Identification of genetic defects in the atoxigenic biocontrol strain Aspergillus flavus K49 reveals the presence of a competitive recombinant group in field populations

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Contamination of corn, cotton, peanuts and tree nuts by aflatoxins is a severe economic burden for growers. A current biocontrol strategy is to use non-atoxigenic Aspergillus flavus strains to competitively exclude field toxigenic Aspergillus species. A. flavus K49 does not produce aflatoxins and cyclopiazonic acid (CPA) and is currently being tested in corn-growing fields in Mississippi. We found that its lack of production of aflatoxins and CPA resulted from single nucleotide mutations in the polyketide synthase gene and hybrid polyketide-nonribosomal peptide synthase gene, respectively. Furthermore, based on single nucleotide polymorphisms of the aflatoxin biosynthesis omrA gene and the CPA biosynthesis dmaT gene, we conclude that K49, AF36 and previously characterized TX9-8 form a biocontrol group. These isolates appear to be derived from recombinants of typical large and small sclerotial morphotype strains. This finding provides an easy way to select future biocontrol strains from the reservoir of non-atoxigenic populations in agricultural fields.

1. Introduction

Aflatoxins, a group of related toxic and carcinogenic bis-furan metabolites, are produced by Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius and a few other aspergilli (Varga et al., 2010). Contamination of crops such as corn, cotton, peanuts, and tree nuts by aflatoxins represents a significant global risk to human and animal health and can cause devastating economic losses due to strict regulatory limits placed on dissemination of contaminated products (Liu and Wu, 2010). Ingestion of high doses of aflatoxins can cause acute aflatoxicosis, and aflatoxin B1 is an established etiological agent of human liver cancer in many underdeveloped countries. Regulation setting limits of allowable levels of total aflatoxin and aflatoxin B1 in foods and feeds is in place in most developed countries (van Egmond et al., 2007). Strategies to prevent preharvest aflatoxin contamination of susceptible crops include introduction of non-atoxigenic A. flavus strains to competitively exclude toxigenic aspergilli. This strategy has shown much promise for control of aflatoxin contamination in corn, peanuts and cottonseed (Abbas et al., 2006; Cotty, 1994; Dorner, 2009). The mechanism by which a non-atoxigenic strain interferes with aflatoxin accumulation of toxigenic strains has not been definitively elucidated (Huang et al., 2011; Mehl and Cotty, 2011).

To date, three non-atoxigenic A. flavus strains have been confirmed to reduce aflatoxin contamination of crops in the United States. AF36 (ATCC 96045) isolated in Arizona (Cotty, 1994) was the first to obtain full Section three registration from the U.S. Environmental Protection Agency (EPA) (Cotty et al., 2007). This biocontrol strain has been applied on cotton in Arizona and on corn in Texas for years; it is also being tested on pistachio in California (Robens, 2008). AF36 has a defect in the early aflatoxin biosynthesis gene, pksA, and is unable to produce the polyketide precursor necessary for aflatoxin formation (Ehrlich and Cotty, 2004). NRRL21882 isolated from peanut in Georgia is the active ingredient of Afla-Guard®, a biocontrol agent marketed by Syngenta. EPA has issued a Section three registration for use of Afla-Guard® to control aflatoxin contamination in peanuts (Dorner, 2009). NRRRL21882 is missing the entire aflatoxin and cyclopiazonic acid (CPA) gene clusters and therefore is unable to produce both mycotoxins (Chang et al., 2005, 2009). The third prospective strain is K49 (NRRRL30797), which was isolated from corn in Mississippi (Abbas et al., 2006). K49 is currently being tested in corn-growing fields in Mississippi for biocontrol of aflatoxin production. K49 still has several aflatoxin genes (Accinelli et al., 2008) but does not produce aflatoxins and CPA. The genetic basis for its inability to produce these toxins was not previously determined. A few other promising A. flavus biocontrol candidate strains including TX9-8 (Chang and Hua, 2007), AF051 (Jiang et al., 2009), Td06 (Degola et al., 2011), and isolate 51 (Huang et al., 2011) also have been identified.

