Characterization and disruption of the *cipC* gene in the ochratoxigenic fungus *Aspergillus carbonarius*.

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Abstract

Aspergillus carbonarius is considered the most important ochratoxin A (OTA) producing fungi among those causing OTA contamination in grapes and grape derived products. CipC is a small protein with unknown function that was previously found to be highly up-regulated in an OTA producer strain of *A. carbonarius* in comparison to a non OTA producer strain. In this study, *cip*C was characterized and disrupted via *Agrobacterium tumefaciens*-mediated transformation in an ochratoxigenic *A. carbonarius* strain in order to study whether this gene has a role in OTA production. Sequence analysis indicated that the promoter region of *cip*C contains putative binding sites for transcription factors that regulate the utilization of nutrients, the stress response and detoxification processes, all factors that can influence mycotoxin biosynthesis. Although the Δ*cipC* mutant grew similarly to the wild type strain, the null mutant showed a much higher OTA production. Moreover, when *A. carbonarius* was grown under the oxidative stress conditions imposed by the presence of hydrogen peroxide, *cipC* gene expression was up regulated. These results indicate that *cipC* is not directly involved in OTA biosynthesis, but sequence analysis of the *A. carbonarius cipC* gene promoter and the phenotype of the Δ*cipC* disrupted mutant suggest that CipC could be a stress response protein that would be up regulated concomitantly with OTA production.

1. Introduction

Ochratoxin A (OTA) is a nephrotoxic, carcinogenic, teratogenic and immunotoxic mycotoxin produced by several *Penicillium* and *Aspergillus* species (Creppy, 1999; Kuiper-Goodman & Scott, 1989; Petzinger & Ziegler, 2000; Pfohl-Leszkowicz & Manderville, 2007). OTA has been classified by the International Agency for Research on Cancer (IARC, 1993) in group 2B (possible human carcinogen). This mycotoxin can be found in a wide range of food commodities, including cereal-based products, coffee, species, nuts, olives, grape-derived products, beans, figs and cocoa (Battilani, Magan, & Logrieco, 2006; Perrone et al., 2007). Grapes and wines are among the commodities with the greatest toxin content, second only to cereals (Bau, Bragulat, Abarca, Minguez, & Cabañes, 2005; Belli et al., 2004). *Aspergillus carbonarius* is considered the main OTA-producing fungus in grapes and grape derived products (Battilani et al., 2006; Perrone et al., 2007).

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), Karoleiwez and Geisen (2005), Bachaet al. (2009) and Gallo et al. (2009) have described PKS genes involved in OTA biosynthesis in *Aspergillus ochraceus*, *Penicillium nordicum*, *Aspergillus westerdijkiae* and *A. carbonarius*, respectively. Additionally, other putative OTA biosynthetic genes have been reported, including two P450 monoxygenase genes in *A. ochraceus* (John O'Callaghan, Stapleton, & Dobson, 2006), a nonribosomal peptide synthetase, a halogenase, a phenylalanine t-RNA synthetase, a methylase and a fragment with homology to ABC transporter genes in *P. nordicum* (Färber & Geisen,

2004). Recently, the implication of a nonribosomal peptide synthetase on OTA biosynthesis in *A. carbonarius* has also been indicated by Gallo et al.(2012).

Different techniques such as Differential Display Reverse Transcriptase-PCR (DDRT-PCR) (Färber & Geisen, 2004), cDNA-AFLP (Botton et al., 2008) and Suppression Subtractive Hybridization (SSH) (Crespo-Sempere, González-Candelas, & Martínez-Culebras, 2010) have been used to identify genes putatively involved in OTA biosynthesis in several fungal species. Recently, we performed a proteomic analysis using two-dimensional electrophoresis (2-DE) combined with MALDI-TOF mass spectrometry to detect changes in the abundance of a large number of proteins in two closely related strains of *A. carbonarius* that differ in their OTA-producing potential (Crespo-Sempere, Gil, & Martínez-Culebras, 2011). Among the differentially expressed proteins identified in that study, a homologue of CipC, a small conserved protein with unknown function, stood out. The name CipC derives from concanamycin-induced protein because it was up-regulated in *Aspergillus nidulans* in response to the antibiotic concanamycin A (Melin, Schnürer, & Wagner, 2002). CipC exhibited the greatest up-regulation in the OTA-producing strain (126.5 fold). RT-qPCR analysis also revealed overexpression of the *cipC* gene (184.5 fold), confirming that overproduction of this protein with unknown function is regulated at the transcriptional level.

In the present study we have examined whether *cipC* has a role in OTA production. For that purpose we have cloned and characterized the *cipC* gene from *A. carbonarius*. Additionally, we have deleted the *cipC* gene in the ochratoxigenic *A. carbonarius* W04-40 strain by targeted gene replacement using *A. tumefaciens*-mediated transformation (ATMT). Finally, after the

phenotypic characterization of the *cipC* null mutant (Δ*cipC*), we discuss a possible relationship between *cipC* and OTA production.

