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Molecular Characterization of Atoxigenic *Aspergillus flavus* Isolates Collected in China

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Aspergillus flavus **strains were isolated from peanut fields of Liaoning, Shandong, Hubei and Guangdong Provinces in China, and identified through phenotypic and molecular approaches. Of the 323 *A. flavus* strains isolated, 76 strains did not produce aflatoxins detectable by UPLC. The incidence of atoxigenic *A. flavus* strains decreased with increase in temperature and increased with increase in latitude in different geographical locations. Amplification of all the aflatoxin genes in the aflatoxin gene cluster in the atoxigenic isolates showed that there were 25 deletion patterns (A-Y), with 22 deletion patterns identified for the first time. Most of the atoxigenic *A. flavus* isolates with gene deletions (97%) had deletions in at least one of the four genes (*aflT*, *nor-1*, *aflR*, and *hypB*), indicating that these four genes could be targeted for rapid identification of atoxigenic strains. The atoxigenic isolates with gene deletions, especially the isolates with large deletions, are potential candidates for aflatoxin control.**

Keywords: aflatoxins, *Aspergillus flavus*, atoxigenic isolate, deletion pattern, gene cluster, biological control

Introduction

Aflatoxins are toxic and carcinogenic secondary metabolites produced by various *Aspergillus* species, such as *A. flavus* and *A. parasiticus* (Payne and Brown, 1998), and contaminate many economically important crops such as corn, cotton, peanuts and tree nuts (Guzmán-de-Peña and Peña-Cabriales, 2005), resulting in serious food and feed safety problems all over the world, with significant economic losses every year (Dorner, 2004). Aflatoxins are a family of toxic compounds, of which aflatoxin B_1 (AFB_1) is the most carcinogenic compound (Egmond, 1989). Thus, the level of aflatoxins in food and feed is strictly limited and monitored in more than 100 countries (Egmond *et al*., 2007). The maximum tolerable limit for AFB_1 is below 20 μg/kg in food products (Egmond and Jonker, 2004).

Aflatoxins are extremely stable compounds and it is very hard to remove them from contaminated food and feed stuffs by detoxification through physical, chemical or even biological approaches (Inan *et al*., 2007; Guan *et al*., 2008; Kabak, 2009). Thus, significant attention has been given to the preharvest control of aflatoxins. Several strategies, such as breeding resistant germplasms, applying pesticides to avoid fungal infections, and a series of agronomic practices like effective tillage, have been used to reduce aflatoxin contamination, but the effects are not obvious, or they lead to environmental problems (Brown *et al*., 1999; Tubajika and Damann, 2001). Therefore, more effective and environmentally friendly approaches are needed to control toxigenic *Aspergillus* strains in the cultivation system.

A. flavus strains isolated from different regions of the world have wide differences in their aflatoxin-producing potentials, and atoxigenicity in the strains range from 0% to over 80% (Vaamonde *et al*., 2003; Pildain *et al*., 2004; Razzaghi-Abyaneh *et al*., 2006; Giorni *et al*., 2007; Mauro *et al*., 2013). Recently, the application of competitive atoxigenic *A. flavus* or *A. parasiticus* strains in field conditions has achieved significant control over aflatoxin contamination, as atoxigenic *Aspergillus* strains can competitively displace the natural toxigenic *Aspergillus* populations present in the field by occupying their niches. In many cases, this strategy can reduce more than 90% of the aflatoxin contamination in peanuts, maize and cottonseed (Dorner and Cole, 2002; Dorner *et al*., 2003; Dorner, 2004; Jaime-Garcia and Cotty, 2006). Two atoxigenic *A. flavus* strains, AF36 and NRRL 21882, are currently registered as biopesticides by the EPA (US Environmental Protection Agency) for use in controlling aflatoxin contamination in the USA (Dorner, 2004; Das *et al*., 2008). Strains isolated from different regions have their own adaptive traits, thus, diverse atoxigenic strains are being exploited for their highly competitive nature, in controlling aflatoxin worldwide (Garber and Cotty, 1997; Dorner, 2004).

