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The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in *Aspergillus parasiticus* 439

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Abstract

A detection system based on reverse transcription PCR (RT-PCR) has been developed to monitor aflatoxin gene expression in *Aspergillus parasiticus*. Total RNAs of aflatoxigenic *A. parasiticus* 439 grown in aflatoxin permissive and non-permissive media were amplified and monitored over time by RT-PCR with specific primers designed from two genes of the aflatoxin biosynthetic pathway. Gene transcription in both media was assessed by monitoring the house keeping β-tubulin gene and aflatoxin production was correlated with transcription by thin layer chromatography. This RT-PCR technique has the potential to be employed as a tool to investigate the effects of a variety of physiological factors on the transcription of the aflatoxin genes.

Keywords: Aflatoxin; *Aspergillus parasiticus*; RT-PCR

1. Introduction

Aflatoxins are polyketide secondary metabolites produced by the important food contaminating species *Aspergillus flavus* and *Aspergillus parasiticus*. The four main aflatoxins produced, aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) are difuranocoumarin derivatives and potent liver carcinogens for a wide variety of animal species, including humans (Smith et al., 1994). While the aflatoxin biosynthetic pathway has been extensively characterised, the large body of information on the physiological factors involved in aflatoxin production and their regulatory role, particularly at the level of transcription, has yet to be fully understood (Payne and Brown, 1998). The biosynthesis of aflatoxin appears to be regulated by several interlinked mechanisms that include transcriptional regulatory elements and physiological factors that affect fungal metabolism. The complex induction and regulation of aflatoxin biosynthesis has been extensively reviewed by Payne and Brown (1998). There is growing evidence to suggest that gene expression is involved

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in the regulation of multiple parts of the aflatoxin biosynthetic pathway. Chang et al. (1995) have demonstrated that the coordinate transcription of three structural genes of the aflatoxin gene cluster, *nor1*, *ver1* and *omtA*, is activated by the *aflR* gene product, AFLR. The *aflR* gene itself also appears to be autoregulated and encodes a Cys_2His_2 zinc finger DNA binding protein which is characteristic of some fungal transcriptional activators (Woloshuk et al., 1994; Chang et al., 1995). AFLR presumably regulates aflatoxin biosynthesis by binding to the promoters of the structural genes of the aflatoxin gene cluster although these binding sites have not been elucidated. A second putative regulatory locus, *afl1*, has also been identified, which appears to positively regulate transcription of *nor1*, *ver1* and *omtA* (Woloshuk et al., 1995).

There is clear evidence that fungal development and aflatoxin biosynthesis are linked and may share regulatory elements. Variants of *A. flavus* and *A. parasiticus* with altered morphology and reduced sporulation did not produce detectable levels of aflatoxin intermediates and lacked *aflR*, *nor1* and *omtA* transcripts (reviewed in Payne and Brown (1998)). Physiological and nutritional factors affecting aflatoxin biosynthesis have been extensively studied (Zaika and Buchanan, 1987; Luchese and Harrigan, 1993; Gqaleni et al., 1997; Payne and Brown, 1998). These determinants include carbon and nitrogen sources, adenylate concentration and mycelial energy charges, NAD and NADPH levels, pH, water activity and temperature. However it has been difficult to unravel the specific action of these determinants as they may have a complex interaction affecting aflatoxin biosynthesis. A framework for assessing these physiological and nutritional factors was proposed by Payne and Brown (1998) whereby physiological parameters impacting specific physiological stages of fungal growth and development need to be correlated with genes known to be expressed and regulated at these different stages.

Here we describe a system for monitoring aflatoxin production and aflatoxin gene expression based on reverse transcription PCR (RT-PCR). RT-PCR allows the detection of mRNAs transcribed by specific genes by the PCR amplification of cDNA intermediates synthesised by reverse transcription. Two pairs of oligonucleotide primers were designed from the coding regions of the structural gene *ord1*, which encodes a cytochrome P450 monooxygenase involved in the conversion of the penultimate aflatoxin pathway intermediate *O*-methylsterigmatocystin to AFB_1, and the intronless regulatory gene *aflR* that positively regulates the transcription of the aflatoxin biosynthetic genes (Payne et al., 1993; Chang et al., 1995; Prieto and Woloshuk, 1997). Total RNA was used as a template to synthesise cDNAs with random primers and M-MLV reverse transcriptase and the resulting cDNA was amplified by PCR using the specific primers. In addition, the gene transcription of a housekeeping gene, β-tubulin, was monitored by RT-PCR.

