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Proteome analysis of the fungus *Aspergillus carbonarius* under ochratoxin A producing conditions

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ABSTRACT

Aspergillus carbonarius is an important ochratoxin A producing fungus that is responsible for mycotoxin contamination of grapes and wine. In this study, the proteomes of highly (W04-40) and weakly (W04-46) OTA-producing *A. carbonarius* strains were compared to identify proteins that may be involved in OTA biosynthesis. Protein samples were extracted from two biological replicates and subjected to two dimensional gel electrophoresis analysis and mass spectrometry. Expression profile comparison (PDQuest software), revealed 21 differential spots that were statistically significant and showed a two-fold change in expression, or greater. Among these, nine protein spots were identified by MALDI-MS/MS and MASCOT database and twelve remain unidentified. Of the identified proteins, seven showed a higher expression in strain W04-40 (high OTA producer) and two in strain W04-46 (low OTA producer). Some of the identified amino acid sequences shared homology with proteins involved in regulation, amino acid metabolism, oxidative stress and sporulation. It is worth noting the presence of a protein with 126.5 fold higher abundance in strain W04-40 showing homology with protein CipC, a protein with unknown function related with pathogenesis and mycotoxin production by some authors. Variations in protein expression were also further investigated at the mRNA level by real-time PCR analysis. The mRNA expression levels from three identified proteins including CipC showed correlation with protein expression levels. This study represents the first proteomic analysis for a comparison of two *A. carbonarius* strains with different OTA production and will contribute to a better understanding of the molecular events involved in OTA biosynthesis.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* and *Penicillium* genera with nephrotoxic, carcinogenic, immunotoxic, genotoxic and teratogenic effects (Creppy, 1999; Kuiper-Goodman and Scott, 1989; Petzinger and Ziegler, 2000; Pfohl-Leszkowicz and Manderville, 2007). Food commodities contaminated by OTA include cereal-based products, coffee, spices, nuts, olives, grape-derived products, beans, figs and cocoa (Battilani et al., 2006; Magnoli et al., 2007; Perrone et al., 2007; Sánchez-Hervás et al., 2008; Santos et al., 2010; Taniwaki et al., 2003).

Grapes and wines have been reported among the food commodities contaminated by OTA with the greatest OTA intake, second only to cereals (Bau et al., 2005; Belli et al., 2004). Several studies have highlighted *Aspergillus* section *Nigri* (black aspergilli) as the main group responsible for OTA contamination of grapes, must and wine (Battilani et al., 2006; Martínez-Culebras et al., 2009; Perrone et al., 2007). Among black *Aspergillus* species, *Aspergillus carbonarius* seems to be predominantly responsible for OTA production in grapes, wine, and dried vine fruits. The reported percentages of OTA-producing strains in *A. carbonarius* as well as OTA production levels are higher than those reported for other black aspergilli such as *Aspergillus niger* and *Aspergillus tubingensis* (Battilani et al., 2006; Martínez-Culebras and Ramón, 2007).

OTA molecule consists of a polyketide derived from a dihydroisocoumarin moiety, linked to the amino acid phenylalanine via an amide linkage. However, as yet, little information is available about the biosynthetic pathway of OTA in any ochratoxigenic fungal species and only a few genes have been reported. These include polyketide synthase (PKS) genes in different *Aspergillus* species (Bacha et al., 2009; Gallo et al., 2009; Karolewiez and Geisen, 2005; O'Callaghan et al., 2003), P450 monoxygenase genes in *A. ochraceus* (O'Callaghan et al., 2006), and a non-ribosomal peptide synthetase, two genes encoding a transporting protein and a chloroperoxidase in *Penicillium nordicum* (Geisen et al., 2006). High-throughput approaches have also been used recently to isolate the genes involved in OTA production. These include differential display techniques, such as Differential Display Reverse Transcriptase-PCR (DDRT-PCR) in *P. nordicum* (Färber

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and Geisen, 2004), cDNA-AFLP (Botton et al., 2008) and Suppression Subtractive Hybridization (SSH) (Crespo-Sempere et al., 2010) in *A. carbonarius*.

Proteomic technology, which combines the resolution of two dimensional gel electrophoresis (2D-GE) with the sensitivity of mass spectrometry (MS), allows the sensitive detection and identification of hundreds of proteins (Berndt et al., 1999; Gygi and Aebersold, 2000). This has provided critical insight into the differential accumulation of proteins during specific developmental processes in filamentous fungi (Kim et al., 2007; Melin et al., 2002). Proteomics in OTA-producing fungi could help identify novel proteins and genes involved in OTA formation and production, thereby contributing to a better understanding of the fungal activities and pathways active during mycotoxin production, because proteins are directly related to function. Proteomics has been used to identify proteins from the ochratoxin producing fungus *Aspergillus ochraceus* (Rizwan et al., 2010). However, a proteome comparison between OTA-producing strains and non OTA-producing strains is necessary in order to identify proteins involved in OTA biosynthesis.