To ensure safety in the use of atoxigenic *Aspergillus* strains, the molecular mechanism underlying the atoxigenic nature of these isolated *Aspergillus* strains should be analyzed. This would help us in choosing a suitable and stable strain. As aflatoxin biosynthesis and its regulation are quite complex, involving at least 29 genes clustered in a 70 kb chromosome region (Yu *et al*., 2004a), the mechanisms involved in the atoxigenic nature of these strains are not well understood (Schmidt-Heydt *et al*., 2008). In many cases, atoxigenic *Aspergillus* isolates were found to possess partial or

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Table 1. Summary of *A. flavus* and *A. parasiticus* isolated in this study

Geographic origin	No. of *A. flavus* strains	No. of *A. parasiticus* strains	No. of atoxigenic *A. flavus* strains
Liaoning	9	2	4
Shandong	73	12	29
Hubei	125	2	29
Guangdong	116	4	14
Total	323	20	76

total deletions in the aflatoxin gene cluster (Chang *et al.*, 2005). The atoxigenic isolate AF36 having a mutation in the aflatoxin gene *pksA* has been widely used in the prevention of aflatoxin contamination in cottonseed fields in Arizona (USA) (Ehrlich and Cotty, 2004). However, for safety reasons, strains with larger deletions in the aflatoxin gene cluster were recommended for use in the aflatoxin control program. The loss of the aflatoxin-producing ability in NRRL 21882 was found to be associated with the deletion of the entire aflatoxin gene cluster (Dorner, 2004; Chang *et al.*, 2005). In China, *A. flavus* is the dominant species isolated from either soil or crops (Gao *et al.*, 2007), and Liaoning (North China), Shandong (East China), Hubei (Central China) and Guangdong (South China) are the main peanut-producing provinces with typical climate characteristics. In the present study, we aimed to isolate atoxigenic *A. flavus* strains from peanut fields in Liaoning, Shandong, Hubei and Guangdong provinces

Table 2. A list of the *A. flavus* isolates used in this study

Strains[a]	Geographic origin	Deletion Patterns[b]	Deletion pattern in *norB-cypA* regions[c]	Strains[a]	Geographic origin	Deletion Patterns[b]	Deletion pattern in *norB-cypA* regions[c]
HB-30	Hubei	ND	ND	GD-9	Guangdong	A	I
SD-30	Shandong	ND	ND	GD-10	Guangdong	A	I
HB-1	Hubei	F	I	GD-11	Guangdong	A	I
HB-2	Hubei	K	I	GD-12	Guangdong	A	I
HB-3	Hubei	H	I	GD-13	Guangdong	A	I
HB-4	Hubei	L	I	GD-14	Guangdong	A	I
HB-5	Hubei	R	II	SD-1	Shandong	A	I
HB-6	Hubei	A	II	SD-2	Shandong	A	I
HB-7	Hubei	N	I	SD-3	Shandong	A	I
HB-8	Hubei	A	I	SD-4	Shandong	A	I
HB-9	Hubei	W	III	SD-5	Shandong	A	I
HB-10	Hubei	O	III	SD-6	Shandong	A	I
HB-11	Hubei	X	III	SD-7	Shandong	A	I
HB-12	Hubei	Q	III	SD-8	Shandong	A	II
HB-13	Hubei	Y	III	SD-9	Shandong	A	I
HB-14	Hubei	A	II	SD-10	Shandong	C	II
HB-15	Hubei	A	II	SD-11	Shandong	A	I
HB-16	Hubei	G	II	SD-12	Shandong	A	I
HB-17	Hubei	A	II	SD-13	Shandong	A	I
HB-18	Hubei	D	I	SD-14	Shandong	C	II
HB-19	Hubei	A	II	SD-15	Shandong	A	I
HB-20	Hubei	J	II	SD-16	Shandong	C	I
HB-21	Hubei	E	II	SD-17	Shandong	A	I
HB-22	Hubei	M	II	SD-18	Shandong	C	I
HB-23	Hubei	V	I	SD-19	Shandong	C	I
HB-24	Hubei	S	III	SD-20	Shandong	C	I
HB-25	Hubei	I	II	SD-21	Shandong	C	I
HB-26	Hubei	P	I	SD-22	Shandong	A	I
HB-27	Hubei	D	I	SD-23	Shandong	U	I
HB-28	Hubei	A	I	SD-24	Shandong	A	I
HB-29	Hubei	B	II	SD-25	Shandong	U	I
GD-1	Guangdong	A	I	SD-26	Shandong	A	I
GD-2	Guangdong	T	III	SD-27	Shandong	A	I
GD-3	Guangdong	U	I	SD-28	Shandong	A	I
GD-4	Guangdong	A	I	SD-29	Shandong	A	I
GD-5	Guangdong	A	I	LN-1	Liaoning	A	I
GD-6	Guangdong	A	I	LN-2	Liaoning	U	I
GD-7	Guangdong	A	I	LN-3	Liaoning	A	I
GD-8	Guangdong	A	I	LN-4	Liaoning	A	I

