含有額外調節基因(*aflR-2*)的產毒素黃麴菌在不同生長條件下其毒素合成基因的表現差異

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摘　要

黃麴毒素為重要食物污染菌 *Aspergillus flavus* 及 *A. parasiticus* 所產生之二次代謝產物，*aflR* 基因產物是大部份黃麴毒素生合成基因的轉錄活化子。薄層色層(TLC)分析顯示只在 *A. flavus* 及 *A. parasiticus* 產毒菌株中偵測出黃麴毒素但其它 *Aspergillus* 供試菌株則無法偵測出。利用從 *aflR* 基因密碼區設計的專一性引子成功地增幅 *aflR* 基因之 PCR 產物大約 1352 bp，PCR 結果顯示在所有產毒菌株及一些非產毒菌株中都能增幅出 *aflR* 基因，反之有 4 個非產毒菌株無法增幅出 *aflR* 基因。*Nco*I 限制酵素分析顯示只有 *A. parasiticus* 五菌株(71.43%)具有 *aflR1/2* 基因存在。*AflR* 序列比對分析顯示產毒菌株之 *aflR1/2* 基因密碼區與非產毒菌株間有 98.5/99.6%相似度，比對 *aflR1/2* 基因密碼區序列發現有 20 至 35 個核苷酸改變。將供試菌株培養於適合產毒(YES)與不適合產毒(YEP)之培養液中生長一天，藉由反轉錄聚合酶連鎖反應(RT-PCR)偵測黃麴毒素生合成相關基因(*nor-1, avnA, cypX, vbs, ver-1, omt-1, ord1* 及 *aflR*)之轉錄表現，由結果得知 *A. parasiticus* 產毒菌株在 YES 培養液中，*vbs* 及 *ord1* 會轉錄表現；在 YEP 培養液中，不會有 *vbs* 及 *ord1* 轉錄表現。*A. flavus* 產毒菌株在 YES 培養液中，*omt-1* 會轉錄表現；在 YEP 培養液中，不會有 *omt-1* 轉錄表現。而產毒菌株與非產毒菌株之黃麴毒素產生與 *aflR1/2*、*nor-1*、*avnA*、*cypX* 及 *ver-1* 基因是否轉錄表現並無絕對的相關性。*aflR* 持續性表現可能導致代謝路徑中蛋白質的合成，然而與黃麴毒素的產生並無相關性。因此，在能夠評估 *Aspergillus* spp.是否為黃麴毒素產毒菌株之前，黃麴毒素合成的基因調控和 *aflR* 的重複數之間的差異尚有待進一步釐清。

關鍵字：黃麴毒素、*aflR1/2*、*Aspergillus flavus*、*A. parasiticus*、反轉錄聚合酶連鎖反應(RT-PCR)。

Differential gene expression in aflatoxigenic *Aspergillus* species containing the extra regulatory gene *aflR-2* for aflatoxin synthesis in different growth conditions

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Abstract

Aflatoxins (AF) are polyketide secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* and are important contaminants that often lead to food poisoning. Among *Aspergillus* species, AF were detected only in aflatoxigenic isolates of *A. flavus* and *A. parasiticus* by TLC analysis. *AflR* gene-specific primers were used to amplify 1352-bp PCR products from all aflatoxigenic and some nonaflatoxigenic isolates. RFLP analysis of the *Nco*I-digested DNA region of the *aflR* gene showed that only five of seven *A. parasiticus* isolates (71.43%) consisted of two *aflR* genes, *aflR1* and *aflR2*, the nucleotide sequences of which were 98.5/99.6% identical to those of nonaflatoxigenic isolates, demonstrating a change in 20 to 35 nucleotides. To understand the genetic regulation of AF, the tested isolates were grown in AF non-permissive YEP broth and AF-permissive YES broth for one day and reverse transcription PCR (RT-PCR) was used to study the expression of *nor-1*, *aflR*, *ver-1*, *avnA*, *omt-1*, *ord1*, *vbs*, *cypX* and *β-tublin.* Gene expression differed between culture media and species. *Vbs* and *ord1* were expressed in *A. parasiticus* aflatoxigenic isolates grown in YES, but not YEP, broth, while *omt-1* could be transcribed from *A. flavus* aflatoxigenic isolates grown in YES, but not YEP, broth. Expression of genes *aflR1/2*, *nor-1*, *avnA*, *cypX* and *ver-1* were not correlated with the production of AF. Constitutive expression of *aflR* could lead to synthesis of pathway proteins, which did not relate to AF accumulation. Therefore, differences in gene regulation and duplication require further characterization in order to assess the benefits for aflatoxigenic *Aspergillus* species.

