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Review

PCR detection of aflatoxin producing fungi and its limitations

Robert E. Levin *

Department of Food Science, Massachusetts Agricultural Experiment Station, University of Massachusetts, Amherst, MA 01003, United States

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ABSTRACT

Unlike bacterial toxins that are primarily peptides and are therefore encoded by a single gene, fungal toxins such as the aflatoxins are multi-ring structures and therefore require a sequence of structural genes for their biological synthesis. There is therefore no specific PCR for any one of the four biologically produced aflatoxins. Unfortunately, the structural genes presently in use for PCR detection of aflatoxin producing fungi are also involved in the synthesis of other fungal toxins such as sterigmatocystin by *Aspergillus versicolor* and *Aspergillus nidulans* and therefore lack absolute specificity for aflatoxin producing fungi (Table 1). In addition, the genomic presence of several structural genes involved in aflatoxin biosynthesis does not guarantee the production of aflatoxin by all isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. The most widely used DNA target regions for discriminating *Aspergillus* species are those of the rDNA complex, mainly the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) and the variable regions in the 5′-end of the 28S rRNA gene. Since these sequence regions are unrelated to the structural genes involved in aflatoxin biosynthesis there successful amplification can be used for species identification but do not confirm aflatoxin production. This review therefore presents the various approaches and limitations in the use of the PCR in attempting to detect aflatoxin producing fungi.

Contents

1. Introduction

Aspergillus subgenus *Circumdati* section *Flavi*, also referred to as the *Aspergillus flavus* group, is divided into two groups of species. One includes the aflatoxigenic species, *A. flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* and the other includes the non-aflatoxigenic species *Aspergillus oryzae*, *Aspergillus sojae*, and *Aspergillus tamari*, traditionally used for production of fermented Asian foods.

Aflatoxins (B1, B2, G1, G2) belong to a family of deketides that are produced as secondary metabolites by *A. flavus* and *A. parasiticus*. Aflatoxin B1, a potent liver carcinogen is the predominant aflatoxin produced by these fungi. The biosynthesis of aflatoxin B1 involves 23 enzymatic reactions starting with acetyl-CoA. A total of 15 intermediate precursors are recognized in the pathway (Meyers et al., 1998), involving 25 genes, clustered in a 75-kb DNA region (Bhatnagar et al., 2006). The sequences of the genes involved in aflatoxin biosynthesis appear to be highly conserved among *A. flavus* and *A. parasiticus* (Yu et al., 1995).

There is no specific PCR for any one of the four biologically produced aflatoxins. The late stages of AFB1 synthesis are carried out by two enzymes, a methyltransferase encoded by the *aflP* (*omtA*) gene that converts sterigmatosystin to *O*-methylsterigmatocystin and an oxidoreductase encoded by the *aflQ* (*ordA*) gene that converts *O*-methylsterigmatocystin to aflatoxin B1. Bhatnagar et al. (1991) presented evidence that these later stages of AFB1 and AFB2 synthesis are catalyzed by common enzymes that use separate precursors as substrates for each toxin.

To-date three different systems have been used for detection of aflatoxin producing isolates of these fungi targeting genes involved

* Department of Food Science, University of Massachusetts, Amherst, MA 01003, United States. Tel.: +1 413 545 0187; fax: +1 413 545 1262.
E-mail address: relevin@foodsci.umass.edu.

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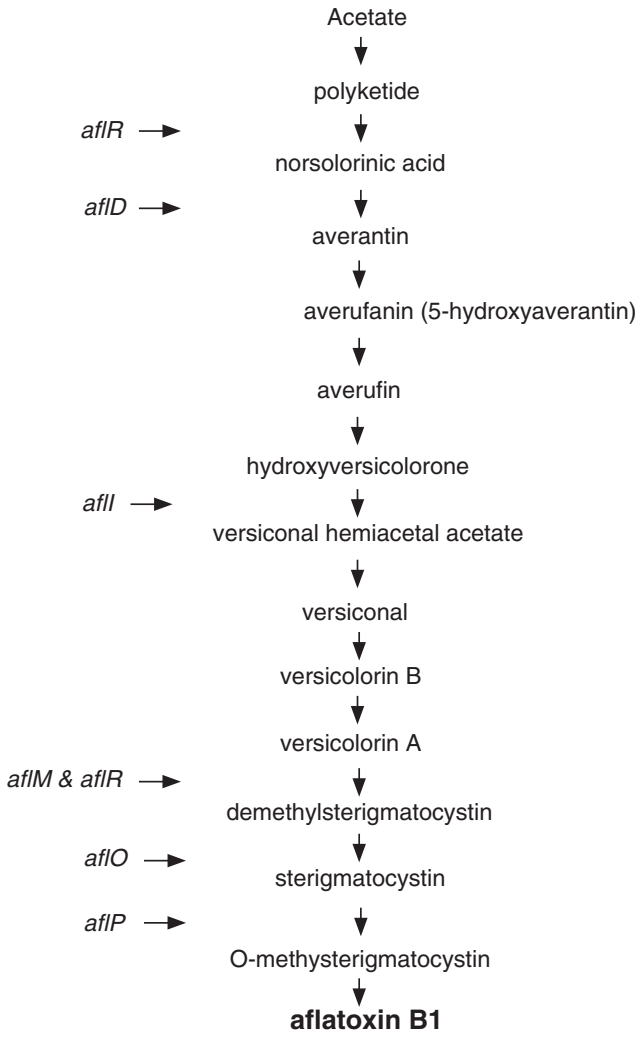

Fig. 1. Diagrammatic illustration of the aflatoxin B1 biosynthesis pathway with genes encoding several metabolic intermediates.

in the biosynthesis of aflatoxins: (1) a multiplex PCR assay targeting the *nor-1*, *apa-2*, and *omt-1* (*omtA*) genes (Shapira et al., 1996), (2) PCR assays targeting the *omt-1*, *nor-1*, and *ver-1* genes individually (Färber et al., 1997) and (3) PCR assays amplifying individual sequences of the *aflRS*, *aflJ*, and *omtB* genes (Rahimi et al., 2008). Both conventional and real-time PCR (Rti-PCR) have made use of these systems.

