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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 ($E_{\text{value}} < 10^{-5}$) 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 unisquences showed homology to genes involved in protein syntesis 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 or 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), Karolewicz 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 monooxygenase 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

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Strains were grown on Petri dishes containing Malt Extract Agar (MEA) medium in the dark at 30 °C 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 10⁶ 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 °C 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 homogeneously grown on CYA pH 5.0 and incubated at 30 °C 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 °C 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 Vínicos 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 Ppolytron 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 ×g 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 °C 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

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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)

187 according to the manufacturer's instructions. The cDNA from the OTA producer
188 strain (W04-40) was used as the tester and the cDNA from the OTA non-
189 producer (W04-46) was used as the driver. The resulting subtracted cDNAs
190 were ligated to plasmid PCR 2.1 (Invitrogen) and the ligation mixtures were
191 transformed into electro-competent *Escherichia coli* DH5α cells (Invitrogen).

192 ~~Plasmid DNA was extracted (GenElute Plasmid Miniprep Kit, SIGMA) from 160~~
193 ~~recombinant colonies and sequenced with a T7 primer using an automated~~
194 ~~sequencer.~~

195 2.6. Sequence analysis

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

205 Results and Discussion

206 3.1. Characteristics of the SSH cDNA library

207 Despite the fact that OTA is an important mycotoxin, not much is known
208 about the biological background of OTA production by ochratoxigenic fungi. The
209 aim of this study was to identify genes involved in OTA biosynthesis and in its

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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).

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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. DNA 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

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(Table 1). 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-acetylglucosaminyltransferase (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.

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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

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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 dehydroisocoumarin 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 isocoumarin 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~~~~mycotoxin~~ 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 fumonisin 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'Brian 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). cAMP 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 (Reverber ~~y~~ⁱ 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 peroxiredoxin ~~e~~ 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 glutathione-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, ~~aflatoxin~~mycotoxin biosynthesis is stimulated by simple glucose-containing sugars, such as sucrose, or derivatives 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 dienelactone 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 dienelactone 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 (C0999 and C137) may encode genes related to the biosynthesis of mycotoxins. C0999 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.

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References

- Abbas, A., Valez, H., Dobson, A.D.W., 2009. Analysis of the effect of nutritional factors on OTA and OTB biosynthesis and polyketide synthase gene expression in *Aspergillus ochraceus*. *International Journal of Food Microbiology* 135, 22-27.
- Abouzied, M.M., Horvat, A.D., Podlesny, P.M., Regina, N.P., Metodiev, V.D., Kamenova-Tozeva, R.M., Niagolova, N.D., Stein, A.D., Petropoulos, E.A., Ganey V. S., 2002. Ochratoxin A concentrations in food and feed from a region with Balkan endemic nephropathy. *Food Additives and Contaminants* 19, 755-764.

526 Alexander, N.J., McCormick, S.P., Hohn, T.M., 1999. TRI12, a trichothecene
 527 efflux pump from *Fusarium sporotrichioides*: gene isolation and expression in
 528 yeast. *Molecular and General Genetics* 261, 977-984.

529 Amnuaykanjanasin, A., Daub, M.E., 2009. The ABC transporter ATR1 is
 530 necessary for efflux of the toxin cercosporin in the fungus *Cercospora*
 531 *nicotianae*. *Fungal Genetics and Biology* 46, 146-158.

532 Bacha, N., Atoui, A., Mathieu, F., Liboz, T., Lebrihi, A., 2009. *Aspergillus*
 533 *westerdijkiae* polyketide synthase gene "aoks1" is involved in the biosynthesis
 534 of ochratoxin A. *International Journal of Food Microbiology* 46, 77-84.

535 Battilani P., Magan N., Logrieco, A., 2006. European research on ochratoxin A
 536 in grapes and wine. *International Journal of Food Microbiology* 111, S2-S4.

537 Bau, M., Bragulat, M.R., Abarca, M.L. Minguéz, S., Cabañes F.J., 2005.
 538 Ochratoxin species from Spanish wine grapes. *International Journal of Food*
 539 *Microbiology* 98, 125-130.

540 Bellí, N., Pardo, E., Marín, S., Farré, G., Ramos, A. J., Sanchis, V., 2004.
 541 Occurrence of ochratoxin A and toxigenic potential of fungal isolates from
 542 Spanish grapes. *Journal of Science of Food and Agriculture* 84, 541-546.

543 Botton, A., Ferrigo, D., Scopel, C., Causin, R., Claudio Bonghi, C., Ramina, A.,
 544 2008. A cDNA-AFLP approach to study ochratoxin A production in *Aspergillus*
 545 *carbonarius*. *International Journal of Food Microbiology* 127, 105-115.

546 Bragulat, M.R., Abarca M.L., Cabañes F.J., 2001. An easy screening method
 547 for fungi producing ochratoxin A in pure culture. International Journal of Food
 548 Microbiology 71, 139-144.

549 Callahan, T.M., Rose, M.S., Meade, M.J., Ehrenshaft, M., Upchurch, R.G.,
 550 1999. CFP, the putative cercosporin transporter of *Cercospora kikuchii*, is
 551 required for wild type cercosporin production, resistance, and virulence on
 552 soybean. Molecular Plant Microbe Interactions 12, 901-910.