Keywords: Aflatoxins; *aflR1/2*; *Aspergillus flavus*; *A. parasiticus*; Reverse transcription PCR (RT-PCR)

Introduction

As one of the most common mycotoxins, aflatoxins (AF) are polyketide-derived toxins and potent liver carcinogens produced primarily by two fungal species, *Aspergillus flavus* Link: Fr. and *A. parasiticus* Speare which are most often found on infected crops such as corn, peanuts, cottonseed, and treenuts (Bhatnagar et al., 2003; Bhatnagar et al., 2006; Jaime Garcia and Cotty, 2006). Among the four main aflatoxins: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2

(AFG_2), AFB_1 is the most toxic and is a potent known carcinogen (Chu, 1991; Doster and Michailides, 1994). Aflatoxin production is regulated by more than 25 genes involved in the biosynthesis of aflatoxins that are present within a 70-kb DNA region in the chromosome of *A. flavus* and *A. parasiticus* (Yu et al., 2004; Bhatnagar et al., 2006).

Aspergillus Section *Flavi*, commonly referred to as the *A. flavus* group, includes *A. flavus*, *A. nomius* Kurtzman and Hesseltine, *A. oryzae* (Ahlb.) Cohn, *A. parasiticus*, and *A. sojae* Sakaguchi and Yamada, *A. tamarii* Kita (Chang et al., 1995a). *A. sojae* and *A. oryzae* are widely used in koji preparation in the Orient and differ from wild strains of *A. flavus* and *A. parasiticus* primarily as a result of long-term domestication (Wicklow et al., 1990; Hara et al., 1992). Differentiation of members of the *A. flavus* group and detection of aflatoxigenic molds have been extensively investigated (Yuan et al., 1995; Geisen, 1996; Shapira et al., 1996; Farber et al. 1997). Recently, four DNA fragments (*nor*-1, *ver*-1, *omt*-1 and *apa*-2) were demonstrated to differentiate aflatoxigenic strains from some non-aflatoxigenic strains by the PCR method (Chen et al., 2002). In *A. parasiticus*, the RT-PCR method has been used to characterize aflatoxin gene expression, such as *ord*1 and *aflR* (Sweeney et al., 2000) and *aflD*, *aflO*, and *aflP* (Scherm et al., 2005). A multiplex reverse transcription-polymerase chain reaction (RT-PCR) protocol was developed to discriminate aflatoxin-producing from aflatoxin-non-producing strains of *A. flavus* (Degola et al., 2007).

Among these genes, a regulatory gene, *aflR* (previously named *afl*-2 for *A. flavus* and *apa*-2 for *A. parasiticus*) was shown to encode AflR protein, which is a binuclear zinc-finger DNA-binding protein and a transcriptional activator of most aflatoxin biosynthetic genes as well as itself. The same zinc finger domain, CTSCASSKVRCTKEKPACARCIE RGLAC of AflR is present in all *A. sojae*, *A. flavus* and *A. parasiticus* isolates examined as well as in some *A. oryzae* isolates (Woloshuk et al., 1994; Chang et al., 1995b; Liu and Chu, 1998). According to the nucleotide sequence variation in the 5'-untranslated and zinc finger region, these four species can be separated into two groups: group I includes *A. oryzae* and *A. flavus*, which contain the T-G-A-A-X-C fingerprint, and group II includes *A. parasiticus* and *A. sojae*, which contain the C-C-C-C-C-T fingerprint at the corresponding position. Furthermore, two nucleotides at positions 290 (C or T) and 2132 (G or A) can be used to further distinguish *A. flavus* from *A. oryzae* and *A. parasiticus* from *A. sojae*, respectively (Chang et al., 1993, 1995a).

There has been no large scale investigation of the relationship between sequence variability in *aflR-1/2* and copy number from aflatoxigenic and nonaflatoxigenic strains of the tested *Aspergillus* spp. Previous studies (Yu et al., 1995; Liang et al., 1996) showed that different aflatoxin pathway genes and their order vary among *Aspergillus* spp. due to duplication and deletion. Deletion of *aflR-1* inhibits the production of aflatoxin precursors, suggesting that *aflR-2* could not complement the function of *aflR-1* (Chang and Yu, 2002). However, the gene composition and expression pattern of *aflR-1/2* in *A. flavus*, *A. oryzae*, *A. parasiticus* and *A. sojae* has not been reported.

The objectives of this study were: (1) to evaluate the *aflR-1/2* composition of four species in *Aspergillus* Section *Flavi*; (2) to examined sequence variability in *aflR-1/2* from aflatoxigenic and nonaflatoxigenic strains of the tested *Aspergillus* spp.; (3) to compare aflatoxin biosynthetic genes

nor-1, *ver-1*, *avnA*, *omt-1*, *ord1*, *vbs* and *cypX* was analyzed in the tested *Aspergillus* spp.

Materials and Methods

Fungal strains and culture

Twenty strains of *Aspergillus* spp. and their characteristics are listed in Table 1. Among them, *A. niger* was isolated from peanut kernels (Chiou, 1997), and *A. oryzae* soysauce was a koji mold for soysauce fermentation (Chen et al., 2002). The other strains were purchased from the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). All strains were maintained on slant cultures containing Yeast Malt Agar (YMA; 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% dextrose, 1% agar) at 4°C or grown in Yeast Malt Broth (YMB; YMA without 1% agar). A conidial suspension (10^6 spore/ml) of all samples was incubated in 2 ml microfuge tubes prepared as reported by Chen et al. (2002) at 28°C for 3 days.

