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Genetic Variability of Non-aflatoxigenic *Aspergillus flavus* Isolates by using Aflatoxin Biosynthesis Genes

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The four aflatoxin (AF) biosynthetic pathway genes included regulatory gene *aflR* and the structural genes *aflD*, *aflM*, *aflP* were evaluated in 59 non-aflatoxigenic *Aspergillus flavus* isolates isolated from wheat, corn and sorghum grains. The isolates were placed into four groups based on their DNA banding pattern. group I constituted by twenty seven isolates (45.8%) correspond to the complete set of genes. group II constituted by fifteen isolates (25.5%) showed three DNA banding pattern clustered in four profiles: *nor-1, ver-1* and *omt-A* was the most frequent profile (11.9%) followed by *nor-1, omt-A* and *aflR* (8.5%) and *nor-1, ver-1* and *aflR* (3.4%), last profile *ver-1*, *omt-A* and *aflR* . group III constituted by thirteen isolates (21.9%) yielded two DNA banding pattern grouped in three characteristic profiles: *nor-1* and *ver-1* (13.5%), *ver-1* and *omt-A* (6.7%), *nor-1* and *aflR* (1.7). group IV constituted by four isolates (6.8%) showed two DNA banding pattern clustered in two profiles. Our data show a high level of genetic variability in non-aflatoxigenic *A. flavus* isolates.

Key words: Non-aflatoxigenic *Aspergillus flavus*, *nor-1*, ver-1, *omt-A* and *aflR* genes.

Aflatoxins (AFs) are hepatotoxic, teratogenic, mutagenic and carcinogenic mycotoxins produced by members of *Aspergillus* section *Flavi* mainly *Aspergillus flavus* and *Aspergillus parasiticus*. The most potent of the four naturally occurring AFs (B1, B2, G1 and G2) is aflatoxin B1 (AFB1), which is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1982; Abd El-Aziz *et al*., 2013) *Aspergillus flavus* is the most common causal agent of aflatoxins (AFs) contamination of crops (Klich, 2007). *Aspergillus flavus* is the most abundant aflatoxin-producing species in agricultural soils and have the potential to cause considerable contamination of crops (Horn, 2005). About 40% of the naturally occurring isolates of *A. flavus* lack the ability to produce AF and different isolates show large differences in the levels of AF produced. In fact, aflatoxin biosynthesis pathway may become unstable in these fungi (Cary and Ehrlich, 2006). Aflatoxin gene cluster including more than 18 enzymatic stages and at least 25 genes residing in a 75 kb cluster in the third fungal chromosome (Zeng *et al.*, 2012). Aflatoxin biosynthesis in *A. flavus* is strongly dependent on the activities of regulatory proteins and enzymes encoded by four genes named *aflR*, *nor-1*, *ver-1* and *omt-A*. By using specific PCR-based methods, the aflatoxigenic *A. flavus* isolates always show the complete gene set, whereas non-aflatoxigenic isolates lacking one, two, three or four PCR products indicating that the genes do not exist in these strains or that the primer binding sites changed. Interestingly, some *A. flavus* strains show a complete set of genes but do not produce aflatoxins (Criseo *et al*., 2001). Aflatoxigenic *A. flavus* isolates always show, by using a multiplex PCR-

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system, four DNA fragments specific for aflR, nor-1, ver-1, and omt-A genes. Non-aflatoxigenic *A. flavus* strains give variable DNA banding pattern lacking one, two, three or four of these genes. Recently, it has been found and reported that some aflatoxin non-producing *A. flavus* strains show a complete set of genes. Because less is known about the incidence of structural genes *aflR*, *nor-1*, *ver-1* and *omt-A* in aflatoxin non-producing strains of *A. flavus* (Criseo *et al*., 2008). In previous studies only a few nonaflatoxigenic *A. flavus* strains were examined for molecular diversity (Shapira *et al*., 1996; Criseo *et al*., 2001; Criseo *et al*., 2008).

In this study, we evaluate the presence and the frequencies of the PCR products corresponding to amplification of *aflR*, *nor-1*, *ver-1* and *omt-A* genes in non-aflatoxigenic *A. flavus* strains isolated from wheat, corn and sorghum and feed commodities.