553 Chang PK., Yu J.J., Bhatnagar, D., Cleveland T.E., 2000. Characterization of
 554 the *Aspergillus parasiticus* major nitrogen regulatory gene, areA Source:
 555 Biochimica et Biophysica Acta-gene Structure and Expression 149, 263-266.

556 Cecarini, V., Gee, J., Fioretti, E., Amici, M., Angeletti, M., Eleuteri, A.M., Keller,
 557 J.N., 2007. Protein oxidation and cellular homeostasis: Emphasis on
 558 metabolism. Biochimica et Biophysica Acta-Molecular Cell Research 1773, 93-
 559 104.

560 Diatchenko, L., Lau, Y.F., Ambell, P.A., Chenchik, A., Moqadam, F., Huang, B.,
 561 Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. and Siebert, P., 1996.
 562 Suppression subtractive hybridization. A method for generating differentially
 563 regulated or tissue-specific cDNA probes and libraries. Proceedings of the
 564 National Academy of Sciences 93, 6025-6030.

565 Dobson, A., O'Callaghan, J., 2009. Detection of ochratoxin A producing fungi.
 566 US Patent 7560234. University College Cork, National University of Ireland
 567 Cork.

568 Dowzer, C.E.A., Kelly, J.M., 1989. Cloning of the *creA* gene from *Aspergillus*
569 *nidulans*: a gene involved in carbon catabolite repression. *Current Genetics* 15,
570 457-459.

571 ~~Ehrlich, K.C., Cary, J.W., Montalbano, B.G., 1999. Characterization of the~~
572 ~~promoter for the gene encoding the aflatoxin biosynthetic pathway regulatory~~
573 ~~protein. *Biochimica et Biophysica Acta - Gene Structure and Expression* 1444,~~
574 ~~412-417.~~

575 Färber, P., Geisen, R., 2004. Analysis of Differentially-Expressed Ochratoxin A
576 Biosynthesis Genes of *Penicillium Nordicum*. *European Journal of Plant*
577 *Pathology* 110, 661-669.

578 Gallo, A., Perrone, G., Solfrizzo, M., Epifani, F., Abbas, A., Dobson, A.D.W.,
579 Mulè, G., 2009. Characterisation of a *pks* gene which is expressed during
580 ochratoxin A production by *Aspergillus carbonarius*. *International Journal of*
581 *Food Microbiology* 129, 8-15.

582 Garrard, L.J., Goodman J.M., 1989. 2 genes encode the major membrane-
583 associated protein of methanol-induced peroxisomes from *Candida-boidinii*.
584 *Journal of Biological Chemistry* 264, 13929-13937.

585 Geisen, R., Schmidt-Heydt, M., Karolewicz, A., 2006. A gene cluster of the
586 ochratoxin A biosynthetic genes in *Penicillium*. *Mycotoxin Research* 22, 134–
587 141.

588 Georgianna, D.R., Payne, G.A., 2009. Genetic regulation of aflatoxin
589 biosynthesis: From gene to genome. *Fungal Genetic and Biology* 46, 113-125.

590 Harris, J.P., Mantle, P.G., 2001. Biosynthesis of ochratoxins by *Aspergillus*
591 *ochraceus*, *Phytochemistry* 58, 709-716.

592 He, M.S., Price, G.R., O'Brian, D.R., Georgianna, D.R., Payne G.A., 2007.
 593 Improved Protocols for the functional analysis in the pathogenic fungus
 594 *Aspergillus flavus*. BMC Microbiology 7, 104.
 595 Horiguchi, H., Yurimoto, H., Goh, T.K., Nakagawa, T., Kato, N., Sakai, Y., 2001.
 596 Peroxisomal catalase in the methylotrophic yeast *Candida boidinii*: Transport
 597 efficiency and metabolic significance. Journal of Bacteriology 183, 6372-6383.
 598 Hynes, M.J., 1975. Estudios on role of AreA gene in regulation of nitrogen
 599 catabolism in *Aspergillus nidulans*. Australasian Journal of Biological Sciences
 600 28, 301-213.
 601 Jowsey, I.R., Smith, S.A., Hayes, J.D., 2003. Expression of the murine
 602 glutathione S-transferase alpha 3 (GSTA3) subunit is markedly induced during
 603 adipocyte differentiation: activation of the GSTA3 gene promoter by the pro-
 604 adipogenic eicosanoid 15-deoxy-Delta (12,14)-prostaglandin J(2). Biochemical
 605 and biophysical Research Communications 312, 1226-1235.
 606 Juvvadi, P.R, Chivukula S., 2006. Putative calmodulin-binding domains in
 607 aflatoxin biosynthesis-regulatory proteins. Current Microbiology 52, 493-496
 608 Luke, M.M., DellaSeta, F., DiComo, C.J., Sugimoto, H., Kobayashi, R., Arndt, T.,
 609 1996. The SAPs, a new family of proteins, associate and function positively with
 610 the SIT4 phosphatase. Molecular and Cellular Biology 16, 2744-2755.
 611 Karolewicz, A., Geisen, R., 2005. Cloning a part of the ochratoxin A biosynthetic
 612 gene cluster of *Penicillium nordicum* and characterisation of the ochratoxin
 613 polyketide synthase gene. Systematic and Applied Microbiology 28, 588–595.