[a] The isolates coded with HB, GD, SD, and, LN were collected from the peanut-cropped soil from Hubei, Guangdong, Shandong, and Liaoning provinces, respectively. The isolates HB-30 and SD-30 are toxigenic *A. flavus* collected from the peanut-cropped soil.
[b] Deletion patterns in the aflatoxin gene cluster (see Fig. 1 for Patterns A–Y). The abbreviation ND indicates that the isolate did not have deletion in the cluster analysed by PCR assays.
[c] The deletion Patterns I, II, or III in the *norB–cypA* regions indicate a 0.3-kb, 0.8-kb or no PCR product, respectively, as analyzed using the primer pair *norB-cypA*-F/*norB-cypA*-R.

in China, and to analyze the deletions in the aflatoxin gene cluster to select suitable atoxigenic *A. flavus* strains as potential aflatoxin control agents.

Materials and Methods

A. flavus isolation and identification

A. flavus strains were isolated from peanut-cropped soils collected from different provinces (Liaoning, Shandong, Hubei, and Guangdong) in China, using the dilution plating method on modified rose Bengal agar (M-RB). After 3.5 days, *A. flavus* colonies produced green conidia on M-RB. All isolates were preserved in malt extract agar (MEA) medium at 4°C (Cotty, 1994) (Tables 1 and 2).

All isolates were identified by phenotypic and molecular approaches as described previously (Zhang *et al*., 2013). Briefly, the strains were initially identified by their characteristic growth patterns on AFPA (*A. flavus* and *A. parasiticus* agar; Cotty, 1994) and were further confirmed by sequencing their calmodulin genes (Rodrigues *et al*., 2011).

Aflatoxin analysis

To determine the aflatoxin producing ability of these isolates, they were cultured in aflatoxin-inducing medium (150 g sucrose peptone/L, 20 g yeast extract/L, 10 g peptone/L, pH 5.9) (Barros, 2007) by inoculating flasks with 1×10^5 spores and then agitating them at 200 rpm (30°C, 7 days) in the dark. Aflatoxin production was analyzed by culturing the isolates on Yeast Extract Sucrose (YES) medium (Yeast Extract 20 g/L, Sucrose 150 g/L, Agar 15 g/L) for 7 days, as described by Rodrigues *et al*. (2009). After extraction as previously reported (Barros, 2007; Rodrigues *et al*., 2009), aflatoxins were detected by UPLC (Waters ACQUITY UPLC I-Class, Waters Corp, USA). UPLC was performed on an LC-C18 reversed-phase column (50 × 2.1 mm, 1.7 µm particle size, Waters ACQUITY UPLC BEH C18, Waters Corp, Milford, USA) and detection was performed with a fluorescence detector (Waters ACQUITY UPLC Fluorescence, Waters Corp) using an excitation wavelength of 365 nm and an emission wavelength of 440 nm. The mobile phase was methanol/H_2O (45:55, v/v) at a flow rate of 1 ml/min. The limit of detection of the UPLC method was 1 µg/kg.

