Inhibitory effect of acetosyringone on two aflatoxin biosynthetic genes

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Aims: The objective of this study was to determine if acetosyringone affected the expression of aflatoxin biosynthetic genes.

Methods and Results: Two genes, nor1 and ver1, representing genes whose products are involved in early and late steps in aflatoxin biosynthesis, were examined. Two GUS (β-glucuronidase) reporter constructs, nor1::GUS (pGAP12) and ver1::GUS (pGAP13), were used to study the effect of acetosyringone on expression of aflatoxin biosynthetic (AF) genes, nor1 and ver1. The product of nor1 is involved in the formation of norsolorinic acid, the first stable intermediate in the aflatoxin pathway. The ver1 gene codes for the enzyme catalyzing the formation of demethylsterigmatocystin, an intermediate late in the AF pathway. GUS activities of these two reporter constructs were inhibited by 80% in the presence of 2 m mol l\(^{-1}\) acetosyringone.

Conclusions: Aflatoxin production in a toxigenic strain 42-12 was also shown to be inhibited by acetosyringone to the same level. The levels of inhibition in aflatoxin production and gene transcription are congruous in these three strains.

Significance and Impact of the Study: Recent studies have indicated that some phenolics act as signal molecules in plant microbial interactions. Concentration of acetosyringone is shown to increase about ten fold when certain metabolically active plant tissues are wounded. The knowledge gained can be applied to develop strategies in plant breeding programs. The compound may be useful for studying molecular mechanism of modulating aflatoxin biosynthesis.

INTRODUCTION

Aspergillus flavus produces aflatoxin B\(_1\) (AFB\(_1\)), a potent hepatotoxin and carcinogen. Contamination of food by AFB\(_1\) has long been recognized as a serious health hazard to both humans and animals (Ellis et al. 1991; Scudamore 1994). More than 25 genes are involved in the synthesis of AFB\(_1\) via the polyketide pathway (Payne and Brown 1998). Most of the genes involved in aflatoxin biosynthesis have been recently identified. The nor1 gene codes for norsolorinic acid reductase and the ver1 gene codes for the enzyme involved in the conversion of versicolorin A to demethyl-sterigmatocystin. GUS reporter gene constructs made with the promoters of nor1 and ver1 genes were fused immediately upstream of the β-glucuronidase (GUS) gene (uidA) from Escherichia coli and were used to quantify gene transcription (Flaherty et al. 1995).

Acetosyringone, identified as a phenolic signal molecule in Agrobacterium tumefaciens—plant interactions, affects the virulence (vir) gene expression (Stachel et al. 1985; Kalogeraki and Winans 1998). In Pseudomonas syringae (Quigley and Gross 1994), certain phenolic compounds activate syringomycin biosynthetic gene expression (syrB and syrD). It has been reported that cutinase production in Monilinia fructicola is inhibited by phenolics on fruit surfaces (Bostock et al. 1999). Most recently acetosyringone has been demonstrated to inhibit aflatoxin production by Aspergillus flavus (Hua et al. 1999).
The objective of this study was to determine if acetosyringone affected the expression of aflatoxin biosynthetic genes. Two genes, *nor1* and *ver1*, representing genes whose products are involved in early and late steps in aflatoxin biosynthesis, were examined.

**MATERIALS AND METHODS**

**Fungal cultures**

*Aspergillus flavus* strains 12–19 and 13–22 were transformants of *A. flavus* 656–2 containing GUS gene constructs of *nor1::GUS* (pGAP12) and *ver1::GUS* (pGAP13), respectively. The strains were provided by G. A. Payne, North Carolina State University, NC, USA. The toxigenic strain *A. flavus* 42–12 (NRRL-25347), isolated from a pistachio nut, was used to examine the effect of acetosyringone on aflatoxin production. The fungi were maintained on potato-dextrose agar (PDA). Fungal spores were suspended in Tween-80 water (0.05% Tween 80) and the number determined using a haemocytometer.

**Sources of chemicals**

Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) was purchased from the Aldrich Chemical Company (Milwaukee, WI). Methylumbelliferone (MU), 4-methylumbelliferyl β-glucuronide (MUG) and aflatoxin B1 were purchased from Sigma Chemical Co. (St. Louis, MO). Potato dextrose agar (PDA) was purchased from DIFCO Laboratories (Detroit, MI).

