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Identification of *Aspergillus flavus* Isolates for Developing Biocontrol Agent Based on the Gene-Defects in the Aflatoxin Biosynthesis Gene-Cluster and Flanking-Regions

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***Aspergillus flavus* is distributed throughout the world, but are more common in warm climate zones, thus the risk of infection is more in hot and dry weather conditions like that of Gujarat. We investigated defects in the aflatoxin gene cluster in 81 non-aflatoxigenic *A. flavus* isolates collected from different peanut growing fields of Gujarat state (India). PCR assays using aflatoxin-gene-specific primers grouped these isolates into 53 deletion patterns and three groups. It was revealed that 84% (group 2) and 11% (group 3) of the non-aflatoxigenic isolates had 1-7 and 19-27 gene-defects respectively. However 5% (group 1) of the non-aflatoxigenic isolates were found to have no gene-defects. No isolate was found defective for all the genes of aflatoxin biosynthesis gene cluster and flanking region. Thus, deletions in the gene-cluster among non-aflatoxigenic *A. flavus* isolates are not unusual, and the deletion patterns were quite diverse. Screening of isolates using gene specific PCR was found quite effective for the identification of gene-defects in *A. flavus*. Four most defective, group 3 isolates were identified, which can be used as bio-control agents in the form of "cocktails" for the Indian peanut growing areas.**

Key words: Aflatoxins, biocontrol strategy, gene-specific PCR, Pathway regulators, sugar utilization genes.

A. flavus is economically very important because it produces hepato-carcinogenic secondary metabolite aflatoxin[1]. Aflatoxins (AFs), a group of polyketide-derived furanocoumarins, were discovered in *A. flavus* about 40 years ago after an outbreak of Turkey X disease in England[2]. AFs contamination was more prevalent during times of high heat and drought, which may stress the host plant thereby facilitating *A. flavus* infection[3,4]. Currently, incidences of AF contamination of crops are limited to tropical and sub-tropical areas (between latitudes 40 °N and 40 °S) around the world[5] which also covers the major peanut growing area of the world[6].

AFs infestation continues to be a potential threat to food-, feed- and consumer-safety and to the world export markets[1,7]. Maximum levels of AFs have been set at levels below 20 ppb by most countries but allowable threshold levels may vary[8,9]. Although *A. flavus* infection does not affect the peanut yield significantly, but AF contamination leads to decrease in quality, thus incurs economic losses[1]. Development and maturation of peanut pods occurs inside the soil, therefore *A. flavus* might infect the pods or seeds at pre-harvest, during harvest or post-harvest stages[10].

AF biosynthesis involves complex inter-connecting network of pathway specific regulators,

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global regulators as well as some environmental and cultural factors[11, 12]. In *A. flavus* and *A. parasiticus*, the AF pathway genes were clustered within a 70-80 kb region on chromosome III nearly 80 kb away from telomere region[12, 13]. This pathway have been extensively studied and at least 27 enzymatic steps have been characterized or proposed to be involved in bioconversion of intermediates to AFs[12, 14]. The genes, their enzymes and pathway involvement are summarized in Fig. 1.

A. flavus is an assorted assemblage of strains which include aflatoxigenic and non-aflatoxigenic strains with cosmopolitan distribution. A very promising strategy currently being used for the reduction of pre-harvest AF contamination is the introduction of non-aflatoxigenic *A. flavus* strains into the crop environment[15]. Moreover, there are evidences that the isolates initially found non-aflatoxigenic, may produce AF when exposed to different environmental conditions[12]. Therefore, it is crucial to identify non-aflatoxigenic isolates at genetic level for their future use as region specific bio-control agent.

Non-aflatoxigenic isolates can be identified by PCR using primers targeting different structural and transcription regulatory genes of AF biosynthesis pathway[13]. Detail study of whole AF gene cluster and flanking regions was conducted by Chang *et al*.[16] in order to be sure about the non-aflatoxigenicity of *A. flavus* isolates along with their reasons.

A total of 81 non-toxigenic and 5 toxigenic isolates, collected from different groundnut growing regions of Gujarat (India) which were previously analysed for aflatoxigenicity[17], were taken for the present investigation with following objectives:

i. Identification of defects in AF gene-cluster and flanking-regions in the *A. flavus* isolates using 32 gene-specific primers.
ii. Identification of most suitable non-aflatoxigenic *A. flavus* isolates for its use as biocontrol agents.

MATERIALS AND METHODS

Fungal Population and Mycelial Collection

The *A. flavus* cultures used in this study were previously isolated from the farmers' fields (under peanut based cropping system) from 09 districts of Gujarat state, India and characterized for aflatoxigenicity. These isolates were maintained by the Crop Protection Division of Directorate of Groundnut Research, Junagadh, India. Out of 86 selected isolates (Table 1), isolate ID 1 to 81 were reported as non-aflatoxigenic, whereas 05 aflatoxigenic isolates (ID 82-86) were used as positive control[17]. All the isolates were grown on potato dextrose agar (PDA) slant cultures for genomic DNA isolation. Conidia were harvested from 7- days old slant cultures, grown at 28 ^{o}C and inoculated into 50 ml Yeast extract-Peptone-Dextrose broth and incubated at 25 ^{o}C (48-72 h) with shaking (150 rpm). After proper growth, the mycelial suspension was filtered through a Buchner funnel with sterile Whatman No. 1 filter paper. Mycelia was rinsed twice with sterile distilled water and properly packed using aluminum foil followed by freezing at -80 ^{0}C.