MATERIALS AND METHODS

Isolates of *A. flavus*

59 isolates of non-aflatoxigenic *A. flavus* isolated from wheat (18), corn (21) and sorghum (20) were used in this work.

Isolation of fungal DNA

Non-aflatoxigenic *A. flavus* isolates were cultured on double-layer media on 50-mm Petri dishes, one solid and the other liquid. The solid base medium was PDA as a film, and the top medium, liquid, was 1200 ¼L peptone yeast glucose. Fungi were incubated at 25°C for two days. Fungal mycelia (50 mg) were scraped using slide cover slips and transferred to 1.5-mL sterile Eppendorf tubes for DNA isolation. DNA was extracted from 50 mg fresh mat according to Amer *et al*. (2011).

PCR reaction

Four pairs of primers were designed on the basis of the sequences of non-aflatoxigenic *A. flavus* aflatoxin biosynthetic genes *nor-1, ver-1, omt-A, aflR*. Amplification was performed in 25 ¼L reaction, containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 3.4 mM $MgCl_2$, 0.25 mM dNTPS, 0.4 mM of each primer, 2 U Taq DNA polymerase (BioLabs), and 25 ng genomic DNA. Amplification parameters (*aflR*, *nor-1*, *ver-1*, *omt-A*) consisted of 1 cycle 95 °C (1 min), 65 °C, (30 s), 72 °C (30 s) and 34 cycles at 94°C (30 s), annealing at 65°C (30 s), and extension at 72°C (6 min). PCR products were separated by 1.5% agarose gel, stained with ethidium bromide in 1X TAE buffer (Tris-acetate EDTA, pH 8.0) at 100 V for 50 min, using a 100-bp ladder DNA marker (Intron, Korea). The DNA gel was scanned for band Rf using a gel documentation system (AAB Advanced American Biotechnology, Fullerton, CA, USA). Sequences of primers are listed in Table 1.

RESULTS

Amplification patterns of aflatoxin biosynthesis genes

Primers pairs were designed for this study to target four aflatoxin biosynthetic genes: the one regulatory genes *aflR*, and the structural genes *nor-1*, *ver-1* and *omt-A*. As shown in (Table 2). The presence or absence of the aflatoxin biosynthetic genes in the genomes of all non-aflatoxigenic *A. Flavus* isolates was separated into ten groups on the basis of PCR products.

Table 1. Sequences of the nucleotide primers used in this study

Primer code	Target gene	Primer sequences	PCR product size (bp)
nor-1	*nor-1*	5'-ACCGCTACGCCGGCACTCTCGGCAC-3'	400
nor-2		5'-GTTGGCCGCCAGCTTCGACACTCCG-3'	
ver-1	*ver-1*	5'-GCCGCAGGCCGCGGAGAAAGTGGT-3'	737
ver-2		5'-GGGGATATACTCCCGCGACACAGCC-3'	
omt-1	*omt-A*	5'-GTGGACGGACCTAGTCCGACATCAC-3'	799
omt-2		5'-GTCGGCGCCACGCACTGGGTTGGGG-3'	
aflR-1	*aflR*	5'-TATCTCCCCCCGGGCATCTCCCGG-3'	1032
aflR-2		5'-CCGTCAGACAGCCACTGGACACGG-3'	
AflJ-giR		5'-CGGTCAGGATGTTACTAAGC-3'	

Table 2. Amplification patterns of aflatoxin biosynthesis genes[a]

