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Full Length Research Paper

The uncertainty of assessing aflatoxin B_1-producing ability using *aflR* gene in *Aspergillus* species

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***Aspergillus* section *Flavi* consists of many species which are very similar in morphology and very important in human health and food industry. *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus toxicarius* are notorious aflatoxigenic species. *Aspergillus oryzae* and *Aspergillus sojae* are non-aflatoxigenic species and widely used for Oriental fermented food. The presence of the *aflR* gene and aflatoxin B_1 (AFB1) production ability was assessed by PCR detection and HPLC analysis, respectively. The investigation of AFB1 and amplification of the *aflR* gene in thirty five authentic strains of *Aspergillus* section *Flavi* showed that (1) all the AFB1-producing strains were detected to possess the *aflR* gene; (2) some non AFB1-producing *Aspergillus* section *Flavi* strains are constitutional, which did not tested to possess the *aflR* gene; (3) some strains used in Oriental fermentation have the *aflR* gene but they are non AFB1-producing aspergilli. The conclusion is that it is uncertain to judge the aflatoxigenicity of an *Aspergillus* strain using the *aflR* gene as an indicator.**

Key words: *Aspergillus*, aflatoxin, *alfR* gene, HPLC, PCR.

INTRODUCTION

Aflatoxins are the mycotoxins causing the most concern worldwide because of their carcinogenic properties and occurrence as natural contaminants of a large number of agricultural commodities. Aflatoxins are a group of chemically related difuranocoumarin compounds produced by species of *Aspergillus* section *Flavi*. Aflatoxin B_1 (AFB1) is hepatotoxic and one of the most potent animal carcinogens (Conning, 1983).

Aspergillus section *Flavi* comprises morphologically very similar and biotechnologically very important species. *Aspergillus flavus*, *A. parasiticus*, and *A. toxicarius* are capable of producing aflatoxins (Qi, 1998; Samson, 1994). However, *A. oryzae* and *A. sojae* are widely used for Oriental fermented food, which are non-aflatoxigenic species and were regarded as domesticated varieties of the wild species *A. flavus* and *A. parasiticus*, respectively (Wicklow, 1984).

In the investigations of the aflatoxin biosynthetic pathway, it was found that the aflatoxin-related genes are clustered within a 75 kb region of the genome (Trail et al., 1995; Woloshuk and Prieto, 1998; Yu et al., 1995). The *aflR* gene, which regulates these clustered genes for aflatoxin biosynthesis, has been identified in *A. flavus*, *A. parasticus*, *A. sojae* and *A. oryzae* (Ehrlich et al., 1999; Trail et al., 1995; Woloshuk et al., 1994). Published data makes clear that aflatoxin biosynthesis requires functional *aflR* gene product and a fully functional aflatoxin biosynthetic cluster (Georgianna and Cary, 2009). The genes for aflatoxin biosynthesis are present, but not expressed in the non-aflatoxin-producing fungi *A. oryzae* and *A. sojae* (Klich et al., 1995, 1997; Lee et al., 2006; Watson et al., 1999; Woloshuk et al., 1994).

Can the *aflR* gene be applied for assessing possible aflatoxin-production in *Aspergillus* species? The present study investigated the relationship between the presence of the *aflR* gene and the AFB1-producing ability of these aflatoxin-producing and non-aflatoxin-producing species

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Table 1. *Aspergillus* strains investigated.

Species	CGMCC Strain and its isolated source and time	[a] *aflR* gene fragment	[b] AFB1 production (μg/ml)
A. flavus	3.417, Soy sauce, 1952	–	–
	3.881, Liquor koji, 1958	+	–
	3.3554, Soil, 1969	+	–
	3.4069, Soil, 1969	+	752
	3.6150, Soil, 2001	+	–
	3.6151, Soil, 2001	+	–
	3.6153, Wheat, 2001	+	–
	3.6303, Maize, 2001	+	–
	3.6304, Maize, 2001	+	–
	3.6434, Soil, 2001	–	–
A. oryzae	3.381, Thick Soy sauce, 1952	+	–
	3.424, Soy koji, 1952	–	–
	3.427, Soy koji, 1952	–	–
	3.428, Soy koji, 1952	–	–
	3.801, koji, 1957	–	–
	3.802, koji, 1957	–	–
	3.863, Soy koji, 1957	+	–
	3.2067, undecanted wine, 1960	+	–
	3.2068, undecanted wine, 1960	+	–
	3.2073, undecanted wine, 1960	+	–
	3.2140, undecanted wine, 1960	+	–
	3.2792, Fermented soybean, 1966	–	–
A. parasiticus	3.6155, Rice, 2001	+	52
	3.6156, Soil, 2001	+	–
A. sojae	3.495, Soy sauce, 1952	+	–
	3.880, Liquor koji, 1958	+	–
A. tamarii	3.3977, Soil, 1969	–	–
	3.4067, Soil,1969	–	–
	3.6350, Mushroom, 2001	–	–
	3.6416, Soil, 2001	–	–
A. toxicarius	[c] 3.4407, unknown, 1983	+	5020
	3.6157, Soil, 2001	+	127
	3.6158, Soil, 2001	+	240
A. zhaoqingensis	3.4626, Soil, 1989	–	–