614 Kim, H., Woloshuk, C.P., 2008. Role of AREA, a regulator of nitrogen
615 metabolism, during colonization of maize kernels and fumonisin biosynthesis in
616 *Fusarium verticillioides*. Fungal Genetic and Biology 45, 947-953.

617 ~~Kusomoto, K., Nogata, Y., Ohta, H., 2000. Directed deletions in the aflatoxin~~
618 ~~biosynthesis gene homolog cluster of *Aspergillus oryzae*. Current Genetics 37,~~
619 ~~104-111.~~

620 Martínez-Culebras, P.V., Ramón, D., 2007. An ITS-RFLP method to identify
621 black *Aspergillus* isolates responsible for OTA contamination in grapes and
622 wine. International Journal of Food Microbiology 113, 147-153.

623 Martínez-Culebras, P.V., Crespo-Sempere, A., Sanchez-Hervas, M.,
624 Elizaquivel, P., Aznar, R., Ramón, D., 2009. Molecular characterization of the
625 black *Aspergillus* isolates responsible for OTA contamination in grapes and
626 wine in relation to taxonomy of *Aspergillus* section *Nigri*. International Journal of
627 Food Microbiology 132, 33-41.

628 Medina, A., Mateo, E.M., Valle-Algarra, F.M., Mateo, F., Mateo, R., Jimenez,
629 M., 2008. Influence of nitrogen and carbon sources on the production of
630 ochratoxin A by ochratoxigenic strains of *Aspergillus* spp isolated from grapes.
631 International Journal of Food Microbiology 122, 93-99.

632 Mendez-Albores, A., Martinez-Bustos, F., Veles-Medina, J.J., Moreno-Ramos,
633 C., Del Rio-Garcia, J.C., Moreno-Martinez, E., 2009. Effect of citric acid addition
634 on aflatoxin degradation and on functional properties of extruded sorghum
635 products. Interiencia 252-258.

636 Motohashi, H., O'Connor, F., Katsuoka, F., Engel, J.D., Yamamoto, M., 2002.
637 Integration and diversity of the regulatory network composed of Maf and CNC
638 families of transcription factors. *Gene* 294,1-12.

639 O'Brian, G.R., Georgianna, D.R., Wilkinson, J.R., Yu, J., Abbas, H.K.,
640 Bhatnagar, D., Cleveland, T.E., Nierman, W., Payne, G.A., 2007. The effect of
641 elevated temperature on gene transcription and aflatoxin biosynthesis.
642 *Mycologia* 99, 232-239.

643 O'Callaghan, J., Caddick, M.X., Dobson, A.D.W., 2003. A polyketide synthase
644 gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*.
645 *Microbiology* 149, 3485–3491.

646 O'Callaghan, J., Stapleton, P.C., Dobson, A.D.W., 2006. Ochratoxin A
647 biosynthetic genes in *Aspergillus ochraceus* are differentially regulated by pH
648 and nutritional stimuli. *Fungal Genetics and Biology* 43, 213–221.

649 Pel, H.J., de Winde, J.H., Archer, D.B., Dyer, P.S., Hofmann, G., Schaap, P.J.,
650 Turner, G., de Vries, R.P., Albang, R., Albermann, K., Andersen, M.R.,
651 Bendtsen, J.D., Benen, J.A.E., van den Berg, M., Breestraat, S., Caddick, M.X.,
652 Contreras, R., Cornell, M., Coutinho, P.M., Danchin, E.G.J., Debets, A.J.M.,
653 Dekker, P., van Dijck, P.W.M., van Dijk, A., Dijkhuizen, L., Driessen, A.J.M.,
654 d'Enfert, C., Geysens, S., Goosen, C., Groot, GSP., de Groot, P.W.J.,
655 Guillemette, T., Henrissat, B., Herweijer, M., van den Hombergh, J.P.T.W., van
656 den Hondel, C.A.M.J.J., van der Heijden, R.T.J.M., van der Kaaij, R.M., Klis,
657 F.M., Kools, H.J., Kubicek, C.P., van Kuyk, P.A., Lauber, J., Lu X., van der
658 Maarel, M.J.E.C., Meulenberg, R., Menke, H., Mortimer, M.A., Nielsen, J.,

659 Oliver, S.G., Olsthoorn, M., Pal, K., van Peij, N.N.M.E., Ram, A.F.J., Rinas, U.,
 660 Roubos, J.A., Sagt, C.M.J., Schmoll, M., Sun, J.B., Ussery, D., Varga, J.,
 661 Vervecken, W., de Vondervoort, P.J.J.V., Wedler, H., Wosten, H.A.B., Zeng,
 662 A.P., van Ooyen, A.J.J., Visser, J., Stam, H., 2007. Genome sequencing and
 663 analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nature
 664 Biotechnology 25, 221-231.

