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黃麴毒素調控蛋白質 AFLR 的表達、純化及羧基端功能之探討

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I.中文摘要

來自於*Aspergillus parasiticus*的*aflR* 基因與黃麴毒素的生化合成有關。為了探討aflR基因在不同種之間的存在及表達與否，４株非產毒性的*A. flavus*、5株產毒性的*A. flavus*、8株*A. oryzae*及４株*A. sojae* 選作為本文之材料。綜合南方點墨法及PCR反應的結果，*aflR*基因可以在以下菌株的染色體DNA中測得：75%的非產毒性*A. flavus*，100% 的產毒性*A. flavus*，75%的*A. oryzae*和50%的*A. sojae*。在誘發黃麴毒素生長的環境下，綜合反轉錄PCR(RT-PCR)及RNase protection assay(RPA)的結果，*aflR*的mRNA只在３株產毒性的*A. flavus*及１株*A. oryzae*的RNA樣品中被偵測到，而所有的非產毒性*A. flavus*及*A. sojae*則呈現陰性反應。所有的非產毒性*A. flavus*、*A. oryzae*及*A. sojae*在誘發黃麴毒素生長的培養環境下，皆沒有產生可偵測到之黃麴毒素濃度。

關鍵字： *aflR*基因，基因表達，黃麴毒素，*Aspergillus*種

II. Abstract

The *aflR* gene from *Aspergillus parasiticus* is involved in the regulation of aflatoxin biosynthesis. To investigate the presence and expression of *aflR* gene in species belonging to *Aspergillus* Section Flavi, twenty-four strains were examined, including three *A. parasiticus* strains serving as positive and negative controls, four nonaflatoxigenic *A. flavus*, five aflatoxigenic *A. flavus*, eight *A. oryzae* strains and four *A. sojae* strains. In combination of the results from Southern-hybridization analysis and polymerase chain reaction (PCR), the *aflR*-homolog gene was detected in the genomic DNAs obtained from 75% of non-alfatoxigenic *A. flavus*, 100% of aflatoxigenic *A. flavus*, 75% of *A. oryzae* and 50% of *A. sojae* strains. Growing under aflatoxin-inducing conditions, the *aflR* transcript was only found in the RNA preparations from three of the five aflatoxigenic *A. flavus* and one of the eight *A. oryzae* strains with the application of reverse transcriptase(RT)-PCR and RNase protection assay (RPA); no signal was detected in samples prepared from nonaflatoxigenic *A. flavus* and *A. sojae*. None of the nonaflatoxigenic *A. flavus*, *A. oryzae* and *A. sojae* isolates produced detectable levels of AFB_1. Our results also suggest that the hybridization techniques, Southern analyses and RPA, are more sensitive and reliable than PCR for studying the existence of *aflR* homolog and transcript in various Aspergillus isolates.

Key words: *aflR* gene, gene expression, aflatoxin, *Aspergillus* species

INTRODUCTION

Aflatoxins are a series of toxic and carcinogenic metabolites produced by *Aspergillus flavus* and *A. parasiticus*, which contaminate many agricultural commodies such as corn, peanut, tree nuts and cotton seeds (CAST, 1989). Aflatoxin B_1 (AFB_1) is known to be one of the most potent naturally occurring carcinogens (Chu, 1991). Because the presence of aflatoxins in food and feed is a potential hazard to human and animal health, extensive research has been conducted to study the mechanisms of gene expression in these aflatoxigenic fungi and to further elucidate the aflatoxin biosynthetic pathway.