Table 1. Correlation of *aflR1/2* genes and relative amount of aflatoxin in aflatoxigenic and non-aflatoxigenic *Aspergillus* strains

Strain	DNA detected		Transcript detected		Aflatoxin produced	Relative amount of aflatoxin (%)
	aflR1	*aflR2*	*aflR1*	*aflR2*		
A. parasiticus CCRC 30160 (= ATCC 26864)	+[a]	−	+	−	$B_{1,2}$, $G_{1,2}$	100
A. parasiticus CCRC 30150 (= ATCC 16869)	+	+	+	+	B_1, $G_{1,2}$	87
A. flavus CCRC 30290 (= ATCC 44618)	+	−	+	−	$B_{1,2}$	81
A. parasiticus CCRC 30172 (= ATCC 15517)	+	+	+	+	$B_{1,2}$, $G_{1,2}$	76
A. parasiticus CCRC 30164 (= ATCC 26691)	+	+	+	+	$B_{1,2}$, $G_{1,2}$	71
A. parasiticus CCRC 30117 (= ATCC 26692)	+	+	+	+	B_1, G_1	23
A. flavus CCRC 30231 (=ATCC 32592)	+	−	+	−	B_1, G_1	15
A. parasiticus CCRC 30228 (= ATCC 36537)	+	+	+	+	−	0
A. flavus CCRC 30010 (= ATCC 10124)	+	−	+	−	−	0
A. flavus CCRC 30119 (=ATCC 15547)	+	−	+	−	−	0
A. flavus CCRC 30166 (= ATCC 26945)	+	−	+	−	−	0
A. flavus CCRC 30300 (= ATCC 36061)	+	−	+	−	−	0
A. flavus CCRC 30291 (Taiwan isolate)	−	−	NA	NA	−	0
A. oryzae CCRC 30120 (= ATCC 11494)	+	−	+	−	−	0
A. oryzae CCRC 30123 (=ATCC 14587)	+	−	+	−	−	0
A. oryzae CCRC 33705 (Taiwan isolate)	−	−	NA	NA	−	0
A. sojae CCRC 30103 (= ATCC 9362)	+	−	+	−	−	0
A. sojae CCRC 30227 (= ATCC 11906)	+	−	+	−	−	0
A. sojae CCRC 30419 (= ATCC 42249)	−	−	NA	NA	−	0
A. *niger*	−	−	NA	NA	−	0

[a] +: presence; −: absence; NA: not available

Aflatoxin analysis by thin-layer chromatography

For aflatoxin analysis, 0.5 ml broth was transferred into a 1.5 ml microfuge tube. After addition of 0.6 ml chloroform, the mixture was vortexed for 5 min to extract aflatoxins. Nitrogen was used to remove chloroform from 0.5 ml of chloroform extract in a 1.5 ml microfuge tube under a hood. Finally, the residue was dissolved in 10 µl chloroform and separated with a thin-layer chromatography (TLC) plate (DC-Alufolien Kieselgel 60F254, E. Merck, Darmstardt, Germany) in solvent

(acetone/chloroform, 87/17, v/v). After separating, unique fluorescent spots were viewed under an ultraviolet box (Chen et al. 2002). A standard aflatoxin solution containing B_1, B_2, G_1 and G_2 (Supelco, Bellefonte, PA) was used as positive control.

Preparation of mycelial clumps and DNA extraction

Mycelial clumps were harvested by centrifugation (8000 x *g*, 1 min at 20°C) and washed two times with 1 ml deionized water. The clumps were immersed in liquid nitrogen and immediately ground into powder. To extract chromosomal DNA, 400 µl of lysis buffer and 1 µl RNase A solution were added and the mixture was heated to 65°C for 15 min. DNA was then purified using a DNA extraction kit (DNeasy Plant Mini Kit, Qiagen GmbH, Hilden, Germany). Isolated DNA was dissolved in 50 µl of acetate-EDTA (AE) buffer and stored at – 20°C until use.

Differentiation of aflR-1/2 genes by polymerase chain reaction - Restriction fragment length polymorphism (PCR-RFLP) analysis

aflR-1/2 genes were amplified using the designed primer pair: AflR-198 (5'-CCTCCACGATGGTTGACC ATA-3')/AflR-1549 (5'-GGCTTTTCTTCATTCT CGATGC-3') according to the nucleotide sequence of the *aflR-1* (L26222, *A. parasiticus*) and *aflR-2* (AF110766, *A. parasiticus*) gene (Table 2). 20 µl of PCR mixture included 10 µM of each primer, 2 µg DNA template, 5 units of Taq polymerase solution (Genemark, Taiwan), 2.5 mM of dNTPs, 2 µl reaction buffer and 3 µl deionized water. PCR conditions were 35 cycles of 1 min at 94°C, 30 sec at 57°C and 1.5 min at 72°C using the GeneAmp PCR system 2400 (Applied Biosystem, Foster City, CA, USA). The PCR product was then purified with the Gene-SpinTM 1-4-3 DNA Extraction Kit (Protech Technology, Taipei, Taiwan). According to the PCR-RFLP method used to readily distinguish *aflR-1* from *aflR-2* (Cary et al., 2002), 0.6 µg of purified PCR product was digested with 1 U of *Nco*I restriction enzyme (New England BioLabs, Beverly, MA, USA) at 37°C for 4 hours and electrophoresed on 1.8% Agarose gels. The *Nco*I restriction fragment length polymorphism was recorded after staining with ethidium bromide. In addition, purified PCR products were cloned into pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) and sequenced using dye- terminators (BigDye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems, Foster City, CA, USA). Sequence comparison was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Table 2. Primer pairs used for amplification of aflatoxin biosynthesis genes