2. Materials and methods

2.1. Fungal strains, media, and culture conditions

The OTA-producing *A. carbonarius* strain W04-40 was isolated from a Spanish vineyard by Martínez-Culebras and Ramon (2007) and deposited in the Institute of Agrochemistry and Food Technology ofthe Spanish National Research Council (IATA-CSIC). The *Agrobacterium tumefaciens* AGL-1 strain was kindly provided by L. Peña (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain).

A. carbonarius was grown on Petri dishes containing Malt Extract Agar (MEA) medium (2 % (w/v) malt extract, 0.1 % (w/v) peptone, 2 % (w/v) glucose and 1.5 % (w/v) agar) in the dark at 28 °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, Holland) and were adjusted to 10^6 conidia/mL using a haemocytometer. One hundred microliters of the conidial suspension was homogeneously spread on Petri dishes containing Czapeck Yeast Extract Agar (CYA) medium (0.5 % (w/v) yeast extract, 0.3 % (w/v) $NaCO_3$, 3 % (w/v) sucrose, 0.13 % (w/v) $K_2HPO_4 \cdot 3H_2O$, 0.05 % (w/v) $MgSO_4 \cdot 7H_2O$, 0.05 % (w/v) KCl, 0.001 % (w/v) $FeSO_4 \cdot 7H_2O$, 0.001 % (w/v) $CuSO_4 \cdot 5H_2O$, 0.001 % (w/v) $ZnSO_4 \cdot 7H_2O$, and 1.5 % (w/v) agar) and sub-cultured in the dark at 28 °C. To study the expression of *cipC* under oxidative stress, *A. carbonarius* was grown

in the presence of different concentrations of hydrogen peroxide (Sigma-Aldrich, UK), added to the CYA medium after sterilization.

2.2. Genomic DNA extraction

The rapid DNA extraction protocol described by Cenis (1992) was followed with minor modifications. Cultures were grown for 1 day at 28ºC in 500 µl of Czapeck's Yeast medium. Mycelium was recovered after 10 min of centrifugation at 17500 X*g* and 300 µl of extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. The mycelium suspension was disrupted with five 2.8 mm stainless steel beads (Precellys, Bertin Technologies) for 2 minutes in a cell disruptor (Mini BeadBeater-8, Biospec). After centrifugation at 17500 X*g* for 10 min, 150 µL of 3 M sodium acetate (pH 5.2) was added to the supernatant. The supernatant was incubated at -20ºC for 10 minutes and centrifuged (17500 X*g*, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by adding 1 volume of isopropyl alcohol. After 5 minutes of incubation at room temperature, the DNA suspension was centrifuged (17500 X*g*, 10 min). The DNA pellet was washed with 70**%** ethanol to remove residual salts. Finally, the pellet was air-dried and the DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

2.3. Characterization of the *cipC* gene

We had previously obtained a partial sequence of the *cipC* gene using degenerated oligonucleotides (Crespo-Sempere, Gil, et al., 2011). The

complete DNA sequence of *cipC*, together with its promoter and terminator sequences, was cloned using the Universal Genome Walker kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. PCR products were purified with the UltraClean PCR Clean-up DNA Purification kit (MoBio, USA) and directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, UK) in an Applied Biosystems automatic DNA sequencer model 373A. The gene-specific primers used for PCR amplification and sequencing are given in Table 1 (from CIPC.G to CIPC.S and AP1-AP2).

A computational structural search was carried out for the ORF using the Pfam database (Finn et al., 2008) (http://pfam.sanger.ac.uk/). Protein sequences of the CipC family were aligned using ClustalW. The genetic distances were calculated using the Poisson correction model and the phylogenetic inference was obtained by the neighbor-joining (NJ) method (Saitou & Nei, 1987). The NJ tree and the statistical confidence of a particular group of sequences, evaluated by bootstrap test (1000 pseudoreplicates), were performed using the program MEGA 4.0 (Tamura, Dudley, Nei, & Kumar, 2007). The search for putative binding sites of transcription factors was done with Transcription Element Search System (TESS) version 2.0 (Schug, 2008) using TRANSFAC database version 6.0, JASPAR 20060301, IMD v1.1 and CBIL/GibbsMatv1.1 (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

2.4. Construction of the *cipC* gene replacement plasmid

Amplified fragments around 1500 bp from the promoter and terminator regions were cloned into the plasmid vector pRF-HU2 (Frandsen, Andersson,