More than 20 genes are involved in aflatoxin biosynthesis and some of them have been identified and cloned (Bennett et al., 1994; Matsushima et al., 2001a); they include a regulatory gene locus *aflR* from *A. flavus* and *A. parasiticus* (Chang et al., 1993; 1995). The *aflR* gene encodes a putative protein, AFLR, with a molecular weight of 47 kDa (Liu et al., 1997). A putative DNA-binding domain of the GAL4-type binuclear zinc finger motif is identified within the AFLR (Woloshuk et al., 1994). Previous studies indicate that AFLR is a transcriptional activator of expression of several aflatoxin biosynthetic structural genes (Chang et al., 1995; Ehrlich et al., 1999; Payne et al. 1993). Disruption of the *aflR* gene in aflatoxigenic *A. parasiticus* strains results in an inability to produce aflatoxin, strongly suggesting that this gene is essential for aflatoxin biosynthesis (Cary et al., 2000)

Aspergillus parasiticus, *A. flavus*, *A. oryzae*, and *A. sojae* are species belonging to *Aspergillus* Section *Flavi*. Although a high degree of DNA homology is found among these four species, *A. oryzae* and *A. sojae* do not produce aflatoxin and are used for the production of various industrial enzymes and fermented foods in eastern Asia (Kusumoto et al., 2000). Therefore, it is important to know whether *aflR* gene and its transcript are present in the non-aflatoxigenic *Aspergillus* species or not. Homologs of *aflR* gene were found in certain *A. oryzae* and *A. sojae* (Kusumoto et al., 1998; Watson et al., 1999). In contrast, Southern analysis showed that genomic DNA from *A. sojae*, but not from *A. oryzae*, hybridized with *aflR* probe (Klich et al., 1995). Directed deletions of aflatoxin biosynthesis gene cluster in *A. oryzae* was also reported (Kusumoto et al., 2000). In view of *aflR* gene expression, Klich et al. (1997) demonstrated that *aflR* gene transcript was detected in some *A. sojae* isolates. The *aflR* mRNA and protein were also found in certain *A.oryzae* and *A. sojae* (Liu et al., 1998). Nevertheless, several studies showed that there was no detectable transcripts of *aflR* in examined *A. oryzae* or *A. sojae* strains (Kusumoto et al., 1998; Matsushima et al., 2001b)

Since the detection methods and examined strains are not the same in each studying group, the information about whether the presence and expression of *aflR* gene in various *Aspergillus* species is controversial. Besides, the factors contributing to the differential ability in aflatoxin production between aflatoxigenic and non-aflatoxigenic *A. flavus* strains are not clear. In the present study, we applied two methods, PCR and Southern analysis, to identify the presence of *aflR* homolog in certain *A. flavus*, *A. oryzae* and *A. sojae* strains commercially available in Taiwan. We also assessed the presence of *aflR* transcript in these species with reverse transcription (RT)-PCR and RNase protection assay (RPA). The results regarding the aflatoxigenic potential of examined strains in an inducing synthetic medium will provide useful information for further basic research and industrial application.

MATERIALS AND METHODS

Fungal strains. *Aspergillus parasiticus* SU-1 and AFR14 were kindly provided by Dr. Jeffrey Cary at Southern Regional Research Center, U. S. Department of Agriculture. SU-1 is a typical aflatoxin producer and AFR 14 is an *aflR* knockout mutant (Cary et al., 2000). All other strains, including one *A. parasiticus* strain, nine *A. flavus* strains, eight *A. oryzae* strains and four *A. sojae* strains, were purchased from Culture Collection and Research Center at Taiwan. All strains were maintained on potato dextrose agar at 4^{o}C for production of conidia.

Culture medium and condition

Peptone-miniral salts (PMS) medium served as the non-aflatoxin-supporting medium. Glucose-mineral salts (GMS) medium (peptone was replaced by glucose in PMS medium) was used as the aflatoxin-supporting medium (Abdollahi and Buchanan, 1981). Frozen spores (kept at –20℃ in 15% glycerol for up to 1 month) were inoculated into PMS medium and grown at –29℃ for 60 h on a rotatory shaker (150rpm).The resulting mycelia were filtered through two layer of cheesecloth and washed with sterile 0.85% NaCl . The wet weight of the mycelia was determined after they were squeezed and blotted with paper towels. Around 1 g of mycelia was subjected to genomic DNA extraction as described later. A portion of the mycelia (0.8 to 1g) was transferred to a flask containing 100ml of fresh GMS medium and cultured on a shaker (150rpm) at 29℃ for another 24 h. Mycelia were collected by filtration through cheesecloth and then immediately frozen in liquid nitrogen for RNA isolation. Culture filtrates were saved for determining the levels of AFB_1.

