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Genes differentially expressed by *Aspergillus carbonarius* strains under ochratoxin A producing conditions

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Abstract

Aspergillus carbonarius is an important ochratoxin A (OTA)-producing fungus that is responsible for toxin contamination of grapes and wine, coffee and cocoa. A suppression subtractive hybridization (SSH) approach was performed with two strains of A. carbonarius, antagonistic in their OTA-production ability, to identify genes whose expression is linked with the ability to produce OTA. BlastX analysis identified 109 differentially expressed sequences putatively involved in the production of OTA, with significant similarities (E_value<10^{-5}) to sequences deposited in the NCBI non-redundant protein database. Of the 109 ESTs, 26% were involved in regulation processes, 15% corresponded to hypothetical proteins, 12% were involved in stress response and detoxification, 9% corresponded to transport and secretion processes, 7% corresponded to amino acid metabolism, 7% were involved in hydrolysis of energy reserves and 5% involved in secondary metabolism. Other unisequences showed homology to genes involved in protein synthesis and general metabolism. According to their sequence similarities to genes in the NCBI database, the possible functional roles they might play in the production and regulation of OTA are discussed. Worth noting is the high percentage of genes involved in regulation, including specific and global regulators. It is also important to note the high percentage of genes involved in the response to stress and detoxification.
1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic, immunotoxic, genotoxic and teratogenic effects, which has been associated with Balkan Endemic Nephropathy (Abouzied et al., 2002). OTA is receiving increasing attention for its toxic effects and high incidence in a wide range of food commodities. These include cereal-based products, coffee, spices, nuts, olives, grape-derived products, beans, figs and cocoa (Battilani et al., 2006; Perrone et al., 2007).

Among the commodities that can be contaminated by OTA, grapes and wines are those with the greatest toxin content, second only to cereals (Belli et al., 2004; Bau et al., 2005). Aspergillus species belonging to section Nigri, in particular Aspergillus carbonarius and species belonging to the Aspergillus niger aggregate, have been identified as the main fungi responsible for OTA contamination in grapes and wine (Battilani et al., 2006; Perrone et al., 2007; Martínez-Culebras et al., 2009). A. carbonarius is considered as being the most responsible for OTA contamination in grapes because the reported percentages of OTA-producing strains in this species are higher than those reported for other members of the A. niger aggregate (Battilani et al., 2006; Martínez-Culebras and Ramón, 2007).

Structurally, the mycotoxin consists of a polyketide derived from a dihydroisocoumarin moiety, linked to the amino acid phenylalanine via an amide linkage. Based on the molecular structure of OTA it is clear that a number of enzymatic reactions are likely to be required for its biosynthesis. These include a polyketide synthase (PKS) for the synthesis of the polyketide dihydroisocoumarin, a cyclase, a chloroperoxidase of halogenase, an esterase
and a peptide synthase for the ligation of the phenylalanine to the dihydroisocoumarin (Harris and Mantle, 2001). However, as yet, little information is available about the biosynthetic pathway of OTA in any fungal species and only a few genes have been reported. O’Callaghan et al. (2003), Karolewiez and Geisen (2005), Bacha et al. (2009) and Gallo et al. (2009) have described PKS genes involved in OTA biosynthesis in *Aspergillus ochraceus*, *Penicillium nordicum*, *Aspergillus westerdijkiae* and *A. carbonarius*, respectively. Additionally, other putative OTA biosynthetic genes have been reported, including two P450 monoxygenase genes in *A. ochraceus* (O’Callaghan et al., 2006), a non-ribosomal peptide synthetase, together with two genes encoding a transporting protein and a chloroperoxidase in *P. nordicum* (Geisen et al., 2006).

These previous studies have focused on the genes directly involved in the OTA biosynthetic pathway. However, few reports concerning a global characterization of OTA biosynthesis that include regulation are available. Differential display techniques, such as Differential Display Reverse Transcriptase-PCR (DDRT-PCR) and microarray hybridization have been used successfully for OTA biosynthesis studies in *P. nordicum* (Färber and Geisen, 2004). The differential conditions were achieved by growing the same strain on two different media, either supporting or inhibiting OTA production. However, with such an approach, the apparent differential expression could be due to differences in the media that are not linked to OTA production, potentially biasing the results. Recently, Botton et al. (2008) used a cDNA-AFLP approach to identify genes differentially expressed in two strains of *A. carbonarius* that contrasted in their ability to produce OTA.
In this study, a suppression subtractive hybridization (SSH) approach (Diatchenko et al., 1996), which is an extremely efficient method for the isolation of differentially-expressed genes, was used to isolate genes differentially expressed in two closely related strains of A. carbonarius differing in their OTA-producing capabilities. Martínez-Culebras and Ramón (2007) previously described the two strains that we have used in this work, namely W04-40 (high OTA producer) and W04-46 (low OTA producer), which were used as tester and driver, respectively. We analyzed the differentially expressed genes for homology and classified them into functional categories. Finally, we discuss the possible functional roles of the genes identified in the production and regulation of OTA.

2. Materials and methods

2.1. Strains

Two strains of A. carbonarius antagonist in their ability to produce OTA—previously characterized by Martínez-Culebras and Ramón (2007), namely W04-40 (OTA high-producing strain) and W04-46 (OTA low-producing strain), were used because of their differential production of OTA.

A. carbonarius strains W04-40 and W04-46 were isolated from the same Spanish vineyard and deposited in the Institute of Agrochemistry and Food Technology of the Spanish National Research Council (IATA-CSIC). They had been previously identified by ITS-RFLP profiles, showing the same pattern and were also tested for OTA production (Martínez-Culebras and Ramón, 2007).

2.2. Media and growth conditions
Strains were grown on Petri dishes containing Malt Extract Agar (MEA) medium in the dark at 30 ºC for 6 days to achieve conidia production. Conidia were collected with a sterile solution of 0.005% (v/v) Tween 80 (J.T. Baker, Deventer, Holland) and were adjusted to $10^6$ conidia/mL using a haemocytometer. 100 µL of the conidial suspension was homogeneously spread on Petri dishes containing Czapeck Yeast Extract Agar (CYA) medium and sub-cultured in the dark at 30 ºC for 5 days. OTA production was monitored by HPLC (see below) in order to determine the optimum OTA-production phase to collect mycelia for RNA extraction.

