MOLECULAR IDENTIFICATION OF AFLATOXIN-PRODUCING ASPERGILLUS STRAINS IN MAIZE SEED-MATERIAL

Grajdieru C.

Institute of Genetics, Physiology and Plant Protection

kgrejdieru@mail.ru

Abstract: Toxigenic fungi are part of soil microbiota and natural inhabitants of agroecosystems and crops. Aflatoxins produced by several *Aspergillus* species are hazardous biological compounds known as potent carcinogens. Using PCR-based assays, a successful identification of toxigenic *A. flavus* and *A. parasiticus* strain was performed. Fungal genome sequences associated with aflatoxin production as target sequences were proved to be effective for identification of toxigenic *Aspergillus* species.

Keywords: aflatoxin, PCR, *Aspergillus parasiticus*, *Aspergillus flavus*, molecular identification.

Introduction

Toxigenic fungi are part of soil microbiota and natural inhabitants of agrocenoses and crops. Mycotoxins play a role in intra- and interspecific relations between filamentous fungi providing certain advantage in competitive interactions and host plant infestation [1]. However, most of these compounds are harmful for animals and humans as they cause serious adverse effects on vital systems [2].

Among others, aflatoxins are considered the most dangerous and potent biological compounds that can induce carcinogenesis in mammals [3,4]. Therefore, they present an emerging risk for human health and their concentration is strictly regulated in many countries, including Republic of Moldova [5,6]. They are produced mainly by *Aspergillus* species that are common contaminants of grains during storage [7]. Current mycotoxin identification methods are mainly chromatography and ELISA-based assays [4,7]. They are effective but imply the occurrence of contamination and a certain toxin concentration for detection. Assessment of emerging risk of aflatoxin hazard prior to food contamination should be considered more justified in terms of human health and agrocenoses' phytosanitary state.

Current paper provides a protocol for identification of aflatoxin-producing *A. flavus* and *A. parasiticus* strains using fungal genome sequences associated with aflatoxin production as target sequences.

Materials and methods

Plant material and DNA extraction. Samples of maize seed material from Institute of Genetics, Physiology and Plant protection Gene Bank (laboratory of Plant Genetic Resources) were collected for analysis. Total DNA was extracted using ISO 21571:2005 protocol with modifications [8]. DNA concentration was quantified using agarose gel electrophoresis.

Primer design and amplification. Primers for nested-PCR were designed using Primer3 software based on specific *Aspergillus* genome sequences associated with aflatoxin production contained in GenBank database (Tab.1).

Table 1. Primers used for identification of toxigenic *Aspergillus* strains

Primer	Primer sequence	Species	Target sequence [9]
afaq1(F)	ACTGCCCAAAGGTGCTCTTC	A. flavus	U81806.1 <i>Aspergillus</i> flavus OMST-oxidoreductase (ord1) gene, promoter region and complete cds <i>Aspergillus</i> flavus isolate AF36 aflatoxin biosynthesis gene cluster, complete sequence
afaq4(R)	TACAAACCGACCGGGGCATA		
aqfaq3(F)	ACACCCGTCCATTGATTCTCCTA		
aqfaq4	GTTTCTGGCGGGGAGTCTTTT		
apap1(F)	TTGCTCGGTAGTGCCATGTT	A. parasiticus	DQ390914.1 <i>Aspergillus</i> parasiticus strain IC73 O-methyltransferase A (aflP) gene, partial cds; and aflP-aflQ intergenic region, genomic sequence
apap4(R)	GGCTTCCATAACACATATTCTCCAA		
aqpap2(F)	CCGCGAAAGAACAAACAGAGA		
aqpap3(R)	AACACATATTCTCCAACCTTCTTGCT		
afap1(F)	CTTTGTTCGGTAGTGCCATCTTGA	A. flavus	FJ877830.1 <i>Aspergillus</i> flavus strain IC289 O-methyltransferase A (aflP) gene, partial cds; and aflP-aflQ intergenic spacer, partial sequence
afap4(R)	GCCATAGCACATATTCTCCAACCT		
aqfap2(F)	GTGTCGGGTGTGCCTATTTAACC		
aqfap3(R)	AAGGCTTTCGGTCGGTTGATG		

The amplification was performed in a 25µl mix containing 66 mM Tris-HCl (pH 8.4), 16 mM (NH₄)₂SO₄, 2,5 mM MgCl₂, 0,1% Tween 20, 7% glycerol, 100 µg/ml-1 BSA, 0,2 mM of each dNTPs, 1,2 U Taq DNA polymerase (Thermo Fisher Scientific), 5 pM of each primer and 50 ng of DNA.

Nested-PCR protocol included two-round amplification of 30 cycles each (Tab.2).

Table 2. Nested-PCR protocol used for identification of toxigenic *Aspergillus* strains

Round	Initial denaturation	Denaturation	Annealing	Elongation	Terminal elongation
I	3 min, 95oC	30 sec, 95oC	30 sec, 60oC	30 sec, 72oC	7 min, 72oC
II	-	30 sec, 95oC	30 sec, 60oC	30 sec, 72oC	7 min, 72oC

Visualization. The products of amplification were separated in 1,5% agarose gel at the 6V/cm in a TBE migration buffer (pH 8.0) with ethidium bromide, viewed in the UV (302 nm) and photographed. Amplicon length was estimated using DNA ladder (Thermo Fisher Scientific) and GelAnalyzer2010 software.

Results and discussions

Methyltransferase A and oxidoreductase are enzymes engaged in aflatoxin biosynthetic pathways of *A. flavus* and *A. parasiticus*. *A. parasiticus* toxigenic strain matched a 120 bp amplicon generated during nested-PCR.

Figure 1. Amplicon generated using aqpap2-aqpap3 primer pairs; M - DNA ladder (100 bp)

For *A. flavus* aflatoxin-producing strains' identification primers for methyltransferase A and oxidoreductase sequences were used. With methyltransferase A as target sequence a 152 bp amplicon matched the *A. flavus* aflatoxin-producing strain in analyzed samples.

Figure 2. Amplicons generated using aqfap2-aqfap3 primer pairs; M - DNA ladder (100 bp)
For oxidoreductase as target sequence, *A. flavus* presence matched the 134 bp amplicon.

Figure 3. Amplicons generated using aqfaq2-aqfaq3 primer pairs; M - DNA ladder (100 bp)
All generated amplicons matched the expected lengths.

Conclusions

Maize seed material was infested with toxigenic aflatoxin-producing strains of *A. flavus* and *A. parasiticus*. Generated primers were specific for identification of *A. flavus* and *A. parasiticus* aflatoxin-producing strains via PCR using different fungal genome regions as target sequences. Molecular methods of toxigenic *Aspergillus* species identification are effective for their monitoring in seed-material during storage. Identification of toxigenic fungi using genome sequences associated with mycotoxins' biosynthetic pathways is justified for effective assessment of mycotoxin contamination.

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