Kristensen, & Giese, 2008), a binary vector designed to be used with the USER friendly cloning technique (New England Biolabs), as described previously (Crespo-Sempere, López-Pérez, Martínez-Culebras, & González-Candelas, 2011). The specific primers for the promoter and terminator regions included 9 bp long 2- deoxyuridine containing overhangs, CIPC-PF (5'-GGTCTTAAUGCGTACCCAGCCCGATAGAC-3'), CIPC-PR (5'-GGCATTAAUTTTGTGGATGAGGGAGAAGAAGG-3'), CIPC-TF (5'-GGACTTAAUACATTGTTTCTTTGGTCGTAGGTG-3') and CIPC-TR (5'-GGGTTTAAUTCATGAAGGACCACATGATGAG-3'), which ensured directionality in the cloning reaction (Fig.1B). The promoter and terminator regions were amplified with EcoTaq DNA Polymerase (Ecogen, Spain). Cycling conditions consisted of an initial denaturation step at 94 °C for 3 min, 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min and a final elongation step at 72 °C for 10 min. Both DNA inserts and the treated vector were mixed together and incubated with the USER (uracil-specific excision reagent) enzyme (New England Biolabs, USA) to obtain plasmid pRFHU2-CIPC (Fig.1A). An aliquot of the mixture was used to transform chemical competent *E. coli* DH5α cells. Kanamycin resistant transformants were screened by PCR. Proper fusion was confirmed by DNA sequencing. Then, plasmid pRFHU2-CIPC was introduced into electrocompetent *A. tumefaciens* AGL-1 cells.

2.5. Fungal transformation

Transformation of *A. carbonarius* was done as described previously (Crespo-Sempere, López-Pérez, et al., 2011) using *A. tumefaciens* AGL-1 cells carrying the plasmid pRFHU2-CIPC. Equal volumes of IMAS-induced bacterial

culture (de Groot, Bundock, Hooykaas, & Beijersbergen, 1998) and conidial suspension of *A. carbonarius* (10^6 conidia/mL) were mixed and spread onto nitrocellulose membrane filters, which were placed on agar plates containing the co-cultivation medium (same as IMAS, but containing 5 mM instead of 10 mM of glucose). After co-cultivation at 26 °C for 40 h, the membranes were transferred to CYA plates containing Hyg B (100 µg/ml) as the selection agent for fungal transformants, and cefotaxime (200 µg/mL) to inhibit growth of *A. tumefaciens* cells. Hygromcycin resistant colonies appeared after 3 to 4 days of incubation at 28 °C.

2.6. Confirmation of *cipC* deletion

Disruption of *cipC* in transformants was confirmed by PCR analyses. Primer binding sites are detailed in Fig.1B. The insertion of the selection marker was checked with the primer pair Hmbr1 (5'-CTGATAGAGTTGGTCAAGACC-3') and Hmbf1 (5'-CTGTCGAGAAGTTTCTGATCG-3'). Deletion of *cipC* was confirmed with a primer pair designed within the sequence of the gene CIPC.A2 (5'-AGGAGCACAAGGCTAAGTTCACC-3') and CIPC.B2 (5'-GCTCACGGTCGACGAAGTC-3'). Real-time genomic PCR analysis was carried out in order to determinate the number of T-DNA molecules that have been integrated in the genome of transformants following basically the procedure established by Solomon et al. (2008). The primers used for quantitative real time PCR (qPCR) were designed close to the selection marker within the promoter region of *cipC* gene using the OLIGO Primer Analysis Software V.5 (Fig.1B). The primer sequences were CIPC-GT (5'-GAGGAGCTCAGCCTTCCATG-3') and CIPC-HT (5'-

GAGCTTCCTCACCCTGTGGTC-3'). qPCR reactions were performed in a LightCycler 480 System (Roche, USA) using SYBR Green to monitor DNA amplification. qPCR efficiency (E) for each pair of primers was calculated from the slopes of the standard curve using the LightCycler software (Rasmussen, 2001). The number of T-DNA that have been integrated in the genome was calculated based on E and the Crossing point (Cp) value of transformant versus the wild type strain, and normalized in comparison to a reference gene that is present with the same copy number in both wild type and transformant. This can be whatever gene except *cipC*. We chose the pyruvate carboxylase (*pyc*) gene (GenBank accession Nº GW328015) using primers pyc-F (5'-GCAGGCCAAGAAGTGTGGTG-3') and pyc-R (5'-TGCTGGGGTTCAGCATGTC-3'). The number of T-DNA copies that have been integrated in the genome of the transformant was calculated according to the following equation based on Pfaffl (2001) and Rasmussen (2001):

$$\text{copy number} = \frac{(E_{cipC})^{\Delta cipC\ (\text{wild type} - \text{transformant})}}{(E_{pyc})^{\Delta pyc\ (\text{wild type} - \text{transformant})}}$$

Three technical replicates were done for each knockout mutant candidate, and PCR reaction quality was checked by analyzing the dissociation and amplification curves.

2.7. RNA isolation and cDNA synthesis

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 grounded to a fine powder 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 dodecyl sulfate (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; Switzerland) 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 X*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 X*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 by adding 300 µL of 12 M LiCl. After centrifugation at 27,200 X*g* for 60 min, the precipitated 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 an agarose gel. Total RNA was treated with DNase (TURBO DNase, Ambion, USA) to remove contaminating genomic DNA. 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, USA).