Primer	Gene	Primer sequence	Optimal annealing temperature (°C)	PCR/RT-PCR product (bp)	Reference
AflR-198	*aflR*	5'-CCTCC ACGATGGTTGACCATA-3'	57	1352/—	AF110766[a]
AflR-1549		5'-GGCTT TTCTTCATTCTCGATGC-3'			
nor-501	*nor-1*	5'-ACCGCTACGCCGGCACTCTCGGCAC-3'	65	400/400	Farber et al., 1997
nor-900		5'-GTTGGCCGCCAGCTTCGACACTCCG-3'			
aflR-620	*aflR*	5’-CGCGCTCCCAGTCCCCTTGATT-3’	59	630/630	Sweeney et al., 2000
aflR-1249		5’-CTTGTTCCCCGAGATGACCA-3’			
ver-623	*ver-1*	5'-GCCGCAGGCCGCGGAGAAAGTGGT-3'	65	538/487	Farber et al., 1997
ver-1160		5'-GGGGATATACTCCCGCGACACAGCC-3'			
avnA-5462	*avnA*	5’-CTAGCTGTCGTCCAATGCAA-3’	62	987/927	AB076804[a]
avnA-6449		5’-TGTTGGCCTTTTCATTCTCC-3’			
omt-848	*omt-1*	5'-GGCCCGGTTCCTTGGCTCCTAAGC-3'	62	1254/1024	L25836[a]
omt-2101		5'-CGCCCCAGTGAGACCCTTCCTCG-3'			
ord-1508	*ord1*	5’-TTAAGGCAGCGGAATACAAG-3’	58	719/598	Sweeney et al., 2000
ord-2226		5’-GACGCCCAAAGCCGAACACAA A-3’			
vbs-496	*vbs*	5’-CACTTTGGCATGTACGGATG-3’	62	852/799	U51327[a]
vbs-1347		5’-CTCCTGTTGCACGATTCTGA-3’			
cypX-6673	*cypX*	5’-CCGGCGATGAAACTATCAAT-3’	62	590/535	AF169016[a]
cypX-7262		5’-ACCCCACTGCATTATCATTG-3’			
tub-440	*β-tublin*	5’-GGTAACCAAATAGGTGCCGCT-3’	62	1300/998	Sweeney et al., 2000
tub-1740		5’-TAGGTCTGGTTCTTGCTCTGGATG-3’			

[a] Primer designed in this study according to the genomic DNA nucleotide sequence from the accession number of GenBank.

Gene expression analysis of aflatoxin biosynthetic genes

Conidial suspensions (10^6 spore/ml) of *Aspergillus* strains were inoculated in 2 ml microfuge tubes containing 700 µl of either Yeast Extract Sucrose (YES; 2% yeast extract, 15% sucrose) or Yeast Extract Peptone (YEP; 2% yeast extract, 15% peptone) and incubated at 28°C in darkness under stationary conditions for 4 days. 1–4 days after inoculation, mycelial dry weight was determined and total RNA was isolated according to the procedure of Gromoff et al. (1989) modified by Collins and Dobson (1997). Total RNA was resuspended in 50 µl sterile distilled water treated with DEPC. The DNA was removed by treatment with RQ1 RNase-free DNAse (Promega, Madison, WI, USA). The DNAse I was subsequently inactivated by incubation at 65°C for 10 min and the treated RNA was measured spectrophotometrically at 260 nm and stored at - 80°C. cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA). 2 µg of each cDNA and 10 µM of each appropriate primer to amplify *nor-1*, *aflR*, *ver-1*, *avnA*, *omt-1*, *ord1*, *vbs*, *cypX* and *β-tublin* (Table 2) were then used in each PCR reaction under the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing temperature dependent on the primer pair and extension at 72°C for 1.5 min; and final extension step at 72°C for 7 min. 10 µl of each RT-PCR product was subsequently separated on a 1.8% Tris-acetate-EDTA (TAE) agarose gel and visualized after staining with ethidium bromide (1 µg ml^{-1}). The intensity of each transcript was quantitated using TotalLab image analysis software (Nonlinear USA Inc, NC, USA).