Aflatoxin analysis

The concentrations of AFB_1 in filtrates were determined with a direct competitive enzyme-linked immunosorbent assay (ELISA) as described by Chu et al. (1987)

Isolation of genomic DNA and Southern analysis

Genomic DNA was isolated from cells with the DNeasy plant kit (Qiagen) according to the manufacture's protocol. DNA (10 g) was digested with EcoRI and electrophoresed on a 1% agarose gel in a Tris-acetate-EDTA electrophoresis buffer. The digested DNA was transferred to a nylon membrane (Schleicher & Schuell) and fixed with UV cross-linking. The 0.5-kb *aflR* probe derived from a 1.9-kb BamHI fragment of genomic DNA was generated by EcoRI digestion (Chang et al., 1993) and then labeled with [α-^{32}P]dATP with a nick translation system (Invitrogen Inc. Carlsbad, CA). Prehybridization and hybridization of blots were carried out by standard protocols (Sambrook et al., 1989). Blots were hybridized with label probes at 56^{o}C overnight and then washed twice in 2 x SSC-0.1% SDS for 10 min and once in 0.1%SSC-0.4%SDS for 10 min at 60^{o}C. Detection of image was with X-ray film (Kodak BioMax MS) or with a Fuji BAS-1500 phosphoimager.

Isolation of RNA and reverse transcription

Total cellular RNA was isolated from collected fungal mycelia with TRI reagent as described by the manufacturer (Molecular research, Cincinnati, OH). The purified RNAs were suspended in formamide and store at –20^{o}C for the following RNase protection assay or for reverse transcription.

Reverse transcription of *aflR* mRNA into first strand cDNA was conducted with superscript^{TM} preamplification system (Life Technologies). Briefly, RNA (5 μg) was reversely transcribed at 42 ^{o} C for 1 h in a 19 μl total volume that included 0.5 μg of Oligo(dT)$_{12\text{-}18}$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mM each dNTP and 200 U of SuperScript II reverse transcriptase. Finally, 1 μl of RNase H (2 U/μl) was added to the reaction and incubated at 70^{o}C for 10 min. Negative controls including all the above components except the reverse transcriptase were run in parallel. The product of each reaction was stored at –20^{o}C until used for the PCR reaction.

Polymerase chain reaction (PCR).

PCR for amplification of *aflR* cDNA and genomic DNA were conducted with primers (5'-TACATGGTCTCCAAGCGGATG-3' and 5'-GATGACCATATCAGTCGTCAT-3') , *AmpliTaq* DNA polymerase and a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk,

Conn.). PCR mixtures were heated at 94°C for 3 min and then subjected to 34 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min. A final 7-min extension step at 72°C was also included. A 10-μl portion of each PCR product was electrophoresed on 1.2% agarose in 1 x Tris-acetate-EDTA buffer and then visualized after the gels were stained with ethidium bromide (0.5 μg/ml).

RNase protection assay (RPA)

The RPA was performed as described by Liu and Chu (1998) with a slight modification. For the generation of an *aflR*-specific cRNA probe, the plasmid pGEM-7Zf(+) harboring *aflR* cDNA from *A. flavus* was linearized with BamHI and transcribed in vitro by use of T7 RNA polymerase with [α-^{32}P]rCTP. The riboprobe was used to protect a 189-nucleotide fragment corresponding to the zinc finger motif in the putative AFLR protein.

