 on Aflatoxin Production by *A. parasiticus* SU-1 after Nutritional Shift

concn of cp2 (μg/mL of medium)	concn of aflatoxin B_1 (ng/mL of culture filtrate)		mycelial dry wt (g)
	36 h[a]	48 h	48 h
0	7000 (3700)[b]	13000 (3300)	0.26
2.6	nd[c]	2000 (870)	0.28
26	nd	970 (360)	0.28
39	54 (7)	83 (9)	0.27
52	0.5 (0.2)	7.9 (5)	0.29
78	nd	3.2 (1)	0.26

[a] Time zero is the time of shift from PMS to GMS and when cp2 was added to the cultures. [b] Numbers are averages of two repetitions performed in each of two experiments, and numbers in parentheses are standard deviations. [c] nd, not determined.

Table 2. Effect of Cp2 (52 μg/mL of Culture Medium) on Growth of *A. parasiticus* after Nutritional Shift

time of harvest[a] (h)	dry wt (g)	
	treated	untreated
0	nd[b]	0.31[c]
10	0.31	0.31
24	0.29	0.31
36	0.37	0.36
48	0.58	0.51

[a] Time of shift from PMS to GMS is considered $t = 0$ and when cp2 was added to the cultures. [b] This value was not determined (nd); it is the same treatment as the untreated sample at time zero. [c] Data are presented as the means of three trials, and no significant variation between trials was observed.

Effect of Cp2 on Gene Transcription, Aflatoxin Production, Sporulation, and Mycelial Growth. To confirm the results of the TLC bioassay, the effect of cp2 on mycelial growth, sporulation, and transcription of aflatoxin biosynthetic genes was determined. Northern hybridization analyses were performed on total RNA extracted from treated mycelia in the nutritional shift assay (Figure 2). Total RNA samples collected 36 and 48 h after the nutritional shift and the addition of cp2 revealed that transcription of *nor-1* and *ver-1* was significantly inhibited in samples containing cp2 at 52 μg/mL, although expression of *benA* was not affected.

Cp2, at concentrations from 2.6 to 260 μg/mL, did not affect sporulation of strain SU-1 (Student's *t* test, $P < 0.01$).

Aflatoxin B_1 levels decreased as concentrations of cp2 in culture filtrates increased for both sampling times (Table 1). The lowest concentration of cp2 tested, 2.6 μg/mL, produced an 84% decrease in aflatoxin production in 48 h cultures. There were no significant trends found in the effect of increasing concentrations of cp2 on mycelial growth as determined by mycelial dry weights (Table 1). The effect of cp2 on growth over time was evaluated with a cp2 concentration of 52 μg/mL of culture medium (Table 2). There was no significant increase in mycelial weight until 48 h, when there was a significantly higher growth ($P < 0.01$) in both the

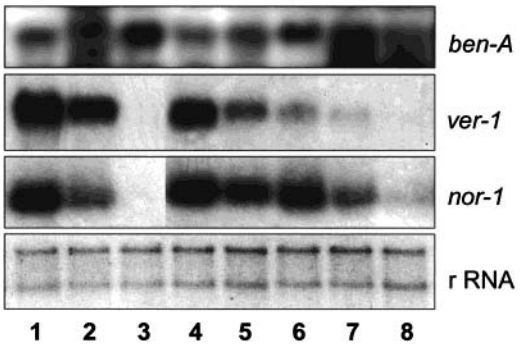

Figure 2. Effect of cp2 on expression of transcripts of the aflatoxin biosynthetic genes *nor-1* and *ver-1* and the housekeeping gene, *benA*, in *A. parasiticus* SU-1. Cultures were grown for 48 h in PMS and transferred to GMS with various concentrations of cp2 ($t = 0$). Total RNA was isolated from cultures at $t = 36$ h (lanes 1−3) and $t = 48$ h (lanes 4−8). Lanes were equally loaded as indicated by rRNA levels. Concentrations of cp2 in GMS medium were as follows: (lanes 1 and 4) control; (lanes 2 and 7) 39 μg/mL; (lanes 3 and 8) 52 μg/mL; (lane 5) 2.6 μg/mL; (lane 6) 26 μg/mL.

treated and untreated cultures. These results suggest that cp2 does not affect mycelial growth as measured by mycelial weight.

DISCUSSION

We developed a TLC bioassay to screen for natural compounds that inhibit the transcription of aflatoxin biosynthetic genes. Our assay provides a simple means for preliminary identification of compounds that affect the transcription of aflatoxin biosynthetic compounds and assists in the tracking of the compounds through purification. In addition, the assay can be used to detect fungitoxic compounds and may be useful in detecting compounds that inhibit sporulation.

The availability of several fusion constructs linking the GUS reporter gene with promoters from aflatoxin biosynthetic genes facilitated the development of the assay. Other workers have reported the use of the GUS reporter gene to follow colonization of seeds by *Aspergillus* spp. (Brown et al., 1995) and to monitor expression of genes associated with aflatoxin biosynthesis in culture (Fernandes et al., 1998; Flaherty et al., 1995; Liang et al., 1997). In addition, the GUS gene fusions in *Aspergillus* spp. have been used to assay for compounds that affect expression of aflatoxin biosynthetic genes in maize extracts (Woloshuk et al., 1997). This bioassay could also be adapted to screen for compounds inhibiting other mycotoxins where promoters for the mycotoxin biosynthetic genes have been identified.

The TLC bioassay was used to identify and purify the novel compound, cp2, that inhibits aflatoxin biosynthesis at the transcriptional level. Determination of the structure of cp2 is underway. Cp2 holds promise for use in controlling aflatoxin production because its inhibitory effects on aflatoxin B_1 biosynthesis could be detected by ELISA in cultures treated with as little as 2.6 μg/mL. There was no discernible effect of cp2 on the transcription of *benA*, a constitutively expressed gene, nor was there a consistent effect on mycelial growth. Sporulation was not affected in culture in concentrations up to 260 μg/mL, but visual analysis of the TLC bioassay revealed some inhibition (Figure 1). This discrepancy may be due to increased concentrations of cp2 in the bioassay. However, neither sporulation nor concentration of cp2 was quantified in the bioassay. Regulation of sporulation and mycotoxin production has been shown to have a common regulatory point in several *Aspergillus* species. Both sporulation and aflatoxin biosynthesis require the inactivation of the FadA G α protein-dependent signaling pathway (Hicks et al., 1997). Cp2 may affect the aflatoxin pathway after this point or affect another regulatory mechanism. Further studies are necessary to determine the mode of action of cp2.

A new generation of control strategies may be developed from natural compounds that affect the toxin-producing processes in mycotoxigenic fungi. Applications of cp2 to mycotoxin control include direct application to stored grain. Use of cp2 in transgenic plants will require determination of the mechanism of synthesis of the compound in pepper plants, cloning of the genes involved in the pathway, and studies of the methods for introducing and effectively expressing those genes in other plants. These studies are underway.

SAFETY

Aflatoxin is a known carcinogen and strains of *Aspergillus* producing aflatoxin must be treated accordingly.

ABBREVIATIONS USED

FC, flash chromatography; GMS, glucose mineral salts medium; GUS, β-glucuronidase; PMS, peptone mineral salts medium; TLC, thin-layer chromatography; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt; YES, 2% yeast extract and 6% sucrose medium.

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