2.3. Extraction and detection of OTA from culture

OTA was extracted using a variation of a simple method, previously set up previously by Bragulat et al. (2001). The isolates were homogenously grown on CYA pH 5.0 and incubated at 30 ºC for 7 days. Briefly, three agar plugs (6 mm in diameter) were obtained from each A. carbonarius culture and placed in a vial containing 500 µl of methanol. After 60 mins, the extracts were shaken and filtered (Millex® SLHV 013NK, Millipore, Bedford, MA, USA) into another vial and stored at 4 ºC until chromatographic analysis. Separation, detection and quantification were performed by injecting 20 µl of extract from each vial into a HPLC system consisting of a Dionex model P680A pump (Sunnyvale, CA, USA), connected to a Dionex model RF-2000 programmable fluorescence detector.

For the determination of OTA, C18 reversed-phase columns (150 x 4.6 mm i.d., 5 µm particle size Kromasil C18, Análisis Vínicos S.L., Tomelloso, Spain), connected to Kromasil C18 pre-columns (10 x 4.6 mm i.d., 5 µm particle
sizes, Análisis Vinicos S.L) were used. For the chromatographic separation of OTA, the mobile phase was acetonitrile:water:acetic acid, (57:41:2 v/v/v) under isocratic elution over 10 mins, at a flow rate of 1 ml/min. OTA was determined by fluorescence detection at an excitation wavelength of 330 nm and an emission wavelength of 460 nm. The ochratoxin standard was obtained from A. ochraceus (Sigma-Aldrich).

2.4. RNA isolation and first-stranded cDNA synthesis

For RNA extraction, strains were homogeneously grown on Petri dishes containing CYA medium in the dark at 30 °C for 2 days. Mycelia were collected from cultures, frozen in liquid nitrogen and then stored at −80°C before nucleic acid extraction.

RNA was isolated from 1 g of mycelium that had been pulverized to a fine powder with a mortar and pestle in the presence of liquid nitrogen. Pulverized mycelium was added to a pre-heated mixture of 10 mL of extraction buffer: 100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% (w/v) sodium-n-lauroylsarcosine (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1% β-mercaptoethanol and 5 mL of Tris-equilibrated phenol. After homogenization with a homogenizer Polytron PT 45/80 (Kinematica AG) for 1 min, the extract was incubated at 65 °C for 15 mins and cooled, before adding 5 mL of chloroform:isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3900 xg for 20 mins at 4 °C and the aqueous phase was re-extracted with 10 mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). Nucleic acids were precipitated by adding 2 volumes of cold ethanol and centrifuged immediately at
27200 ×g for 15 mins. The resulting pellet was dissolved in 900 µL of TES (10 mM Tris-HCl, 5 mM EDTA, 0.1% SDS, pH 7.5) and RNA was precipitated overnight at −20 ºC with 300 µL of 12 M LiCl. After centrifugation at 27200 ×g for 60 mins, the precipitate was re-extracted with 250 µL of 3 M sodium acetate (pH 6.0) to remove residual polysaccharides and, finally, dissolved in 200 µL of water. RNA concentration was measured spectrophotometrically and verified by ethidium-bromide staining of the gel. Finally, poly(A) RNA was purified with Dynabeads Oligo(dT)$_{25}$ (Invitrogen). First-stranded cDNA synthesis was carried out using the SuperScript III (RT) kit (Invitrogen), according to the manufacturer’s instructions.

2.5. Suppression subtractive hybridization

Suppression subtractive hybridization (SSH) is an effective approach to identify those genes that vary in expression levels during different biological processes. The theory behind subtraction is simple. First, mRNA from two different populations corresponding to different biological processes or situations are converted to cDNA. We refer to the cDNA that contains specific (differentially expressed) transcripts as tester, and the reference cDNA as driver. Tester and driver cDNAs are hybridized and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester but absent from the driver mRNA.

Two µg of mRNA from the _A. carbonarius_ strains W04-40 and W04-46 were subjected to SSH using the PCR-Select cDNA subtraction kit (Clontech).
according to the manufacturer’s instructions. The cDNA from the OTA producer strain (W04-40) was used as the tester and the cDNA from the OTA non-producer (W04-46) was used as the driver. The resulting subtracted cDNAs were ligated to plasmid PCR 2.1 (Invitrogen) and the ligation mixtures were transformed into electro-competent *Escherichia coli* DH5α cells (Invitrogen). Plasmid DNA was extracted (GenElute Plasmid Miniprep Kit, SIGMA) from 160 recombinant colonies and sequenced with a T7 primer using an automated sequencer.

2.6. Sequence analysis

Plasmid DNA was extracted (GenElute Plasmid Miniprep Kit, SIGMA) from 160 recombinant colonies and sequenced with a T7 primer using an automated sequencer. Vector and adaptor sequences were removed and the resulting sequences checked for redundancy using the program Seqtools 8.4 (http://www.seqtools.dk). Homologous sequences in the databases were identified by BLAST searches. Matches with E-values less than $1 \times 10^{-5}$ were considered significant and classified into broad functional categories, mainly based on their putative function as described in the KEGG classification system (www.genome.ad.jp/kegg).

Results and Discussion

3.1. Characteristics of the SSH cDNA library

Despite the fact that OTA is an important mycotoxin, not much is known about the biological background of OTA production by ochratoxigenic fungi. The aim of this study was to identify genes involved in OTA biosynthesis and in its
genetic regulation in *A. carbonarius*, the species most responsible for OTA contamination in grapes, by constructing an SSH library. The screening was performed with two closely related strains of *A. carbonarius*, according to their ITS-RFLP patterns, that differed in their ability to produce OTA. This approach was chosen as an alternative to that adopted by O’Callaghan et al. (2003) and Färber and Geisen (2004) for *A. ochraceus* and *P. nordicum*, respectively, in order to avoid the isolation of genes whose expression is induced by the differential growing conditions, irrespective of OTA production. Recently, a similar experimental set-up using two antagonist strains for OTA production was also adopted by Botton et al., (2008) to conduct a cDNA-AFLP approach to study OTA production in *A. carbonarius*. Here, we used an SSH approach, which is an extremely efficient method for the isolation of differentially expressed genes, putatively involved in OTA biosynthesis and its regulation. Although an SSH approach has previously been carried out by O’Callaghan et al. (2003) in the OTA-producing fungus *A. ochraceus*, data from only six clones were published. This is the first report where an SSH approach has been used to generate an overall picture of the genes putatively involved in mycotoxin biosynthesis in an OTA-producing fungus.