2. Materials and methods

2.1. Growth of Aspergillus parasiticus cultures

The aflatoxigenic isolate of *Aspergillus parasiticus* was isolated from wheat in 1986 by Dr. M. Stuart, Botany Dept., University College Dublin, Ireland. *A. parasiticus* 439 was grown on malt extract agar slants (Difco Laboratories, Detroit, MI, USA) at 28°C until sporulation occurred (7–10 days). An inoculum of 1.8×10^7 conidia ml^{-1} was harvested with sterile 0.1% Tween 80 (v/v). Fifty millilitres of either Yeast Extract Sucrose (YES; 2% yeast extract, 15% sucrose) or Yeast Extract Peptone (YEP; 2% yeast extract, 15% peptone) in 250-ml Erlenmeyer flasks was inoculated with 0.5 ml of conidial suspension and incubated at 28°C in darkness under stationary conditions for 5 days. At 2–5 days triplicate cultures were assayed for aflatoxin production by TLC analysis, the mycelial dry weight determined and total RNA and genomic DNA isolated.

2.2. Extraction of aflatoxin and TLC analysis

Chloroform (50 ml) was added to each culture and the contents were filtered through Whatman No. 1 filter paper on a Büchner funnel. The mycelium retained on the filter was air dried at 90°C for 12 h and weighed. The chloroform phase was concentrated by rotary evaporation and the residue redissolved in 1 ml methanol. Aliquots (10 μl) were spotted on TLC plates (Silica gel G60, Merck, Darmstadt, Germany) and resolved with known

AFB_1, AFB_2, AFG_1 and AFG_2 standards (500 ng) in a toluene:ethyl acetate:formic acid (5:4:1) solvent system. The resolved samples were subsequently quantified by UV densitometry with a scanning densitometer (UVP, Cambridge, UK) in reflectance mode at 312 nm using Phoretix software (UVP).

2.3. Total genomic DNA isolation

Genomic DNA was isolated by a modification of a procedure described by Varadarajan and Prakash (1991). Frozen fungal mycelium (0.5 g fresh weight) harvested from 5-day cultures was pulverised with liquid nitrogen in a sterilised mortar and pestle and suspended in 15 ml extraction buffer (100 mM Tris–HCl (pH 7.4), 500 mM NaCl, 50 mM EDTA, 1% SDS and 4.375 mM β-mercaptoethanol). The extraction mixture was incubated at 65°C for 30 min with occasional gentle mixing. Polysaccharides and proteins were precipitated at −20°C by addition of 5 ml 5 M potassium acetate (pH 4.2) and pelleted at $20,000 \times g$ for 20 min. The supernatant was filtered through two layers of sterilised Miracloth (Calbiochem, CA, USA) and the DNA precipitated at −20°C by addition of 10 ml ice cold isopropanol. The DNA was pelleted at $20,000 \times g$ for 20 min, resuspended in 700 μl TE (50 mM Tris–Cl, 10 mM EDTA (pH 8.0)) and treated with RNase A (10 mg ml^{-1}; Sigma, St. Louis, MO, USA) for 30 min at 37°C. The DNA solution was centrifuged at $12,000 \times g$ for 10 min and the DNA precipitated by addition of 75 μl 3 M sodium acetate (pH 5.2) and 500 μl isopropanol. The DNA pellet was recovered by centrifugation at $12,000 \times g$, washed twice with 80% ethanol, air dried and resuspended in 100 μl TE (10 mM Tris–Cl, 10 mM EDTA (pH 8.0)).

2.4. Total RNA isolation

Total RNA was isolated according to the procedure of Gromoff et al. (1989) modified by Collins and Dobson (1997). Frozen fungal mycelia were pulverised with liquid nitrogen in a sterilised mortar and pestle, suspended in 0.5 vol. extraction buffer (0.6 M NaCl, 10 mM EDTA (pH 8.0), 100 mM Tris–Cl (pH 8.0), 4% SDS) and 0.5 vol. phenol:chloroform:isoamylalcohol (25:24:1, pH 5.2) and shaken vigorously for 20 min. The suspension was centrifuged at 14,000 rev./min and the upper supernatant phase extracted twice with phenol:chloroform:isoamylalcohol. Total RNA was precipitated by the addition of 0.75 vol. of 8 M LiCl and incubated at 4°C for more than 3 h. The solution was vortexed briefly and total RNA collected by centrifugation at $15,400 \times g$ for 15 min. The RNA pellet was resuspended in 400 μl sterile distilled water treated with diethyl pyrocarbonate (DEPC) and precipitated by the addition of 40 μl 3 M sodium acetate treated with DEPC and 1 ml 100% ethanol. The mixture was held at −20°C for 2 h and the total RNA collected by centrifugation at $15,400 \times g$. The RNA pellet was washed with 75% ethanol and resuspended in 50 μl sterile distilled water treated with (DEPC).