The main objective of the present study was to use the 2D-GE technique combined with MALDI-MS/MS to detect changes in the abundance of a large number of proteins in two closely related strains of *A. carbonarius* differing in their OTA-producing potential. Martínez-Culebras and Ramón (2007) previously described the two strains used in this work, namely W04-40 (high OTA producer) and W04-46 (low OTA producer). These strains were also used as tester and driver in a SSH approach to identify differentially expressed genes in OTA production (Crespo-Sempere et al., 2010). Differentially expressed proteins were identified by consulting the MASCOT database. The possible functional roles of the proteins identified in OTA production and regulation are also discussed. Finally, the genes corresponding to the identified proteins were partially sequenced and their expression levels evaluated using reverse transcriptase (RT)-PCR analyses.

2. Materials and methods

2.1. Strains, culture media and growth conditions

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

Strains were grown on Petri dishes containing Malt Extract Agar (MEA) (2% malt extract, 1% peptone, 2% glucose and 1.5% agar) medium in the dark at 30 °C for 6 days to attain 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. Then, 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 2 days.

2.2. Extraction of proteins

The extraction method developed in this study is based on a previously published method for plant tissues (Hurkman and Tanaka, 1986). Briefly, mycelium was collected from plates, frozen in liquid nitrogen and ground to a fine powder. Two grams of ground mycelium was suspended in 6 mL of extraction buffer [0.7 M sucrose, 2% (w/v) β-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride] and homogenized twice with a Polytron PT 45/80 (Kinematica AG) 2000 rpm 30 s. An equal volume of water-saturated phenol (pH = 8) was then added. After 30 min with shaking at 4 °C, the phases were separated by centrifugation (10,000*g*, 15 min). The phenol phase was recovered and re-extracted with an equal volume of extraction buffer twice. Proteins were precipitated from the phenol phase by addition of 5 volumes of 0.1 M ammonium acetate in methanol and incubated at −20 °C overnight. The precipitate was washed three times with methanol (−20 °C) and three more times with acetone (−20 °C). The pellet was dried and solubilized with pre-isoelectric focusing buffer (7 M urea, 2 M thiourea and 1% (w/v) NP-40). All samples were loaded immediately after preparation, although it was found that similar two dimensional (2-D) gel patterns were obtained when samples were stored at −70 °C.

2.3. Two-dimensional gel electrophoresis

Samples containing 75 μg of protein were loaded per gel. Protein content was previously determined by 2-D Quant kit (GE Healthcare) using bovine serum albumin (BSA) as standard. Immobilized pH gradient (IPG) strips (24 cm GE Healthcare) with pH range from 4 to 7 were used for protein separation. IPG strips were rehydrated with the samples containing 2-D buffer [7 M urea, 2 M thiourea, 1% (w/v) NP-40, 4% dithiothreitol (DTT), 1% ampholytes (Pharmalite 4–7, GE Healthcare) and 0.002% bromophenol blue] overnight at room temperature. Focusing was performed in six steps (500 V, 2 h; 500–1000 V, 2 h; 1000–5000 V, 2 h; 5000–8000 V, 4 h; 8000 V, 8 h). The focused IPG strips were equilibrated for 15 min in 50 mM Tris–HCl, pH 8.8, 6 mM urea, 30% glycerol, 2% (w/v) SDS containing 2% (w/v) DTT, and then for 15 min in the same buffer containing 2.5% iodeacetamide. After that, samples were resolved in SDS-PAGE with 12.5% of polyacrylamide on a vertical system (Ettan DALT six; GE Healthcare, 26 cm × 20 cm × 1 mm). Electrophoresis conditions were 1 W per gel until dye front reached the bottom of the gel. Sets of three gels were used for each sample.

2.4. Protein visualization and images analysis

The gels were fixed for 1 h in 50% v/v methanol, 10% v/v acetic acid followed by staining with SYPRO Ruby (BioRad) overnight. Background stain was removed by incubation in 10% v/v acetic acid, 7% v/v methanol for 1 h. Gels were stored in water at 4 °C. Gel images were obtained using a high resolution scanner (Typhoon FLA 9000, GE Healthcare). The resulting gel image files were analyzed by image software (PD Quest, Biorad). Spot intensity levels were normalized by expressing the intensity of each protein spot in a 2-D gel as a proportion of the total protein intensity detected for the entire gel. The analysis was carried out on two biological replicates of independent triplicates of gels for each sample, strains W04-40 and W04-46. Differentially regulated spots between high and low OTA-producing strains were considered potentially significant by Student's *t*-test ($p<0.05$), showing two-fold change in expression, or greater.

2.5. Protein identification by MALDI-MS/MS

Spots were excised manually and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany). The digestion protocol followed Shevchenko et al. (2007) with minor variations: proteins in the gel plugs were reduced by 10 mM DTT (GE Healthcare, Uppsala, Sweden) in 50 mM ammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, MO, USA) and alkylation was carried out with 55 mM iodoacetamide (Sigma Chemical) in 50 mM ammonium bicarbonate. The pieces of gel were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a nitrogen stream. Modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 8 ng/μl in 50 mM ammonium bicarbonate was added to the dry gel and digestion was carried out at 37 °C for 8 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical) was added for peptide extraction.