Results

Aflatoxigenic nature

To evaluate aflatoxin production among *Aspergillus* species, all tested strains were grown in YM broth for 3 days and subjected to aflatoxin extraction. Differences in aflatoxin production and types were determined by TLC separation and UV irradiation of reference standard toxins (Table 1). Only *A. flavus* CCRC 30231, CCRC30290, *A. parasiticus* CCRC 30117, CCRC30160, CCRC30172, CCRC30164 and CCRC30150 synthesized the aflatoxin. Four types of aflatoxins were found in two species (Table 1). Among *A. parasiticus* strains, at least two types of aflatoxins appeared in each strain, including aflatoxin B_1, B_2, G_1 and G_2 in strains CCRC 30160, CCRC 30164 and CCRC 30172; aflatoxin B_1, G_1 and G_2 in strain CCRC30150, and aflatoxin B_1 and G_1 in CCRC30117. In *A. flavus*, aflatoxin G_1 was only produced in CCRC 30231 and aflatoxin B_1 and G_1 appeared in CCRC30290. In addition, these four strains varied in relative aflatoxin quantity.

RFLP and sequence analysis of aflR-1/2 genes

To further investigate the variation in gene *aflR* and its homologs among members of *Aspergillus* Section *Flavi*, an approximate 1,352-bp PCR product was amplified from most (16/19) strains, being especially abundant in all *A. parasiticus* strains (7/7), by primer pair AflR-198/AflR-1549 (Table 1). However, twelve of these strains could not produce aflatoxin (Table 1). After *Nco*I digestion of PCR products, two PCR-RFLP patterns, i.e. *aflR-1* (fragment 1,352 bp) and *aflR-1/2* (fragments 1,352, 866, and 486 bp), appeared (Fig 1). Among all of the *Aspergillus* Section *Flavi* strains tested, only *A. parasiticus* CCRC 30160, *A. sojae* CCRC 30103 and CCRC 30227, *A. oryzae* CCRC 30120 and CCRC

30123 encoded the *aflR-1* gene; aflatoxigenic *A. parasiticus* CCRC 30117, CCRC 30150, CCRC 30164, CCRC 30172 and non-aflatoxigenic *A. parasiticus* CCRC 30228 contained *aflR-1* and a duplicated *aflR-2* gene; no strains contained the *aflR-2* gene alone.

Sequence alignment of the *aflR-1* sequence from aflatoxigenic, non-aflatoxigenic *A. flavus* strains and L32577 (*A. flavus*) showed 99.6 and 99.3% identity, respectively. Nucelotide sequences of *aflR-1 and aflR-2* were compared between strains and genes. High sequence homology for *aflR-1* was obtained between *A. flavus* with 99.2–99.6% identity and *A. parasiticus* with 98.6–99.7% identity. However, a much more diverse nucleotide sequence demonstrating 97-97.2% identity was found between *aflR-1* and *aflR-2*. Sequence variations were frequently presented in 11 positions for *aflR-1*, at nucleotides 259, 262, 264, 445,454,493, 866, 891, 1337, 1352 and 1452, and 5 positions for *aflR-2*, at nucleotides 449, 460, 526, 879, and 1337 (Table 3 and 4). Despite point mutations, the sequence similarity of the *aflR* gene from aflatoxigenic and non-aflatoxigenic strains showed that the *aflR-1* coding region has more difference than the *aflR-2* coding region. In addition, a TGCTCA insertion was located at nucleotides 533 and 534 for *A. sojae* CCRC 33103, but not in *A. flavus*, *A. parasiticus* or *A. oryzae*.

Differential gene expression in aflatoxin biosynthesis by RT-PCR analysis

Transcript levels of the housekeeping gene *β-tubulin* remained constant from day 1 to 4 in cultures grown in both YES and YEP. The production of aflatoxin was not associated with *aflR* expression in some *Aspergillus* strains (Table 1 and 5). Both *aflR1/2* genes were expressed in YES and YEP cultures. However, the latter medium did not support aflatoxin biosynthesis. The highest transcript levels of *aflR* were detected in YES cultures at day 4 and in YEP culture at day 2 (Table 5). Further analysis of gene expression involved in aflatoxin production in YES broth found that *nor-1*, *aflR*, *ver-1*, *avnA*, *omt-1*, *ord1*, *vbs* and *cypX* were expressed in five *A. parasiticus* aflatoxigenic strains, and *nor-1*, *aflR*, *ver-1*, *omt-1*, *ord1*, *vbs* and *cypX*, but not *avnA*, were expressed in two aflatoxigenic *A. flavus* strains (Table 6). For non-aflatoxigenic strains grown in YES broth, most of these genes, especially *ord1* and *vbs*, were not expressed. The present results may provide a better way to differentiate between aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi*, and to identify the key genes involved in the aflatoxin biosynthesis pathway.