DNA extraction

To extract genomic DNA, each isolate was grown in YES liquid medium cultures by inoculating flasks with 1×10^5 spores. After cultivation at 200 rpm (30°C) for 5 days in the dark, mycelia were collected by filtration through Whatman filter paper, and washed with distilled water. Genomic DNA was extracted from individual mycelia using benzyl chloride with a few modifications (Zhang *et al*., 2013). Briefly, mycelia were disrupted in polyethylene tubes using a pestle, resuspended in 750 µl of extraction buffer (100 mM Tris-HCL, pH 8.0 and 150 mM EDTA, pH 8.0), followed by addition of 150 µl of 10% SDS and 450 µl of benzyl chloride.

Table 3. Primers used for detecting the presence of aflatoxin genes

Gene	Forward primer	Reverse primer
norB-cypA	GTGCCCAGCATCTTGGTCCA	AGGACTTGATGATTCCTCGTC
aflT	ATGACATGCTAATCGACGAG	AGGCGCATGCTACGGATC
pksA	ACTTTGAGGGCGTTCTGTGC	CTTTCGGTGGTCGGTGATTC
hypE	TGACTCGCAAGCCAGACC	TCACGCCACCAAGCACCA
nor-1	AGCACGATCAAGAGAGGCTC	GATCTCAACTCCCCTGGTAG
hypD	ACCACGCCCTTACCTTCTT	AACAGTCCCGACAGAGCAG
fas-2	TCCTATCCAGTCCACCTCGTA	CACATCTTTGTCTTGCCCGC
fas-1	ACAATCGAATGACAACACTC	CCACCGAATCCACTACCTACA
aflR	ATGGTCGTCCTTATCGTTCTC	CCATGACAAAGACGGATCC
aflJ	CTTCAACAACGACCCAAGGTT	AGATGAGATACACTGCCGCA
adhA	CCTCGTGGGAGAGCCAAATC	GGAGCAAGAAGGTTACAGCG
estA	CGATGGGACTGACGGTGATT	ACCACGCCGCTGACTTTAT
norA	GTGTTCGTGTGTCGCCCTTA	GTCGGTGCTTCTCATCCTGA
ver-1	CATCGGTGCTGCCATCGC	CCTCGTCTACCTGCTCATCG
hypC	GACTGTCCATGCCTCTTTCA	AACAGTCCCGACAGAGCAG
verA	CCGCAACACCACAAGTAGCA	AAACGCTCTCCAGGCACCTT
avnA	GCGATAGAACTGACAAAGGCA	GAATGAGTCTCCAAAGGCGAG
verb	TTCAGTGACAAAGGTCTTCGC	GGCAGCGTT ATTGAGCATCT
hypB	GTTCGGTTATGCCCAAGGT	CAGCCAACGGAAAGAGTGC
avfA	ATTCAAATCCTCGTTCGGTCG	TAGCCCGTTGGTTGTGTTCC
omtB	ACAGACGATGTGGGCAAACG	ACGCAGTCCTTGTTAGAGGTG
omtA	CAGGATATCATTGTGGACGG	CTCCTCTACCAGTGGCTTCG
ordA	AAGGCAGCGGAATACAAGCG	ACAAGGGCGTCAATAAAGGGT
vbs	AACGAGCAGCGTAAGGGTCT	TCAGCCAGAGCATACACAGTG
cypX	GGAGCCTACCATTCGCAACA	GGCTTTGACGAACAGATTCCG
moxY	TGCTACTGGAACGAAGACCG	CGACGACAACCAAACGCAA
ordB	GCTGCTACTGGAATGAAGACC	ATGCGACGACAACCAAACG
hypA	CGCAAGACGGCAGAGATACT	GCTCCTTCAGTTCCACACCA

The extracted DNA was quantified by electrophoresis in 1% (w/v) agarose gels with 1× TAE buffer containing 0.1‰ GelRed Nucleic Acid Stain (10000×, Biotium, USA). DNA was diluted for further use in PCR reactions at a concentration of 50 ng/μl.