665 Peplow, A.W., Meek, I.B., Wiles, M.C., Phillips, T.D., Beremand, M.N., 2003.
 666 *Tri16* Is Required for Esterification of Position C-8 during Trichothecene
 667 Mycotoxin Production by *Fusarium sporotrichioides*. Applied and Environmental
 668 Microbiology 69, 5935-5940.

669

670 Perrone, G., Susca, A., Cozzi, K., Ehrlich, J., Varga, J., Frisvad, J.C., Meijer,
 671 M., Noomin, P., Mahakarnchanakul, W., Samson, R.A., 2007. Biodiversity of
 672 *Aspergillus* species in some important agricultural products. Studies in
 673 Mycology 59, 53-66.

674 Reverberi, M., Zjalic, S., Ricelli, A., Punelli, F., Camera, E., Fabbri, C., Picardo,
 675 M., Fanelli, C., Fabbri, A., 2008. Modulation of antioxidant defence in
 676 *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for *ApyapA*
 677 gene. Eukaryotic Cell 25, 988-1000.

678 Roze, L.V., Miller, M.J., Rarick, M., Mahanti, N., Linz, J.E., 2004a. A novel
 679 cAMP-response element, CRE1, modulates expression of *nor-1* in *Aspergillus*
 680 *parasiticus*. Journal of Biological Chemistry 26, 27428-27439.

681 Roze, L.V., Beaudry, R.M., Keller, N.P., Linz, J.E., 2004b. Regulation of
682 aflatoxin synthesis by FadA/cAMP/protein kinase A signaling in *Aspergillus*
683 *parasiticus*. *Mycopathologia* 158, 219-232.

684 Saxena, M., Mukerji, K.G., Raj, H.G., 1988. Positive correlation exists between
685 glutathione s-transferase activity and aflatoxin formation in *Aspergillus-flavus*.
686 *Biochemical Journal* 254, 567-570.

687 Saxena, J., Munimbazi, C., Bullerman, L.B., 2001. Relationship of mould count,
688 ergosterol and ochratoxin A production. *International Journal of Food*
689 *Microbiology* 71, 29-34.

690 Schlömann, M., Ngai, K.L., Ornston, L.N., Knackmuss, H.J., 1993. Dienelactone
691 hydrolase from *Pseudomonas-cepacia*. *Journal of Bacteriology* 175, 2994-3001.

692 |
693 ~~Schwarz, G., Mendel, R.R., 2006. Molybdenum cofactor biosynthesis and~~
694 ~~molybdenum enzymes. *Annual Review of Plant Biology* 57, 623-647.~~

695
696 Sies, H., 1993. Strategies of antioxidant defense. *Biochemical* 15, 213–219.

697 Steyn, P.S., Holzapfel, C.W., 1970. The biosynthesis of the ochratoxins,
698 metabolites of *Aspergillus ochraceus* *Phytochemistry* 9, 1977-1983.

699 Tanaka, A., Tsuge, T., 2000. Structural and functional complexity of the
700 genomic region controlling AK-toxin biosynthesis and pathogenicity in the
701 Japanese pear pathotype of *Alternaria alternata*. *Molecular Plant Microbe*
702 *Interactions* 13, 975-986.

Teichert, S., Wottawa, M., Schoenig, B., Tudzynski, B., 2006. Role of the *Fusarium fujikuroi* TOR kinase in nitrogen regulation and secondary metabolism. Eukariotic Cell 5, 1807-1819.

Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, A., Orejas M., Mungroo J., Peñalva, M.A., Arst, H.N., 1995. The Aspergillus PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO Journal 14, 779-790.

Wong, K.H., Hynes, M.J., Todd, R. B., Davis, M.A., 2007. Transcriptional control of nmrA by the bZIP transcription factor MeaB reveals a new level of nitrogen regulation in *Aspergillus nidulans*. Molecular Microbiology 66, 534-551.

Yu, J., Chang, P.K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., 2004. Clustered pathway genes in aflatoxin biosynthesis. Applied. Environmental Microbiology 70, 1253–1262.

Table 1. Summary of 109 sequenced inserts from the SSH library, enriched with the high OTA-producing strain of *A. carbonarius* (W04-40).

724

725 **Fig. 1.** Production of OTA after 5 days growth of the high OTA-producing strain
726 of *A. carbonarius* (W04-40) and the low OTA-producing strain (W04-46). The
727 means of three separate experiments is shown.

728

729 **Fig. 2.** Functional classification of cDNAs representing putative genes
730 expressed by *A. carbonarius* during OTA production. The percentage in each
731 one of the categories is shown (refer to Table 1).