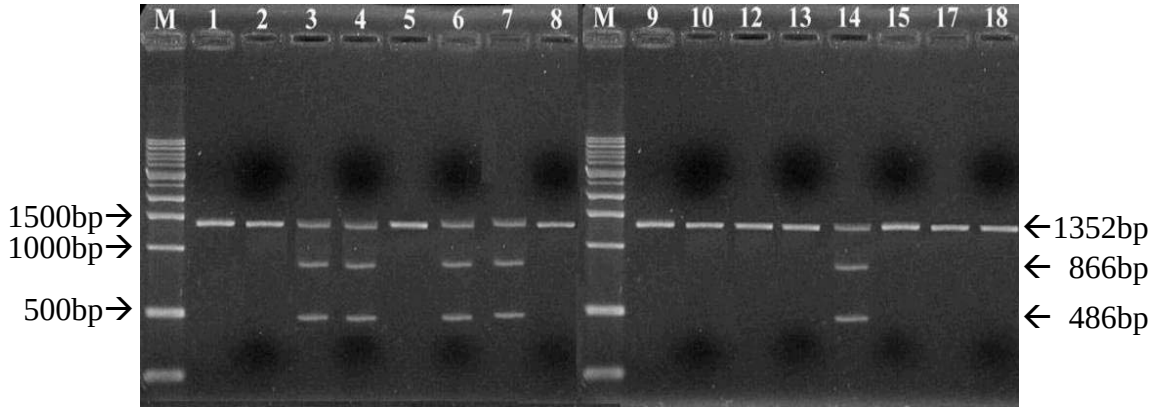

Fig. 1. Restriction fragment length polymorphism analysis of the *aflR* gene of the tested *Aspergillus* strains using *Nco*I restriction enzyme. Lane M, 0.2-14 kb Gen-KB DNA ladder (GeneMark, Taiwan); lane 1, *A. flavus* CCRC30231; lane 2, *A. flavus* CCRC30290; lane 3, *A. parasiticus* CCRC30117; lane 4, *A. parasiticus* CCRC30150; lane 5, *A. parasiticus* CCRC 30160; lane 6, *A. parasiticus* CCRC30164; lane 7, *A. parasiticus* CCRC 30172; lane 8, *A. flavus* CCRC30010; lane 9, *A. flavus* CCRC30119; lane 10, *A. flavus* CCRC30166; lane 12, *A. flavus* CCRC30300; lane 13, *A. sojae* CCRC30227; lane 14, *A. parasiticus* CCRC 30228; lane 15, *A. sojae* CCRC30103; lane 17, *A. oryzae* CCRC30120; lane 18, *A. oryzae* CCRC30123.

Table 3. Comparison of nucleotide sequences containing the coding region of *aflR-1*

Strains	259	262	445	454	1352
A. flavus CCRC 30231 (AF[a])	CA**A** (Gln) [c]	AC**T** (Thr)	CC**G** (Pro)	AG**A** (Arg)	**C**GA (Arg)
A. flavus CCRC 30290 (AF)	CA**A** (Gln)	AC**T** (Thr)	CC**G** (Pro)	AG**A** (Arg)	**C**GA (Arg)
A. flavus CCRC 30119	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. flavus CCRC 30166	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. flavus CCRC 30300	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. flavus CCRC 30010	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. parasiticus CCRC 30172 (AF)	CA**G** (Gln)	AC**C** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. parasiticus CCRC 30150 (AF)	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. parasiticus CCRC 30164 (AF)	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. parasiticus CCRC 30160 (AF)	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. parasiticus CCRC 30117 (AF)	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. parasiticus CCRC 30228	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. parasiticus (AF110766)	CA**G** (Gln)	AC**C** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**C**GA (Arg)
A. sojae CCRC 30227	CA**G** (Gln)	AC**T** (Thr)	CC**A** (Pro)	AG**T** (Ser)	**T**GA (stop)

[a]AF: aflatoxigenic

[b]DNA sequence comparison of *aflR-1* sequences among the tested strains of *Aspergillus* spp. and *A. parasiticus* (AF110766).

[c]Amino acids in parentheses represent the codon encoding products.

Table 4. Comparison of nucleotide sequences containing the coding region of *aflR-2*

Strains	449	460	526	879	1337
A. parasiticus CCRC 30172 (AF[a])	**G**AG (Gln)[c]	CC**T** (Pro)	CA**G** (Gln)	C**G**G (Arg)	**C**TG (Leu)
A. parasiticus CCRC 30150 (AF)	**G**AG (Gln)	CC**T** (Pro)	CA**G** (Gln)	C**T**G (Leu)	**C**TG (Leu)
A. parasiticus CCRC 30164 (AF)	**A**AG (Lys)	CC**C** (Pro)	CA**G** (Gln)	C**T**G (Leu)	**C**TG (Leu)
A. parasiticus CCRC 30160 (AF)	**G**AG (Gln)	CC**T** (Pro)	CA**G** (Gln)	C**T**G (Leu)	**C**TG (Leu)
A. parasiticus CCRC 30117 (AF)	**G**AG (Gln)	CC**T** (Pro)	CA**A** (Gln)	C**T**G (Leu)	**T**TG (Leu)
A. parasiticus CCRC 30228	**G**AG (Gln)	CC**T** (Pro)	CA**G** (Gln)	C**T**G (Leu)	**C**TG (Leu)
A. parasiticus (AF110766)	**G**AG (Gln)	CC**T** (Pro)	CA**G** (Gln)	C**T**G (Leu)	**C**TG (Leu)
A. sojae CCRC 30227	**G**AG (Gln)	CC**T** (Pro)	CA**G** (Gln)	C**T**G (Leu)	**C**TG (Leu)

[a]AF: aflatoxigenic

[b]DNA sequence comparison of *aflR-2* sequences among the tested strains of *Aspergillus* spp. and *A. parasiticus* (AF110766).