Populations of A. flavus in agricultural fields consist of complex communities that exhibit considerable genetic diversity based on phylogenetic and vegetative compatibility group analyses (Barros et al., 2006; Mehl and Cotty, 2010). Surveys of A. flavus isolates from various geographic...
regions have revealed significant differences in their aflatoxin-producing ability including many isolates that do not produce aflatoxins (Donner et al., 2010; Horn and Donner, 1999). The currently used biocontrol strains have been selected from this non-aflatoxinogenic natural reservoir. In the present study, we identified the genetic defects associated with the inability of K49 to produce aflatoxins and CPA. Our data suggest that known (AF36) and prospective (K49) biocontrol strains possess a shared phylogeny and that certain unique genetic polymorphisms allow for future selection of promising biocontrol strain candidates.

2. Materials and methods

2.1. Fungal strains

A. flavus K49 and AF36 are biocontrol strains currently being tested on and applied to agricultural fields, respectively. TX9-8 isolated from a Texas cotton field is a competitive strain identified previously from laboratory tests (Chang and Hua, 2007). In this study, S-morphotype was used to refer to aflatoxinogenic isolates that produce abundant small sclerotia and have the type I deletion in the cyPA-norB region of the aflatoxin gene cluster (Chang et al., 2006; Ehrlich et al., 2004). Non-aflatoxinogenic isolates with the type I deletion that produce large sclerotia presumably due to the inability to make aflatoxin and channeling the common precursor, acetate, to sclerotial formation (Chang et al., 1999) were referred as non-aflatoxinogenic L-morphotype. The S-morphotype A. flavus isolates are AF12, AF70, CA28, CA42, CA43, CA44, CA10-18s, and V44-36s. The non-aflatoxinogenic L-morphotype A. flavus isolates are LA4-5, MS1-1, MS5-6, NC3-6, NM1-6, SC3-5, SC6-9, TX21-9 and VA2-1, TX3-25s, TX12-10-2s and TX13-21s are atypical aflatoxinogenic A. flavus isolates that produce small sclerotia but do not produce CPA (Chang et al., 2006). A. flavus NRRL3357, a typical aflatoxinogenic L-morphotype isolate that has the type II cyPA-norB deletion pattern, and A. oryzae RIB40 are genome sequenced strains (Machida et al., 2008; Payne et al., 2006). Selected A. flavus isolates used in further studies (see Section 2.4) are listed in Table 1.

2.2. Preparation of genomic DNA, PCR and sequencing

Approximately 10⁶ conidia were inoculated into 1 ml Potato Dextrose Broth (EMD, Darmstadt, Germany) in a 2-ml microtube. The tube was incubated horizontally at 30 °C for 18 to 24 h until the formation of a thin layer of mycelial mat. The mycelia were harvested and processed using a Scientific Industries' Disruptor Genie™ (ZYM RESEARCH, Orange County, California, USA). Fungal genomic DNA was prepared using the ZR Fungal/Bacterial DNA Kit™ (ZYM RESEARCH). PCR was performed under the following conditions in a Perkin Elmer GeneAmp PCR System 2400. Fifty pmol of each primer and 10 ng genomic DNA were added to 50 μl Platinum® Blue PCR Supermix (Invitrogen, Carlsbad, California, USA) and subjected to 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2.0 min. PCR fragments were sequenced at Iowa State University DNA Sequencing and Synthesis Facility (Ames, Iowa, USA). Gene sequence assembly was made with DNASTAR Lasergene 8 (DNASAR, Madison, Wisconsin, USA). DNA sequence alignment was performed with DNAMAN version 5 (Lynnon Corporation, Vandreuil, Quebec, Canada) or online with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. Determination of genetic defect in the pksA gene, mating type, cyPA-norB deletion pattern, and single nucleotide polymorphisms (SNPs) of dmaT