An aliquot of the above digestion solution was mixed with an aliquot of α- cyano-4-hydroxycinnamic acid (Bruker-Daltonics) in 33% aqueous acetonitrile and 0.25% trifluoroacetic acid. This mixture was deposited onto a 600 μm AnchorChip pre-structured MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. MALDI-MS/MS data were obtained in an automated analysis loop using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device (Suckau et al., 2003). Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1000 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Mass data were analyzed with flexAnalysis software (Bruker-Daltonics). MALDI-TOF mass spectra were internally calibrated using two trypsin autolysis ions with $m/z = 842.510$ and $m/z = 2211.105$; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800–3200 m/z region. MALDI-MS and MS/MS data were combined through the BioTools program (Bruker-Daltonics) to search for a nonredundant protein database (NCBInr;~4.8×10^6 entries; National Center for Biotechnology Information, Bethesda, US; or SwissProt; ~2.6×10^5 entries; Swiss Institute for Bioinformatics, Switzerland) using the MASCOT software (Matrix Science, London, UK) (Perkins et al., 1999).

2.6. RNA isolation and 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 stored at −80 °C before nucleic acid extraction.

RNA was isolated from 1 g of mycelium previously finely powdered with a mortar and pestle with 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*-laurylsarcosine (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1% β-mercaptoethanol and 5 mL of Tris-equilibrated phenol. After homogenization with a Polytron PT 45/80 (Kinematica AG) for 1 min, the extract was incubated at 65 °C for 15 min and cooled, before adding 5 mL of chloroform:isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3900*g* for 20 min 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 27,200*g* for 15 min. 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 27,200*g* for 60 min, 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. Total RNAs were treated with DNase (TURBO DNase, Ambion) to remove contaminating genomic DNA. Absence of genomic DNA was confirmed by PCR, using primers designed with an intron sequence of Beta Tubulin (BT1–BT3) detailed in Table 1. Single-strand cDNA was synthesized from 10 μg of total RNA, using SuperScript III reverse transcription kit and an oligo(dT), according to the manufacturer's instruction (Invitrogen).

2.7. Quantification of relative gene expression by real-time RT-PCR

Partial gene sequences were obtained using degenerated oligos (details in Table 1). Gene-specific primer sets were designed for gene expression analysis by OLIGO Primer Analysis Software V.5 to amplify PCR fragments between 71 and 182 bp in length (Table 3). Real-time RT-PCR reactions were performed in a LightCycler system (Roche) using SYBR Green to monitor cDNA amplification. Real-time PCR efficiencies were calculated from the slopes in the LightCycler software (Rasmussen, 2001). Thus, the corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10[-1/\text{slope}]$. The relative expression ratio of the previous target genes was calculated based on E and the CP deviation of the sample *A. carbonarius* strain W04-40 versus a control W04-46 and expressed in comparison to the ribosomal S18 gene (reference gene), according to the following equation (Pfaffl, 2001; Rasmussen, 2001): $\text{ratio} = (\text{Etarget})\Delta\text{CPtarget}(\text{control} - \text{sample}) / (\text{Eref})\Delta\text{CPref}(\text{control} - \text{sample})$. Three technical replicates were done for each combination of cDNA and primer pair, and PCR reaction quality was checked by analyzing the dissociation and amplification curves.

2.8. Data analysis

To calculate the normalised relative gene expression levels (fold induction), data were analyzed using the Relative Expression Software Tool (REST), with the mathematical model based on mean threshold cycle differences between the sample and the control group (Pfaffl et al., 2002). REST was also used for a randomisation test with a pair-wise reallocation to assess the statistical significance of the differences in expression between the control and treated samples (significance at $p \leq 0.05$).

3. Results and discussion

3.1. Comparison of high and low OTA-producing strains proteomes

The aim of this study was to identify proteins involved in OTA biosynthesis using a proteomic analysis in *A. carbonarius*. Screening was performed on two *A. carbonarius* strains with similar ITS-RFLP patterns, but differing in their ability to produce OTA (Martínez-Culebras and Ramón, 2007); the high OTA producer (W04-40) and the low OTA producer (W046). Recently, the same *A. carbonarius* strains

Table 1
Degenerate and, beta tubulin primers used in this study. Protein spot numbers, primer names and primer sequences are indicated.

Protein	Forward		Reverse	
	Primer name	Primer sequences (5′ to 3′)	Primer name	Primer sequences (5′ to 3′)
4719	ACET.A	TGAAGATYAAGGAGATGAAGGA	ACET.B	CCRTACCAGAAGAGRACCTC
1612	ACOB.A	ATCCTSWCCACCATCTTCAACAC	ACOB.B	GTRTCRGCVGTGAARATCTC
4723	AMINO.C	ASARGGCYTGGGGTACCGA	AMINO.F	GCCATGAAYTCSGARTGGAAC
2107	CIPC.A	ATGGGTTGGTTCGACRRMGAC	CIPC.B	TAGWAGTCCTGGSSGGARGC
6507	HELI.A	GAGATGCTKTCKCGTGGTTTCAC	HELI.B	CCTTACGACCGAAACGACCAC
5822	LEU.A	ACYCCRGCCCAAACGTC	LEU.B	TGYTCCCAKGAWGCATTGGT
7403	OXI.A	CCBRCCGACAAGTACCAG	OXI.B	TCSGTCATGATACGVCGCAG
1313	PER.A	TCCTTCTTGAGGTTGGTGAAGC	PER.B	CTCAGAAGAAGTTCGGCGATG
3702	TUB.A	ACTCCGACTGCAGYTTCATG	TUB.B	GAATTCACCYTCCTCCATACCCTC
beta tubulin	BT1	GCAGACCATCTCTGGTGAGCAC	BT3	AGTTGTTACCAGCACCGGACTG

were used to identify differentially expressed genes involved in OTA production via a SSH approach (Crespo-Sempere et al., 2010).This paper is the first to report the use of proteome analysis to generate an overall picture of the proteins putatively involved in mycotoxin biosynthesis in an OTA-producing fungus.