Genomic DNA sequences homologous to RNA targets present in the PCR reaction may interfere with the detection of RNA by RT-PCR, so the RNA was treated with DNAse I (Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions. The DNAse I was subsequently inactivated by incubation at 65°C for 10 min and the treated RNA was quantified spectrophotometrically at 260 nm and stored at −80°C until required.

2.5. Reverse transcriptase PCR

Total RNA was used as the template to generate first strand cDNA in 20 μl reaction mixtures containing: 1 μg of total RNA, 40 ng random hexamer primers, 0.5 mM of each dNTP (Roche Diagnostics), 2 μg BSA (Promega, Madison, WI, USA), 40 U of RNasin ribonuclease inhibitor (Promega), 1× RT buffer and 200 U M-MLV reverse transcriptase (Promega). Reactions were incubated at 37°C for 60 min and terminated at 65°C for 10 min. Each RT reaction (2 μl) was added to 50 μl PCR reaction mixtures containing: 50 pmol of each appropriate primer (Table 1), 5 μl of 10× KCl Taq buffer (Bioline, London, UK), 100 μM of each dNTP and 1.25 U Taq polymerase (Bioline). PCR amplification of specific *ord1*, *aflR* and β-tubulin cDNAs was performed under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles consisting of denaturation at 94°C for 30 s, annealing at a temperature dependent on the primer pair (Table 1) and extension at 72°C for 30 s. After the cycles the PCR reaction was ended with an extension step at 72°C for 10 min. The primers were designed from conserved regions of the target genes and successful-

Table 1
Details of the targeted genes, oligonucleotide primer sequences, annealing temperatures and the product lengths in base pairs (bp)

Primer	Gene	EMBL accession no.	Primer sequence (5′→3′)	Optimal annealing temperature (°C)	PCR product length (bp)	RT-PCR product length (bp)
aflR620			CGCGCTCCCAGTCCCCTTGATT			
aflR1249	*aflR*	L32577	CTTGTTCCCCGAGATGACCA	59	630	630
ord1508			TTAAGGCAGCGGAATACAAG			
ord2226	*ord1*	U81806	GACGCCCAAAGCCGAACACAAA	58	719	598
tub440			GGTAACCAAATAGGTGCCGCT			
tub1740	β-tubulin	M38265	TAGGTCTGGTTCTTGCTCTGGATG	62	1300	598

ly amplified PCR products in the following taxa; *aflR* and *ord1* (*Aspergillus parasiticus* IHEM 4383 and *Aspergillus oryzae* 358), β-tubulin (*Aspergillus ochraceus* IMI 132429, *Aspergillus nidulans* FGSC A89, *Aspergillus sojae* IMI 287278, *A. parasiticus* IHEM 4383 and *A. oryzae* 358). To distinguish between cDNA and genomic DNA targets the *ord1* and β-tubulin primers were designed from coding regions flanking introns (*aflR* is an intronless gene) so that the decreased size of the RT-PCR product (PCR product derived from cDNA) relative to the PCR product derived from genomic DNA could be discerned (Table 1). This strategy allowed the detection of false positives resulting from contamination of the RNA treated with DNase I with genomic DNA from foreign cellular material or carryover contamination from previous PCR reactions and also assessed the efficiency of the DNase I treatment of the isolated RNA. RT-PCR and PCR products (10 μl) were subsequently resolved on 2% TAE agarose gels and visualised by post staining with ethidium bromide (1 μg ml^{-1}). The RT-PCR and PCR products were sequenced to confirm their identities.

3. Results

3.1. TLC analysis

Direct extraction of fungal cultures and culture fluid with chloroform and the subsequent TLC analysis enabled the detection and identification of AFB_1, AFG_1 and AFG_2 as clearly resolved fluorescent bands which corresponded to the respective aflatoxin standards (Fig. 1A). AFB_1, AFG_1 and AFG_2 were detected by TLC analysis at concentrations of 72±30, 484±20 and 140±10 μg mg^{-1} mycelium dry weight, respectively, cultures grown on YES for 2 days (Fig. 1A). AFB_1, AFG_1 and AFG_2 concentrations reached a maximum of 169±20, 842±60 and 413±30 μg mg^{-1} mycelia dry weight, respectively, after 4 days in cultures grown