A kinetic study of OTA production was initially carried out in order to establish the optimal conditions for the construction of the SSH library. OTA production was quantified over 5 days of growth in CYA medium for both previously selected *A. carbonarius* strains; the high OTA producer strain (W04-40) and the low OTA producer strain (W04-46) (see Material and Methods). There was a clear difference in OTA production between strains W04-40 and W04-46, confirming their differential ability in producing OTA (Figure 1).
Production of OTA in strain W04-40 increased from the time of inoculation to 72 h following inoculation, whereafter the rate of accumulation levelled off. For this reason, 48 h was selected as the optimum point to study differences in gene expression between the two strains. Colony appearance, growth rate, and yield of conidia for W04-40 were also compared with those of W04-46. There were no significant differences between these two strains in colony growth rates or the yield of conidia, and colony colours were nearly identical (data not shown).

After establishing the optimal conditions, an SSH cDNA library, enriched for sequences differentially expressed in the OTA producer strain W04-40, was constructed. mRNA from the high OTA producer W04-40 (tester) was subtracted against mRNA from the low OTA producer W04-46 (driver). To identify the bacterial colonies that possessed cloning vectors with inserts, the plasmid DNA was extracted and sequenced. The cDNA library was converted into plasmid clones for library screening and analysis. Examination of 160 random cDNA clones indicated that 88% of the recombinant clones had inserts ranging in size from 0.12 to 0.9 kb, with an average insert size of 0.40 kb. Following sequence analysis using the program Seqtools 8.4, 138 ESTs were identified. Blast searches against the Genome Databases indicated that 118 of the above genes had matches in the databases and 20 ESTs were categorized as having no match in the databases. Self-BLAST analysis showed 11 sequences among the 138 ESTs, each one represented by two ESTs, with a redundancy level of 8%. This enabled the identification of 116 non-redundant ESTs (Table 1). Among these, 109 ESTs had matches in the databases. We found 11 clusters among the 138 ESTs, each one represented by two ESTs, with a redundancy level of 8%. This enabled the overall identification of 109 non-redundant ESTs.
Based on homology analysis, the 11 genes whose transcripts are represented by two ESTs encode a GTP-binding protein (GTPBP1) related to elongation factor 1 alpha, (C001, C013), a fluconazole-resistance protein (FLU1) that is an MFS transporter (C050, C139), an acid alpha-amylase (C142, C154), an N-acetylglicosaminyltransferase (C079, C064), a 123 K-chain alpha-trehalose-phosphate synthase (C048, C121), a hypothetical verprolin-related protein (C087, C107), three hypothetical proteins and two proteins without homology in the databases.

3.2. Categorization of ESTs representing the genes expressed by A. carbonarius during OTA production

A BlastX analysis was performed on the 138 EST clones (putative genes). 14.49% of the EST clones showed no significant similarity to entries in the public protein databases, highlighting the paucity of knowledge of gene expression in filamentous fungi. Most of the annotated EST clones showed the highest similarities with sequences from Aspergillus species, and especially with A. niger. There is no doubt that the availability of the A. niger genome (Pel et al., 2007) facilitate assessments of the genes that are predicted to have a role in OTA biosynthesis. Although genes with unknown function or hypothetical proteins were found (14.67%), significant proportions of genes involved in regulation (25.68%), oxidative stress and detoxification (11.8%), transport and secretion (9.17%), amino acid metabolism (7.34%) and hydrolytic processes (7.34%) were also found (Table 1; Figure 2). These data suggest that, despite the low level of redundancy obtained, overall the SSH technique was successful due to the high percentage of ESTs involved in regulation of transcription and
translation. In a failed SSH, a much higher percentage of constitutive genes with a high level of expression would be obtained.

3.3. Amino acid metabolism

Amino acid metabolism is clearly involved in OTA biosynthesis by fungi. OTA is a composite mycotoxin consisting of the pentaketide dehydroisocumarin and the amino acid phenylalanine. Thus, during OTA biosynthesis the metabolic flux of phenylalanine has to be re-routed from protein biosynthesis to OTA biosynthesis. Färbet and Geisen (2004) found induction of a phenylalanine-tRNA synthase in an OTA-producing strain of *P. nordicum*. They concluded that the up-regulation of this enzyme by the fungus is a consequence of ensuring the availability of enough phenylalanine-tRNA for protein biosynthesis. Two ESTs identified in our analysis, clones C014 and C049, showed similarity to an acetamidase from *A. nidulans* and a dopa-decarboxylase (DDC3) from *Aspergillus oryzae*, respectively. Both are involved in the metabolism of several amino acids, including phenylalanine. It is also worth noting the importance of enzymes related to biosynthesis of methionine, because the carboxyl group at position 4 in the isocumarin structure of the OTA molecule is derived from the amino acid methionine (Steyn et al., 1970). EST C091 showed strong similarity to the alpha subunit of assimilatory sulphite reductase Met10 from *Saccharomyces cerevisiae*. Met10 catalyzes the fourth step in the sulphate assimilation pathway that leads to the biosynthesis of methionine and cysteine. Furthermore, EST C157 showed high similarity to an adenosylhomocysteinase that is also involved in the biosynthesis of methionine.
3.4. Regulation-Transcription-Transduction

Mycotoxin biosynthetic pathways are usually controlled by transcription factors, like the AflR and AflS proteins in the case of aflatoxins (Georgianna et al., 2009) or the genes tri6 and tri10 in the case of trichothecenes (Peplow et al., 2003). Among these transcription factors, AflR and tri6 are zinc finger proteins, which play a very important role in the regulation of aflatoxins and trichothecenes, respectively (Georgianna et al., 2008). In the present study, ESTs C028, C061 and C073 encode putative zinc finger-domain transcription factors that might be involved in the induction or repression of genes for OTA biosynthesis. Zinc finger proteins were also found in a cDNA-AFLP approach to studying OTA production in A. carbonarius (Botton et al., 2008).