2. Aflatoxin genes used for PCR amplification

The *nor-1* gene encodes norsolorinic acid reductase and converts norsolorinic acid to averantin (Chang et al., 1992) (Fig. 1, Table 1). The *ver-1* gene encodes versicolorin A dehydrogenase, and converts versicolorin A to sterigmatocystin (Chang et al., 1992; Skory et al., 1992) (Fig. 1, Table 1). The *omt-1* gene encodes sterigmatocystin-*o*-methyltransferase and is required for conversion of demethylsterigmatocystin and dehydrodemethylsterigmatocystin to sterigmatocystin and dihydrosterigmatocystin respectively (Motomura et al., 1999; Yu et al., 2000). The *afl-2* (*aflR*) gene in *A. flavus* and its homolog in *A. parasiticus apa-2* (*apaR*) regulate aflatoxin biosynthesis (Liu and Chu, 1998; Payne et al., 1993) by controlling the expression of the *nor-1* and *ver-1* genes (Woloshuk et al., 1994). Evidence for an antisense transcript (*aflSRAS*) derived from the opposite strand of *aflR*, suggests that the *aflR* locus involves some form of antisense regulation (Woloshuk et al., 1994). These two genes appear to be homologous. Woloshuk et al. (1994) proposed that because of the ability of these genes to regulate aflatoxin biosynthesis, that they be designated *aflR*. DNA hybridization of the *aflR* gene with genomic digests of seven polypeptide-producing fungi revealed similar sequences in three other species related to *A. flavus*: *A. parasiticus*, *A. oryzae*, and *A. sojae* (Woloshuk et al., 1994). These authors maintained that these four species represent morphological and physiological variants of a single species. Kurtzman et al. (1986) reported that *A. flavus* and *A. oryzae* have 100% DNA complementarity and *A. parasiticus* and *A. sojae* have 91% complementarity.

Liu and Chu (1998) studied the role of the *aflR* regulatory gene and its product, AFLR, in the biosynthesis of aflatoxin in species of *Aspergillus*. Western blot and ELISA assays revealed that aflatoxin B1 accumulation was directly related to AFLR expression and was regulated by various environmental and nutritional conditions, including temperature, air supply, carbon source, nitrogen source, and zinc availability. Expression of the aflatoxin biosynthetic pathway structural gene, *omtA*, was found to be regulated by the presence of AFLR. Analysis of non-aflatoxin-producing strains of *A. flavus*, *A. sojae*, and *A. oryzae* grown in a medium suitable for aflatoxin B1 production indicated that both *aflR* mRNA and AFLR production occurred; however, *omtA* mRNA production was not detected in these aflatoxin negative strains.

3. PCR detection of aflatoxin producing fungi

Geisen (1996) developed a multiplex PCR assay to amplify the aflatoxin biosynthetic genes: norsolorinic acid reductase (*nor-1*), versicolorin A dehydrogenase (*ver-1*), and sterigmatocystin O-methyltransferase (*omt-A*) from *A. parasiticus*. The primers nor1/nor2, ver1/ver2, and omt1/omt2 (Table 2) were utilized. The reaction yielded a triplet banding pattern with aflatoxin producing strains of *A. flavus*, and *A. parasiticus*, and also with sterigmatocystin producing strains of *A. versicolor*. The pattern of aflatoxin negative *A. flavus*

Table 1
Genes used for the identification of aflatoxigenic fungi by PCR and RT-PCR.

Gene	Synonym	Enzyme	Step in AF biosynthesis pathway	References
aflD	*nor-1*	Norsolinic acid reductase	Norsolorinic acid to averantin	Chang et al. (1992)
aflI	*avfA*	Averufin oxidase	Averufin to versiconal hemiacetal acetate	Yu et al. (2000)
aflM	*ver-1*	Versicolorin A dehydrogenase	Versicolorin A to demethylsterigmatocystin	Skory et al. (1992)
aflO	*omtB*	*O*-methyltransferase	Demethylsterigmatocystin to sterigmatocystin	Yu et al. (2000)
aflP	*omtA*, *omt-1*	*O*-methyltransferase	Sterigmatocystin to *O*-methylsterigmatocystin	Bhatnagar et al. (1991); Yu et al. (1993)
aflQ	*ordA*	Oxidoreductase	*O*-methylsterigmatocystin to aflatoxin B1	Bhatnagar et al. (1991)
aflR	*afl-2*, *apa-2*	Transcription factor containing a zinc cluster DNA binding motif	Positive regulator of AFB1 and 2 biosynthesis	Chang et al. (1992); Chang et al. (1993); Payne et al. (1993); Woloshuk et al. (1994)
aflS	*aflJ*	Transcription factor coactivating AFLR	Positive regulator of AFB2 biosynthesis Required for conversion of norsolinic acid, sterigmatocystin, and O-methylsterigmatocystin to aflatoxin	Meyers et al. (1998)