To investigate the *A. carbonarius* proteins involved in OTA production, total proteins were extracted from 2 day-old mycelia grown on CYA plates and separated by 2D-GE. The optimum OTA-production phase to collect mycelia for protein extraction had previously been determined (Crespo-Sempere et al., 2010). It is also worth mentioning that there were no significant differences between these two strains in colony growth rates and yield of conidia (data not shown). The 2-D gel for two biological replicates was separated technically three times. Thus, a total of twelve gels, six for each strain, were conducted. Good reproducibility of spot intensities was obtained among the replicates of each strain. Comparison of 2-D images indicated that there were both qualitative and quantitative differences between protein profiles of the high and low OTA-producing *A. carbonarius* strains. Approximately 450 spots were matched on each of the 12 gels (data not shown). A typical 2-D gel illustrating the resolution of spots is presented in Fig. 1. Differential protein expression between strain W04-40 (high OTA producer) and strain W04-46 (low OTA producer) was statistically significant by Student's *t*-test ($p<0.05$) and showed a two-fold change in expression, or greater, for 21 of the 450 proteins (Fig. 2). Of these 21 differentially expressed proteins, 13 were up-regulated and eight were down-regulated in strain W04-40 (Fig. 3). Among these, only nine protein spots were identified by MALDI-MS/MS and MASCOT database as listed in Table 2, and 12 remain unidentified. The relatively low number of proteins identified highlights the paucity of knowledge about proteins and gene expression in filamentous fungi. All of the identified proteins corresponded with proteins from *Aspergillus* species, and especially with *Aspergillus niger*. There is no doubt that the availability of the *A. niger* genome (Pel et al., 2007) will facilitate evaluation of the genes and proteins predicted to play a role in OTA biosynthesis.

Of the identified proteins, seven were up-regulated (Figs. 2,3, Table 2) including one hydrolase, one aminotransferase, CipC, ubiquinone reductase, a mitochondrial peroxiredoxin, an acoB protein and a protein related to the translation initiation factor IF-4A. The two down-regulated proteins included an acetylglutamate kinase and a tubulin (Figs. 2,3, Table 2).

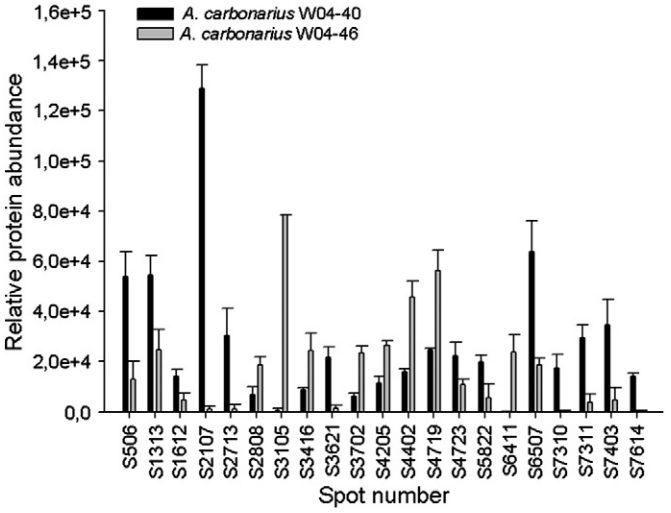

Fig. 2. Protein abundance for differentially displayed spots from 2-D gel. The T-ticks on the top of each bar indicate the standard error.

3.2. Possible functional roles of the identified proteins

Identified protein from spot 5822 exhibited homology with a leukotriene-A4 hydrolase (Table 2). Leukotriene belongs to the oxylipin family, which are oxidized polyenoic fatty acids able to regulate different cellular processes including asexual and sexual development, infectious processes and toxin production (Gao and Kolomiets, 2009; Tsitsigiannis and Keller, 2007). Several studies have demonstrated that oxylipins are able to modulate host–pathogen interaction and toxin synthesis in some mycotoxigenic fungi. They include aflatoxin and sterigmatocystin in *Aspergillus flavus* and *Aspergillus nidulans* (Burow et al., 1997), T2 toxin in *Fusarium sporotrichioides* (McDonald et al., 2004), fumonisin B1 in *Fusarium verticillioides* (Gao et al., 2007) and OTA in *Aspergillus ochraceus* (Reverberi et al., 2010a). Inhibition of oxylipin formation can be correlated with a remarkable inhibition of OTA biosynthesis, as suggested by results obtained in other OTA-producing fungi such as *A. ochraceus* (Reverberi et al., 2010a). It is reasonable to speculate that a leukotriene hydrolase might play a role in OTA biosynthesis by hydrolyzing leukotriene molecules.