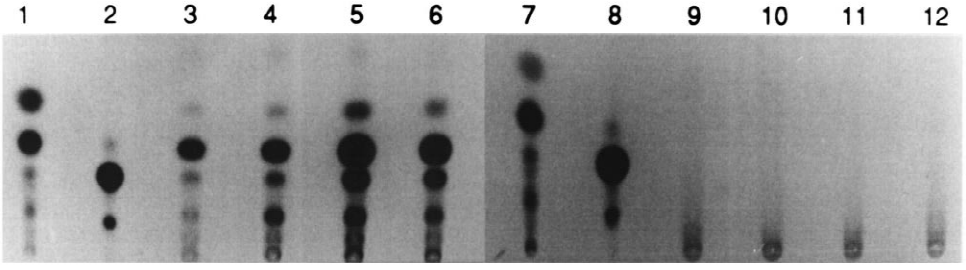

Fig. 1. TLC analysis to detect aflatoxin production in *A. parasiticus* 439 grown in aflatoxin-permissive YES media and aflatoxin non-permissive YEP media (YEP). Lanes 1 and 7, AFB_1 and AFG_1 standard (500 ng each); lanes 2 and 8, AFG_2 standard (500 ng); lanes 3–6, 10 μl extract of YES-grown cultures harvested at days 2 to 5, respectively; lanes 9–12, 10 μl extract of YEP-grown cultures harvested at days 2 to 5, respectively.

on YES which was consistent with the fungal cultures reaching idiophase. Aflatoxins were absent in cultures grown on YEP and AFB_2 was not detected in cultures on YES (Fig. 1A).

3.2. Reverse transcription PCR

RT-PCR enabled the detection of the three specific gene transcripts in *A. parasiticus* 439. Fig. 2A shows that transcripts for the housekeeping gene β-tubulin were detected by RT-PCR after 2 days incubation in cultures grown on both YES and YEP. Transcript levels of β-tubulin were significantly higher in YES which was consistent with the higher mycelial yield obtained with YES (maximum yield of 236.8±7.16 mg at day 3).

Transcript levels of β-tubulin remained constant from day 3 to 5 in cultures grown in both YES and YEP. The production of aflatoxin in *A. parasiticus* 439 was correlated with the detection of transcripts for *ord1* and *aflR* by RT-PCR (Fig. 2B and C). Transcription of both aflatoxin genes was observed in cultures grown in YES but was absent in YEP which did not support aflatoxin biosynthesis. *ord1* and *aflR* were detected in cultures grown in YES at day 2 with the highest transcript levels detected at day 5 for *ord1* and day 3 for *aflR*.

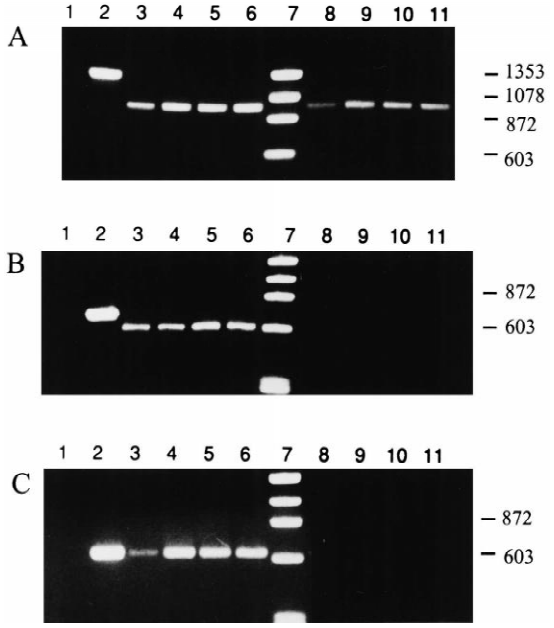

Fig. 2. Transcript levels of β-tubulin (Fig. 2A), *ord1* (Fig. 2B) and *aflR* (Fig. 2C) detected by RT-PCR in *A. parasiticus* 439 grown in aflatoxin-permissive YES media and aflatoxin non-permissive YEP media. Lane 1, negative control PCR reaction (genomic DNA template absent); lane 2, positive control PCR product (20 ng genomic DNA template); lanes 3–6, RT-PCR products amplified from YES grown cultures harvested at days 2 to 5, respectively; lane 7, molecular weight marker; lanes 8–11, RT-PCR products amplified from YEP grown cultures harvested at days 2 to 5, respectively.