**Culture media and growth conditions for the GUS assay**

Peptone-mineral salts (PMS) medium was used as the aflatoxin nonproducing medium and glucose-mineral salts (GMS) medium supplemented with 10 mmol l⁻¹ leucine was used as the aflatoxin inducing medium (Abdollahi and Buchanan 1981). Acetosyringone stock solution was added to GMS medium to give final concentrations of 0.5, 1 and 2 mmol l⁻¹. Spore suspensions from the fungal strains 12–19 or 13–22 were inoculated into PMS medium to give 10⁵ spores ml⁻¹ in a microfuge tube and grown horizontally at 28°C for 64 h. Fungal mycelia were collected by centrifugation, and PMS medium was removed and replaced with GMS containing various concentrations of acetosyringone, then incubated at 28°C for 30 h. The resulting mycelia were centrifuged at 14 000 r.p.m. in an Eppendorf microfuge for 5 min, washed with GUS extraction buffer (Gallagher 1992; Flaherty *et al.* 1995) and the harvested mycelia stored at −20°C.

**Quantitative GUS assays**

Frozen mycelia were resuspended in 0.5 ml of GUS extraction buffer in a microfuge tube, then disrupted by ultra-sonication in a Branson sonifier. The resulting crude enzyme extracts were centrifuged at 14 000 r.p.m. in an Eppendorf microfuge for 5 min at 4°C and the supernatants assayed for GUS activity using MUG as a substrate (Gallagher 1992; Flaherty *et al.* 1995). The enzyme product MU was measured with a Pharmacia DQ200 fluorometer (Hoefer Scientific, San Francisuc, CA). Protein was measured by the method of Bradford with bovine serum albumin as a standard (Bradford 1976).

**Aflatoxin extraction and determination**

Acetosyringone stock solution was added to molten PDA to final concentrations of 0.5, 1 or 2 mmol l⁻¹. Five milliliters of agar media were dispensed into each Petri dish (60 mm × 10 mm). Triplicate Petri dishes were inoculated with 5 μl of a spore suspension (1 × 10⁵ spores ml⁻¹). The inoculated Petri dishes were incubated at 28°C in the dark for 10 d. Aflatoxin was extracted and analysed (Rodriguez and Mahoney 1994; Hua *et al.* 1999) by high performance liquid chromatography (HPLC) on a Hewlett Packard model 1050 Chem Station (Hewlett-Packard Company, Palo Alto, CA). Aflatoxin B1 was used as standard.

**RESULTS AND DISCUSSION**

**Effect of acetosyringone on GUS activity**

Both *nor1* and *ver1* genes were coordinately induced in GMS. Mycelial extracts from control cultures, i.e. no acetosyringone in the GMS medium, showed high GUS activities. The enzyme activity of the crude extract from 13–22 was about 20 times higher than 12–19, indicating that the transcriptional rate of *ver1* promoter is higher than that of *nor1* promoter. In the presence of 0.5, 1 and 2 mmol l⁻¹ acetosyringone, the GUS activities of 13–22 and 12–19 were both decreased in a concentration dependent manner. As shown in Fig. 1(a), GUS activity of 13–22 was reduced by 1 mmol l⁻¹ acetosyringone from 27.2 to 15.1 μmol mg protein⁻¹ min⁻¹ corresponding to 62% of repression. When the concentration of the compound was increased to 2 mmol l⁻¹, the GUS activity was reduced to approximately 20% of the control culture, thus accounting for 80% repression. Figure 1(b) shows the pattern of repression in strain 12–19 by acetosyringone was similar to that of 13–22.

Inhibition of aflatoxin biosynthesis

Aflatoxin production by the toxigenic strain 42–12 was reduced as the concentrations of the acetosyringone increased in GMS medium. Figure 2 shows that the fungal culture produced 28 \( \mu \text{g} \) AFB\(_1\) plate\(^{-1}\) in GMS medium containing 2 mmol l\(^{-1}\) acetosyringone whereas the control culture produced 158 \( \mu \text{g} \) AFB\(_1\) plate\(^{-1}\). This is equivalent to 82% of inhibition. The GUS activities in both reporter constructs, 13–22 and 12–19 were shown to be reduced by about 80%. The levels of inhibition in aflatoxin production and gene transcription are congruous in these three strains.

**Fig. 1** Effect of acetosyringone on GUS activity. The fungal cultures of *A. flavus* 13–22 and 12–19 were grown in PMS for 64 h, then in GMS containing 0.5, 1 and 2 mmol l\(^{-1}\) acetosyringone for 30 h. Enzyme extracts were prepared according to the procedure described in the method section. GUS activities of the mycelial extracts were determined by measuring the enzyme product MU using a DQ 200 fluorometer. Values are means of three replicates and the bars represent standard deviation. Specific enzyme activity is \( \mu \text{mol MU mg protein}^{-1} \text{min}^{-1} \). (a) ver1::GUS and (b) nor1::GUS

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**Fig. 2** Inhibition of AFB\(_1\) production by acetosyringone. Effects of 0.5–2 mmol l\(^{-1}\) acetosyringone on AFB\(_1\) levels of the toxigenic strain *A. flavus* 42–12. Values are means of three replications and bars indicate standard deviation

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**REFERENCES**