Mycotoxin production is also responsive to general environmental factors, such as carbon and nitrogen sources, temperature, light, and pH. Signals generated in response to the environment are also typically relayed through zinc-finger proteins, including CreA for carbon signaling (Dowzer and Kelly 1989), PacC for pH signaling (Tilburn et al., 1995) and AreA for nitrogen signaling (Hynes, 1975). In relation to the latter, it has been observed that organic nitrogen activates aflatoxin biosynthesis while nitrate inhibits it (Georgianna et al., 2009). Noteworthy is the presence of AreA binding sites in the intergenic region between aflatoxin regulator genes aflR and aflS (Chang et al., 2000). Presence of nitrate results in active AreA binding to this region, leading to an increased expression of aflS and repression of aflatoxin biosynthesis (Georgianna et al., 2009). It has also been hypothesized that biosynthesis of fumonisins B1 is regulated by AreA (Kim and Woloshuk, 2008). In the present study, EST C009 showed homology to a negative regulator of the
transcription factor AreA. Accordingly, the protein encoded by EST C009 could negatively regulate AreA under OTA-production conditions. EST C093 showed weak similarity to a basic-region leucine zipper (bZIP) transcription factor (Motohashi et al., 2002) that, at least in A. nidulans, regulates the nitrogen metabolic repression regulator protein (Wong et al., 2007). Additionally, bZIP transcription factors may also regulate the expression of genes involved in the antioxidant response element (ARE) (Jowsey et al., 2003).

The biosynthesis of mycotoxins has many layers of regulation. Some are specific to the pathway, such as the transcription factors described above, whereas others have a more global effect on the regulation of secondary metabolism. Target of Rapamycin (TOR) is a protein kinase that constitutes the point of convergence for a number of cellular signaling pathways, including cell growth in response to nutrients, hormones, and stresses. It has recently been implicated in the biosynthesis by Fusarium fujikuroi of some secondary metabolites, such as gibberellins (Teichert et al., 2006). In the present study, we have identified several ESTs (C008, C088, C090 and C135) related to TOR. EST C088 showed similarity to TSC2, which is an upstream regulator of TOR. EST C090 showed homology to a protein (SAP190) that associates with the phosphatase SIT4 that is also involved in TOR regulation (Luke et al., 1996). Finally, ESTs C008 and C135 are related to the phosphatase PP2A complex that is also involved in TOR regulation. EST C008 showed homology with subunit B’ of PP2A and EST C135 with an inhibitor of PP2A. Interestingly, the results obtained in the present study correlate with those of Botton et al. (2008), who found that the phosphatase PP2A regulatory subunit B was down-
regulated in the ochratoxin-low-producing strain of *A. carbonarius* compared to
the high-producing strain. Thus, the four ESTs related with TOR found in the
present study suggest the possible involvement of this regulation system in
OTA production.

ESTs C002, C052, C143 and C153 displayed similarity with protein
kinases that might be involved in MAP kinase-dependent signal transduction.
Differential expression for several MAPK genes was observed in a comparison
of aflatoxin production using different temperatures of fungal growth (O’Brien et
al., 2007). Possible regulation may also be exerted throughout the G protein
signaling, since EST C069 encodes a calmodulin implicated in signal
transduction cascades activated by Ca\(^{2+}\)/calmodulin-dependent phosphorylation
and dephosphorylation. The relevance of this signal transduction during aflatoxin
biosynthesis has previously been noted (Juvvadi and Chivucula, 2006). In
addition, a down-regulated calmodulin was found in a low OTA production strain
of *A. carbonarius* in the study of Botton et al. (2008). C-AMP is another
important signaling molecule that is also involved in aflatoxin production. At
least 10 genes examined so far from the aflatoxin cluster appear to have CRE1
(cAMP-response element) sites in their promoters (Roze et al., 2004a, 2004b).
In the present analysis, a cAMP-dependent protein (EST C010) was also
identified. Finally, other sequences were shown to encode putative RNA and
DNA regulators (ESTs C003, C007, C067 and C082) and proteins involved in
protein synthesis/metabolism (C001, C004, C0013, C015, C032, C089 and
C098). In particular, it is interesting to note that the redundant ESTs C001 and
C013 showed similarity to GTPBP1, a GTP-binding protein related to the
translation elongation factor 1 alpha. Further clones, ESTs C089 and C098,
may also code for the translation elongation factor alfa (EF-1). Interestingly, similar clones were identified as differentially expressed in the OTA-producing fungi *A. carbonarius* and *P. nordicum* (Färber and Geisen, 2004; Botton et al. 2008). These data suggest a correlation between the elongation factor 1 alpha and OTA production.

### 3.5. Stress response and detoxification

Research over the past decade has made it clear that oxidative stress stimulates mycotoxin production and anti-oxidants have an inhibitory effect (Reverbery et al., 2008; Georgianna et al., 2009). In the present study, *A. carbonarius* ESTs represent a wide range (11.92%) of genes related to stress and detoxification (Table 1). EST C005 showed homology with the enzyme superoxide dismutase (SOD), which reduces the radical superoxide to form hydrogen peroxide and oxygen and can, therefore, act as an antioxidant (Cecarini et al., 2007). A knockout of a superoxide dismutase gene in *A. flavus* has been shown to increase aflatoxin production (He et al., 2007). EST C105 putatively encodes a mitochondrial peroxiredoxina with peroxidase activity (Amstrong, 1997). EST C122 putatively encodes a NADPH-quinone reductase, which plays an important role in oxidative stress resistance (Sies, 1993). EST C147 encodes a glutation-S-transferase (GST) whose activity detoxifies endogenous compounds. GST may also bind toxins and plays a role in the transport of proteins. Additionally, a positive correlation exists between GST activity and aflatoxin formation in *A. flavus* (Saxena et al. 1988). The correlation between oxidative stress and peroxisome proliferation is clear. Here, ESTs C037 and C083 showed homology with the proteins Pm27 and PEX3 from *S.*
cerevisiae and Pichia angusta, respectively, which are involved in peroxisome biogenesis. EST C054 encodes a protein similar to PMP20, a membrane protein from the peroxisome identified in Candida boidinii, which also has antioxidant activity (Horiguchi et al., 2001; Garrard and Goodman, 1989). Finally, it has become increasingly clear that molecular chaperones not only facilitate protein folding, but also regulate a number of cellular processes, including stress response. Chaperones encoded by five unisequences were identified (ESTs C017, C034, C106, C140 and C158).