Fungal DNA Isolation

Frozen mycelia (50 mg) is added in pre-heated 600 µL of extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA and 2% SDS) and grounded using Fastprep24 grinder (MP Biomedicals, India) then incubated in a water bath (65 °C for 1 h) with occasional shaking. DNA was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) and precipitated with 0.7 volume of chilled ethanol and vacuum dried. Finally, TE Buffer (pH=8.0) was used to re-dissolve the pellets and treated with RNaseA (37 ^{o}C for 1.5 h) to make it RNA free[18]. DNA concentrations and purity was determined by taking absorbance at 260 and 280 nm using NanoDrop, and integrity of DNA was examined on agarose gel (0.8%).

PCR Reactions

The *A. flavus* isolates were screened using 32 gene specific primer pairs (Table 2, Fig. 1) of Chang *et al.*[16], targeting different AF gene cluster and flanking regions using polymerase chain reaction (PCR) to identify the defects if any. The PCR mixtures (10 µl) contained 2 µl template DNA (10 ng), 1 µl of 10x Taq buffer+$MgCl_2$ (15 mM), 1.0 µl of dNTP (2 mM), 0.4 µl of primers (25 p moles each, Forward and Reverse), 0.13 µl of Taq polymerase (Genei 3U) and final volume was made using sterile double distilled water. Amplification

was performed in 0.2 ml (each tube) thin walled PCR plates (96 wells plate^{-1}) in a thermal cycler (Eppendorf).

The samples were initially incubated at 94.0 ºC for 2 min followed by 5 cycles of 94 ºC for 60 s, 60-55 ºC for 60 s (1.0 ºC reduction per cycle) and 72 ºC for 2 min. This was followed by another 35 cycles of 94 ºC for 60 s, 55 ºC for 60 s and 72 ºC for 2 min. Final Extension was done at 72 ºC for 10 min and then holding at 4°C indefinitely. Amplified product (10 µl) was then mixed with 3 µl of 6x loading dye (Fermentas) and analyzed using 1.2% Agarose gel at constant power 100 volts (2.0-2.5 h) and stained with Ethidium Bromide. The amplification was documented using automated gel documentation system (Fujifilm FLA-5000). Gel scoring and analysis was done using the software Gel Compare II (Applied Maths, Kortrijk, Belgium). Amplification for each marker was performed twice independently to ensure the fidelity.

Amplicons Size Comparison and Identification of Gene-Defects

In NCBI, Nucleotide BLAST was performed using the sequence of forward primer of all the 32 genes so as to find out the GenBank IDs which are then downloaded and *in silico* PCR was performed with appropriate primer sets using the software 'Sequence Manipulation Suite'. Further, the size of amplified fragments were recorded and compared with the amplicons size obtained in the present study. From the gel pictures, band size obtained for each primer-set was measured using 'AlphaEaseFC' software. Compared to the control, for non-aflatoxigenic isolates, either absence of amplification or difference in the amplicons sizes over expected-size was considered as defect in the gene.

RESULTS AND DISCUSSION

Gene-defect Pattern and Grouping of Isolates

Very diverse gene-specific PCR amplification pattern was obtained for 81 non-aflatoxigenic isolates (Fig. 1). Absence of gene-specific amplification in certain *A. flavus* isolates may be due to some defect(s) in the gene(s)[16]. Four non-aflatoxigenic isolates did not show any defect in the genes studied, while none of the isolates were found to have all the pathway genes with defects. A maximum of 27 gene-defects was recorded in isolate number 50 (NRCG 06019), out of 32 genes screened. Similarly Chang *et al*.[16] also showed that in non-aflatoxigenic *A. flavus* isolates, deletion of a part or the entire AF gene cluster is not rare and the resulting patterns are diverse. In other closely relates species (*A. oryzae* and *A. sojae*) also AF has not been detected and they were also found defective for several AF biosynthesis pathway genes[19, 20, 21]. Kusumoto *et al*.[19] also recorded deletions in the AF gene cluster in some *A. oryzae* isolates and categorized them different groups.

However, in the present investigation, 53 types of gene-defects patterns were observed which are broadly classified into three groups (Fig. 2, Table 3). This scenario is different from the directional deletion proposed for closely related *A. oryzae* isolates[19]. Similarly, loss of AF-producing ability in *A. flavus* 649-1 has been suggested to be associated with a large deletion in the AF gene cluster[22]. The amplicon size of *norB-cypA* gene region is found to be 856 bp (type II deletion) and 856 or 295 bp (type I deletion)[16]. However in 11 non-aflatoxigenic isolates this region was not amplified (Fig. 2) which could be due to some other deletion or because of absence of primer binding site. This needs further confirmation.