4. Discussion

TLC analysis of fungal cultures and culture fluids showed that aflatoxin biosynthesis in *A. parasiticus* 439 was induced by the presence of glucose but not by peptone. This observation is consistent with previous reports where, in contrast to other secondary metabolites, glucose induced and supported aflatoxin biosynthesis whilst analogues of glucose that are not readily metabolised failed to do so (Abdollahi and Buchanan, 1981; Luchese and Harrigan, 1993). Aflatoxin is thought to be synthesised extramitochondrially from acetate and malonyl precursors derived from the catabolism of simple carbohydrates by the glycolytic and pentose phosphate pathways (Hsieh and Mateles, 1970; Payne and Brown, 1998). Glucose may regulate aflatoxin biosynthesis through catabolite repression of enzymes generating NADPH and in the Krebs cycle (Buchanan and Lewis, 1984). However, although carbon source and utilisation may be one of the most important determinants of aflatoxin biosynthesis, little is known about the mechanisms of carbohydrate regulation (Payne and Brown, 1998).

Transcription analyses of the aflatoxin pathway genes have traditionally employed northern hybridisation analysis (Chang et al., 1995; Feng and Leonard, 1995; Cary et al., 1996; Flaherty and Payne, 1997; Prieto and Woloshuk, 1997). Although Northern hybridisation analysis and RNAse protection assays are well established and reliable techniques for the detection of mRNAs, their sensitivity may be insufficient for the detection of low levels of gene transcription. A previous study reported that *ord1* transcripts were not detected by Northern analysis of total RNA in *A. parasiticus* but required the laborious isolation of considerable amounts (7 μg) of poly $(A)^+$ RNA to detect transcription (Prieto

and Woloshuk, 1997). In contrast, the RT-PCR method allowed the detection of *ord1* transcripts from 1 μg total RNA in this study. RT-PCR has been demonstrated to be 10^3–10^4 times more sensitive than Northern hybridisation analysis (Gilliland et al., 1990; Sooknanan et al., 1993). The use of nested RT-PCR can also further increase detection sensitivity or overcome the sensitivity limitations of RT-PCR if they arise (Green et al., 1998). RT-PCR can also easily allow the distinction between very similar mRNAs as the specificity of the primers to the target(s) can be increased by designing very specific primers or by increasing the stringency of primer annealing (Gettemy et al., 1998; Verma and Upadhyaya, 1998). In addition, several specific mRNAs may be detected simultaneously in a single RNA sample by multiplex RT-PCR (Verma and Upadhyaya, 1998; Halminen et al., 1999). RT-PCR is a more rapid, simpler and safer alternative to hybridisation techniques as hazardous chemicals, radiolabel and autoradiography are not involved. In addition, the amount and purity of total RNA required is substantially less as detection sensitivities as low as 10 bacterial cells per ml can be obtained with RT-PCR from a crude cellular lysate (Vaitilingom et al., 1998). The technique is therefore superior in studies where the amount of tissue is limiting and the isolation of large concentrations of intact RNA is unfeasible or in studies where the production of large amounts of toxic waste is undesirable e.g. aflatoxigenic cultures.

The RT-PCR approach outlined here has proven to be a rapid, sensitive, highly specific and non-isotopic alternative to conventional techniques for the detection of *aflR*, *ord1* and β-tubulin mRNA transcripts from *A. parasiticus*. The transcription of other genes involved in the pathway may also be easily monitored using RT-PCR as many of the structural genes of the aflatoxin gene cluster have been cloned and sequenced. RT-PCR primers designed from aflatoxin genes of *A. parasiticus* may also be employed to monitor the transcription of aflatoxin genes of *A. flavus* as aflatoxin gene homology is very high between these two aflatoxigenic species (Payne and Brown, 1998).

Although AFLR specifically regulates the transcription of the aflatoxin structural genes, little is known about the global cellular factors that may regulate aflatoxin production. Collins and Dobson (1997) successfully demonstrated the induction and regulation of laccase gene transcription in the white rot fungus, *Trametes versicolor*, when cultured under a variety of physiological conditions and in the presence of known laccase inducers with a RT-PCR method similar to the one described here. Doohan et al. (1999) developed a RT-PCR assay to successfully study the expression of a specific gene involved in trichothecene biosynthesis in *Fusarium*. It is therefore envisaged that the RT-PCR technique described here has the potential of being able to detect a number of different mRNA transcripts from aflatoxin genes when the producing fungus is cultured under a variety of different physiological conditions affecting aflatoxin biosynthesis.

One important overall objective of the molecular and genetic aflatoxin research is to apply molecular biological techniques to help eliminate or control the biosynthesis of aflatoxin in crop plants. Assessing the affect of specific determinants on the transcription of the aflatoxin genes with the RT-PCR technique presented here may identify factors or agents, such as natural plant products which may be incorporated into preharvest strategies for aflatoxin control.

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