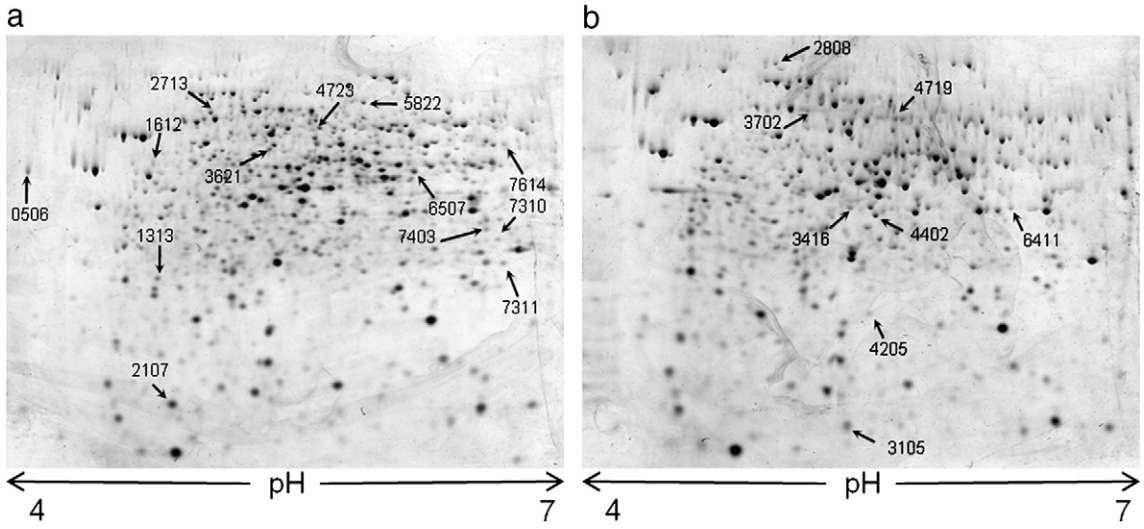

Fig. 1. 2-D gel electrophoresis patterns of the proteins of *A. carbonarius* strains W04-40 (part a) y W0-46 (part b) cultured in OTA production conditions. Proteins were separated in pH 4–7 IPG-strips for the first dimension and in acrylamide 12.5% gradient gels for the second dimension as described in the Materials and methods. Upregulated proteins in the OTA-producing strain (W04-40) and in the non-OTA-producing-strain (W04-46) are localized with arrows and marked with spot ID (also listed in Table 2). Only one of six independent runs from each strain is shown.

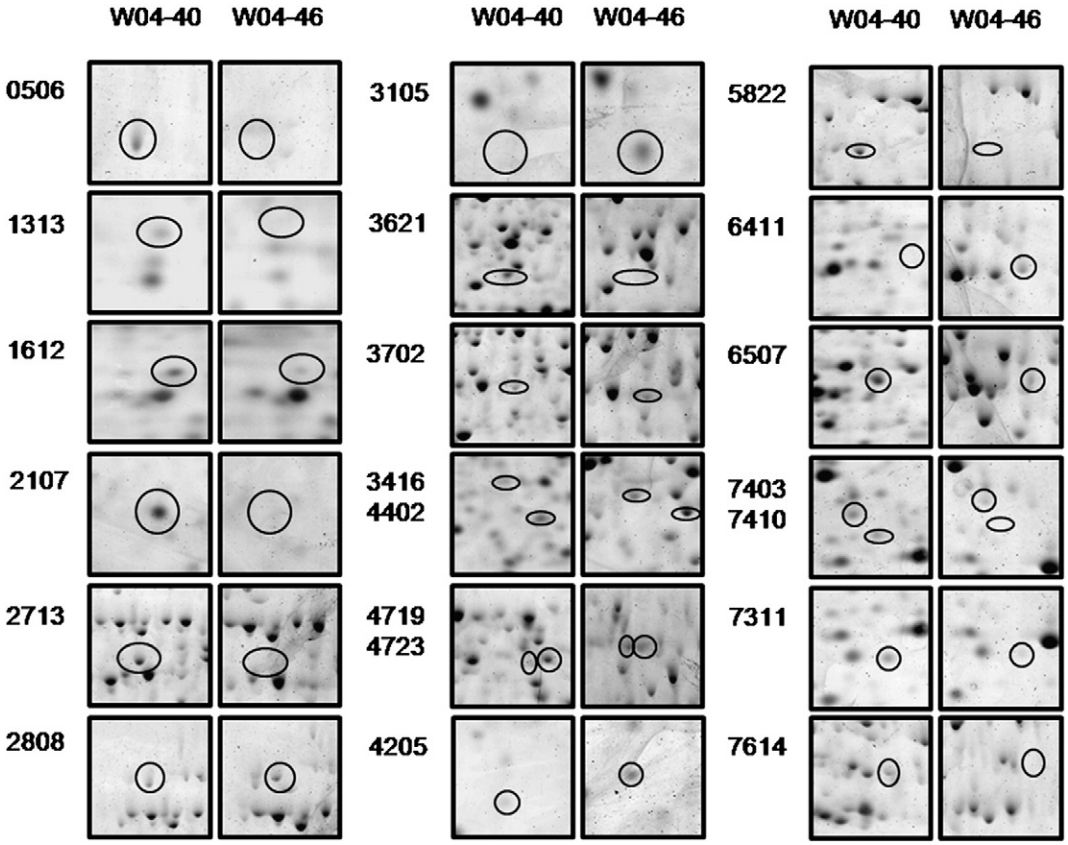

Fig. 3. Images of 2-D gels from proteins up-regulated and down-regulated from W04-40 (high OTA producer) and W04-46 (low OTA producer).