It has been shown that the populations of *A. flavus* in any agricultural environment do contain abundant numbers of non-aflatoxigenic isolates[23]. Genetic drift may be a driving force for the loss of the AF gene cluster in non-aflatoxigenic *A. flavus* isolates when AFs have lost their adaptive value in nature[16]. Moreover larger effective population sizes also tend to increase mean population mutation and recombination rates which may lead to the evolution of new VCGs, with lost AF-producing ability[24]. Chang *et al*.[16] also determined sequence breakpoints associated with various deletions in the AF gene cluster of *A. flavus* isolates.

Grouping of Isolates Based on Gene-Defects

Based on number of defective genes, the non-aflatoxigenic isolates were broadly classified in to three groups i.e. Group 1 (no defects), 2 (1-7 defects) and 3 (>7 defects), details of which are discussed in the following section.

Table 1. Collection site details and number of defects in the *Aspergillus flavus* isolates used in study

ID/NRCG Acc. No.	District/ Taluka*	Latitude; Longitude**	No. of defects	ID/NRCG Acc. No.	District/ Taluka*	Latitude; Longitude**	No. of defects	ID/NRCG Acc. No.	District/ Taluka*	Latitude; Longitude**	No. of defects
1/1009	Junagadh/ Manavadar	21.507499; 70.121098	2	30/2029	Amreli/ Amreli	21.583711; 71.188662	3	59/8016	Surendranagar/ Vadhavan	22.708423; 71.619959	2
2/1012	Junagadh/ Keshod	21.310246; 70.230316	2	31/2031	Amreli/ Rajula	21.040888; 71.450143	4	60/8018	Surendranagar/ Vadhavan	22.699554; 71.648111	2
3/1018	Junagadh/ Keshod	21.310006; 70.226669	1	32/2039	Amreli/ Kunkavav	21.419473; 71.451602	1	61/8019	Surendranagar/ Lakhatar	22.855178; 71.79913	1
4/1026	Junagadh/ Maliyahatina	21.172567; 70.281086	4	33/3005	Bhuj/ Nakhatrana	23.347142; 69.258842	1	62/8021	Surendranagar/ Lakhatar	22.864629; 71.788445	2
5/1036	Junagadh/ Mangrol	21.136466; 70.113115	3	34/3007	Bhuj/ Nakhatrana	23.348403; 69.257813	1	63/10001	Junagadh/ Keshod	21.283536; 70.236969	1
6/1038	Junagadh/ Mangrol	21.121695; 70.110927	6	35/3024	Bhuj/ Bhuj	23.233302; 69.672804	4	64/10004	Junagadh/ Veraval	20.93122; 70.371552	5
7/1040	Junagadh/ Visavadar	21.348863; 70.733929	2	36/3028	Bhuj/ Mandavi	21.675974; 71.625731	5	65/10005	Junagadh/ Veraval	20.910295; 70.40082	2
8/1041	Junagadh/ Talala	21.032196; 70.527163	1	37/3029	Bhuj/ Mandavi	21.675675; 71.619551	5	66/10006	Junagadh/ Veraval	20.92673; 70.370436	1
9/1045	Junagadh/ Kodinar	20.798084; 70.686293	25	38/5016	Bhavnagar/ Talaja	21.350621; 72.050378	1	67/10007	Junagadh/ Kodinar	20.797723; 70.704703	2
10/1046	Junagadh/ Una	20.814773; 71.012621	1	39/5020	Bhavnagar/ Talaja	21.358095; 72.036109	2	68/1037	Junagadh/ Mangrol	21.121294; 70.110197	1
11/1047	Junagadh/ Una	20.820007; 71.044292	0	40/5025	Bhavnagar/ Mahuva	21.072447; 71.767759	1	69/2019	Amreli/ Dhari	21.332234; 71.035023	1
12/1048	Junagadh/ Una	20.819927; 71.044035	0	41/5028	Bhavnagar/ Mahuva	21.072487; 71.753082	1	70/2028	Amreli/ Rajula	21.032997; 71.454134	1
13/1049	Junagadh/ Visavadar	21.350302; 70.766459	1	42/5029	Bhavnagar/ Mahuva	21.072727; 71.747761	1	71/3015	Bhuj/ Anjar	23.264197; 69.689058	1
14/1051	Junagadh/ Talala	21.031242; 70.524802	2	43/5033	Bhavnagar/ Mahuva	21.089385; 71.740294	3	72/3019	Bhuj/ Anjar	23.108855; 70.018991	1
15/1052	Junagadh/ Talala	21.055066; 70.531754	2	44/5036	Bhavnagar/ Talaja	21.349432; 72.044821	2	73/3020	Bhuj/ Anjar	23.108796; 70.020279	1
16/1058	Junagadh/ Manavadar	21.531334; 70.144958	3	45/6006	Sabar Kantha/ Talod	23.357478; 72.958403	22	74/3025	Bhuj/ Bhuj	23.232395; 69.671259	1