A part of the pksA gene encoding the polyketide synthase for aflatoxin biosynthesis was amplified from K49 by PCR with primers of 5′-cagacccgcagcagtac-3′ and 5′-actgccagctggtgagc-3′. The mating-type specific primers were derived from A. flavus MAT1-1 (GenBank ID: EU357934) and MAT1-2 (EU357936). The primers were MAT1-1F: 5′-atggaacacgacagtccc-3′, MAT1-1R: 5′-tcaacaagtggaaacagtc-3′, MAT1-2F: 5′-atcgagaagcactatac-3′ and MAT1-2R: 5′-ttcctagagcagtcaa-3′. The primer set of 5′-gtgcccagcatcttggtcca and 5′-agaggctggatccctgcg was used to examine unique deletion patterns in the cyPA-norB region found in the A. flavus aflatoxin gene cluster (Ehrlich et al., 2004). A portion of the dnaT gene encoding the cyclooctaetoctryptophanyl dimethylallyl transferase for CPA biosynthesis (Chang et al., 2009) was amplified by PCR with primers of 5′-gtctccgatgctcgtggg-3′ and 5′-gatagagctgcgctcgg-3′.

2.4. Sequencing of the complete CPA gene cluster of selected A. flavus isolates

Based on the dmaT SNP profiles selected isolates (Table 1) were further analyzed for their CPA gene cluster sequences. The CPA gene cluster of A. flavus NRRL3357 is about 16 kb and consists of three biosynthetic genes: maoA, dmaT and pks-nrps. Using the NRRL3357 genome information as a reference (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html), we synthesized a series of primers that gave consecutive PCR fragments of 1.0 to 1.5 kb with an overlap of about 100 nucleotides to each of the adjoining fragments. In the cases of sequence gap(s) due to the lack of PCR products, new sets of primers were synthesized based on resolved CPA gene cluster regions.

3. Results

3.1. Identification of genetic defects associated with K49’s inability to produce aflatoxins and CPA

Our sequence analyses with BLAST on available sequence data in GenBank showed that published partial nucleotide sequences of five genes from A. flavus K49 are identical to those from AF36 (AY510455) including nor1 (aflD, EF565463), avnA (aflG, EF565465), omtA (aflP, EF565464), aflR (EF565466) and aflJ (EF565467). These K49 genes, however, have different degrees of nucleotide identity to some of the corresponding genes of A. flavus AF13 (AYS10451), AF70 (AYS10453) or NRRL3357. To determine if the previously identified pksA defect in AF36 (Ehrlich and Cotty, 2004) and other non-aflatoxinogenic A. flavus isolates is also present in K49, we sequenced a 0.4 kb partial pksA gene region of K49 that would include this genetic mutation. The pksA gene of K49 had the identical G to A point mutation at nucleotide position 591 (Fig. 1), which introduces a TGA stop codon that causes truncation of the resulting polyketide synthase. The GenBank ID for the partial pksA gene of K49 is JN575332.

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Table 1

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Geographic location</th>
<th>Source</th>
<th>AF/CPA</th>
<th>MAT</th>
<th>omtA</th>
<th>cyPA-norB</th>
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<tbody>
<tr>
<td>NRRL3357</td>
<td>USA</td>
<td>Peanut</td>
<td>Y/Y</td>
<td>1-1</td>
<td>*</td>
<td>II</td>
</tr>
<tr>
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<td>Corn</td>
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<td>1-2</td>
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</tr>
<tr>
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<td>Arizona</td>
<td>Cottonseed</td>
<td>N/Y</td>
<td>1-2</td>
<td>=</td>
<td>1</td>
</tr>
<tr>
<td>TX-9</td>
<td>Texas</td>
<td>Soil</td>
<td>N/N</td>
<td>1-2</td>
<td>=</td>
<td>1</td>
</tr>
<tr>
<td>AF70</td>
<td>Arizona</td>
<td>Cottonseed</td>
<td>Y/Y</td>
<td>1-1</td>
<td>~</td>
<td>1</td>
</tr>
<tr>
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<td>Pistachio</td>
<td>Y/Y</td>
<td>1-2</td>
<td>=</td>
<td>1</td>
</tr>
<tr>
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<td>Georgia</td>
<td>Soil</td>
<td>Y/Y</td>
<td>1-1</td>
<td>~</td>
<td>1</td>
</tr>
<tr>
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<td>California</td>
<td>Pistachio</td>
<td>Y/Y</td>
<td>1-1</td>
<td>~</td>
<td>1</td>
</tr>
<tr>
<td>NC-6</td>
<td>North Carolina</td>
<td>Soil</td>
<td>N/N</td>
<td>1-1</td>
<td>#</td>
<td>1</td>
</tr>
</tbody>
</table>

* Production of aflatoxin and cyclopiazonic acid. Y: positive and N: negative.