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

Partial gene sequences from a superoxide dismutase (*sod*) and a peroxiredoxin (*prx*) genes from *A. carbonarius* were obtained from previous work (Crespo-Sempere et al., 2010), which correspond to GenBank accession numbers GW327919 and GW327996, respectively. Gene-specific primer sets were designed for gene expression analysis (SODf/SODr for *sod*, PRXf/PRXr for *prx* and CIPC.A2/CIPC.B2 for *cipC*) with OLIGO Primer Analysis Software V.5 to amplify PCR fragments of 67 (*prx*), 182 (*cipC*) and 183 (*sod*) bp in length (Table 1). Real-time RT-PCR reactions were performed in a LightCycler 480 System (Roche) using SYBR Green to monitor cDNA amplification. The ribosomal 18S RNA gene was used as a reference gene. To calculate the normalized relative gene expression levels (fold induction), data were analyzed using the Relative Expression Software Tool (REST) and the mathematical model based on mean threshold cycle differences between the sample and the control group (Michael W. Pfaffl, Horgan, & Dempfle, 2002). REST was also used for a randomization test with a pair-wise reallocation to assess the statistical significance of the differences in expression between the control and treated samples (significance at $p \leq 0.05$).

2.9. Determination of vegetative growth

For growth assessment, CYA plates were inoculated centrally with 5 µl of conidia suspensions (10^6 conidia/mL) from the wild-type strain of *A. carbonarius* and the Δ*cipC* knockout transformant. Two perpendicular diameters of the growing colonies were measured daily over four days until the colony reached the edge of the Petri dish. The assay was performed in triplicate.

2.10. Extraction and detection of OTA from culture

OTA was extracted using a variation of a simple method described previously (Bragulat, Abarca, & Cabanes, 2001). The isolates were grown on CYA and incubated at 28 °C (Pitt & Hocking, 1997). Separation, detection and quantification of OTA were performed by injecting 20 µl of extract from each vial into an HPLC system consisting of a Dionex model P680A pump (Sunnyvale, USA) connected to a Dionex model RF-2000 programmable fluorescence detector and to a DionexPDA-100 photodiode array detector. For determination of OTA, a C18 reversed-phase column (150×4.6 mm i.d., 5 µm particle size Kromasil C18 (Análisis Vínicos S.L., Spain), connected to a precolumn Kromasil C18 (10×4.6 mm i.d., 5 µm particle sizes, Análisis Vínicos S.L.) were used. For chromatographic separation of OTA, the mobile phase was acetonitrile: water: acetic acid, (57:41:2 v/v/v) under isocratic elution during 10 min, 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, USA).

2.11. Statistical analyses

All comparisons were analyzed by One way ANOVA followed by the Tukey's honestly significant different test (HSD), using Statgraphics Centurion Version XVI. Significance was defined as $p<0.05$.

3. Results and Discussion

CipC is a protein with unknown function, which has been previously related with different stress processes in fungi such as pathogenesis, nitrogen

starvation and mycotoxin production. However, its biological function remains to be elucidated. Recently, the proteomes of a highly (W0-40) and a weakly (W0-46) OTA-producing *A. carbonarius* strains were compared to identify proteins that may be involved in OTA production (Crespo-Sempere, Gil, et al., 2011). This proteomic study highlighted CipC as the protein with the highest induction in strain W04-40. The *cip*C gene also showed a higher transcriptional level in the OTA-producing strain, suggesting a possible role in OTA production.

3.1. Sequence analysis and characterization

We sequenced a 3,646 bpDNA fragment that contained the *cipC* gene together with 1,550 bp and 1,631bp upstream and downstream regions, respectively (GenBank accession N^{o}. KC348440). *cipC* contained a 465 bp ORF interrupted by two introns of 77 and 55 bp that encode a protein of 110 amino acids. The introns were verified by comparison with cDNA sequences having characteristic 5' (GT) and 3' (AG) intron splicing sites of fungal genes (Kupfer et al., 2004).

A BlastP search indicated that CipC shows homology to CipC of *A. niger* CBS 513.88, with an identity of 81% (XP_001393387.1). Additionally, in order to detect functional domains, we searched CipC against the Pfam database. The aminoacid sequence presented a significative match with a family of eukaryotic proteins with unknown function (DUF3759). Proteins of this family are typically between 107 and 132 amino acids in length. According to the Pfam database, the DUF3759 protein family presents two possible domain organizations. Although most of the protein sequences belonging to this family contain only a single domain, DUF3759 (PF12585), the presence of this domain has also been

reported linked to glutathione synthetase domains, which catalyze the final step of glutathione synthesis, an important antioxidant molecule (Pócsi, Prade, & Penninckx, 2004).