17/1059	Junagadh/ Manavadar	21.497557; 70.137835	3	46/6007	Sabar Kantha/ Talod	23.361404; 72.954712	23	75/6005	SabarKantha/ Talod	23.343832; 72.935143	4
18/1060	Junagadh/ Manavadar	21.501719; 70.110971	4	47/6013	Sabar Kantha/ Talod	23.361286; 72.934842	2	76/2026	Amreli/ Rajula	21.04261; 71.453447	5
19/1062	Porbandar/ Porbandar	21.665405; 69.663448	4	48/6015	Sabar Kantha/ Talod	23.359119; 72.936859	7	77/2027	Amreli/ Rajula	21.044012; 71.454649	20
20/1064	Porbandar/ Porbandar	21.645941; 69.700012	3	49/6016	Sabar Kantha/ Talod	23.355731; 72.93364	2	78/2033	Amreli/ Amreli	21.61642; 71.203938	19
21/1065	Junagadh/ Mangrol	21.115572; 70.102043	3	50/6019	Sabar Kantha/ Talod	23.34659; 72.934713	27	79/2036	Amreli/ Amreli	21.621048; 71.216984	24
22/1067	Junagadh/ Mangrol	21.138547; 70.117922	3	51/7002	Jamnagar/ Kalavad	22.206438; 70.37065	3	80/3035	Bhuj/ Mandavi	21.682993; 71.628799	20
23/1069	Junagadh/ Vanthali	21.462015; 70.305805	2	52/8002	Surendranagar/ Surendranagar	22.701296; 71.616268	1	81/3036	Bhuj/ Anjar	23.107582; 70.021641	22
24/1077	Junagadh/ DGR Farm	21.482253; 70.440828	2	53/8003	Surendranagar/ Surendranagar	22.687835; 71.606483	5	82/4005	Anand/ Umreth	22.698129; 73.122232	0
25/2002	Amreli/ Bagasra	21.488972; 70.949573	2	54/8004	Surendranagar/ Surendranagar	22.692982; 71.608286	1	83/04010	Anand/ AAU	22.529405, 72.971535	0
26/2011	Amreli/ Amreli	21.577156; 71.239815	2	55/8008	Surendranagar/ Sayala	22.535767; 71.477695	2	84/06003	SabarKantha/ Talod	23.351121, 72.966042	0
27/2014	Amreli/ Dhari	21.320001; 71.034079	0	56/8009	Surendranagar/ Chuda	22.480213; 71.678367	3	85/06012	SabarKantha/ Talod	23.364123, 72.937416	0
28/2017	Amreli/ Dhari	21.320962; 71.037855	0	57/8011	Surendranagar/ Chuda	22.484208; 71.682744	2	86/05011	Bhavnagar/ Talaja	21.352142, 72.044864	0
29/2024	Amreli/ Amreli	21.569812; 71.207371	2	58/8013	Surendranagar/ Lakhatar	22.85585; 71.798186	1				

*Taluka is also known as *tahsil,* or *mandal,* is an administrative division in some countries of South Asia including India. **The latitude (N) and longitude (E) coordinates are presented in decimal-decimal or numerical form.

Table 2. Details of primer sets (Chang *et al*. [16]) and amplicons size comparison between PCR and *in silico* PCR for various aflatoxin producing genes and its flanking regions

Designation	Forward primer (5'-3')	Reverse primer (5'-3')	PCR amplicon size (bp)	*In silico* amplicon size (bp)
C1	CGTTCCAGTAGTTCGTATCG	CATCGTAAACGTTGACACAG	614	618
C2	TCGCCTTGTTCTCGCTATAC	ACACCTGATAGCGAGAGTTC	674	677
C3	GCGATCTGTAACACTACACA	GCCATACGATTCCCAAGTCT	633	642
alfF-alfU (norB-cypA)	GTGCCCAGCATCTTGGTCCA	AGGACTTGATGATTCCTCGTC	856, 295	1840
aflT	ATGACATGCTAATCGACGAG	AGGCGCATGCTACGGATC	1138	1141, 867
alfC (pksA/pksL)	ACTTTGAGGGCGTTCTGTGC	CTTTCGGTGGTCGGTGATTC	494	515
alfD (nor1)	AGCACGATCAAGAGAGGCTC	GATCTCAACTCCCCTGGTAG	356	-
alfA (fas2/hexA)	TCCTATCCAGTCCACCTCGTA	CACATCTTTGTCTTGCCCGC	664	663
alfB (fas1/hexB)	ACAATCGAATGACAACACTGC	CCACCGAATCCACTACCTACA	581	580
aflR	ATGGTCGTCCTTATCGTTCTC	CCATGACAAAGACGGATCC	627	627
alfS (aflJ)	CTTCAACAACGACCCAAGGTT	AGATGAGATACACTGCCGCA	420	436, 376
alfH (adhA)	CCTCGTGGGAGAGCCAAATC	GGAGCAAGAAGGTTACAGCG	413	433
alfJ (estA)	CGATGGGACTGACGGTGATT	ACCACGCCGCTGACTTTAT	521	530
alfE (norA)	GTGTTCGTGTGTCGCCCTTA	GTCGGTGCTTCTCATCCTGA	750	771, 714
alfM (ver-1)	CATCGGTGCTGCCATCGC	CCTCGTCTACCTGCTCATCG	622	643
alfN (verA)	CCGCAACACCACAAGTAGCA	AAACGCTCTCCAGGCACCTT	407	424
alfG (avnA)	GCGATAGAACTGACAAAGGCA	GAATGAGTCTCCAAAGGCGAG	518	541
alfL (verB)	TTCAGTGACAAAGGTCTTCGC	GGCAGCGTT ATTGAGCATCT	454	474
alfI (avfA)	ATTCAAATCCTCGTTCGGTCG	TAGCCCGTTGGTTGTGTTCC	485	491
alfO (omtB/dmtA)	ACAGACGATGTGGGCAAACG	ACGCAGTCCTTGTTAGAGGTG	610	613, 440
alfP (omtA)	CAGGATATCATTGTGGACGG	CTCCTCTACCAGTGGCTTCG	595	594, 423
alfQ (ordA)	AAGGCAGCGGAATACAAGCG	ACAAGGGCGTCAATAAAGGGT	396	411, 410, 353
alfK (vbs)	AACGAGCAGCGTAAGGGTCT	TCAGCCAGAGCATACACAGTG	632	629
alfV (cypX)	GGAGCCTACCATTCGCAACA	GGCTTTGACGAACAGATTCCG	396	394
alfW (moxY)	TGCTACTGGAACGAAGACCG	CGACGACAACCAAACGCAA	592	599
alfX (ordB)	GCTGCTACTGGAATGAAGACC	ATGCGACGACAACCAAACG	600	604
alfY (hypA)	CGCAAGACGGCAGAGATACT	GCTCCTTCAGTTCCACACCA	583	587
nadA	TGACGAGGCCTGCGAGCTGT	AAGCCTCTTCAGAACGGTCA	559	570
hxtA	TGTCCTCACCTCTGGCGTAT	AGACCAACCACTCTTATGGGC	676	684
glcA	AGACACAGTCATCGCCTGTT	GGTGCGAATAGGTGCAGGTA	661	660
sugR	TCAGCTGAAGCGCTCGAGAG	GTATTGCCGCACTATGTATG	592	600
C4	ATCGTGCAGACAGGAACAC	GGTGCCTTGGCCTATGCGCT	583	-