3.6. Transport-secretion

In toxin-producing fungi, transporters are critical for detoxification by excreting mycotoxins to the outside of the cells, enabling them to produce these compounds continuously. Proteins related with transporters encoded by at least 10 unisequences were identified in A. carbonarius, reflecting increased or altered transport, metabolic and/or assimilatory activities during OTA production (Table 1). The redundant ESTs C050 and C139 and the EST C072 encode the proteins FLU1 and Atr1, respectively, which are transporters belonging to the major facilitator superfamily (MFS). MFS proteins are required for the transport of the trichothecene and cercosporine toxins in Fusarium sporotrichioides, Cercospora kikuchii and Cercospora nicotianae (Alexander et al., 1999; Callahan et al., 1999; Calabrese et al., 2000; Amnuaykanjanasin and Daub, 2009). Finally, other proteins involved in transport processes were identified in our study. They include proteins involved in the traffic of secretory pathway vesicles (ESTs C041, C047 and C150), K⁺ transport (ESTs C095 and C118)
and permeases associated with the transport of lactose and nicotinic acid (ESTs C110 and C130).

3.7 Hydrolysis of energy reserves

Several environmental conditions modulate mycotoxin biosynthesis, including carbon source. Eight ESTs encoding hydrolases during OTA production have been identified in *A. carbonarius* (Table 1). The redundant ESTs C142 and C154 encode an alpha-amylase, which is a glycosyl hydrolase, involved in starch degradation. ESTs C018 and C120 correspond to an alpha-glucosidase and a beta-glucosidase, respectively, while EST C128 is an endo-1,4-beta-xylanase. The aforementioned hydrolytic enzymes identified in *A. carbonarius* suggest their importance to the hydrolysis of nutrient reserves during OTA production. Usually, aflatoxinmycotoxin biosynthesis is stimulated by simple glucose-containing sugars, such as sucrose, or derivates of glucose, such as fructose (Georgianna et al., 2009). However, there is no consensus about OTA biosynthesis stimulation by sugars. Medina et al. (2008) reported a correlation between the presence of sucrose and glucose in a basal media (BM) and production of OTA by *Aspergillus* species. On the other hand, when fructose or lactose were used as a carbon source in that study, no OTA was detected. In contrast, in the study by Abbas et al. (2009) of *A. ochraceus*, different carbon sources including glucose, sucrose, maltose, galactose, xylose and glycerol appeared to repress OTA production when the fungus was grown in OTA-permissive PDY medium. In contrast, lactose induced OTA production. Apart from the different *Aspergillus* isolates used, there are other possible reasons for the disagreement in results reported by different researchers.
Laboratory practices used in extraction and detection of OTA (culture medium, incubation time and/or temperature) might lead to different results. A consensus in laboratory practices is needed in order to analyze the effect of nutritional factors on OTA biosynthesis. Other hydrolases identified were ESTs C079 and C111 encoding a N-acetylglucosaminyltransferase and an enzyme with sugar transferase activity, respectively. Interestingly, a protein with similarity to a dienalactone hydrolase family protein (EST C159) was also identified. Protoanemonin is a toxic metabolite that may be formed during the degradation of some chloroaromatic compounds such as OTA. This toxic metabolite can be transformed by the dienalactone hydrolase of Pseudomonas (Schlömann et al., 1993). Therefore, EST C159 might also play a role in detoxification processes.

3.8. Secondary metabolism

Aspergillus species are excellent producers of secondary metabolites. Beneficial secondary metabolites include food additives such as citric acid, antibiotics such as penicillin, and cholesterol-reducing drugs such as lovastatin. In contrast, the repertoire of fungal secondary metabolites also includes mycotoxins. It is interesting to note that two ESTs (C0099 and C137) may encode genes related to the biosynthesis of mycotoxins. C0099 showed homology to the gene moxY or aflW, which is a monooxygenase involved in the biosynthesis of aflatoxins (Yu et al., 2004). Similarly, C137 corresponds to the regulated protein AKtR-1, which is involved in the biosynthesis of the toxin AK in Alternaria alternata (Tanaka et al., 2000). Other interesting clones uncovered here were ESTs C086 and C058. The former showed homology to a specific precursor of the acyl-CoA dehydrogenases (ACD). ACDs are a family of
mitochondrial enzymes that oxidize straight- or branched-chain acyl-CoAs in the metabolism of fatty acids or branched chain amino acids. Interestingly, a sequence from the up-regulated acyl-CoA dehydrogenase gene in OTA production conditions has recently been used to develop a patented method for the detection of OTA-producing fungi (Dobson and O'Callaghan, 2009). EST C058 encoded for a metal esteril oxidase involved in ergosterol biosynthesis, which is a component of the fungal cell membrane. Interestingly, ergosterol and OTA production have been correlated (Saxena et al., 2001). Finally, EST C123 showed homology to a pyruvate carboxylase involved in the biosynthesis and degradation of citric acid. Recently, Mendez-Albores et al. (2008) reported an effect of citric acid in aflatoxin degradation. In addition, the antioxidant effect of citric acid is well know and therefore, might also play an antioxidant role in OTA production.

In conclusion, our study represents an important contribution to the identification of genes putatively involved in OTA production in A. carbonarius, and paves the way for further investigations. We have identified a total of 109 differentially expressed genes using SSH and, according to their sequence similarities to genes in the NCBI database, we have speculated on the possible functional roles some might play in both the production and regulation of OTA synthesis and metabolism. We show that the SSH approach is feasible for generating an overall picture of genes that may be involved in OTA production. Worth noting is the high percentage of genes involved in regulation processes, including important transcriptional factors such as zinc finger proteins, a regulator of AreA and a bZIP transcription factor. Other genes involved in a more global regulation process are also present. It is also important to note the
high percentage of genes involved in stress responses and detoxification. An enhanced knowledge of genes involved in OTA biosynthesis represents a key advance in the study of environmental and nutritional influences on OTA production. In addition, this study could contribute to further development of improved specific detection and quantification methods, which are required to control OTA contamination in the grape wine industry.