The CipC amino acid sequence of *A. carbonarius* together with a total of 84 CipC amino acid sequences from different fungi and yeast obtained from the database were subjected to phylogenetic analysis (Fig. 2). The CipC sequence from *A. carbonarius* clustered together with the sequence of *A. niger* (A2QT35). However, another CipC sequence from *A niger* (A2QMX6) belonging to the DUF3759 family clustered separately within the dendrogram. Similarly, several proteins belonging to the DUF3759 family were found in other fungi, indicating that it is possible to find several CipC proteins in fungal genomes: *Aspergillus clavatus* (2 proteins), *Aspergillus flavus* (6), *Aspergillus fumigatus* (3), *A. niger* (2), *Aspergillus oryzae* (4), *Candida albicans* (5), *Candida dubliniensis* (2), *Candida tropicalis* (2), *Emericella nidulans* (3), *Laccaria bicolor* (3), *Metarhizium robertsii* (2), *Neosartorya fischeri* (3), *Paxillus involutus* (7), *Penicillium marneffei* (2) and *Trichophyton equinum* (2). The disparity in the number of cipC proteins for different fungi is accompanied by a diverse phylogenetic distribution of the representatives from each species. Thus, in some instances, the different CipC proteins from the same fungus grouped together, as those of *A. fumigatus*, but in other cases they are located in distant branches. Unfortunately, the possible roles of the different cipC members are unknown, as no deletion mutant has been reported yet.

Sequence analysis of the promoter region of *cipC* revealed multiple potential binding sites for transcription factors that regulate the utilization of different carbon and nitrogen sources as well as macrominerals and trace

minerals (CP1, MIG1, PHO_4, PUT3, REB1, SEF1, NIT2, GCR1, FACB and UAY) (Table 2). Interestingly, Teichert et al. (2004) observed that when the glutamine synthetase gene was deleted in *Gibberella fujikuroi*, another important mycotoxin producer fungus, *cipC* was down regulated. They also observed that this deletion had a significant impact on the transcriptional control of primary and secondary metabolism. Moreover, Böhmer et al. (2007) described an induction of *cipC* when *Ustilago maydis* was grown with glucose (Glu) and ammonium (NH_4^+) and transferred to a medium containing arabinose (Ara) and nitrate (NO_3^-). These results suggest that *cipC* might be controlled by transcription factors that regulate the utilization of different carbon and nitrogen sources.

A second group of binding sites for transcription factors that regulate the stress response and detoxification (AP1, ACE1, HSF, MSN4, SKO1 and GCN4) (Table 2) within the promoter of *cipC* was also identified. Noteworthy is the fact that the majority of the transcription factors with binding sites within the promoter of *cipC*, regulate elements with high influence on the biosynthesis of mycotoxins such as carbon and nitrogen sources (Abbas, Valez, & Dobson, 2009; Ferreira & Pitout, 1969; Medina et al., 2008), metals (Steele, Davis, & Diener, 1973), oxidative stress (Reverberi, Ricelli, Zjalic, Fabbri, & Fanelli, 2010), pH (transcription factor PacC) (Esteban, Abarca, Bragulat, & Cabañes, 2005; John O'Callaghan et al., 2006) and sporulation (transcription factor abaA) (Guzmán-de-Peña & Ruiz-Herrera, 1997).

3.2. Disruption of *cipC* gene

ATMT was successfully applied to *A. carbonarius* using the binary vector pRFHU2-CIPC. T-DNA integration was confirmed by PCR analyses based on expected genomic patterns using locus specific primers (Fig. 3). Only one out of 68 monosporic transformants had the PCR pattern corresponding to the deletion of the *cipC* gene. Thus, gene replacement efficiency by homologous recombination obtained was 1.5%. Real-time genomic PCR analysis was also carried out in order to determinate the number of copies of the T-DNA that had been integrated into the genome. A mutant with ectopic insertions was also included as a control. Copy number was calculated according to the equation detailed above using the Efficiency of *cipC* and *pyc* amplification and Crossing points for the wild type, the Δ*cipC* mutant and the mutant with ectopic insertions (Table 3). The Δ*cipC* deletion mutant only harbors a copy (0.945 according to the equation) of the T-DNA, which, as shown previously, replaces the original *cipC* gene. However, the mutant with ectopic insertions harbors four copies (4.08 as calculated at Table 3). Although Southern-blot analysis had been traditionally used to determinate gene copy number, the use of qPCR has proven to be a suitable, more accurate and faster technique (Solomon et al., 2008). As far as we are aware, this is the first time that ATMT is used for gene replacement in *A. carbonarius*.

3.3. Phenotypical analysis of the Δ*cipC* deletion mutant of *A. carbonarius*

No statistical differences in growth and colony morphology were observed in the Δ*cipC* mutant when compared to the wild type strain on non-selective media (CYA plates). Thus, by day four, the diameter of the Δ*cipC* mutant colony was 67.5 ± 0.8mm and the wild type reached 68.7 ± 1.2 mm.

However, the Δ*cipC* mutant showed a much higher OTA production than the wild type strain (Fig. 4) in the same medium. The production of OTA after two days of incubation in CYA medium, without hygromycin, was 3.69 fold higher in the Δ*cipC* mutant with respect to the wild type strain. This difference increased at days 3 and 4 to 7.86 and 14.66 fold, respectively. Thus, the wild type produced 0.21 µg of OTA /g of growth medium at day 4, while the Δ*cipC* mutant produced 3.12 µg/g. The higher OTA production of the Δ*cipC* mutant clearly indicates that CipC is not part of the OTA biosynthetic pathway. However, in a previous proteomic study carried out by our research group (Crespo-Sempere, Gil, et al., 2011) we observed that CipC exhibited a high up-regulation in a OTA-producing strain compared to a non OTA-producing strain of *A. carbonarius* and this high induction was also observed at the transcriptional level. Both results seem contradictory, as higher OTA production in the OTA producer strain is accompanied by a higher level of CipC protein, whereas the null mutant, which lacks the *cipC* gene, shows even a higher OTA production.