Total RNA samples were hybridized with 5×10^5 cpm of ^{32}P-labeled *aflR* cRNA probes in a solution containing 80% formamide, 100 mM Tris-HCl (pH 7.4), 2.5 N NaCl, and 10 mM EDTA at 47°C for 16 to 18 h. Each hybridized sample was digested with RNase A at 37°C for 60 min, treated with proteinase K at 37°C for 20 min, extracted with phenol-chloroform, and precipitated with isopropanol at −20°C. After centrifugation, the pellets were resuspended in sequencing gel loading buffer. Protected fragments were resolved on a 6% polyacrylamide-8 M urea denaturing gel. The image of the gel was detected with a Fuji BAS-1500 phosphoimager.

RESULTS

The presence of *aflR*-homolog genes in the *Aspergillus* isolates

In the present study, two techniques, Southern hybridization and PCR, were performed to explore the presence of *aflR*-homolog genes in the genomes of *Aspergillus* Section Flavi isolates. Genomic DNAs were prepared from the fungal species listed in Table 1 and 2. Results from the Southern analysis of *A. parasiticus* and *A. flavus* strains with the *aflR* probe are shown in Fig 1 A. The EcoRI-digested genomic DNAs from *A. parasiticus* SU1 and CCRC 30169 showed an *aflR*-specific hybridization whereas *aflR disruptant* AFR14 did not. On the other hand, according to the information obtained from CCRC in Taiwan, four of the nine *A. flavus* strains (CCRC30107, CCRC30010, CCRC30203 and CCRC30014) are considered to be non-aflatoxigenic and the other five (CCRC30115, CCRC30112, CCRC30173, CCRC30231 and CCRC30212) are aflatoxigenic under aflatoxin-producing conditions. However, *aflR* genes were detected in all of the examined *A. flavus* isolates except *A. flavus* CCRC30203. Although *A. oryzae* and *A. sojae* are considered to be non-aflatoxigenic and widely used in food industry, the *aflR* probes hybridized, at least to some extent, to the six strains of eight examined *A. oryzae* and two strains of the four examined *A. sojae* (Table 2).

When genomic DNA from each strain was used as template in PCR amplification, certain aflatoxigenic and non-aflatoxigenic aspergilli gave a PCR product of about 650 bp, whose size was identical to that predicted from the nucleotide sequence of *aflR* (651 bp). Sequence analysis of the 650-bp product identified this fragment corresponding to positions172 to 822 in the *A. parasiticus aflR* nucleotide sequence (Chang et al., 1995). The *aflR* disruptant AFR14 showed no PCR product as predicted, but the other two aflatoxin-producing *A. parasiticus* (SU1 and CCRC30169) did. One of the four nonaflatoxigenic *A. flavus* and four of the five aflatoxigenic *A. flavus* generated 650-bp

PCR products (Fig. 1B). Besides, 37.5 % of *A. oryzae* and 75 % of *A. sojae* strains failed to generate any detectable signal in PCR amplification (Table 2).

The presence of *aflR* transcript in the *Aspergillus* isolates

To study the expression of *aflR* gene in various strains, two methods, RT-PCR and RPA, were adopted for detecting the existence of *aflR* transcripts in fungal mycelia. RT-PCR method has been used in examining the *aflR* mRNA in certain *A. oryzae* isolates (Kusumoto et al., 1998); the transcripts of *aflR*-homolog genes in the test strains were examined by PCR coupled with reverse transcription, using primers specific to the *aflR* coding region. However, various DNA sequences of *aflR*-homolog genes in different *Aspergillus* species and the specificity of primers may lead to a false negative result in RT-PCR. In addition, since the putative AFLR is supposed to be a protein for regulating the formation of secondary metabolites, *aflR* transcripts are presence in trace amounts in fungal cells and difficult to be detected in either RT-PCR or Northern hybridization. Solution hybridization-RPA was considered to have a detection sensitivity five to ten times higher than that of Northern hybridization. Therefore, in the current study RPA with riboprobes complementary to the zinc finger motif and downstream region of *aflR* gene was used to detect low levels of *aflR* mRNA in various *Aspergillus* strains.