Acknowledgements

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M., Fanelli, C., Fabbri, A., 2008. Modulation of antioxidant defence in
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Table 1. Summary of 109 sequenced inserts from the SSH library, enriched with the high OTA-producing strain of A. carbonarius (W04-40).
Fig. 1. Production of OTA after 5 days growth of the high OTA-producing strain of *A. carbonarius* (W04-40) and the low OTA-producing strain (W04-46). The means of three separate experiments is shown.

Fig. 2. Functional classification of cDNAs representing putative genes expressed by *A. carbonarius* during OTA production. The percentage in each one of the categories is shown (refer to Table 1).
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<th>Unigene identification</th>
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<th>Transcript abundance</th>
<th>Annotation (putative function)</th>
<th>Organism and gene Accession Nº</th>
<th>E-value</th>
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<td>450</td>
<td>1</td>
<td>Dopa decarboxylase 3 DDC3 from Aspergillus oryzae patent W09960136-A1 (histidine metabolism; tyrosine metabolism;phenylalanine metabolism; tryptophan metabolism;alkaloid biosynthesis)</td>
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RNA binding protein-\textit{Schizosaccharomyces pombe}
Nucleolar protein Nsr1 involved in pre-rRNA processing-\textit{Saccharomyces cerevisiae} (binds to histone H2B)
Nonhistone chromosomal protein Nhp6b-\textit{Saccharomyces cerevisiae}
Serine/threonine kinase par-1- \textit{Caenorhabditis elegans}
Nitrogen metabolic repression regulator hnmr
\textit{cAMP}-regulated phosphoprotein/endosulfine conserved region-\textit{Schizosaccharomyces pombe}
GTP binding protein (GTPBP1)-\textit{Homo sapiens} (related to elongation factor 1 alpha)
Autophagic death protein Aut7/IDI
C2H2-type zinc-finger protein-\textit{Schizosaccharomyces pombe}
Peptide chain release factor 2 PrfB PrfB belongs to the prokaryotic and mitochondrial release factors family- \textit{Streptomyces coelicolor}
Serine/threonine protein kinase (Kin4)
C-x8-C-x5-C-x3-H type zinc finger protein
Histone H2A
Calmodulin calM-\textit{Aspergillus nidulans}
C2H2 finger domain protein
Nuclear polyadenylated RNA-binding protein Nah3-\textit{Saccharomyces cerevisiae}
Gigas protein gig-\textit{Drosophila melanogaster} (related to GTPBP1)
Translation elongation factor 1 alpha- \textit{Podospora anserina}
bZIP transcription factor L-Maf- \textit{Gallus gallus}
Translation elongation factor EF-1 alpha subunit, putative-\textit{Saccharomyces cerevisiae}
Myosin-like protein Mlp1- \textit{Saccharomyces cerevisiae} (implicated in nuclear transport)
Translation elongation factor Ela1- \textit{Saccharomyces cerevisiae}
Ubiquitin activating protein Uba1 - \textit{Saccharomyces cerevisiae}
Inhibitor of PP2A SET- \textit{Homo sapiens}
Nucleolar RNA-associated proteins-\textit{Aspergillus nidulans} (strong similarity to hypothetical protein YGR090w from patent WO200039342)
Elongation factor 1 alpha- \textit{Aspergillus nidulans} (related to elongation factor 1 alpha)
Serine/threonine kinase par-1- \textit{Aspergillus nidulans}
Serine/threonine protein kinase involved in cell cycle control
Mitochondrial superoxide dismutase
14-3-3 protein homolog artA- \textit{Aspergillus nidulans}
Heat shock protein spsB
Peroxisomal membrane protein Prnp27- \textit{Saccharomyces cerevisiae}
Peroxisomal membrane protein Pmp20- \textit{Candida boidinii}
Postsynaptic receptor clustering and molybdenum cofactor biosynthesis protein gephyrin-\textit{Rattus norvegicus}
Peroxisomal membrane protein PEX3- \textit{Pichia angusta}
Mitochondrial peroxiredoxin PRX1

\textbf{Mitochondrial peroxiredoxin PRX1}

\textbf{Peroxisomal membrane protein PEX3}

\textbf{Postsynaptic receptor clustering and Pmp27- \textit{Saccharomyces cerevisiae}}

\textbf{Heat shock protein spsB}

\textbf{Peroxisomal membrane protein Pmp20- \textit{Candida boidinii}}

\textbf{Postsynaptic receptor clustering and molybdenum cofactor biosynthesis protein gephyrin- \textit{Rattus norvegicus}}

\textbf{Peroxisomal membrane protein PEX3- \textit{Pichia angusta}}

\textbf{Mitochondrial peroxiredoxin PRX1}
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<td>Secretion-associated G-actin binding protein sarA</td>
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C090  GW327985  252  1  Bud formation factor Sap190 -Saccharomyces cerevisiae
C097  GW328010  295  1  Tubulin beta chain beta-tubulin - Aspergillus flavus
C102  GW327993  498  1  Con-8 - Neurospora crassa (expressed early during conidial differentiation)
C127  GW328019  439  1  Meiotic sister-chromatid recombination protein Msc1 - Saccharomyces cerevisiae
C151  GW328042  396  1  Spore-wall fungal hydrophobin dewA - Aspergillus nidulans
C156  GW328047  225  1  Vacular (H+)-ATPase G subunit