[c]Amino acids in parentheses represent the codon encoding products.

Discussion

Clustering genes and the pathway-specific regulatory gene *aflR* for aflatoxin biosynthesis in *A. parasiticus* and *A. flavus* have previously been determined (Chang et al., 1995a; Trail et al., 1995; Yu et al., 1995; Ehrlich et al., 1999). However, not all *A. parasiticus* and *A. flavus* strains produced aflatoxins (Table 1). Significantly, 3 out of 12 non-aflatoxigenic strains lost the *aflR* gene and no such deletion was observed in aflatoxigenic strains. Therefore, this *aflR* deletion could be a differential characteristic of non-aflatoxigenic strains (Chen et al., 2002). Previously, *Hinc*II and *Pvu*II-digested PCR-RFLP patterns of the *aflR* gene could be used to distinguish *A. flavus* and *A. parasiticus* (Somashekar et al., 2004). However, the presence and expression of *aflR-1* was not associated with the production of aflatoxin in *A. oryzae*, *A. sojae*, *A. flavus* as well as *A. parasiticu* (Table 1, Chang et al., 1995a; Wei, 2003). Such non-aflatoxigenic phenotypes might be due to a regulatory malfunction, possibly at the level of the positive regulator *aflR* (Watson et al., 1999) or mutations in the upstream elements of *aflR* (Chang et al., 1999).

Recently, Chang and Yu (2002) proposed that duplication of the aflatoxin cluster is not a prevalent event. The copy of duplicated *aflR* is predicted to encode a protein that is defective in its nuclear localization domain (Cary et al., 2002). However, target fragments of *aflR-1/2* were observed in 5 out of 7 tested strains of *A. parasiticus* (Table 1, Fig 1). Apparently, this was more prevalent for *aflR-1/2* in *A. parasiticus*, but not in strains of *A. flavus*, *A. sojae* and *A. oryzae.* These results suggest that duplication of aflatoxin clustering is a recent event. It is believed that *A. flavus, A. oryzae, A. parasiticus* and *A. sojae* represent morphological and physiological variants of a single species. A duplicated *aflR-2* gene cannot be used as a marker to differentiate aflatoxin and non-aflatoxin producers and strains among *A. flavus*, *A. oryzae*, *A. sojae* and *A. parasiticus*. Similar to the result of Ehrlich et al. (2003) for *A. flavus* L, *A. flavus* SB, *A. flavus* SBG, *A. bombycis*, *A. nomius* and *A. pseudotamarii* isolates, highly sequence identity (more than 98.7%) of *aflR-1* was observed among aflatoxigenic and non-aflatoxigenic *A. flavus* and *A. parasiticus* strains. The difference (97.1-97.2% sequence identity) between *aflR-1* and *aflR-2* suggests that *alfR-2* has evolved since duplication and become functionless due to amino acid changes (Table 1, Cary et al., 2002). However a relationship between *aflR-1/2* composition and the relative amount of aflatoxin production in aflatoxigenic *Aspergillus* strains was not found (Table 1).

Table 5 - Expression of *aflR1/2* genes in *Aspergillus* strains grown in YES and YEP broth

Day	YES culture												YEP culture											
	Aflatoxigenic strains							Nonaflatoxigenic strains					Aflatoxigenic strains							Nonaflatoxigenic strains				
	A[a]	B	C	D	E	F	G	H	I	J	K	L	A	B	C	D	E	F	G	H	I	J	K	L
aflR1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	3[b]	3	3	3	3	3	3	1	3	3	3	3	3	3	3	3	5	4	4	1	1	4	5	4
2	4	4	3	1	1	1	1	4	4	1	4	4	3	3	3	2	4	4	5	3	4	1	5	5
3	4	3	4	4	4	3	3	3	3	4	1	3	3	4	4	0	5	4	3	4	4	1	4	4
4	6	6	5	6	3	4	3	5	5	5	1	5	5	4	3	3	4	3	3	3	3	4	0	3
aflR2	−	−	+	+	−	+	+	−	−	−	+	−	−	−	+	+	−	+	+	−	−	−	+	−
1	NA	NA	0	0	NA	0	0	NA	NA	NA	0	NA	NA	NA	1	1	NA	2	1	NA	NA	NA	2	NA
2	NA	NA	2	1	NA	1	1	NA	NA	NA	0	NA	NA	NA	1	1	NA	2	3	NA	NA	NA	1	NA
3	NA	NA	2	1	NA	2	2	NA	NA	NA	0	NA	NA	NA	0	0	NA	0	1	NA	NA	NA	2	NA
4	NA	NA	0	2	NA	2	2	NA	NA	NA	0	NA	NA	NA	2	2	NA	0	1	NA	NA	NA	0	NA