** Mating-type gene.

† Single nucleotide polymorphisms based on omtA (Chang et al., 2006). The symbol indicates “=“ identical; “~” nearly identical to the pattern indicated by “=“; “#” and “*” distinct from others.

‡ The deletion patterns in cyPA-norB have been used to infer ancestral sclerotial genotypes of A. flavus isolates. Type I deletion corresponds to S-genotype and type II deletion corresponds to L-genotype (Chang et al., 2006).
3.2. Genetic characterization of K49 based on mating type, cypA-norB deletion pattern, and single nucleotide polymorphisms of dmaT

K49 had the MAT1-2 mating-type gene and the type I cypA-norB deletion pattern identical to those of AF36 (Table 1). To determine the relationship of K49 with other A. flavus strains having the same cypA-norB deletion pattern, we first compared their dmaT SNP profiles. The 765-bp region of dmaT amplified encodes mainly the N-terminal half (1-244) of the predicted DmaT protein. In this comparison, we included NRRL3357, which has MAT1-1 and the type II cypA-norB deletion pattern, A. oryzae RIB40, and three rare aflatoxigenic A. flavus isolates that do not produce CPA. Fig. 2 shows that based on dmaT SNPs these isolates resolve into six clades. K49, AF36 and TX9-8 are in the same clade with NRRL3357.

3.3. The complete CPA gene clusters of selected A. flavus isolates

To delineate the relationship among clades, we further compared the sequences of the complete CPA gene cluster of selected isolates from four clades (B, C, D and F), and NRRL3357. We divided the CPA cluster sequence into two parts for comparison: part 1 is the 5′ untranslated region (UTR) of the maoA gene, and part 2 encompasses the rest of the CPA cluster that includes the three genes of maoA, dmaT and pks-nrps, intergenic regions, and the distal 3′ UTR of pks-nrps (Table 2). The sequence of the 5′ UTR of K49, AF36 and TX9-8 is 99.5% and 97.0% identical to that of the S-morphotype isolates and distinct from those of NRRL3357 (Chang et al., data not shown). In contrast, while K49, AF36 and TX9-8 are nearly identical to NRRL3357 for the rest of the region of the CPA cluster, these isolates are only about 98% identical to the other examined A. flavus isolates that have the type I cypA-norB deletion pattern. Clade F, represented by NC3-6, is distinct from other A. flavus isolates in the CPA cluster gene sequence; its overall identity to others is about 50%. NC3-6, however, has nearly 100% identity to that of A. oryzae RIB40, an industrial strain used widely in food fermentation.
Table 2
Comparison of two regions of the CPA gene cluster among A. flavus isolates.

<table>
<thead>
<tr>
<th>3357</th>
<th>K49</th>
<th>AF36</th>
<th>TX</th>
<th>AF70</th>
<th>CA28</th>
<th>CA10-9</th>
<th>CA43</th>
<th>NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 s</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>3357</td>
<td>97.0</td>
<td>97.0</td>
<td>97.0</td>
<td>97.1</td>
<td>97.6</td>
<td>97.1</td>
<td>97.1</td>
</tr>
</tbody>
</table>

On top right are comparisons based on the 0.4-kb proximal 5′ UTR of the CPA gene cluster. On bottom left are comparisons based on the 16.4-kb region including the 15.9-kb CPA gene cluster plus the 0.5-kb 3′ UTR. The GenBank IDs for the CPA gene cluster of AF36, AF70, CA28, CA10-9, CA43, K49, NC3-6 and TX9-8 are JN712209 to JN712216, respectively. The corresponding CPA gene cluster region in NRRL3357 is on supercontig 7 from 2261243 to 2278260.