Figure1
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Figure2

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Table

Unisequence identification	GenBank Accession No	cDNA insert size bp	Transcript abundance	Annotation (putative function)	Organism and gene Accession N°	E-value
Amino acid metabolism						
C014	GW327927	566	1	Acetamidase amdS- <i>Aspergillus nidulans</i> (arginine, proline and phenylalanine metabolism)	<i>Aspergillus niger</i> CBS 513.88 XM_001400659	9,00E-86
C049	GW327951	450	1	Dopa decarboxylase 3 DDC3 from <i>Aspergillus oryzae</i> patent WO9960136-A1 (histidine metabolism; tyrosine metabolism;phenylalanine metabolism; tryptophan metabolism; alkaloid biosynthesis)	<i>Aspergillus niger</i> CBS 513.88 XM_001389507	4,00E-32
C059	GW327959	441	1	Glutamine synthase Gln1- <i>Saccharomyces cerevisiae</i> (involved in glutamate metabolism and nitrogen metabolism)	<i>Aspergillus niger</i> CBS 513.88 XM_001389265	3,00E-74
C091	GW327986	492	1	Alpha subunit of assimilatory sulphite reductase Met10 - <i>Saccharomyces cerevisiae</i> (biosynthesis of methionin and cysteine)	<i>Aspergillus niger</i> CBS 513.88 XM_001394608	1,00E-107
C112	GW328003	443	1	Acetolactate synthase protein from patent EP257993-A - <i>Saccharomyces cerevisiae</i> (valine and isoleucine biosynthesis)	<i>Aspergillus niger</i> CBS 513.88 XM_001397691	4,00E-93
C124	GW328016	611	1	Ornithine decarboxylase ODC - <i>Paracoccidioides brasiliensis</i> (urea cycle and metabolism of amino groups;arginine and proline metabolism; alkaloid biosynthesis II)	<i>Aspergillus niger</i> CBS 513.88 XM_001401573	1,00E-59
C133	GW328025	522	1	Alpha-isopropylmalate synthase Leu4 - <i>Saccharomyces cerevisiae</i> (leucine biosynthesis)	<i>Aspergillus niger</i> CBS 513.88 XP_001389717	3,00E-78
C157	GW328048	376	1	Adenosylhomocysteinase – <i>Homo sapiens</i> (biosynthesis of methionin)	<i>Aspergillus niger</i> CBS 513.88 XM_001392278	1,00E-58
Hydrolases of energy reserves						
C018	GW327931	231	1	Extracellular alpha-glucosidase aglU- <i>Aspergillus niger</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001402016	8,00E-43
C040	GW327944	606	1	Hypothetical S-layer protein - <i>Clostridium thermocellum</i> (involved in attachment of the cellulosome to the cell surface)	<i>Aspergillus niger</i> CBS 513.88 XM_001389321	2,00E-11
C064	GW327963	281	2	Protein B12K8.10- <i>Neurospora crassa</i> . Similar to N-acetylglucosaminyltransferase from <i>R. norvegicus</i>	<i>Aspergillus niger</i> CBS 513.88 Complete genome AM270309	4,00E-32
C079	GW327974	422	2	Protein B12K8.10- <i>Neurospora crassa</i> . Similar to N-acetylglucosaminyltransferase from <i>R. norvegicus</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001388866	3,00E-84
C111	GW328002	147	1	Enzyme with sugar transferase activity from patent JP11009276-A - <i>Acremonium</i> sp. (glycosyl hydrolases)	<i>Aspergillus niger</i> CBS 513.88 XM_001389473	7,00E-16
C120	GW328009	603	1	Beta-glucosidase bg11- <i>Aspergillus niger</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001398779	1,00E-96
C128	GW328020	215	1	Endo-1,4-beta-xylanase CAA93120.1 - <i>Ascochyta pisi</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001397609	1,00E-69
C142	GW328033	576	2	Acid alpha-amylase- <i>Aspergillus niger</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001394298	3,00E-66
C154	GW328045	286	2	Acid alpha-amylase- <i>Aspergillus niger</i>	<i>Aspergillus niger</i> CBS 513.88	4,00E-20
C159	GW328050	581	1	Dienelactone hydrolase family protein	<i>Aspergillus fumigatus</i> Af293 XM_742790	1,00E-72
Secondary metabolism						
C058	GW327958	623	1	Methyl sterol oxidase Erg25 - <i>Saccharomyces cerevisiae</i> (involved in the ergosterol biosynthesis pathway)	<i>Aspergillus niger</i> CBS 513.88 XM_001390479	1,00E-105
C086	GW327981	532	1	Short/branched chain specific acyl-CoA dehydrogenase precursor ACADSB - <i>Homo sapiens</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001389661	1,00E-150
C099	GW328012	252	1	Monoxygenase moxY- <i>Aspergillus parasiticus</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001400931	2,00E-19
C123	GW328015	291	1	Pyruvate carboxylase pyc- <i>Aspergillus niger</i> (involved in the biosynthesis and degradation of acid citric)	<i>Aspergillus niger</i> CBS 513.88 XM_001401540	4,00E-50
C137	GW328029	182	1	AK-toxin regulating protein AktR-1 - <i>Alternaria alternata</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001401153	5,00E-08
Regulation-Transcription-Transduction						
C001	GW328053	146	2	GTP binding protein (GTPBP1)- <i>Homo sapiens</i> (related to elongation factor 1 alpha)	<i>Aspergillus niger</i> CBS 513.88 XM_001401247	3,00E-31
C002	GW327916	326	1	Protein kinase PK12 belong to the LAMMER family	<i>Aspergillus niger</i> CBS 513.88 XM_001389399	9,00E-9