[a]A: *A. flavus* CCRC 30231, B: *A. flavus* CCRC 30290, C: *A. parasiticus* CCRC 30117, D: *A. parasiticus* CCRC 30150, E: *A. parasiticus* CCRC 30160, F: *A. parasiticus* CCRC 30164, G: *A. parasiticus* CCRC 30172, H: *A. flavus* CCRC 30010, I: *A. flavus* CCRC30119, J: *A. flavus* CCRC 30166, K: *A. parasiticus* CCRC 30228, L: *A. sojae* CCRC 30227

[b]0, 1, 2, 3, 4, 5 and 6 showed a relative amount of 0%, 1-5%, 6-10%, 11-25%, 26-50%, 51-75% and 76-100% of transcripts, respectively. NA: not available; +: gene expression; −: no gene expression

Table 6. Expression of aflatoxin biosynthetic genes in the tested *Aspergillus parasiticus* and *A. flavus* strains were detected by RT-PCR in YES broth cultures 24 hr after incubation at 28°C

Strain	Gene expression								
	nor-1	*aflR*	*ver-1*	*avnA*	*omt-1*	*ord1*	*vbs*	*cypX*	*β-tublin*
A. flavus CCRC 30231 (AF[a])	+	+	+	−	+	+	+	+	+
A. flavus CCRC 30290 (AF)	+	+	+	−	+	+	+	+	+
A. flavus CCRC 30010	+	−	−	−	−	−	−	−	+
A. flavus CCRC 30119	+	+	−	−	−	−	−	−	+
A. flavus CCRC 30166	+	+	−	−	−	−	−	−	+
A. parasiticus CCRC 30117 (AF)	+	+	+	+	+	+	+	+	+
A. parasiticus CCRC 30150 (AF)	+	+	+	+	+	+	+	+	+
A. parasiticus CCRC 30160 (AF)	+	+	+	+	+	+	+	+	+
A. parasiticus CCRC 30164 (AF)	+	+	+	+	+	+	+	+	+
A. parasiticus CCRC 30172 (AF)	+	+	+	+	+	+	+	+	+
A. parasiticus CCRC 30228	+	+	+	+	+	−	−	+	+
A. parasiticus CCRC 30227	+	+	−	−	−	−	−	−	+

[a]AF: aflatoxigenic; ＋: gene expression; －: no gene expression

The *aflR* gene has been reported to be useful for distinguishing species of the *Aspergillus* Section *Flavi* (Chang, et al., 1995a). However, sequence comparison further assists the differentiation of these species. Earlier, Wei (2003) indicated that T-G-A-A-X-C and C-C-C-C-C-T at position 90, 89, 72, 61, 43 and 102 could not differentiate all the examined strains in the *Aspergillus* Section *Flavi*. Sequence analysis of *aflR-1* showed that mutations at position 259, 445 and 454 from A-G-A to G-A-T were associated with the change from aflatoxigenic to non-aflatoxigenic in *A. flavus* strains (Table 3). In *A. parasiticus*, no clear association of mutations with the change from aflatoxigenic to non-aflatoxigenic strains was observed. In *A. sojae* CCRC 33103, the TGCTCA insertion in the *aflR-1* gene resulted in an increase in the HAHA motif that was not found in *A. flavus*, *A. parasiticus* and *A. oryzae* and a pretermination stop codon (**T**GA) in *A. sojae aflR-1* at nucleotide 1352 replaced the original arginine codon (**C**GA) found at the same position in *A. parasiticus aflR-1* (Table 3). Such a pretermination defect formed a truncated protein without the last 62 amino acids (Watson et al., 1999). This defect is apparently associated with non-aflatoxigenicity of *A. sojae* (Matsushima et al., 2001; Takahashi et al., 2002; Chang, 2004).

AflR, coding for a protein shown to be involved in the transcriptional activation of most of the structural genes in the *Aspergillus parasiticus* aflatoxin gene cluster, is also a part of the same cluster (Cary et al., 2000). The reverse transcription PCR technique was employed as a tool to investigate gene regulation at the level of transcription (Sweeney et al., 2000). RFLP analysis showed that both genes *aflR*1/2 were transcribed at different levels depending on the culture conditions, with higher and lower expression observed for *aflR2* in YES and YEP, respectively (Table 5), confirming the study of Cary et al. (2002). Differences were also observed in gene expression. For example, *vbs* and *ord1* were transcribed in *A. parasiticus* aflatoxigenic isolates when grown in YES broth, but not YEP broth. *Omt-1* could be transcribed in *A. flavus* aflatoxigenic isolates grown in YES broth, but not YEP broth. The production of AF in aflatoxigenic isolates did not correlate with transcripts of *aflR1/2*, *nor-1*, *avnA*, *cypX* and *ver-1* genes (Table 6). Constitutive expression of *aflR* could lead to synthesis of pathway proteins, which did not relate to AF accumulation. Therefore, differences in gene regulation and

duplication require further characterization in order to assess the benefits for aflatoxigenic *Aspergillus* species, such as *A. parasiticus*.

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