(-) No amplification

Table 3. Classification of non-aflatoxigenic *A. flavus* isolates based on number of defective genes

Groups	Defective genes	Number of isolates	Accession numbers
Group 1	0	4(5%)	01047, 01048, 02014, 02017
Group 2	1-7	68(84%)	01009, 01012, 01036, 01026, 01038, 01018, 01040, 01046, 01049, 01051, 01052, 01058, 01059, 01059, 01060, 01062, 01063, 01064, 01065, 01067, 01069, 01077, 02002, 02011, 02024, 02029, 02031, 02039, 03005, 03007, 03024, 03028, 03029, 05016, 05020, 05025, 05028, 05029, 05033, 05036, 06013, 06015, 06016, 07002, 08002, 08003, 08004, 08008, 08009, 08011, 08013, 08016, 08018, 08019, 08021, 10001, 10004, 10005, 10006, 10007, 01037, 02019, 02028, 03015, 03019, 03020, 03025, 06005, 02026
Group 3	19-27	9(11%)	01045, 08005, 06007, 06019, 02027, 02033, 02036, 03035, 03036

* Values in the parenthesis are percent of total number of isolates studied

Group 1

As expected, five toxigenic isolates (ID No. 82-86) were part of this group, where no gene defect was observed (Table 3). However, four non-toxigenic isolates [ID No. 11, 12 (from Amreli district), 27, and 28 (from Dhari district)] which accounts for the 5% of total isolates, were also found to have no gene-defects in the AF gene cluster and in its flanking regions (Table 1; Fig. 2). As we know, the AF biosynthetic pathway have been confirmed through either gene disruption or enzymatic studies and details of several biological conversion steps and genes responsible have not yet been deciphered[25]. Woloshuk *et al*.[26] reported highest levels of AF production when fungus invades the seed embryo, where the highest concentrations of simple sugars are present[26].

The genomes of several *Aspergillus* species have recently been sequenced and it is speculated that other global regulators controlling AF biosynthesis will be uncovered[11]. Possibility of genes located outside the AF biosynthetic clusters, regulating AF biosynthesis cannot be ruled out. Environmentally relevant volatiles (i.e. ethylene and crotyl alcohol) were also known to act as probable candidates regulating AF biosynthesis, potentially independent of *aflR*[27]. All or some of these factors could be the reason(s) for the non-aflatoxigenicity of the isolates having intact AF pathway cluster genes and flanking regions. This needs further confirmation based on in-depth gene expression studies.

Group 2

Isolates having 1 to 7 defective cluster gene(s) and flanking regions were pooled together to form Group 2. Under this category, 68 (84%) isolates were grouped (Fig 2). The amplification pattern within this group was very diverse with randomly distributed gene-defects. Previous reports also suggests that the loss of AFs production in many *A. flavus* isolates is probably caused by point mutations[28] or small deletions in AF pathway genes[29]. Since chances of reversion are quite high if defects were less, therefore it is better not to use these isolates for the development of biocontrol agents.

Group 3

Nine isolates (11%) which were found defective for more than 7 (19 to 27) genes are classified as group 3 isolates (Table 3). Isolate with maximum number of defective genes (27 genes and flanking regions) is NRCG 06019 (Fig. 2). It was quite interesting to note that in the isolates studied, out of 32 genes and flanking regions analyzed, we did not found defects in the range of 8-18 or >27 genes and flanking regions. As of now it is not possible to explain the reasons why defects were not normally distributed among all the AF biosynthetic pathway genes and the isolates studied. Isolates of this group could be the better choice for the development of bio-control agents.