[a] The experiment repeated for three times; [b] The determination repeated for two times; [c] Strain originated from Japan, the rest from China.

using *Aspergillus* strains from CGMCC (China General Microbiological Culture Collection).

MATERIALS AND METHODS

Aspergillus strains

Thirty five *Aspergillus* strains based on morphological characteristics from CGMCC were used in this study (Table 1).

Media and culture preparation

The strains were grown on malt extract agar (MEA; powdered. 20 g; peptone, 1 g; glucose, 20 g; agar, 15 g; distilled water of 1 litre) slant culture at 25°C for one week. 2.5 ml sterilized water was added to each tube. The surface of the slant was scraped with

Table 2. Primers used to amplify *aflr* gene fragments.

Primer	Sequence	Primer	Sequence
F1	TCGGTACGTAAACAAGGAAC	R1	TCTGATGGTCGCCGAGTTGA
F2	CCGATTTCTTGGCTGAGT	R2	TCCTCATCCACACAATCC
F3	GCTGTCTGACGGAAGAGCG	R3	ACCATGACAAAGACGGATCC
F4	GCAATCCGCGCGCTCCCAGT	R4	CCGACTCGAGGAACGGGTCG
F5	GGCCTGTGCTCGGTGTAT	R5	GGACTCTGGTGAGAAAAG
F6	CAATGGTAGCAGTAGCGTCT	R6	CATGCTCAGCAAGTAGCCAT

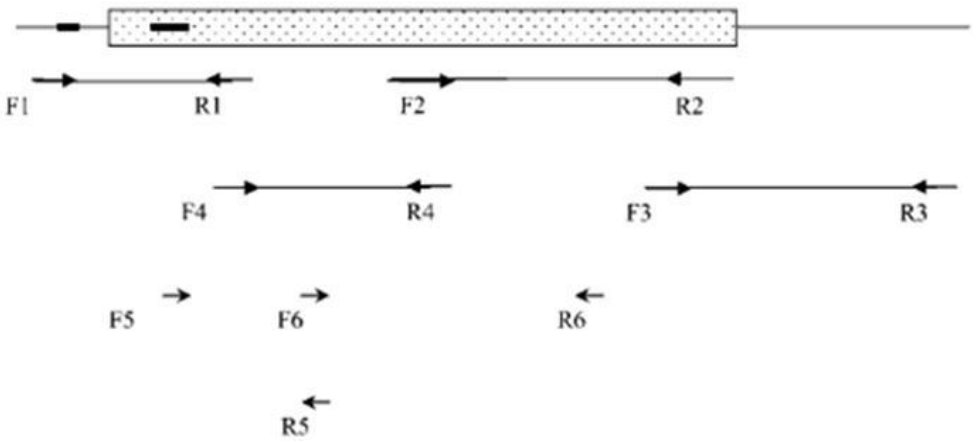

Figure 1. Positions of primers used to amplify segments of the *aflR* gene. Dotted bar shows *aflR* ORF (open reading frame) (Lee et al. 2006)

spore suspension. 100 µl of the spore inoculum (10^4-10^5 c.f.u./ml) was transferred to the 250-ml flask consisting of 40 ml YES medium (yeast extract 40 g and sucrose 160 g l^{-1} in distilled water), which is usually used for the production of fungal toxins (Blanc et al., 1995). The flask cultures were statically incubated at 25°C for 10 days.

Aflatoxin B_1 analysis

Pure aflatoxin B_1 was purchased from Sigma Chemical Company. All solvents used were HPLC grade; all other reagents used were analytical grade.

The fermentation broth was filtered through No.1 Whatman and then membrane filter paper. The filtrate was centrifuged at 12000 g for 30 min. The upper aqueous phase was analyzed.