PCR primers

To detect the presence of aflatoxin genes in the atoxigenic *A. flavus* isolates, 28 pairs of primers were used, as listed in Table 3. The PCR primers used to amplify the gene *norB-cypA* were previously described by Ehrlich *et al.* (2004), the primers for *hypB*, *hypC*, *hypD*, and *hypE* were designed based on their gene sequences using Primer Premier 5.0 (Premier, Canada), and the other primers were based on Chang *et al.* (2005).

PCR conditions

The PCR reaction volume was 20 μl containing 50 ng fungal genomic DNA, 10 ng of a single primer, 10 μl Go Taq polymerase (2×Colorless Go Taq Reaction Buffer, pH 8.5, 400 μM of each dNTP, 3 mM $MgCl_2$, Promega Crop, USA) and 7 μl sterile water. Isolate HB-30 and SD-30, which produce aflatoxins, were used as positive controls in the PCR screenings for deletions in the aflatoxin gene cluster. The PCR cycling conditions were as follows: a 5 min initial denaturation step at 95°C, 30 cycles of amplification (30 sec at 95°C, 30 sec at 55°C, 90 sec at 72°C), and a final elongation step of 7 min at 72°C. PCR was performed using a TaKaRa PCR Thermal Cycler Dice TP600 (TaKaRa, Japan). Each isolate was amplified twice. The PCR products were separated by electrophoresis in 1% (w/v) agarose gels with 1× TAE buffer containing 0.1‰ GelRed Nucleic Acid Stain (10000×, Biotium, USA) and photographed under UV light. The presence or absence of PCR products was recorded for each isolate.

Statistical analysis

PCR products were scored for the gene's presence (1) or absence (0) in the aflatoxin gene cluster from each of the 34 atoxigenic strains with gene deletions. A clustering analysis of the strains was performed using the UPGMA functionality in NTSYSpc 2.10 (Department of Ecology and Evolution, State University of New York, NY, USA).

Results

Atoxigenic *A. flavus* identification

The morphological analysis, which was initially used to identify the strains, revealed that 343 fungi isolated from the peanut-cropped soil of the main peanut-producing provinces (11 isolates from Liaoning, 85 isolates from Shandong, 127 isolates from Hubei and 120 isolates from Guangdong) in China were bright orange on AFPA medium, indicating that they were likely *A. flavus*. The isolates were further confirmed by analyzing their calmodulin genes. Comparing the calmodulin sequences of these isolates using the BLAST algorithm revealed that 323 isolates belonged to *A. flavus* (9 isolates from Liaoning, 73 isolates from Shandong, 125 isolates from Hubei and 116 isolates from Guangdong), and 20 isolates belonged to *A. parasiticus* (2 isolates from Liaoning, 12 isolates from Shandong, 2 isolates from Hubei and 4 isolates from Guangdong) (Table 1). Among the 323 *A. flavus* strains isolated, 76 strains did not produce a detectable level of aflatoxins, as determined by UPLC (Table 1).

Deletions in the aflatoxin gene cluster of the atoxigenic *A. flavus* isolates

All 28 gene fragments used in our study were successfully PCR-amplified for the aflatoxin-producing isolate HB-30 and SD-30, indicating the primers used were appropriate