Defects in the AF Biosynthesis Pathway Genes

For *aflA*, *aflB*, and *aflC* genes (acetate NOR), 17, 09 and 06 isolates respectively, were

found defective and isolate number 50 is found defective for all the three genes. The biocontrol agents, Afla-Guard® lacks genes from *aflA* to the telomeric region[16] whereas; AF36 is found defective for *aflC* gene[30]. It means isolates defective for these genes may be the potential candidates for selection as biocontrol agents.

aflD, *aflE*, and *aflF* are required for the conversion of NOR AVN whereas *aflU* is most likely required for OMST AFG1 and DHOMST AFG2 conversion. However, *aflE* and *aflF* is reported to be often non-functional in *A. flavus* due to deletions[28]. Yu *et al*.[31] also observed that the disruption of *aflE* or *aflF* did not severely influence AF production. But Ehrlich *et al*.[32] reported the involvement of *aflE* in the final two steps in AFB1 formation and *aflF* in AFG1/AFG2 formation.

Based on PCR analysis, 69, 10 and 11 isolates were found defective for *aflD*, *aflE* and *aflF-aflU* genes respectively. Of these, eight isolates (group 3) were found to have all the three defects. However, in isolate number 15, two genes (*viz*. *aflE* and *aflC*) were found defective and still it exhibited non-toxic phenotype. Of all the genes studied *aflD* is found most defective (69 isolates) in the non-aflatoxigenic isolates studied. Further in depth analysis is required to find the reasons why this gene is so prone for the defects.

For *aflG* (AVN→HAVN) and *aflH* (HAVN→OAVN) genes, 06 and 09 isolates respectively, were found defective. But, the enzyme that converts OAVN→AVF has not yet been

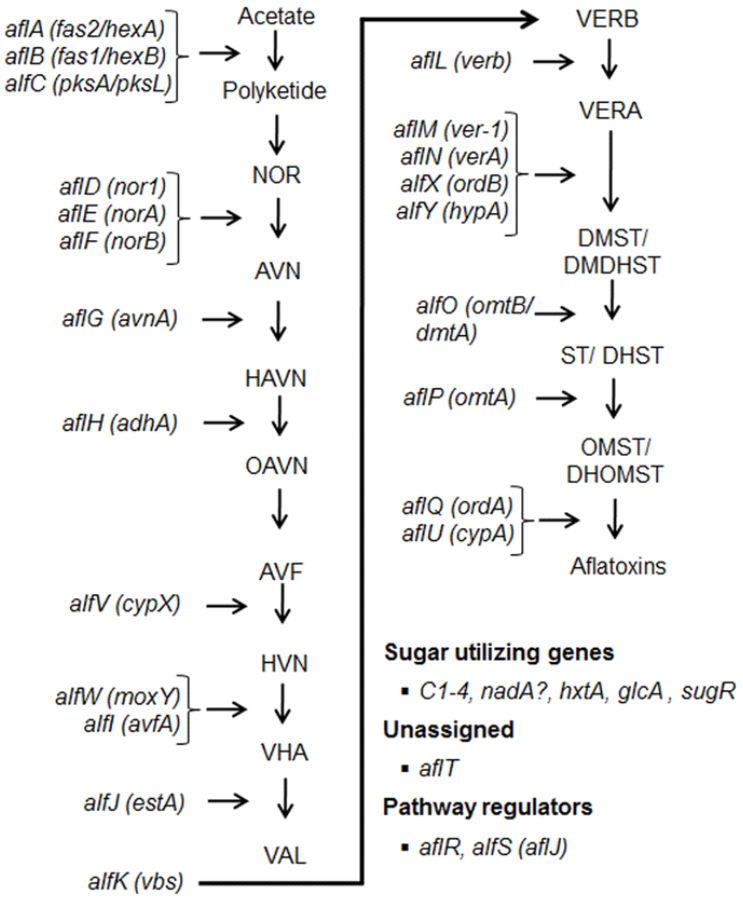

Fig. 1. The aflatoxin biosynthetic pathway. Modified from Yu *et al*.[31]; Do and Choi[43]. (AF B1, B2, G1, G2: aflatoxin B1, B2, G1, G2; AVF: Averufin; AVN: averantin; DHOMST: dihydro-O-methylsterigmatocystin; DHST: dihydrosterigmatocystin; DMDHST: demethyldihydrosterigmatocystin; DMST: demethylsterigmatocystin; HAVN: 5'-hydroxyaverantin; HVN: hydroxyl versicolorone; NOR: Norsolorinic acid; OAVN: oxoaverantin; OMST: O-methylsterigmatocystin; ST: sterigmatocystin; VAL: versiconal; VERA: versicolorinA; VERB: versicolorin B; VHA: versiconalhemiacetal acetate)

identified[25]. *aflI* is required for conversion of AVF→VHA through HVN. Although clear role *aflV* gene in AF biosynthesis is not known, but *aflI* and *aflV* gene products are involved in the ring-closure step in the formation of HVN[25, 33]. Similarly, *aflW* has proposed role in the conversion of HVN→VHA, but no conclusive role could be asserted yet[25]. A total of 09, 08 and 09 non-aflatoxigenic isolates were found defective for *aflI*, *aflV* and *aflW* genes respectively (Fig. 2). Similarly a chromosomal translocation within the *stcW* gene (*A. flavus moxY*) was reported in an echinocandin B-producing strain of *A. nidulans*, which does not produce sterigmatocystin[34].