C003	GW327917	431	1	RNA binding protein- <i>Schizosaccharomyces pombe</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001390309	4,00E-54
C004	GW327918	361	1	Nucleolar protein Nsr1 involved in pre-rRNA processing- <i>Saccharomyces cerevisiae</i> (binds to histone H2B)	<i>Aspergillus niger</i> CBS 513.88 XM_001397587	6,00E-38
C007	GW327920	596	1	Nonhistone chromosomal protein Nhp6b- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001399837	1,00E-47
C008	GW327921	656	1	Serine/threonine kinase par-1- <i>Caenorhabditis elegans</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001392493	3,00E-137
C009	GW327922	160	1	Nitrogen metabolic repression regulator hNmrr	<i>Aspergillus niger</i> CBS 513.88 XM_001396743	1,00E-22
C010	GW327923	334	1	cAMP-regulated phosphoprotein/endosulfine conserved region- <i>Schizosaccharomyces pombe</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001392126	1,00E-24
C013	GW327926	684	2	GTP binding protein (GTPBP1)- <i>Homo sapiens</i> (related to elongation factor 1 alpha)	<i>Aspergillus niger</i> CBS 513.88 XM_001401247	5,00E-136
C015	GW327928	671	1	Autophagic death protein Aut7/IDI-7	<i>Aspergillus fumigatus</i> Af293 XM_745400	2,00E-56
C028	GW327935	554	1	C2H2-type zinc-finger protein- <i>Schizosaccharomyces pombe</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001396868	4,00E-87
C032	GW327938	622	1	Peptide chain release factor 2 PrfB PrfB belongs to the prokaryotic and mitochondrial release factors family- <i>Streptomyces coelicolor</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001396628	5,00E-96
C052	GW327954	666	1	Serine/threonine protein kinase (Kin4)	<i>Neosartorya fischeri</i> NRRL 181 XM_001264275	1,00E-109
C061	GW327961	584	1	C-x8-C-x5-C-x3-H type zinc finger protein	<i>Aspergillus clavatus</i> NRRL 1 XM_001271384	1,00E-48
C067	GW327965	587	1	Histone H2A	<i>Aspergillus niger</i> Y15320	0,00E+00
C069	GW327967	516	1	Calmodulin caM- <i>Aspergillus nidulans</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001402025	0,00E+00
C073	GW327971	338	1	C2H2 finger domain protein	<i>Neosartorya fischeri</i> NRRL 181 XM_001258431	3,00E-43
C082	GW327977	136	1	Nuclear polyadenylated RNA-binding protein Nab3- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CB XM_001397616S 513.88	3,00E-10
C088	GW327983	648	1	Gigas protein gig - <i>Drosophila melanogaster</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001396626	1,00E-116
C089	GW327984	515	1	Translation elongation factor 1 alpha- <i>Podospira anserina</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001398905	0,00E+00
C093	GW327988	483	1	bZIP transcription factor L-Maf- <i>Gallus gallus</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001396789	1,00E-102
C098	GW328011	383	1	Translation elongation factor EF-1 alpha subunit, putative- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus fumigatus</i> Af293 XM_745295.2	8,00E-154
C109	GW328000	430	1	Myosin-like protein Mlp1 - <i>Saccharomyces cerevisiae</i> (implicated in nuclear transport)	<i>Aspergillus niger</i> CBS 513.88 XM_001390505	2,00E-115
C117	GW328052	922	1	Translation elongation factor Ela1 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001390618	8,00E-62
C125	GW328017	189	1	Ubiquitin activating protein Uba1 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001397094	7,00E-21
C135	GW328028	253	1	Inhibitor of PP2A SET - <i>Homo sapiens</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001393766	7,00E-09
C138	GW328030	417	1	Nucleolar RNA-associated proteins-strong similarity to hypothetical protein YGR090w from patent WO200039342-A2- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001395035	3,00E-15
C143	GW328034	155	1	Serine/threonine protein kinase involved in cell cycle control	<i>Aspergillus oryzae</i> RIB40 XM_001821579	8,00E-22
C153	GW328044	381	1	Serine/Threonine protein kinases	<i>Aspergillus niger</i> CBS 513.88 XM_001398952	8,00E-64
Stress response and detoxification						
C005	GW327919	566	1	Mitochondrial superoxide dismutase	<i>Aspergillus terreus</i> NIH2624 XM_001213098	4,00E-158
C017	GW327930	594	1	14-3-3 protein homolog artA- <i>Aspergillus nidulans</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001399043	2,00E-40
C034	GW327940	615	1	Heat shock protein sspB	<i>Aspergillus niger</i> CBS 513.88 XM_001393937	1,00E-97
C037	GW327941	415	1	Peroxisomal membrana protein Pmp27- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001394223	7,00E-88
C054	GW327956	153	1	Peroxisomal membrane protein PMP20- <i>Candida boidinii</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001397368	2,00E-24
C068	GW327966	547	1	Postsynaptic receptor clustering and molybdenum cofactor biosynthesis protein gephyrin- <i>Rattus norvegicus</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001397064	1,00E-89
C083	GW327978	386	1	Peroxisomal membrane protein PEX3- <i>Pichia angusta</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001398336	5,00E-30
C105	GW327996	221	1	Mitochondrial peroxiredoxin PRX1	<i>Aspergillus terreus</i> NIH2624	2,00E-19