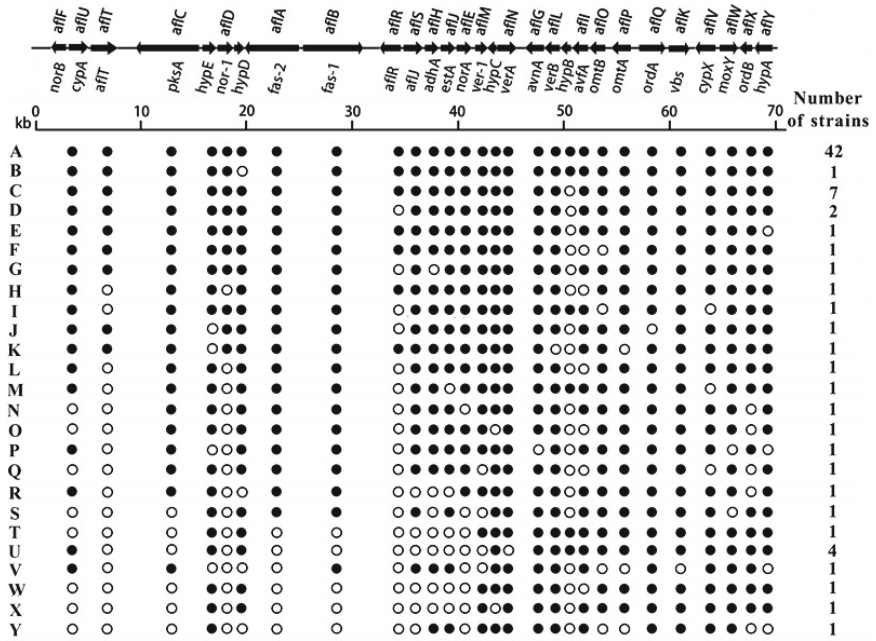

Fig. 1. Deletion patterns (A-Y) in the aflatoxin gene cluster of the atoxigenic *A. flavus* isolates. Solid circles indicate positive PCR products, whereas open circles indicate no PCR products.

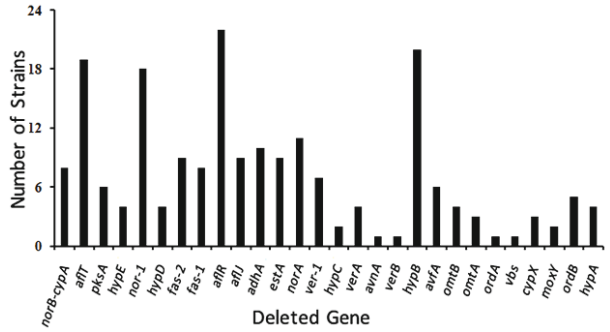

Fig. 2. Frequency of the aflatoxin gene deletion in the 34 atoxigenic *A. flavus* strains.

(data not shown). Based on the differences in deletion patterns in the aflatoxin gene cluster, the 76 atoxigenic *A. flavus* strains were grouped into 25 deletion patterns (Fig. 1 and Table 2). Pattern A indicates no deletions, and there were 42 atoxigenic *A. flavus* isolates that belonged to this pattern, showing that atoxigenic *A. flavus* strains without gene deletions are not rare. One gene deletion was seen in Patterns B (*hypD*) and C (*hypB*), whereas Patterns D (*aflR* and *hypB*) and E (*hypB* and *hypA*) showed two gene deletions. There were more gene deletions seen for patterns F to Y. Pattern Y showed the most gene deletions, with 15 genes deleted. In addition, the PCR primers for *norB-cypA* could amplify a 0.3-kb (Pattern I), or 0.8-kb (Pattern II) fragment in *A. flavus* with these 2 genes, or no fragment (Pattern III) without these 2 genes (Ehrlich *et al.*, 2004). Considering the length of the *norB-cypA* PCR fragment, 54 isolates belonged to Pattern I and 15 isolates belonged to Pattern II, whereas the remaining 7 isolates belonged to Pattern III.

Among the deleted genes, *aflT*, *nor-1*, *aflR*, and *hypB*, were found to have the highest frequencies of loss and were absent in 19, 18, 22, and 20 *A. flavus* strains, respectively (Fig. 2).

Genetic similarity among the atoxigenic *A. flavus* isolates

Based on the presence of aflatoxin genes, a cluster analysis was performed to identify the similarities in the aflatoxin gene cluster among the 34 atoxigenic *A. flavus* isolates with gene deletions. The genetic similarity coefficients (GSC) ranged from 0.57 to 1.00 for the 34 strains. A dendrogram assembled using the unweighted pair-group method with arithmetic averages (UPGMA) grouped the 34 strains into six main clusters (labeled Groups A, B, C, D, E, and F), which were delineated at a GSC value of 0.75 (Fig. 3). Group A had the largest number of strains (20 strains), with 7 strains from Shandong (East China) and 13 strains from Hubei (Central China). Group B had four strains, whereas Groups C, D, and E had one strain per group, and all of the strains in these groups were from Hubei (Central China). Group F included seven strains with two subgroups.