HPLC analysis of aflatoxin B_1 employed the method described in Sobolev (2007). The analysis was performed using a Waters 244, column: Shoex C_{18}, 4 µm, 250 mm × 4 mm, temperature: 28°C, injector volume: 20 µl. Fluorescence detector (Waters 470) set at 365 nm excitation and 440 nm emission wavelength. The mobile phase consisted of water/MeOH (63:37, v: v), the flow rate was 0.15 ml/min. The concentrations of aflatoxin B_1 of samples were calculated by the equation:

$$Cs = Cp \times As / Ap$$

In this equation, Cp and Cs are the concentration of pure aflatoxin B_1 solution and sample solution, respectively, and Ap and As are the area of peaks of pure aflatoxin B_1 solution and sample solution, respectively.

DNA extraction

A liquid medium (MEA without agar) in a 9 cm diametric plate was incubated statically with each *Aspergillus* strain slant inoculum at 25°C for one week. Fresh fungal mycelia were collected from the plate and ground in liquid nitrogen. 0.2 g mycelial powder in 2 ml tubes was resuspended in 800 µl extraction buffer (20 mM EDTA, 1.4 M NaCl, 3% CTAB, 100 mM Tris-HCl pH 8) and incubated at 65 °C for 30 min. The mixture was then extracted with equal volume phenol/chloroform (1:1). After mixing and centrifugation for 15 min at 12000 g, the resulting aqueous phase was added to 10% of the volume of 3 M sodium acetate pH 5.5. DNA was then precipitated by addition of 1 volume of isopropyl alcohol. After washing with 70% ethanol, the DNA was air-dried at room temperature and re-dissolved in ddH_2O. The RNA was removed by incubation with DNase-free RNaseA at 37°C for 30 min. Purity and average fragment size were checked by agarose gel-electrophoresis.

PCR Amplification of *aflR* Gene Fragments

The PCR reactions (20 µl) contained 50 ng total DNA, dNTP at 0.1 mM each, primers at 100 pM each, 1 U DNA polymerase and 1 × reaction buffer. The PCR reactions were performed as per the following programme: 5 min at 94°C; 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; then a final extension at 72°C for 5 min. Six pairs of primers were used to identify *aflR* gene fragments (Table 2, Figure 1). The internal transcribed spacer (ITS) fragment of the rRNA gene was amplified using primers ITS4 and ITS5 as a control for the PCR reaction. Each PCR product was

analysed by electrophoresis on a 0.8% agarose gel.

RESULTS AND DISCUSSION

The results are given in Table 1. The a*flR* gene was amplified in all five aflatoxin-producing strains, including one strain of *A. flavus*, one strain of *A. parasiticus* and three strains of *A. toxicarius*. If no *aflR* gene exists in an *Aspergillus* strain, no aflatoxin is produced. Eight out of ten *A. flavus* strains have the *aflR* gene, but only one strain produced detectable AFB1. In six out of twelve *A. oryzae* strains, no *aflR* PCR products were obtained. However, the *aflR* gene exists in six other strains, while no AFB1 was detected in these strains. Two *A. parasiticus* strains have the *aflR* gene, and one of them produced AFB1. Four *A. tamarii* strains have neither the *aflR* gene nor produced AFB1. All three strains of *A. toxicarius* produced AFB1 as well as having the *aflR* gene. One strain of *A. zhaoqingensis* is also a non- AFB1 producing species without the *aflR* gene.

The results showed that: (1) all the AFB1-producing strains were detected to possess the *aflR* gene; (2) some non AFB1-producing *Aspergillus* section *Flavi* strains are constitutional, which did not possess the *aflR* gene when tested; (3) some strains used in Oriental fermentation have the *aflR* gene but they are non AFB1-producing aspergilli. The conclusion is that it is uncertain to judge the aflatoxigenicity of an *Aspergillus* strain using the *aflR* gene as an indicator.

Domesticated strains differ from “wild” strains primarily because long-term domestication results in the loss of certain taxonomic characters and mycotoxin-producing capability (Wicklow, 1984).

Lee et al. (2006) compared the *aflR* gene sequences of strains in *Aspergillus* section *Flavi* and suggested that the *aflR* gene could be examined to assess aflatoxin production of those fungi for safety reasons. Whereas, the relationship could not be inferred between *aflR* gene and aflatoxin production in the *Aspergillus* section *Flavi* strains because aflatoxin production of some strains did not be tested in their research (Lee et al., 2006). Dehghan et al. (2008) investigated aflatoxin in twenty three clinical sinus *A. flavus* group isolates by TLC method. Although all tested *A. flavus* group isolates could be able to amply *aflR* gene but only five (17.8%) were aflatoxin producer. Their finding is in accordance with our conclusion.

The study clearly reveals that an *Aspergillus* species which has the *aflR* gene may produce AFB1 or may not. So, it is uncertain that using *alfR* gene as a target to assess the aflatoxigenicity of *Aspergillus* when examining possible aflatoxin contamination of grain and crops.

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