Isolate code	*nor-1*	*ver-1*	*omt-A*	*aflR*	Group
W503	+	+	+	+	
W504	+	+	+	+	
W505	+	+	+	+	
W511	+	+	+	+	
W513	+	+	+	+	
W514	+	+	+	+	
W516	+	+	+	+	
W517	+	+	+	+	
W518	+	+	+	+	
C601	+	+	+	+	
C602	+	+	+	+	
C606	+	+	+	+	
C607	+	+	+	+	
C612	+	+	+	+	
C613	+	+	+	+	
C614	+	+	+	+	
S701	+	+	+	+	
S703	+	+	+	+	
S704	+	+	+	+	
S706	+	+	+	+	
S707	+	+	+	+	
S711	+	+	+	+	
S712	+	+	+	+	
S715	+	+	+	+	
S717	+	+	+	+	
S719	+	+	+	+	
S720	+	+	+	+	
W501	+	+	+	-	
W502	+	+	+	-	
C608	+	+	+	-	
C610	+	+	+	-	
C611	+	+	+	-	
C620	+	+	+	-	
C621	+	+	+	-	
W509	+	-	+	+	
W510	+	-	+	+	
C616	+	-	+	+	
C617	+	-	+	+	
C619	+	-	+	+	
W508	+	+	-	+	
C603	+	+	-	+	
W507		+	+	+	
W506	+	+	-	-	
W512	+	+	-	-	
C604	+	+	-	-	
S702	+	+	-	-	
S708	+	+	-	-	
S709	+	+	-	-	
S713	+	+	-	-	
S716	+	+	-	-	
W515	-	+	+	-	
C603	-	+	+	-	
S714	-	+	+	-	
S718	-	+	+	-	
W609	+	-	-	+	
W615	+	-	-	-	
S705	+	-	-	-	
S715	+	-	-	-	
C618	-	-	-	+	

[a] Presence (+) or absence (-) of PCR products of the tested AFs genes

[b] Distribution of isolates in seven amplification patterns

Group I of 27 isolates was characterized by amplification of all four biosynthetic genes. Group II, consisting of fifteen isolates was characterized by amplification three of four aflatoxin biosynthetic genes clustered in four profiles, 1) *nor-1*, *ver-1* and *omt-A* (seven isolates), 2) *nor-1*, *omt-A* and aflR (five isolates), 3) *nor-1*, *ver-1* and *aflR* (two isolates) and 4) *ver-1*, *omt-A* and *aflR*. Group III constituted by thirteen isolates were characterized by amplification two of genes clustered in three profiles, 1) *nor-1* and *ver-1* (eight isolates), 2) *ver-1* and *omt-A* (four isolates) and 3) *nor-1* and *aflR* (one isolate). Group IV consisting of four isolates was characterized by amplification two genes clustered in two profiles, 1) nor-1 (three isolates), 2) *aflR* (one isolate).

Genetic pattern of *A. flavus* isolates

Non-aflatoxinogenic *A. flavus* isolates (59 isolates) presented varying patterns of the aflatoxin biosynthetic pathway (Table 3).

Non- aflatoxinogenic *A. flavus* isolates divided into four groups on the basis of their DNA banding patterns: twenty seven isolates displayed a complete set pattern, fifteen isolates displayed three-banded pattern, thirteen isolates displayed two-banded pattern, four isolates displayed one-banded pattern.

Frequency of single genes and genetic pattern in *A. flavus* isolates

Non-aflatoxigenic isolates showed four groups, first group constituted by twenty seven isolates (45.8%) correspond to the complete set of genes. Second group constituted by fifteen isolates (25.5%) showed three DNA banding pattern clustered in four profiles: *nor-1*, *ver-1* and *omt-A* was the most frequent profile (11.9%) followed by *nor-1*, *omt-A* and *aflR* (8.4%) and the lowest frequent profile (2.5%) *ver-1*, *omt-A* and *aflR*.

Third group constituted by thirteen isolates (21.9%) yielded two DNA banding pattern grouped in three characteristic profiles: *nor-1*, *ver-1* (13.5%) and *ver-1*, *omt-A* (6.7) and *nor-1*, *aflR* (1.7%). Fourth group four isolates (6.8%) showed one DNA banding pattern clustered in two profiles. The *nor-1* gene was the most representative more than (89%) between the four aflatoxin assayed genes followed by *ver-1* (83.1%) and *omt-A* (74.6%). Lower incidence (62.7%) was found for *aflR* gene. The frequencies of banding pattern and respective profiles are showed in (Fig. 1).

Table 3. Origin and genetic pattern of non-aflatoxin producing strains of *A. flavus* isolated from some crops

Crop	N° Isolates	Complete set	Three bands	Two bands	One band	No band
Wheat	18	9	6	3	0	0
Corn	21	7	9	3	2	0
Sorghum	20	11	0	7	2	0

Table 4. Frequency of single genes in non-aflatoxigenic *A. flavus* isolates examined