Discussion

The ability of *A. flavus* to produce aflatoxins varies significantly among different strains, and atoxigenic *A. flavus* strains have been found in many regions all over the world (Chang *et al.*, 2007; Donner *et al.*, 2010). In general, the percentage of isolated atoxigenic *A. flavus* strains can range from 0% to more than 80% (Vaamonde *et al.*, 2003; Pildain *et al.*, 2004; Razzaghi-Abyaneh *et al.*, 2006; Giorni *et al.*, 2007; Mauro *et al.*, 2013). In this study, 76 of the 323 *A. flavus* isolates did not produce detectable aflatoxins. The four provinces where the *A. flavus* strains were isolated are Guangdong (20.13 °N ~ 25.31 °N), Hubei (29.01 °N ~ 33.60 °N), Shandong (34.23 °N ~ 38.24 °N), and Liaoning (38.43 °N ~ 43.26 °N). With the decrease in temperature and increase in latitude in these four provinces (Guangdong, Hubei, Shandong and Liaoning), the percentage of atoxigenic isolates gradually increases (Guangdong: 12.1%, Hubei: 23.2%, Shandong: 39.7% and Liaoning: 44.4%) indicating that the atoxigenic *A. flavus* incidence decreases with the increase in temperature and decrease in latitude. However, for *A. flavus* isolates, an opposite correlation for temperature and latitude with the strain incidence was determined (Cotty, 1997), suggesting that toxigenic *A. flavus* strains are more likely to be

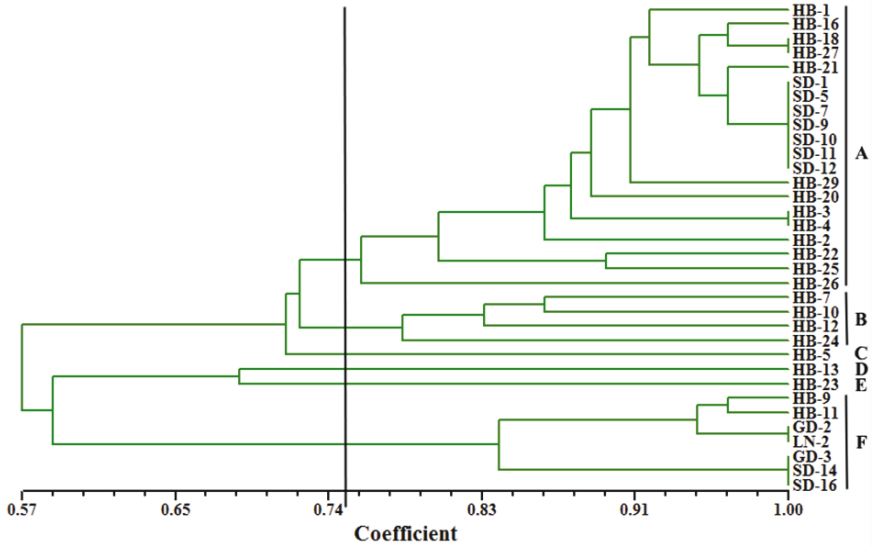

Fig. 3. Cluster analysis of the gene deletions in the aflatoxin gene cluster for the 34 *A. flavus* isolates collected from different provinces in China. The isolates coded with HB, GD, SD, and LN were collected from peanut-cropped soil from Hubei, Guangdong, Shandong and Liaoning provinces, respectively.

isolated from regions with high temperature and low latitude compared to atoxigenic *A. flavus* strains.