Hypothetical proteins
C026  GW327933  333  1  Hypothetical protein An09g04880
C038  GW327942  513  1  Hypothetical protein EAA77948.1-Gibberella zeae
C044  GW327947  394  1  Hypothetical protein An11g02730
C053  GW327955  431  1  Yci-like protein
C057  GW327957  255  2  Hypothetical protein CAD2176.1 -Neurospora crassa
C062  GW327962  521  2  Hypothetical protein CAD2195.1-Neurospora crassa
C065  GW327964  275  2  Hypothetical protein CAD2195.1-Neurospora crassa
C080  GW327975  236  1  Hypothetical protein SCP91.02c - Streptomyces coelicolor
C087  GW327982  180  2  Hypothetical verprolin related protein encoded by B24P7.40 -Aspergillus niger
C092  GW327987  283  1  Hypothetical protein CAD11387.1-Neurospora crassa
C094  GW327989  338  2  Hypothetical protein ES2-Musculus
C096  GW327991  365  1  Hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe
C107  GW327998  309  2  Hypothetical verprolin related protein encoded by B24P7.40- Neurospora crassa (Rossman-fold NAD (P) (+)-binding protein)
C116  GW328007  415  2  Hypothetical protein CAD21276.1 - Neurospora crassa
C126  GW328018  212  1  Hypothetical lipoprotein SCA42.13c - Streptomyces coelicolor
C129  GW328021  393  1  Hypothetical protein CAE76294.1 - Neurospora crassa
C132  GW328024  388  1  Hypothetical protein B24H17.110 -Neurospora crassa
C141  GW328032  580  1  Hypothetical protein
C146  GW328037  572  1  Hypothetical protein C50F7.2 -Caenorhabditis elegans
C152  GW328043  330  2  Hypothetical protein ES2-Musculus

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C039  GW327943  252  2  no blast
C042  GW327946  241  1  no blast
C046  GW327948  297  1  no blast
C051  GW327953  513  2  no blast
C060  GW327960  272  2  no blast
C081  GW327976  512  2  no blast
C084  GW327979  455  1  no blast
C103  GW327994  394  1  no blast
C113  GW328004  306  1  no blast

XM_749842  Aspergillus niger CBS 513.88  3,00E-33
XM_001401893  Aspergillus niger CBS 513.88  2,00E-93
XM_001392599  Aspergillus niger CBS 513.88  2,00E-45
XM_001401542  Aspergillus niger CBS 513.88  2,00E-19
XM_001397716  Aspergillus niger CBS 513.88  2,00E-23
XM_001390100  Aspergillus clavatus NRRL 1  8,00E-17

Aspergillus niger CBS 513.88  4,00E-67
Aspergillus niger CBS 513.88  3,00E-18
Aspergillus niger CBS 513.88  2,00E-54
Aspergillus clavatus NRRL 1  6,00E-33
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Valencia 27th april 2010

Manuscript reference: FOOD-D-10-00093

Genes differentially expressed by Aspergillus carbonarius strains under ochratoxin A producing conditions

Dear Dr. Luca Cocolin,

Thank you very much for your mail with the referees' comments on our above referenced manuscript submitted to ‘International Journal of food Microbiology’. After carefully reading the comments we think that overall you have raised interesting and pertinent comments which have considerably improved the manuscript. Please find below our replies to each of the points raised.

Yours sincerely,

Pedro Vicente Martínez Culebras (Ph. D.)
Reviewer 1

Comments

1. This manuscript deals on the important topics related to understanding the molecular basis of the biosynthesis of Ochratoxin A (OTA) in Aspergillus carbonarius. The SSH approach, used by the authors, is interesting and give new information on 109 differentially expressed sequences between two strains of A. carbonarius antagonist in their ability to produce OTA. However, as both the two strain are able to produce OTA, but at very different level, the information retrieved by this work could be related to the process of regulation and not to the genes directly involved in the biosynthesis of the toxin. In this respect, should be better to use the same technique growing a good producing strain in conducive/suppressive condition for OTA production as made by other authors for A. ochraceus and P. verrucosum/nordicum. However, the results obtained are worthy to be published on this journal and contribute to increase the knowledge and the molecular data on this hot topic for the mycotoxin field.

OTA production by strain W04-46 is very low (see in figure 1). Indeed, we could consider W04-46 to be an OTA non-producer. It is likely that all so-called OTA non-producing strains of A. carbonarius are in fact able to produce a very small amount of OTA. Firstly, we tried to use the SSH technique growing the OTA producing strain in conducive/suppressive condition for OTA production. However, the OTA-producing strain was able to produce a large amount of OTA in spite of the suppressive conditions. In contrast, the use of two strains of A. carbonarius antagonist in their ability to produce OTA avoids this problem. In addition, this approach avoids differential expression due to differences in the media that are not linked to OTA production, which could otherwise bias the results.

2. page 2 line 22: insert that A. carbonarius is also responsible of OTA contamination of coffee and cocoa (DONE)

3. page 6 line 126: delete "previously previously" and write: previously set up by. (DONE)
   page 8 line 172-173: change "OTA non-producer" in "OTA low producer" 

4. page 8 line 172-173: change "OTA non-producer" in "OTA low producer. (DONE)

5. page 11 line 233-235: the period is not clear, how you reach from 138 ESTs the 109? subtracting 11 genes represented by two ESTs 138-22= 116?? Please correct table 1 because in it are listed all the 138 ESTs identified and not the unique 109. We agree that this part was difficult to understand and it has been duly modified. We consider that it might be interesting to maintain all the ESTs in Table 1 because the redundancy in some cases is based on two ESTs belonging to different parts of the same gene. The following paragraph has been included: “Blast searches against the Genome Databases indicated that 118 of the above genes had matches in the
databases and 20 ESTs were categorized as having no match in the databases. Self-BLAST analysis showed 11 sequences among the 138 ESTs, each one represented by two ESTs, with a redundancy level of 8%. This enabled the identification of 116 non-redundant ESTs (Table 1). Among these, 109 ESTs had matches in the databases.