					XM_001214520	
C106	GW327997	297	1	DnaJ protein homolog Scj1- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001390628	5,00E-48
C122	GW328014	271	1	NADPH:quinone reductase and related Zn-dependent oxidoreductases	<i>Aspergillus oryzae</i> RIB40 XM_001818753	6,00E-20
C140	GW328031	733	1	DNA damage checkpoint protein rad24p - <i>Schizosaccharomyces pombe</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001391814	9,00E-28
C147	GW328038	440	1	Glutathione S-transferase	<i>Aspergillus oryzae</i> RIB40 XM_001822925	5,00E-52
C158	GW328049	377	1	TCP-1 chaperonin family, alpha subunit	<i>Gibberella zeae</i> PH-1 XM_385426	2,00E-32
Transport-Secretion						
C041	GW327945	568	1	Secretion-associated GTP-binding protein sarA	<i>Aspergillus niger</i> CBS 513.88 XM_001388795	0,00E+00
C047	GW327949	322	1	GDP dissociation inhibitor in the secretory pathway Gdi1- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001399411	1,00E-112
C050	GW327952	534	2	Fluconazole resistance protein FLU1 MFS transporter- <i>Candida albicans</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001388440	2,00E-45
C072	GW327970	205	1	MFS transporter multidrug efflux system protein-Aminotriazole resistance protein Atr1- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001389800	3,00E-21
C095	GW327990	276	1	Predicted flavoprotein involved in K ⁺ transport	<i>Aspergillus terreus</i> NIH2624 XM_001214404	1,00E-17
C110	GW328001	496	1	Lactose permease Lac12 <i>Kluyveromyces lactis</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001395942	9,00E-96
C118	GW328008	525	1	Voltage-gated potassium channel beta subunit Kv beta 1 - <i>Rattus norvegicus</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001389342	1,00E-131
C130	GW328022	560	1	High-affinity nicotinic acid permease Tna1 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001402426	4,00E-78
C136	GW328026	660	1	Protein Sec7 - <i>Saccharomyces cerevisiae</i> (essential role in vesicular traffic)	<i>Aspergillus niger</i> CBS 513.88 XM_001391268	1,00E-112
C139	GW328027	580	2	Fluconazole resistance protein FLU1 MFS transporter- <i>Candida albicans</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001388440	3,00E-51
C150	GW328041	396	1	p150 component of the COPII coat of secretory pathway vesicles Sec31 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001399273	5,00E-45
General metabolism						
C027	GW327934	570	1	Phosphoglycerate mutase SPAC5H10.03- <i>Schizosaccharomyces pombe</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001394282	1,00E-167
C048	GW327950	258	2	123K chain alpha, alpha-trehalose-phosphate synthase (UDP-forming) Tsl1- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001391909	4,00E-48
C101	GW327992	216	1	Fructose-bisphosphate aldolase Fba1- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001399844	4,00E-23
C121	GW328013	429	2	123K chain alpha, alpha-trehalose-phosphate synthase (UDP-forming) Tsl1- <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001391909	2,00E-65
C145	GW328036	504	1	Phosphopyruvate hydratase ENO1- <i>Candida albicans</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001399041	0,00E+00
Protein synthesis						
C012	GW327925	326	1	Cytoplasmic aspartate-tRNA ligase Aps - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001388949	3,00E-31
C016	GW327929	217	1	Cytoplasmic ribosomal protein of the large subunit L7 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001400693	6,00E-99
C030	GW327937	219	1	Cytoplasmic ribosomal protein of the small subunit S19 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001398839	2,00E-12
C104	GW327995	246	1	Cytoplasmic ribosomal protein of the large subunit L13a - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001398803	2,00E-103
C108	GW327999	179	1	Cytoplasmic ribosomal protein of the large subunit L1 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001392653	2,00E-81
Unclassified						
C022	GW327932	468	1	Extracellular matrix protein AAL47843.1- <i>Fusarium oxysporium</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001397996	5,00E-24
C070	GW327968	487	1	Uracil phosphoribosyltransferase UPRT - <i>Toxoplasma gondii</i>	<i>Aspergillus niger</i> CBS 513.88 XP_001396020.1	4,00E-13
C071	GW327969	408	1	Hypothetical protein SPCC1902.02 Pfam and COG motives suggest a putative ketopantoate reductase- <i>Schizosaccharomyces pombe</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001395814	3,00E-62
C077	GW327972	261	1	Inorganic pyrophosphatase	<i>Aspergillus terreus</i> NIH2624 XM_001208997	9,00E-74
C078	GW327973	626	1	Protein #70 from patent WO9925825-A2- <i>Homo sapiens</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001395763	1,00E-150
C085	GW327980	337	1	Ubiquitin thiolesterase protein OTUB1	<i>Aspergillus fumigatus</i> Af293	2,00E-44