Amino acid metabolism is clearly involved in OTA biosynthesis. Taking into account that phenylalanine is part of the OTA molecule, the metabolic flux of phenylalanine during OTA biosynthesis has to be re-routed from protein to OTA biosynthesis. Färber and Geisen (2004) found phenylalanine-tRNA synthase induction in a *P. nordicum* OTA-producing strain. Crespo-Sempere et al. (2010) found ESTs that might be involved in the metabolism of several amino acids, including phenylalanine. Although proteins involved in amino acid metabolism were not found in the present study, the up-regulated protein from spot 4723 showed homology with an aminotransferase (Table 2). This enzyme might play a role in amino acid phenylalanine transport.

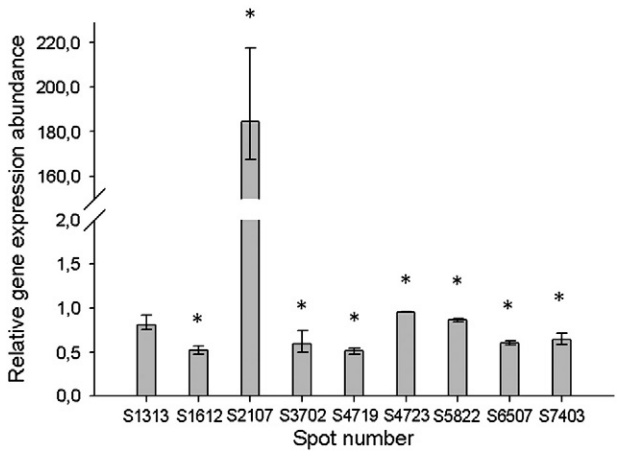

Fig. 4. Relative gene expression abundance for genes corresponding to the identified proteins in W04-40 (high OTA producer) versus W04-46 (low OTA producer). Error bar represents standard deviation. (*) significance $p<0.005$.

Protein spots 7403 and 1313 were up-regulated and identified as NADH-ubiquinone reductase and mitochondrial peroxiredoxin, respectively (Table 2). Both of them were also identified in a proteomic analysis of early phase conidia germination in *A. nidulans* and were potentially associated to stress response and detoxification (Oh et al., 2010). NADH-ubiquinone reductase has also been identified and is predictably involved in cellular redox reactions in *Botrytis cinerea* (Fernández-Acero et al., 2009) and *Verticillium dahliae* (El-Bebany et al., 2010). Research over the past decade has shown that oxidative stress stimulates mycotoxin production whereas anti-oxidants have an inhibitory effect (Georgianna and Payne, 2009; Reverberi et al., 2010b). The latter authors hypothesize that the role of mycotoxin production might be to detoxify the cell of excessive accumulation of reactive oxygen species (ROS). In a previous study, a wide range (11.92%) of genes related to stress and detoxification were identified in *A. carbonarius* using a SSH approach (Crespo-Sempere et al., 2010). This is supported by our results, which show the accumulation of the protein related to electron transport system, such as NADH-ubiquinone reductase, and up-regulation of ROS-removal related proteins, such as mitochondrial peroxiredoxin. Interestingly, the gene encoding for mitochondrial peroxiredoxin was also identified as differentially expressed in the SSH approach above mentioned. Therefore, NADH-ubiquinone reductase and mitochondrial peroxiredoxin might play a role in stress response, detoxification and OTA production.

Protein spot 2107 exhibited the greatest up-regulation (126.5 fold) (Fig. 2) and it was identified as a homologue of CipC, a small conserved protein with unknown function (Table 2). The name CipC derives from concanamycin-induced protein because it was up-regulated in *A. nidulans* in response to the antibiotic concanamycin A, produced by *Streptomyces* species (Melin et al., 2002). It has also been found as a pathogenesis-associated gene in *Verticillum alboatrum* (Mandelc et al., 2009), *Verticillium dahliae*, *Stagonospora nodorum* and

Table 2
Relative protein spot intensities and identification of differentially expressed proteins in the high OTA producer (W04-40) versus the low OTA producer (W04-46).

Spot	[a]Fold	Accession NCBI	Organism	Protein name (putative function)	[b]Mascot *expect*	MW, *Da, theor*	[c]p*I theor*	[d]Matched peptides	[e]Cover. %
5822	3.53	XP_001390581	*Aspergillus niger* CBS 513.88	Unnamed protein product leukotriene-A4 hydrolase	0.0016	75599	5.79	7	10
4723	3.07	XP_659527	*Aspergillus nidulans* FGSC A4	Hypothetical protein AN1923.2 aminotransferase family	2.90E-06	55273	5.59	4	10
2107	126.5	XP_001393387	*Aspergillus niger* CBS 513.88	Hypothetical protein An09g00630 Cip c	1.90E-07	12599	5.09	3	16
7403	7.54	XP_001394604	*Aspergillus niger* CBS 513.88	Hypothetical protein An11g06200 NADH + ubiquinone reductase	4.20E-11	31985	7.68	6	21
1313	3.77	XP_001401704	*Aspergillus niger* CBS 513.88	Hypothetical protein An04g03360 Peroxirredoxin	0.0078	23427	5.2	3	17
1612	3.64	XP_001396183	*Aspergillus niger* CBS 513.88	Hypothetical protein An13g00430 ACO B gene may be a gene that is required for sporulation	5.50E-08	40482	5.63	4	12
6507	3.41	XP_001400296	*Aspergillus niger* CBS 513.88	Hypothetical protein An02g11680 translation initiation factor eIF-4A - *Schizosaccharomyces pombe*	3.60E-08	45104	5.04	4	7
4719	0.46	XP_001395807	*Aspergillus niger* CBS 513.88	Hypothetical protein An12g07580 acetylglutamate kinase/N-acetyl-gamma-glutamyl-phosphate reductase	1.70E-12	99010	8	4	4
3702	0.33	XP_657920	*Aspergillus nidulans* FGSC A4	TBA1_emeni Tubulin Alpha-1 Chain	3.70E-06	51069	4.92	2	6