N° Isolates	%	*nor-1*	*ver-1*	*omt-A*	*aflR*
27	45.8	+	+	+	+
7	11.9	+	+	+	-
5	8.5	+	-	+	+
2	3.4	+	+	-	-
1	1.7	-	+	+	+
8	13.5	+	+	-	-
4	6.7	-	+	+	-
1	1.7	+	-	-	+
3	5.1	+	-	-	-
1	1.7	-	-	-	+
59		53(89.8)	49(83.1)	44(74.6)	37(62.7)

[a] Presence (black box) or absence (grey box) of PCR products of the tested AFs genes

DISCUSSION

In present work, 59 non-aflatoxigenic *Aspergillus flavus* isolates were screened for the presence of four genes (*nor-1, ver-1* and *omt-A and aflR*) of the aflatoxin biosynthesis. The result was the grouping of non-aflatoxigenic isolates lacking one, two, three genes. Our data show a high level of genetic variability in non-aflatoxigenic *A. flavus* isolates. The non- aflatoxigenic *A. flavus* isolates probably results from point mutations (Ehrlich *et al*., 2004) or small deletions in genes essential for aflatoxin production, such as those having a regulatory role (Calvo *et al*., 2004) or being involved in the signaling pathway (Hicks *et al*., 1997).

In the last few years, several PCR-based systems have been developed to revealed genetic variation in *A. flavus* isolates; PCR-based systems included molecular marker (APLP, DAF, RAPD and ISSR) and the aflatoxin biosynthesis gene cluster. Molecular markers are useful for genetic variation in fungi (Majer *et al*. 1996).

Several molecular methods were employed in an attempt to study genetic variability for aflatoxigenic and nonaflatoxigenic isolates of *A. flavus*. Montiel *et al*. (2003) who analysed 24 isolates of *A. sojae*, *A. parasiticus*, *A. oryzae* and *A. flavus* using the AFLP technique, and could clearly separate the *A. sojae* D *A. parasiticus* isolates from the *A. oryzae* D *A. flavus* isolates. Barros *et al*. (2007) reported that AFLP analysis was applied to compare 82 isolates of *A. flavus* and *A. parasiticus*. AFLP analysis indicates that no genotypical differences can be established between aflatoxigenic and nonaflatoxigenic producers in both species analysed.

Ribosomal sequence ITS for 24 isolates of *Aspergillus sojae, A. parasiticus*, *A. oryzae* and *A. flavus* (aflatoxigenic) found some variation between *A. oryzae* and *A. flavus* isolates, but it is difficult to use molecular data to separate the two