6. page 11 line 238: delete the comma after alpha. (DONE)
7. page 13 line 286-287: correct "Georgina" to "Georgianna" (DONE)
8. page 15 line 336: correct "suggest the involvement" to "suggest the possible involvement" (DONE)
9. page 15 line 351: Roze et al 2004 please indicate if is 2004a or 2004b or both (BOTH: It has been indicated)
10. page 16 line 368: correct "Reverbery" to "Reverberi" (DONE)
11. page 16 line 375: correct "peroxiredoxine" to "peroxiredoxin" (DONE)
12. page 16 line 376: Armstrong 1997 is missing in the reference list. This reference has been eliminated from the text.
13. page 18 line 413: correct "hydolases" to "hydrolases" (DONE)
14. page 19 line 448: correct "lovastin" to "lovastatin" (DONE)
15. page 19 line 450-451: correct C009 in C099 (DONE)
16. In the reference list please delete Ehrlich et al 1999, Kusomoto et al 2000 and Schwarz et al 2006 because are not cited in the manuscript. (DONE)
The authors found several classes of genes, which are activated under conditions supportive for ochratoxin A biosynthesis. The discussion about these activated genes is plausible. However it is very curious that no genes which are expected to be directly involved in ochratoxin A biosynthesis, like pks, nrps, chlorinating activities or methylases, esterases etc. were found during this analysis. It was shown that the optimum time to take the samples was after 48 h, but was it ensured that the culture used for RNA extraction did indeed produce the toxin. Despite the kinetic experiment, variations in the optimal time for production may occur depending on the preculture conditions. On the other hand the SSH library might be biased for some reasons or simply the concentrations of the ochratoxin biosynthesis gene transcripts might have been to low to be detected by the conditions used. However other putative secondary metabolite genes, like homologues to the aflatoxin pathway were detected. This however suggests that the conditions used were not optimal to detect ochratoxin A biosynthesis genes, so the relation of the results described here to ochratoxin biosynthesis is not absolute conclusive. Two different approaches could have been used to develop the SSH technique. In the first, the OTA-producing strain could have been used in conductive/suppressive conditions for OTA production. We tried to do this but unfortunately the OTA-producing strain was able to produce an large amount of OTA in spite of the suppressive conditions. The second approach is based on the use of two strains of A. carbonarius antagonistic in their abilities to produce OTA. This avoids the problem noted above as well as avoiding differential expression due to differences in the media that are not linked to OTA production, which could bias the results. For these reasons the second approach was selected to do the SSH technique.

Several questions can be taken into account in order to explain that no genes directly involved in OTA biosynthesis were found. Firstly, it is necessary to indicate that it is possible that differences between the two strains of A. carbonarius antagonistic in their abilities to produce OTA would be due more to genes involved in regulation than genes directly involved in OTA biosynthesis. Additionally, the lack of OTA production in the OTA non-producing strain (W04-46) would be due to mutations in genes located at the end of the OTA biosynthesis pathway. Thus, expression of the genes located before the mutated genes will be subtracted during the SSH experiment. Finally, it is necessary to indicate that a total of 20 ESTs were categorized as having no match in the databases. Some of these genes would be directly involved in OTA biosynthesis.

In relation to the conditions for growing the culture of A. carbonarius for RNA extraction it is worth noting that an identical procedure and conditions were followed in this case to those followed for the kinetic experiment. Moreover, OTA production was analysed in the culture (48 h) used for RNA extraction and similar results of OTA production were found to those obtained from the three replicates used for the kinetic experiment. This information has been added in the text. A culture of 48 h was selected as the optimum point to study differences in gene expression between the two strains taking into account the difference between maximum OTA production (72 h) and maximum gene expression (24 h before the maximum OTA production).

1 Minor comments: Paragraph 2.1: Here it should be described which is the producing is the non-producing strain! Paragraph 2.1. in the Material and methods sections has been changed to: "Two strains of A. carbonarius antagonist in their ability to produce OTA previously characterized by Martínez-Culebras and Ramón (2007), namely W04-40 (OTA high-producing strain) and W04-46 (OTA low-producing strain), were used because of their differential production of OTA."

2 What is a Polytron? It is a homogenizer. This word has been included in manuscript.
Paragraph 2.4: In the heading it is mentioned that cDNA synthesis is described, which however is not the case. Only the RNA isolation procedure is described. cDNA synthesis has been added:
“First-stranded cDNA synthesis was carried out using the SuperScript III (RT) kit (Invitrogen), according to the manufacturer’s instructions.”

Paragraph 2.5: A brief description of the SSH procedure should be given, albeit it is pinpointed to the recommendations of the manufacturer. The following sentence has been added in order to support a brief description of the SSH technique: “Suppression subtractive hybridization (SSH) is an effective approach to identify those genes that vary in expression levels during different biological processes. The theory behind subtraction is simple. First, mRNA from two different populations corresponding to different biological processes or situations are converted to cDNA. We refer to the cDNA that contains specific (differentially expressed) transcripts as tester, and the reference cDNA as driver. Tester and driver cDNAs are hybridized and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester but absent from the driver mRNA.

The last sentence of this paragraph belongs to the next paragraph “Sequencing analysis”. (DONE)

10/227ff: To identify the bacterial colonies.. This sentence is not completely clear. Obviously it is meant the plasmid DNA was isolated from the single E. coli colonies. However what is meant with library screening? Is it the confirmation that the plasmids contains inserts? Yes, this sentence has been modified to read: “To identify the bacterial colonies that possessed cloning vectors with inserts, the plasmid DNA was extracted and sequenced”.

11/first paragraph: Is the word "cluster" the right term for a group of two EST’s? Moreover it is not clear where the 11 clusters came from. The text pinpoint to Table 1. However in this table 23 EST’s with transcript abundance of 2 are listed? The word cluster has been eliminated for sequences represented by just two ESTs. Table 1 has been corrected and 11 sequences represented by two ESTs appear in the transcript abundance column as 2.

14/317: The message of this sentence is not completely correct. The authors state the transcription factors described above (areaA, creA or pacC) are specific pathway transcription factors and others like TOR are global transcription factors. That is not the case areaA, creA or pacC are global transcription factors, because the act on a lot of different pathways regulated by glucose or nitrogen etc! Yes, we agree. In order to write with more precision, the following paragraph has been eliminated: “The biosynthesis of mycotoxins has many layers of regulation. Some are specific to the pathway, such as the transcription factors described above, whereas others have a more global effect on the regulation of secondary metabolism”.

18/420: It cannot be generally said that "mycotoxin" biosynthesis is stimulated by simple glucose-containing sugars or by certain nitrogen sources (13/301). The reference given in both occasions is only dealing with aflatoxin, not with mycotoxins in general! In both sentences 18/420 and 13/301 mycotoxin biosynthesis has been changed to aflatoxin biosynthesis