					XM_749842	
C090	GW327985	252	1	Bud formation factor Sap190 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001401893	3,00E-33
C097	GW328010	295	1	Tubulin beta chain beta-tubulin - <i>Aspergillus flavus</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001392399	2,00E-93
C102	GW327993	498	1	Con-8 - <i>Neurospora crassa</i> (expressed early during conidial differentiation)	<i>Aspergillus niger</i> CBS 513.88 XM_001401542	2,00E-45
C127	GW328019	439	1	Meiotic sister-chromatid recombination protein Msc1 - <i>Saccharomyces cerevisiae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001397716	2,00E-19
C151	GW328042	396	1	Spore-wall fungal hydrophobin dewA - <i>Aspergillus nidulans</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001390100	2,00E-23
C156	GW328047	225	1	Vacuolar (H+)-ATPase G subunit	<i>Aspergillus clavatus</i> NRRL 1 XM_001272340	8,00E-17
Hypothetical proteins						
C026	GW327933	333	1	Hypothetical protein An09g04880	<i>Aspergillus niger</i> CBS 513.88 XM_001393764	4,00E-67
C038	GW327942	513	1	Hypothetical protein EAA77948.1- <i>Gibberella zeae</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001396711	3,00E-18
C044	GW327947	394	1	Hypothetical protein An11g02730	<i>Aspergillus niger</i> CBS 513.88 XM_001397263	2,00E-54
C053	GW327955	431	1	YciI-like protein	<i>Aspergillus clavatus</i> NRRL 1 XM_001271574	6,00E-33
C057	GW327957	255	2	Hypothetical protein CAD21276.1 - <i>Neurospora crassa</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001395123	1,00E-75
C062	GW327962	521	2	Hypothetical protein CAD21295.1- <i>Neurospora crassa</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001390705	1,00E-57
C065	GW327964	275	2	Hypothetical protein CAD21295.1- <i>Neurospora crassa</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001390705	2,00E-44
C080	GW327975	236	1	Hypothetical protein SCF91.02c - <i>Streptomyces coelicolor</i>	<i>Aspergillus niger</i> CBS 513.88 XP_001390166	2,00E-27
C087	GW327982	180	2	Hypothetical verprolin related protein encoded by B24P7.40- <i>Neurospora crassa</i> (Rossman-fold NAD (P) (+)-binding protein)	<i>Aspergillus niger</i> CBS 513.88 XM_001393627	1,00E-25
C092	GW327987	283	1	Hypothetical protein CAD11387.1- <i>Neurospora crassa</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001389287	2,00E-07
C094	GW327989	338	2	Hypothetical protein ES2- <i>Mus musculus</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001398397	1,00E-17
C096	GW327991	365	1	Hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001401451	1,00E-126
C107	GW327998	309	2	Hypothetical verprolin related protein encoded by B24P7.40- <i>Neurospora crassa</i> (Rossman-fold NAD (P) (+)-binding protein)	<i>Aspergillus niger</i> CBS 513.88 XM_001393627	3,00E-39
C116	GW328007	415	2	Hypothetical protein CAD21276.1 - <i>Neurospora crassa</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001395123	4,00E-26
C126	GW328018	212	1	Hypothetical lipoprotein SC4A2.13c - <i>Streptomyces coelicolor</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001391107	1,00E-25
C129	GW328021	393	1	Hypothetical protein CAE76294.1 - <i>Neurospora crassa</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001399831	3,00E-57
C132	GW328024	388	1	Hypothetical protein B24H17.110 - <i>Neurospora crassa</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001400607	2,00E-21
C141	GW328032	580	1	Hypothetical protein	<i>Aspergillus niger</i> CBS 513.88 XM_001399070	2,00E-65
C146	GW328037	572	1	Hypothetical protein C50F7.2 - <i>Caenorhabditis elegans</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001394236	2,00E-12
C152	GW328043	330	2	Hypothetical protein ES2- <i>Mus musculus</i>	<i>Aspergillus niger</i> CBS 513.88 XM_001398397	5,00E-19
No blast						
C011	GW327924	462	1	no blast		
C029	GW327936	116	1	no blast		
C033	GW327939	226	1	no blast		
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		

C114	GW328005	344	1	no blast
C115	GW328006	213	1	no blast
C131	GW328023	468	1	no blast
C144	GW328035	255	1	no blast
C148	GW328039	266	1	no blast
C149	GW328040	140	1	no blast
C155	GW328046	183	1	no blast
C160	GW328051	660	1	no blast

Valencia 27th april 2010

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

Dear Dr. Luca Coccolin,

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 conditions 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)**

Reviewer 2

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 too 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 7/154 What is a Polytron? It is a homogenizer. This word has been included in manuscript.

- 3 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."
- 4 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 .
- 5 The last sentence of this paragraph belongs to the next paragraph "Sequencing analysis".(DONE)
- 6
- 7 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".
- 8 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.
- 9
- 10 14/317: The message of this sentence is not completely correct. The authors state the transcription factors described above (areA, creA or pacC) are specific pathway transcription factors and others like TOR are global transcription factors. That is not the case areA, creA or pacC are global transcription factors, because they 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".
- 11 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