Examined *Aspergillus* strains were first grown in PMS medium for 60 h and then distributed to GMS, an aflatoxin-supporting medium, for another 24 hr. Total RNA samples were then prepared from the fungal mycelia for RT-PCR or RPA. Under aflatoxin-producing condition, only the RNA preparations obtained from *A. parasiticus* SU-1 and CCRC30169, *A. flavus* CCRC30231 and *A. oryzae* CCRC30195 gave a single product of approximately 650 bp after reverse transcription and PCR, while no other RNA samples (*A. parasiticus* AFR14, the other eight strains of *A. flavus*, and five strains of *A. oryzae*) could give any signal in RT-PCR (Fig 2 A, Table 1 and Table 2).

To further confirm the presence of *aflR* transcript, RNA preparations from four *Aspergillus* species were subjected to RPA. The signals of *aflR* mRNA were found in *A. parasiticus* SU-1 and CCRC30169, in *A. flavus* CCRC30112, CCRC30173 and CCRC30231, and also in *A. oryzae* CCRC30195 (Fig 2B, Table 1 and Table 2). No *aflR* transcript was seen in any of the *A. sojae* strains examined (Table 2). The bottom panel of Fig. 2B shows the AFB_1 concentrations in culture filtrate obtained from *A. parasiticus* and *A. flavus* isolates. AFB_1 concentrations higher than 5.8 μg/ ml were detected in *A. parasiticus* SU-1, CCRC30169, and in *A. flavus* CCRC30231 as well. Low levels of AFB_1 were also found in *A. flavus* CCRC30112 and CCRC 30212. All *A. oryzae* and *A. sojae* strains produced no AFB_1 in their culture filtrates.

DISCUSSION

Comparing the results from Southern hybridization with those of PCR, we found that there is a discrepancy in certain isolates, including *A. flavus* CCRC30014, *A. flavus* CCRC30212, *A. oryzae* CCRC30229, CCRC30103, CCRC30174, and *A. sojae* CCRC30103. Genomic DNAs from these strains hybridized with *aflR* probe in Southern analysis, but gave no amplified products in PCR. One of the reasons why no PCR product was generated is that the level of similarity between the primers and the target DNA probably was too low to allow annealing under the PCR amplification conditions used. Another possible explanation is that there may have been mismatches at the 3'end between

the primers and the target DNA, which resulted in less efficient annealing and extension. Due to the sequence variability in *aflR* homologs, Chang et al. (1995) also demonstrated that generation of PCR products from *A. oryzae* was dependent on strain type and the primer pairs used. Therefore, in comparison with PCR amplification, Southern hybridization should be a more efficient and reliable method to detect the existence of *aflR*-homolog gene in various aspergilli.

In view of the methods for studying the presence of *aflR* gene transcripts, solution-hybridization RPA was more sensitive in detecting low levels of *aflR* transcripts than RT-PCR. RNA preparations from three aflatoxigenic *A. flavus* (CCRC30112, CCRC30173 and CCRC30231) showed positive results in RPA using *aflR* riboprobe, but only one of them (CCRC30231) gave a 650-bp PCR product in RT-PCR. One limitation for applying RT-PCR in various species is the similarity between the primers and the reversely-transcribed cDNA. Like *A. parasiticus* SU-1 and CCRC30169, *A. flavus* CCRC30231 produced high amounts of AFB_1 (10 μg/ml) in our aflatoxin-inducing system, so it is highly possible that the level of *aflR* transcripts was abundant enough to give a strong signal in RT-PCR.

In combination of results from Southern analyses with RPA data, we found that 75% of examined non-aflatoxigenic *A. flavus*, 75% of *A. oryzae* and 50 % of *A. sojae* isolates contained the *aflR*-homolog gene, while all of them, except *A. oryzae* CCRC 30195, did not have the *aflR* gene transcribed and produce no detectable levels of AFB_1 even growing in aflatoxin-supporting medium. It is known that loss of *aflR* transcription causes the loss of expression of aflatoxin biosynthesis- related genes, leading to lack of aflatoxin production (Chang et al., 1993, 1995; Payne et al. 1993). However, the reason for the loss of *aflR* expression in non-aflatoxigenic strains remains unknown. As the deduced AFLR protein sequences from *A. parasiticus*, *A. flavus*, *A. oryzae* and *A. sojae* are almost the same (Watson et al., 1999), it is suggested that there is a serious defect in signal transduction for activation of *aflR* gene in nonaflatoxigenic species. Cloning and sequencing the upstream promoter regions of these four *Aspergillus* species may provide a clue about how *aflR* gene expression is regulated.