For *aflJ* (VHA→VAL) gene, only two isolates *viz*. 50 and 79 (group 3), whereas for *aflK* (VAL→VERB) and *aflL* (VERB→VERA) genes, ten isolates each were found defective. *aflM* and *aflN* are involved in the conversion of VERA→DMST. Putatively *aflX* and *aflY* genes were also supposed to be associated in the conversion of VERA→DMST[25, 35]. A total of 11, 05, 09 and 13 isolates were found defective for *aflM*, *aflN*, *aflX* and *aflY* genes respectively. Due to the *aflR*-binding signatures in the promoter regions, *aflU*, *aflX* and *aflY* genes are supposed to be involved in AF formation[35].

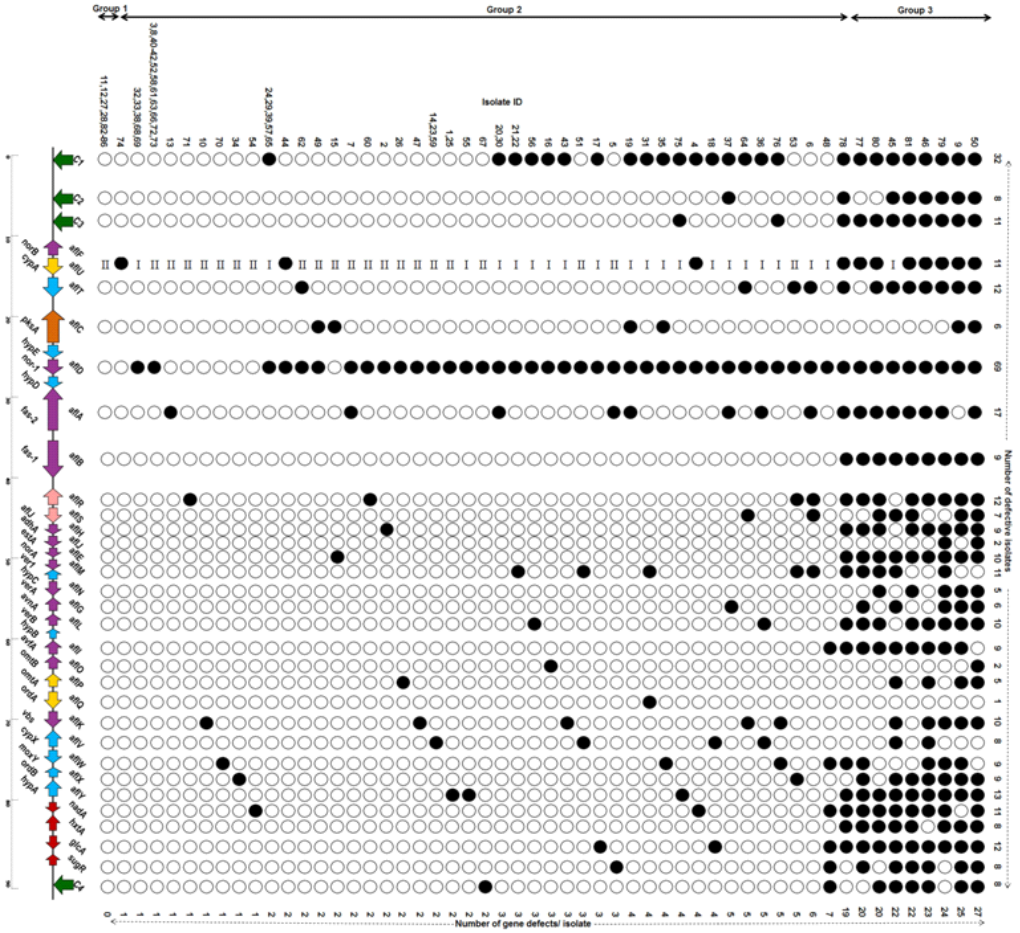

Fig. 2. PCR amplification pattern of 86 isolates for various aflatoxin producing genes and its flanking regions; Where white circles represent positive PCR products; black circles represent absence of PCR amplification, (-) and (=) represent a 295 and 856 bp PCR product (type of deletion) respectively. Schematic representation of AF gene cluster in *A. flavus*, which is located near the telomeric region of the chromosome number 3. The bar on the bottom represents the length in Kb and the direction of transcription is represented as arrows[1, 16, 31, 47]. The new names of each gene are presented on the top while the old names on the bottom

aflO is involved in the conversion of DMST→ST and of DMDHST→DHST whereas, *aflP* for ST→OMST and DMST→DHOMST and *aflQ* for OMST→AFB1/AFG1 and DMDHST→ AFB2/AFG2 conversion. It is interesting to note that, in the present investigation, five genes namely *aflJ*, *aflN*, *aflO*, *aflP* and *aflQ* were found least defective, since only 02, 05, 02, 05 and 01 isolates respectively were found defective for these genes (Fig. 2).