[a] Fold = Relative protein spot intensities of differentially expressed proteins in the high OTA producer (W04-40) versus the low OTA producer (W04-46).
[b] Mascot expect = The expectation value provided by Mascot is the number of times you could expect to get this identification by chance.
[c] p*I* = Isoelectric point.
[d] Matched peptides = Number of peptides matched from protein in MS/MS query.
[e] Cover = percentage of amino acid sequence of protein covered in MS/MS analysis.

Sclerotinia sclerotiorum (Tan et al., 2008). In the human pathogen *Cryptococcus neoformans* CipC was found up-regulated during infection (Melin et al., 2002). In *Aspergillus fumigatus* it was found on the conidial surface and was proposed to constitute an important allergen (Asif et al., 2006). In another study, it was exclusively found in the hyphal morphotype that enables invasive growth during infection (Bauer et al., 2010). On the other hand, CipC is also significantly up-regulated under nitrogen starvation conditions in *Gibberella fujikuroi* (Le Queré et al., 2004; Sexton et al., 2006; Steen et al., 2003; Teichert et al., 2004) and *Ustilago maydis* (Böhmer et al., 2007). Interestingly, nitrogen starvation is one of the environmental factors for disease development in phytopathogenic fungi (Snoeijers et al., 2000) and mycotoxin production (Kohut et al., 2009). Finally, CipC has also been related to mycotoxin production and CipC expression followed a similar expression pattern to the FUM cluster involved in fumonisin biosynthesis by *Fusarium verticilloides* (Pirttilä et al., 2004). Assuming these diverse roles that CipC might play in different fungi, no clear picture emerges for the biological function of CipC-like proteins. However, its strong up-regulation in the high OTA-producing strain W0-40, recorded in the present study, would suggest the potentially important involvement of the CipC protein in OTA production by *A. carbonarius*.

Protein spot 1612 was upregulated and identified as acoB protein, which has been related to sporulation (Table 2). It has been proposed that mycotoxin biosynthesis might be related to fungal development, mainly in relation to conidia formation. Pigments from *Aspergillus* conidia are also polyketide derivatives (Brown et al., 1993) and thus mycotoxins and conidia pigment biosynthesis could share regulatory steps. A relationship between aflatoxin biosynthesis and sporulation in *Aspergillus parasiticus* has been demonstrated in earlier works (Guzmán-de-Peña and Ruiz-Herrera, 1997). Botton et al. (2008) also pointed out that OTA biosynthesis is related to sexual/asexual sporulation in *A. carbonarius*. AcoB gene is one of the three pre-induction genes required for sporulation at high temperatures in *A. nidulans* and it was also demonstrated that acoB was involved in secondary metabolism (Lewis and Champe, 1995). Since there are common signalling pathways connecting mycotoxin biosynthesis and sporulation (Brodhagen and Keller, 2006), a similar role may be also hypothesized for protein acoB gene in *A. carbonarius*.

Protein spot 6507 showed homology with the translation initiation factor eIF-4A from *Schizosaccharomyces pombe* (Table 2). This protein is an ATP-dependent RNA helicase belonging to a diverse family of proteins involved in ATP-dependent unwinding, needed in a variety of cellular processes including splicing, ribosome biogenesis and RNA degradation. To our knowledge, no connections between this protein and mycotoxin production have been described to date.

Finally, protein spots 4719 and 3702 showed homology with acetylglutamate kinase and TBA1_ emeni tubulin alpha-1 chain, respectively. Both of them showed decreased accumulation of their relative protein expressions in the high OTA-producing strain (Fig. 2). Acetylglutamate kinase showed a decrease in the abundance of 0.46 fold in the high OTA-producing strain compared to the low OTA-producing strain (Table 2). This protein is an enzyme related to ornithine metabolism, which represents another likely connection between OTA biosynthesis and sporulation in *A. carbonarius*. A link was established among ornithine, sporulation and aflatoxin biosynthesis in *A. parasiticus* (Guzmán-de-Peña and Ruiz-Herrera, 1997). Ornithine decarboxylase (ODC) is the first and most regulated enzyme involved in polyamine synthesis, essential for differentiation in fungi. If diaminobutane, an ODC inhibitor, is added after spore germination, it blocks sporulation completely and suppresses aflatoxin biosynthesis in *A. parasiticus* (Guzmán-de-Peña and Ruiz-Herrera, 1997). Interestingly, a sequence showing homology with ODC was identified as differentially expressed in the high OTA-producing strain of *A. carbonarius* using the aforementioned SSH approach (Crespo-Sempere et al., 2010). It is difficult to speculate about the exact function of acetylglutamate kinase in relation to OTA production. However, acetylglutamate kinase down-regulation could alter the ornithine metabolism, which might indirectly be involved in the OTA production pathway in *A. carbonarius*.