Aspergillus oryzae CCRC30195 is the only nonaflatoxigenic strain showing the presence of *aflR* mRNA in the current study. Klich et al. (1997) have reported that transcripts of *aflR* occurred in two strains of *A. sojae* although no other aflatoxin-related genes were expressed. Furthermore, Liu et al. (1998) also demonstrate that the presence of *aflR* mRNA in certain fungal isolates is not directly correlated with aflatoxin formation, although the role of *aflR* transcript in the non-aflatoxigenic aspergilli is not clear. In contrast, several evidence show that the transcript of *aflR* homolog was not found in selected *A. oryzae* and *A. sojae* strains (Kusumoto et al., 1998; Matsushima et al., 2001b). This difference may merely reflect the use of different strains in each study. On the other hand, the *aflR* homolog in certain *A. oryzae* strains was proved to have an amber mutation at agrinine 383, it would give rise to a carboxyl terminal-truncated protein upon translation (Watson et al., 1999). The carboxyl- terminal region of AFLR protein is thought to be critical for transcriptional activation of aflatoxin-related genes (Chang et al., 1999; Matsushima et al., 2001a). The RT-PCR method established herein just amplified the cDNA region corresponding to the amino-terminal of AFLR protein, and the riboprobe used in RPA hybridized to and protected any *aflR* transcript comprising a fragment (189 nucleotides) corresponding to the zinc finger motif of AFLR. Since our experimental

systems cannot tell whether the *aflR* transcript detected in *A. oryzae* CCRC30195 is in its full length or not, there is a possibility that truncated AFLR protein in *A. flavus* CCRC30195 leads to the lack of aflatoxin biosynthesis in this strain.

Production of AFB_1 was, however, confined to the *A. parasiticus* and aflatoxigenic *A. flavus* under the culture conditions used, suggesting the safety of applying *A. oryzae* and *A. sojae* in dustrial food fermentation. In addition, only one (CCRC30231) of the five reported aflatoxigenic *A. flavus* isolates produced a significant amount of AFB_1 in our culture conditions. As aflatoxin production and induction of *aflR* gene expression in fungal cultures are regulated by environmental and nutritional factors, such as temperature, oxygen availability, and carbon source (Dutton, 1988; Liu et al., 1998), certain aflatoxigenic strains may not generate AFB_1 until a favorable condition has met.

CONCLUSION

In conclusion, we have showed that the hybridization techniques, Southern analysis and RPA, are more sensitive and reliable than PCR to investigate the presence and expression of *aflR* homolog in various aspergilli. Our data also indicate that more than half of examined isolates have the *aflR*-homolog gene in their genome, but only few of them showed detectable *aflR* transcripts under aflatoxin-supporting conditions. The absence of transcription of *aflR* homolog implies that there are defects in signal transduction for *aflR* activation in these nonaflatoxigenic species. Because a nonfunctional *aflR* homolog may guarantee the safety of aspergilli in terms of their industrial use, further efforts will be made to elucidate the mechanism behind the inability of *aflR* transcription.