Defects in the AF Pathway Regulators

aflR and *aflS* genes, with their independent promoters were involved in the regulation of AF gene expression[11]. An absolute requirement for *aflS* in AF biosynthesis or activation of *aflR* is still doubtful[22, 36]. Of 81 isolates studied, 12 and 07 were found to be defective for *aflR* and *aflS* genes respectively. The non-toxigenic strain of *A. sojae*, was also reported to have a defective *aflR* gene in addition to other defects in the AF pathway structural genes[20]. Further deletion of *aflR* was recorded in 80 out of 210 strains of *A. oryzae*[37]. A number of mutations were also recorded in the *aflR* promoter region and in three ORFs (*aflT*, *norA*, and *verA*) in non-aflatoxigenic *A. oryzae*, strain RIB 40[28]. Keeping these in view, the strains having both gene defects (i.e. strain number 50, 09, 81, 80, and 06) could be the potential candidate for its use as bio-agent.

Defects in the Genes with Ambiguous or Unclear Role

The genes whose pathway involvements are ambiguous or remain unclear are *aflT, aflU, aflV, aflW*, *aflX*, and *aflY*[25]. Of these, except *aflT* other genes were putatively associated for different functions in the pathway which is already discussed. *aflT*, encodes a membrane-bound protein with homology to antibiotic efflux genes, presumed to be involved in AF secretion[38]. This gene was found independent of *aflR* or *aflS* regulation and partial deletion (in *A. oryzae* RIB 40) has shown a little share on the non-productivity of AF[25].

In this study, twelve non-aflatoxigenic isolates were found to be defective for this gene (Fig. 2). Similarly an alcohol dehydrogenase gene (*adh1*), in *A. flavus*, was found to express concurrently with AF pathway genes[39], however no further report is made on its role. Price *et al*.[40] indicated that *aflR* might control the expression of other genes outside the AF biosynthetic cluster (*hlyC* and *niiA*) with *aflR* binding sites. These reports do indicate the possibilities of presence of some more gene(s) which are directly or indirectly regulating the AF biosynthesis in *A. flavus*. This might be one of the reasons for the non-aflatoxigenicity of four group 1 isolates (Table 3).

Defects in the Sugar Utilization Genes

No *aflR*-binding motif was identified in the UTR of the seven sugar utilization genes (C1-4, *hxtA*, *glcA*, and *sugR*) that are found adjacent to the AF gene cluster[12, 40, 41]. Of these C1 gene is found to be most defective (32 isolates) which was followed by *glcA* (12 isolates), C3 (11 isolates), and *hxtA*, C2, *sugR* and C4 (8 isolates each) (Fig. 2). Although available information did not support the direct role of these genes in the AF biosynthesis, but indirect role of some or all of these genes cannot be ruled out.

More recently, the *nadA* gene was shown to be a member of the AF gene cluster[12] rather than belonging to the sugar utilization cluster[33]. Although no *aflR*-binding motif was identified, it was found to play a role in the formation of an intermediate named NADA between OMST and AFG1[41]. Eleven isolates were found to be defective for this gene (Fig. 2). Therefore, it is better to select those isolates which are not only defective for the known genes of AF biosynthetic pathway but also simultaneously defective for the sugar utilizing genes and genes with unassigned functions.

Defects in the *A. flavus* Strains and Biocontrol Strategies

Populations of *A. flavus* in many parts of the world vary considerably in the proportion of isolates that are aflatoxigenic and non-aflatoxigenic[17, 42]. Thus, fundamental knowledge of defects in the AF biosynthesis pathway genes in different native *A. flavus* strains could be useful for the development of biocontrol agents more adapted to specific agro-ecological system[43].

For the management of AF-producing fungi, two non-aflatoxigenic *A. flavus* isolates, Afla-Guard® and AF36, were already registered as biopesticides with the United States Environmental Protection Agency[42]. In peanut, a reduction in AFs to the tune of 96% and 75% respectively was recorded when non-aflatoxigenic agents, Afla-guard®[44] and AFCHG2[45] were used. Therefore, it was aimed to identify specific non-aflatoxigenic

strain(s) which can be used as biocontrol agents in Indian groundnut growing areas which is the second largest in the world.

Considering various gene defects, it is concluded that strain 50 is the most suitable for its use as biocontrol agent (Fig. 2). However, reports do suggest the use of competing fungi as 'cocktails' that include application of multiple strains of non-aflatoxigenic *A. flavus*[9]. In this study, a combination of group 3 isolates (especially 50, 09, 81 and 80) having not only maximum pathway gene defects but also regulatory gene (both *aflR* and *aflS*) defects may be the potential candidate for the development of 'cocktails' (Fig. 2). The extensive deletions identified in the AF gene cluster nonetheless, serve as a safeguard in preventing adverse genetic reversion or recombination[16].

In this study, PCR based screening of non-toxigenic isolates seems quite promising for identifying the isolates with specific AF biosynthesis gene-cluster defects. Deletion of the entire AF gene cluster may be the ultimate consequence in *A. flavus* isolates that no longer produce AFs[46]. Although no single strain was identified having all the gene defects. But the non-aflatoxigenic strains identified, with maximum gene defects will be the prospective biocontrol agents for the long-term protection of crops against AF contamination. However, caution should be taken to prevent undue crop damage or damage to the soil micro-flora.

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