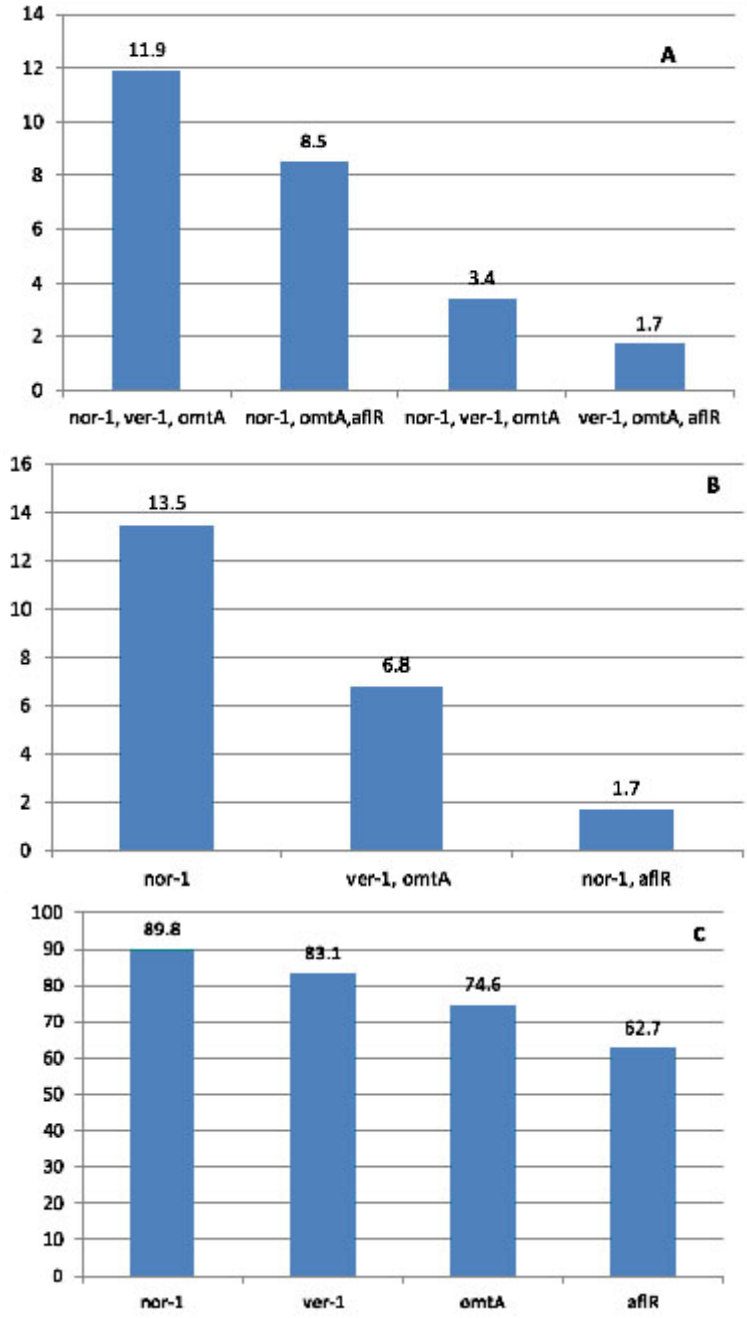

Fig. 1. Frequencies of three (A), two (B) and one (C) genes pattern in non-aflatoxigenic *A. flavus* isolates

species (Montiel *et al*. 2003). Using DNA amplification fingerprinting (DAF) to differentiate aflatoxigenic from nonaflatoxigenic isolates of *A. flavus* but, could not be separated the isolate types (Baird *et al*. 2006).

The ISSR primers produced differential amplification products, varying both in size and band intensity. ISSR revealed higher genetic variabil-ity in several strains of *A. flavus*, even though high interspecific variation was observed (Hatti, 2010; Wang *et al*., 2012, Al-Wadai *et al*. 2013). The utility of DNA markers as RAPD-DNA

employ it as well established sample molecular marker tool for detecting genetic variability for many phytopathogenic fungi (Megnegneau *et al*., 1993) especially *Aspergillus* genera and related species (Wostemeyer and Kreibich, 2002; Lourenço *et al*., 2007; Gashgari *et al*., 2010; Irshad and Nawab 2012; Al-Wadai *et al*. 2013; Mahmoud *et al*, 2013).

Furthermore, several researches have adopted detect an aflatoxin biosynthetic gene and differentiate aflatoxin-producing from non-producing strains of *A. flavus*. Criseo *et al*. (2001) combining sets of primers for *aflR*, *nor-1*, *ver-1* and *omt-A* genes of the aflatoxin biosynthetic pathway, Quadruplex-PCR showed that aflatoxinogenic strains gave a quadruplet pattern, indicating the presence of all the genes involved in the aflatoxin biosynthetic pathway which encode for functional products. Non-aflatoxinogenic strains gave varying results with one, two, three or four banding patterns. 134 non-aflatoxin producing strains of *A. flavus* isolated examined using a multiplex PCR-system, four DNA fragments specific for *aflR*, *nor-1*, *ver-1*, and *omt-A* genes. Forty nine (36.5%) of the examined non-aflatoxigenic *A. flavus* strains showed DNA fragments that correspond to the complete set of genes (quadruplet pattern). Forty three strains (32%) showed three DNA banding patterns grouped in four profiles where *nor-1*, *ver-1* and *omt-A*. Twenty five (18.7%) yielded two DNA banding pattern whereas sixteen (12%) of the strains showed one DNA banding pattern. In one strain, isolated from poultry feed, no DNA bands were found (Criseo *et al*. 2008).

Our results are in agreement with the results reported by Criseo *et al*. (2001) and Criseo *et al*. (2008). In our study, we found that 22 of aflatoxin non-producing *A. flavus* isolates lacking the *aflR* PCR amplicon because in these isolates the *aflR* gene has been lost or mutations occurs within the primer binding sites. This could be due to the location of the aflatoxin gene cluster in the telomeric region of *A. flavus*, that would facilitate gene loss as well as recombination, DNA inversions, partial deletions, translocations and other genomic rearrangements (Carbone *et al*., 2007).

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