4. Discussion

The biocontrol strains, K49 and AF36, have shown great promise in their ability to reduce aflatoxin contamination of corn and cotton in field tests (Abbasi et al., 2006; Cotty, 1994). In a laboratory study, A. flavus TX9-8 was found to prevent aflatoxin production by A. flavus isolates that produce either large or small sclerotia (Chang and Hua, 2007). Our analyses indicate that K49, AF36 and TX9-8, besides containing the same genetic defect in pksA, have identical omrA SNP patterns (Table 1); this omrA SNP pattern differs greatly from other known patterns, such as those of aflatoxigenic and non-aflatoxigenic L-morphotype isolates (Chang et al., 2006). The dmaT SNP pattern of the biocontrol group, however, is identical to that of NRRL3357 (Fig. 2, clade B), an aflatoxigenic isolate having the type II deletion in the cyPA-norB region (L-morphotype). These results suggest that an ancestral recombination event separates the biocontrol group from the S-morphotype A. flavus. Furthermore, all isolates of the biocontrol group have the maoA SNP patterns (Grubisha and Cotty, 2010), but NRRL3357 has MAT1-1 at the non-allelic locus on chromosome VI. The fact that the aflatoxin and CPA gene clusters are adjacent to each other on the same chromosome but are distant from the mating-gene locus suggests that the observed sequence variations in isolates of the biocontrol group resulted from an ancestral sexual recombination. The presence of either MAT1-1 or MAT1-2 among the aflatoxigenic S-morphotype isolates (Table 1) also points to the possibility of sexual recombination in A. flavus and is supported by previous studies (Horn et al., 2009; Ramirez-Prado et al., 2008).

The comparison of respective sequences in the region proximal to the maoA gene (5′ UTR) and in the region distal to the 3′UTR of pks-nrps and dmaT (Table 2) suggests that a crossover between the genomes of an ancestor of NRRL3357 and a strain ancestral to CA28 that has the MAT1-2 gene likely occurred within the proximal sequence of maoA (see Fig. 3). An insertion at the maOA-dmaT intergenic region and a deletion in the sole intron of dmaT are present in K49, AF36, TX9-8 and NRRL3357 but not present in the S-morphotype isolates (Chang et al., data not shown). The conservation of the indels also supports the proposed recombination between aforementioned ancestral strains. Our conclusion is consistent with a proposition that predicts recombination would occur at the junction region of aflatoxin and CPA gene clusters (Moore et al., 2009).

The comparison of the CPA cluster sequences among the A. flavus isolates of the type I cyPA-norB deletion pattern reveals a non-aflatoxigenic A. flavus group (Fig. 2, clade F) that is highly homologous to A. oryzae. Previous studies based on the omrA SNPs also have

Fig. 3. Schematic representation of the proposed ancestral recombination between S and L-morphotype A. flavus strains that resulted in the formation of the biocontrol group. The sequence identity percentages indicate those compared to the corresponding parts in the CPA gene cluster of the biocontrol group (see Table 2).
confirmed that this group is more closely related to A. oryzae than to other groups of A. flavus (Chang et al., 2006; Geiser et al., 2000). Available evidence has suggested that the prospective biocontrol candidate AF051 is a flavus/oryzae strain (Jiang et al., 2009; Lee et al., 2006). Since A. oryzae is routinely used in food fermentation and is accepted as safe for commercial use, an effort should be directed to select other promising biocontrol strains from this A. flavus/oryzae group. Such strains could quickly receive regulatory approval for field application. K49 and AF36, although similar in many ways, exhibited differences in competitiveness against toxigenic A. flavus strains from various sources, such as maize, soybean, soil, ear worm, and air in field tests (Abbas et al., 2011). A multi-strain formulation likely will be the ultimate biocontrol approach for effective management of aflatoxin contamination of crops. To achieve this goal, identification of additional prospective strains either within the biocontrol group or from non-aflatoxigenic A. flavus populations like the isolates recently found in Italy and Nigeria that lack some or all of the aflatoxin biosynthesis genes (Degola et al., 2011; Donner et al., 2010) may be required.

Acknowledgments

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References