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Legends for figures

Fig. 1. The presence of *aflR* gene in *A. parasiticus*, non-aflatoxic *A. flavus* and aflatoxic *A. flavus.* (A) Southern blot analyses of EcoRI-cut genomic DNAs from three strains of *A. parsiticus* and nine strains of *A. flavus*. According to the catalog published by CCRC in Taiwan, four of the *A. flavus* strains are considered to be non-aflatoxigenic; the other five strains are aflatoxigenic. The arrow indicates the hybridized *aflR* fragment. (B) The fungal DNAs of various *Aspergillus* strains were used as templates for PCR with the primers as described in the Material and Methods. The PCR products with the size of 650 bps were electrophoresed in agarose gel and photographed. The strain examined in each lane is as follows. Lane 1, *A. parasiticus* SU1; Lane 2, *A. parasiticus* AFR14; Lane 3, *A. parasiticus* CCRC30169; Lane 4, *A. flavus* CCRC30107; Lane 5, *A. flavus* CCRC30010; Lane 6, *A.flavus* CCRC30203; Lane 7, *A. flavus* CCRC30014; Lane 8, *A. flavus* CCRC 30115; Lane 9, *A. flavus* CCRC30112; Lane 10, *A. flavus* CCRC 30173; Lane 11, *A. flavus* CCRC30231; Lane 12, *A. flavus* CCRC30212.

Fig 2. Detection of *aflR* transcripts in *A. parasiticus* and *A. flavus.* (A) Three *A. parsiticus* and nine *A. flavus* strains were first grown in PMS medium for 60 h and then distributed to GMS medium for another 24 hr. The RNA was prepared from the fungal mycelia, treated with reverse transcriptase, and then used as template for PCR with primers specific for *aflR* gene. The PCR products were electrophoresed in agarose gel and photographed. (B) RNA samples at 25 μg were subjected to RPA with ^{32}P-labeled *aflR* riboprobe. Protected fragments were resolved on a 6% polyacrylamide-8M urea denaturing gels. Images of the gel recorded with a phosphoimager are shown in the top panel. The arrow indicates the protected *aflR* signal with a size of 189 nucleotides. The accumulation of AFB_1 in culture filtrate is shown in the bottom panel. The strain examined in each lane is as follows. Lane 1, *A. parasiticus* SU1; Lane 2, *A. parasiticus* AFR14; Lane 3, *A. parasiticus* CCRC30169; Lane 4, *A. flavus* CCRC30107; Lane 5, *A. flavus* CCRC30010; Lane 6, *A.flavus* CCRC30203; Lane 7, *A. flavus* CCRC30014; Lane 8, *A. flavus* CCRC 30115; Lane 9, *A. flavus* CCRC30112; Lane 10, *A. flavus* CCRC 30173; Lane 11, *A. flavus* CCRC30231; Lane 12, *A. flavus* CCRC30212.

Figure 1.

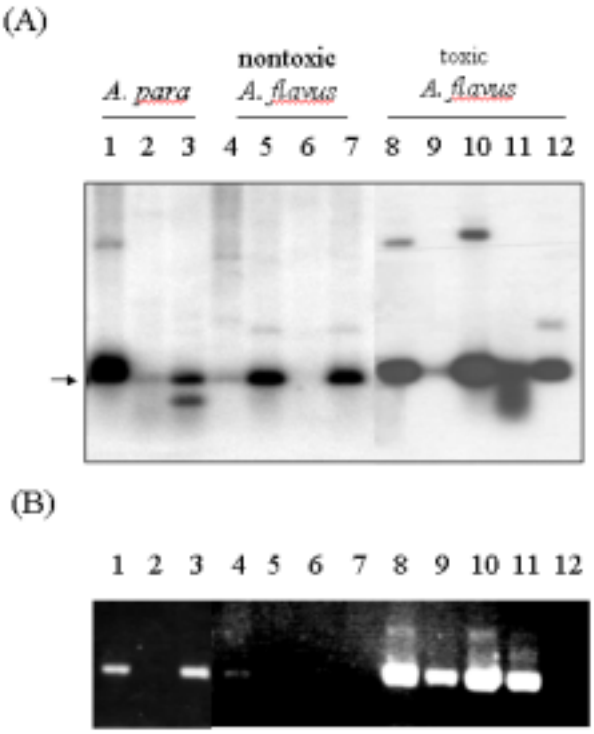

Figure 2.