Reviewing published information about *cipC* we found that this gene is up-regulated in pathogenicity processes in *V. alboatrum* (Mandelc, Radisek, Jamnik, & Javornik, 2009), *V. dahliae, S. nodorum* and *S. sclerotiorum* (Sexton et al., 2006; Tan et al., 2008), during the ectomycorrhizal symbiosis in *Paxillus* (Le Queré et al., 2004), during meningitis infection in the human pathogen *C. neoformans* (Steen et al., 2003), in *G. fujikuroi* and *U. maydis* grown under nitrogen starvation conditions (Bohmer et al., 2007; Teichert et al., 2004), in *A. nidulans* in the presence of an antibiotic inhibitor of proton pumps (Melin et al., 2002), or in *F. verticilloides* and *U. maydis* when they were grown at acidic pH conditions (Pirttilä, McIntyre, Payne, & Woloshuk, 2004; Rodríguez-Kessler et

al., 2012). All these instances are adverse situations and hence, stressful for the fungus. Results obtained from the sequence analysis of *cipC* revealed that in some organisms the functional domain of CipC is linked to a glutathione synthetase domain, which confers an antioxidant function (Bai, Harvey, & McNeil, 2003). Interestingly, we also identified in the promoter of *cipC* multiple potential binding sites to transcription factors that regulate responses to stress situations, nutrient starvation and ambient pH, all of them being elements that affect largely mycotoxins biosynthesis. In fact, there are considerable experimental results that suggest that oxidative stress correlates with mycotoxin production (Jayashree & Subramanyam, 2000; Reverberi et al., 2010; Reverberi et al., 2008). Reverberi et al. (2012) showed that oxidative stressors induced significantly OTA biosynthesis in *A. ochraceus*. Additionally, they found that when the stress response transcription factor (Yap1) was deleted from *A. ochraceus*, the disrupted strain showed a higher quantity of ROS and a higher amount of OTA compared to wild type strain. This effect was also described in *A. parasiticus*, in which the deletion of Yap1 leads to an increase in aflatoxin production (Reverberi et al., 2008). Furthermore, some authors have formulated an oxidative stress theory of mycotoxin biosynthesis (Reverberi et al., 2010).

The potential influence of oxidative stress on *cip*C expression was monitored by growing the OTA-producing *A. carbonarius* strain in CYA medium amended with different concentrations of hydrogen peroxide (50, 500 and 5000 μM) (Fig. 5). A clear up-regulation of *sod and prx* was observed when hydrogen peroxide was added to the media (Fig. 5). As the hydrogen peroxide concentration increased the expresion levels of *sod* and *prx* were higher than in the control (same medium without hydrogen peroxide), reaching 5 and 24 fold

induction, respectively. The expression of *cipC* showed a constant 2 fold induction in comparison with the control, which suggests that *cipC* might be a stress oxidative response gene, but with a different induction pattern.

If OTA production is induced in response to a stressful situation, as it has been shown for in *A. ochraceus* by Reverberi et al. (2012), it would be logical to expect that the same stress would also trigger the expression of *cipC*, as we have observed in a previous work (Crespo-Sempere, Gil, et al., 2011). Accordingly, if a stress response gene is deleted, the stress level would increase leading to an increase inmycotoxin biosynthesis, as occurs in the Δ*cipC* mutant strain obtained in the present study and also in the case of the *A. ochraceus* Δ*Aoyap1* disrupted strain.

4. Conclusions

The higher OTA production by the Δ*cipC* mutant strain clearly demonstrates that CipC is not needed for the synthesis of OTA. Our results suggest that CipC is a stress response protein, whose synthesis is induced under the oxidative stress imposed by hydrogen peroxide. A deeper knowledge of the genes involved in OTA biosynthesis, either directly or indirectly, would help us to elucidate the nutritional and environmental clues that lead to OTA contamination of food commodities.

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Figure captions

Figure 1. A) Physical map of plasmid pRFHU2-CIPC. LB= Left Border, HR2 = Homologous flanking region 2, pTrpC = Tryptophan promoter from *Aspergillus nidulans*, HygR = hygromycin phosphotransferase, TtrpC = Tryptophan terminator from *A. nidulans*, HR1 = Homologous flanking region 1, RB=Right Border, oriV = origin of replication in *E. coli*, KanR = kanamycin resistance, TrfA = replication initiation gene (broad-host-range). B) Diagram of the *cipC* replacement with the *hygR* selectable marker from pRFHU2-CIPC by homologous recombination to generate the Δ*cipC* mutant.

Figure 2. Phylogenetic tree derived from the alignment of 85 proteins that contain the PFAM-DUF3759 domain. The tree was constructed using neighbor joining. The numbers at the nodes give bootstrap values (%) of 1000 iterations. The proteins are designated by their TrEMBL ID number and the species name. Down left: scale of the phylogenetic distances.