TBA1_emeni tubulin alpha-1 chain also showed decreased in the abundance (0.33 fold) (Table 2) in the high OTA-producing strain. Assuming the diverse and important roles that tubulins can play in cells, variations in these proteins may also influence many other cellular activities, some of which might be related to the OTA production process.

3.3. Analysis on selected transcripts of differentially accumulated proteins

To verify the up-regulated or down-regulated expression of the identified proteins at transcription level, total RNAs were extracted

Table 3
List of specific primers used in RT-PCR analysis. Protein spot numbers, primer names, primer sequences, theoretical annealing temperatures of the primers and PCR amplicon sizes are indicated.

Protein	Forward		Reverse		Temperature annealing	Size of PCR product
	Primer name	Primer sequences (5′ to 3′)	Primer name	Primer sequences (5′ to 3′)		
4719	ACET.C	GTGATCGTGAGGGACTGGATG	ACET.D	CAGGTGTGCCAAGGGAGAAG	56 °C	155
1612	ACOB.H	TCGACTTCACCCCGTTGAC	ACOB.I	GGCGGTGAAGATTTCAAGGAG	56 °C	71
4723	AMINO.K	CAAGGGCATGGTTGGTGAG	AMINO.L	TGATGCTGACGTACTTGTAGACCTG	56 °C	76
2107	CIPC.A2	AGGAGCACAAGGCTAAGTTCACC	CIPC.B2	GCTCACGGTCGACGAAGTC	56 °C	182
6507	HELI.C	CCCAGGCCGTCATCTTCTG	HELI.D	GCATGGCAGAGACGGTGAAG	56 °C	90
5822	LEU.C	CCTGTCAAGATACGCCTGATGTC	LEU.D	GCGCATCACGGACAGGTAAG	56 °C	96
7403	OXI.C	AGCGCTTCGAGGTCGTCTAC	OXI.D	GCACCCTCGAACAGACCTG	56 °C	118
1313	PER.A	TCCTTCTTGAGGTTGGTGAAGC	PER.B	CTCAGAAGAAGTTCGGCGATG	56 °C	46
3702	TUB.C	TGTTAACGAGATCACCATGTCCTG	TUB.D	TGGTGCGCTTGGTCTTGAG	56 °C	161
S18	S18A.3	GCAAATTACCCAATCCCGACAC	S18B.3	GAATTACCGCGGCTGCTG	56 °C	132

and analyzed by real-time RT-PCR. EST sequences corresponding to the nine identified proteins (seven up-regulated and two down-regulated) were used to compare protein and transcript expression level. After sequencing part of the genes using degenerate primers (see Materials and methods), sequence data was used to design the primer pairs used for real time RT-PCR (Table 3). Of the seven up-regulated proteins identified, only one gene (CipC) showed a higher transcription level (184.5 fold) in the high OTA-producing strain W04-40, consistent with proteome level observations (Fig. 4). This data confirmed that CipC expression is clearly different in the high and low OTA-producing strains and that suggest a possible role in OTA production. However, there is no conclusive evidence and further work will be required to elucidate this finding.

In contrast to CipC, the rest of up-regulated proteins did not show correlation between transcription and translation expression levels (Fig. 4). The discrepancy between proteomic results and real time RT-PCR analysis was not due to growth stage or growth conditions because all cells and proteins were harvested 48 h after incubation in CYA medium. However, differences between transcription and translation may also be time-dependent. The correlation between transcription and translation is reported to be less than 50% (King and Sinha, 2001). The low correlation between transcription and translation expression levels might imply that the expression of many genes involved in OTA biosynthesis might be controlled directly through translational regulation.

Protein spots 4719 (acetylglutamate kinase) and 3702 (TBA1_emeni tubulin alpha-1 chain), identified as down-regulated, showed a significant decrease in transcription abundance in the high OTA producing strain (W04-40) in relation to the low OTA producer (W04-46). Transcription levels of 0.51 and 0.59 were observed for genes encoding protein spots 4719 and 3702, respectively (Fig. 4). Therefore, a correlation between transcriptional and translational expression patterns was observed in these down-regulated proteins. Expression of these genes might be controlled directly through transcriptional regulation.

In conclusion, this is a first report on the identification of proteins associated with OTA production in *A. carbonarius* by comparative proteomic analysis in both OTA producing and non-producing strains. The proteome analysis identified nine proteins (seven up-regulated and two down regulated) potentially involved in diverse biological functions. We have speculated on the possible functional roles some might play in regulation, amino acid metabolism, oxidative stress and sporulation. A protein was identified as a homologue of CipC, a protein with unknown function, which exhibited the greatest up-regulation in the OTA-producing strain (126.5 fold). RT-PCR analysis also revealed overexpression of the CipC gene, confirming that overproduction of this protein with unknown function is regulated at the transcriptional level and is probably involved in OTA biosynthesis. A comprehensive analysis of mutants lacking the proteins identified in this study may provide new insights into the OTA biosynthesis pathway.

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