Although, the phenomenon of atoxigenic strain isolation is not rare, the mechanisms of losing aflatoxin producing ability for atoxigenic *A. flavus* strains are not entirely clear (Schmidt-Heydt *et al*., 2008). In many cases, there were partial or even entire deletions in the aflatoxin gene cluster in the atoxigenic *A. flavus* isolates. Chang *et al.* (2005) analyzed the presence of 25 genes in the aflatoxin gene cluster of 38 atoxigenic *A. flavus* isolates, and found 8 different deletion patterns. Yin *et al.* (2009) found 5 different deletion patterns by amplifying 11 genes in the aflatoxin gene cluster of 11 atoxigenic *A. flavus* isolates. Donner *et al.* (2010) found 9 different deletion patterns by amplifying 21 genes in the aflatoxin gene cluster of 21 atoxigenic *A. flavus* isolates. In our present study, all of the aflatoxin genes (29 genes) in the aflatoxin gene cluster were analyzed by amplifying 28 fragments, two genes, *norB* and *cypA*, were amplified together using a pair of primers (*norB-cypA*-F/*norB-cypA*-R). The PCR results demonstrated that there were 25 different deletion patterns in the 76 atoxigenic isolates, among which three deletion patterns (A, T, and U) were also detected in previous studies (Chang *et al*., 2005; Yin *et al*., 2009). The remaining 22 deletion patterns (Fig. 1) were identified for the first time. Among all the deletion patterns, the strains from Hubei (Central China) possessed 22 deletion patterns (A, B, D-T, V-W), suggesting that Hubei (Central China) is rich in varieties of atoxigenic strains. Among the 76 atoxigenic strains, 34 strains were found to have gene deletions with 10 isolates having more than 10 genes (over 1/3 of the aflatoxin genes) deleted (Patterns S-Y, Fig. 1 and Table 2). Atoxigenic *A. flavus* strains with gene deletions, especially large deletions, in the aflatoxin gene cluster have been proposed for use in aflatoxin control by competing for the niches occupied by toxigenic strains (Dorner, 2004). Therefore, the 34 atoxigenic strains, especially the 10 isolates with large deletions mentioned above, could be candidates for controlling aflatoxin contamination.

In our study, the four aflatoxin genes of *aflT*, *nor-1*, *aflR*, and *hypB* were found to be most likely deleted in these strains. In total, 97% (33 out of 34) of the atoxigenic strains with gene deletions isolated in this study had at least one of these four genes deleted (Table 2 and Fig. 1), indicating that these four genes will be possible marker genes for the initial, rapid and large-scale identification of atoxigenic strains. *AflT* plays a role in the aflatoxin secretion process, encoding a membrane-bound protein homologous to antibiotic efflux genes (Yu *et al*., 2004a). *Nor-1* (*AflD*) encodes a ketoreductase, which is essential for converting 1'-keto group in norsolorinic acid (NOR) to the 1'-hydroxyl group of averantin (AVN) (Trail *et al*., 1994; Zhou and Linz, 1999). *AflR* encodes a 47-kDa sequence-specific zinc-finger DNA-binding protein and is the key regulator in aflatoxin biosynthesis, required for the transcriptional activation of most of all the structural genes in the aflatoxin gene cluster (Woloshuk *et al*., 1994; Chang *et al*., 1999; Price *et al*., 2006). *HypB*, a hypothetical gene, is assumed to be involved in one of the oxidation steps in the conversion of O-methylsterigmatocystin (OMST) to aflatoxins (Yu *et al*., 2004b; Ehrlich, 2009). Previously, it was also suggested that atoxigenic strains could be identified by detecting the presence of aflatoxin genes (Criseo *et al*., 2001; Mayer *et al*., 2003; Yin *et al*., 2010). The genes used in these studies have some overlap with the results of our study (*aflT*, *nor-1*, *aflR*, and *hypB*). However, the combination of these four genes, especially *hypB*, used for identifying atoxigenic strains, was first proposed in our study

In conclusion, 76 *A. flavus* strains collected from peanut fields of the main peanut-producing provinces in China were found not to produce detectable aflatoxins, of which 34 strains were found to have deletions in the aflatoxin gene cluster. The atoxigenic *A. flavus* strains with gene deletions, especially the 10 isolates with large deletions, could be exploited as the agents for controlling aflatoxin contamination. Among the deleted genes, *aflT*, *nor-1*, *aflR*, and *hypB*, were those most frequently deleted, suggesting that they could be used as marker genes for the rapid identification of atoxigenic strains.

Acknowledgements

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