Figure 3. PCR amplification of the hygromycin resistant cassette in the Δ*cipC* knockout mutant with primers Hmbr1 and Hmbf1 (lane A) and amplification of *cipC* gene with primers CIPC.A2 and CIPC.B2 in the Δ*cipC* knockout mutant and the wild type strain (lane B and C respectively).

Figure 4. OTA production in the Δ*cipC* mutant and wild type strain of *A. carbonarius* (W04-40) inoculated in CYA plates. Error bars indicate standard errors. Letters indicate homogeneous groups (ANOVA, $p < 0.05$).

Figure 5. Relative expression of *sod, prx* and *cipC* in *A. carbonarius* W04-40 grown in the presence of different concentrations of hydrogen peroxide with respect to expression level in the same medium without hydrogen peroxide. Error bars indicate standard errors.

Table legends

Table 1. Gene-specific primers used for PCR amplification and sequencing with the Universal Genome Walker kit and primers used for analysis of gene expression.

Table 2. Potential binding sites to regulatory elements predicted by TESS (http://www.cbil.upenn.edu/cgi-bin/tess) within the promoter of *cipC* (1550 bp).

Table 3. Estimation of the number of T-DNA copies that have been integrated in the genome of the mutants.

Tables

Table 1. Gene-specific primers used for PCR amplification and sequencing with the Universal Genome Walker kit and primers used for analysis of gene expression.

Oligo	Oligo sequence (5'-3')	T melting (ºC)
CIPC.G	GAGCACCAGGCCAAGAACG	66.6
CIPC.H	TGCCTTCATCGACCGTGAG	65.9
CIPC.I	TCTTGGCCTGGTGCTCCTC	66.3
CIPC.J	ACCGCCGATGAACTCGTG	65.8
CIPC.L	CCCGATCGCAGTTTATAGATCG	64.9
CIPC.M	GGCAGTAGTACTCACCGAACCAAC	65.3
CIPC.N	GGATGAGGGAGAAGAAGGGGATAG	66.4
CIPC.O	GCATCTGTCATTGTGCGAATACTC	65.2
CIPC.P	GGATGTTATGTAAGGTTCTGTGTGAG	62.2
CIPC.Q	GATGTGCATCCTGGAGATACAGC	65.3
CIPC.R	TGACATGGCCTTGGCTCTC	64.9
CIPC.S	CCCGACTATCCTTCGCCTACTG	64.9
AP1	GTAATACGACTCACTATAGGGC	59
AP2	ACTATAGGGCACGCGTGGT	71
SODf	CCCGGAACTGACCCTATGC	59.3
SODr	AGGGCTTGAGGGCAATCTG	59.4
PRXf	TCCTTCTTGAGGTTGGTGAAGC	59.6
PRXr	CTCAGAAGAAGTTCGGCGATG	57.8
CIPC.A2	AGGAGCACAAGGCTAAGTTCACC	61.8
CIPC.B2	GCTCACGGTCGACGAAGTC	59.6
S18f	GCAAATTACCCAATCCCGACAC	59.1
S18r	GAATTACCGCGGCTGCTG	58.4

Table 2. Potential binding sites to regulatory elements predicted by TESS (http://www.cbil.upenn.edu/cgi-bin/tess) within the promoter of *cipC* (1550 bp).

Factor	Beginning	Sequence	Functional Features
ABF-2	54	CAAGTTGA	Recognizes divergent sequences (Qasba *et al.*, 1992)
	87	AACCCTGA	
	375	AAAGTTCA	
	455	TACCTGGA	
	481	GACGTGGA	
AP-1	293	TTGGTCA	Involved in response to oxidative stress/oxygen detoxification and metal resistance (Murray *et al.*, 2011)
	753	TGACTCC	
	837	GGAGTCA	
	959	TGAGACA	
BUF	163	AGTGGCAG	Activator or repressor; Involved in replication, ss-DNA-binding protein (Luche *et al.*, 1993)
CP1	125	ATCCCGTG	Required for optimal chromosome segregation and methionine prototrophy (O'Connell *et al.*, 1995)
	286	CCAAT	
	1148	CCAAT	
	1258	CCAAT	
ACE1	36	TCATTTGGTC	Activates copper detoxification and storage genes when copper is in excess (Wegner *et al.*, 2011).
	234	TATTTTCCGC	
	803	GCTTTCCGTG	
	864	TCAGTCCCGG	
HSF	665	GAAGTGTC	Activator, mediates expression of heat-shock genes, whose products maintain protein homeostasis under normal physiological conditions, as well as under conditions of stress (Sakurai and Enoki, 2010)
MCBF	546	ACGCGT	Binds to the MluI cell cycle box of DNA replication genes; may be involved in cell cycle regulation (Raithatha and Stuart, 2005)
	1294	CATACCT	
	1516	CATTCCA	
MIG1	247	GTGGGG	Homologous to CreA/CRE-1, which encodes a carbon catabolite repressor (Ronne, 1995)
	256	GTGGGG	
MSN4	92	TGACGT	Play a major role in the general stress response program by transcribing hundreds of genes following exposure to diverse stress conditions (Sadeh *et al.*, 2011)
NBF	517	ATGGGAA	Regulates phospholipids biosynthetic genes (Lopes and Henry, 1991)
PHO4	240	CCGCGTGG	Controls the induction of the phosphate-responsive gene expression program (Oshima, 1997)
	546	ACGCGTGG	
PUT3	569	GAAGCCGA	Positive activator of the proline utilization pathway (Des-Etages *et al.*, 1996)
	1029	AAAGCCAT	
	1144	GATACCAA	
	1177	AGAGCCAA	
REB1	841	TCACCCT	Involved in termination of rRNA transcription and regulates G1 phase under nitrogen starvation (Rodríguez-Sánchez *et al.*, 2011)
SEF1	285	CCCAATAA	Positive regulator of iron acquisition (Homann *et al.*, 2009).
SKO1	61	AGGACTTA	Represses the yeast-to-hypha transition and regulates the oxidative stress response in *Candida albicans*. (Alonso-Monge *et al.*, 2010)
	752	ATGACTCC	

STE12	913	TGAAAC	Regulates fungal invasive growth (Rispail and Di Pietro, 2010)
USF	127	CCCGTGC	Upstream stimulatory factor activates GATA5 gene transcription by specially binding to the E-box motif of GATA5 promoter (Chen *et al.*, 2012)
	202	CACATCC	
	710	CTCATGC	
	814	CACCTGC	
ADR1	188	TGGGGC	Activator, inactivated by phosphorylation while DNA-binding is retained; positive regulator of peroxisomal protein genes (Simon *et al.*, 1991)
	465	AGGGGT	
	836	TGGAGT	
	880	GGGAGG	
	938	AGGAGG	
	1223	TGGAGC	
	1322	GGGAGT	
	1350	CGGAGG	
	1398	CGGAGG	
GCN4	557	CAGTCA	Involved in stress and nitrogen starvation response (Ecker *et al.*, 2010; Rodrigues-Pousada *et al.*, 2010)
	692	TGACTG	
	750	CGATGACTCC	
	753	TGACTC	
	838	GAGTCA	
MATalpha 1-2	94	ACGTGTTGAC	Activates alpha-specific genes and responds to a-mating pheromone (Sengupta and Cochran, 1991)
	768	CGATGTGGCT	
	827	GCCTGTCAAT	
	910	TGATGAAACC	
NIT2	147	TATCGA	Activator of nitrogen-regulated genes (Fu and Marzluf, 1990). NIT2 can partially complement for lack of AREA in *A. nidulans* (Davis and Hynes, 1987)
	682	GAGATA	
	1373	TATCAT	
	1452	TATCTA	
RAP1	889	CANCCNNNCA	Repressor or activator, depending on context; Participates in heterochromatin boundary-element formation, chromatin opening, meiotic recombination hotspot activity (Morse, 2000)
	1193	CAGCCACTCA	
	1283	CATCCACCCA	
	1360	CANCCNNNCA	
	1496	CATCCACAAA	
GCR1	1241	AGCTTCCAC	Coactivator of RAP1; Stimulates cell growth by participating in nutrient-responsive gene expression on a global level (Barbara *et al.*, 2007)
abaA	1509	CCTTCTTCATTCCACACTT	Activator; necessary for spore differentiation, binding to developmentally regulated genes (Andrianopoulos and Timberlake, 1994)
	1516	CATTCC	
PacC	1267	CTCCGCCAAGAATCCCC	Activator and repressor; in response to alkaline ambient pH, PacC activates expression of alkaline-expressed genes and represses genes required for growth under acidic conditions (Tilburn *et al.*, 1995)
FACB	239	TCCNNNNNNNNNGGA	Transcriptional activator; Controls acetate induction of enzymes specific for acetate utilization as well as glyoxalate bypass enzymes (Todd *et al.*, 1998)
	311	GCANNNNNNNNNNTGC	
	733	GCANNNNNNNNNCGC	
	757	TCCNNNNNNNNCGA	
	868	TCCNNNNNNNNNNGGA	
	1015	TCCNNNNNNNNNGGA	
	1386	TCCNNNNNNNNNNGGA	

UAY	564	CGCNNNNNNCCG	Transcriptional activator; mediates the induction af a number of unlinked genes involved in purine utilization in *A. nidulans* (Suarez *et al.*, 1995).
	1003	CGCNNNNNNCCG	

Table 3. Estimation of the number of T-DNA copies that have been integrated in the genome of the mutants.

Efficiency		**Cp *cipC***			**Cp *pyC***			**Copy number**	
cipC	***pyC***	**Wild type**	***ΔcipC***	**Ectopic**	**Wild type**	***ΔcipC***	**Ectopic**	***ΔcipC***	**Ectopic**
2.007	2.047	21.51±0.21	21.45±0.15	19.56±0.24	19.55±0.07	19.41±0.16	19.63±0.18	0.945	4.08

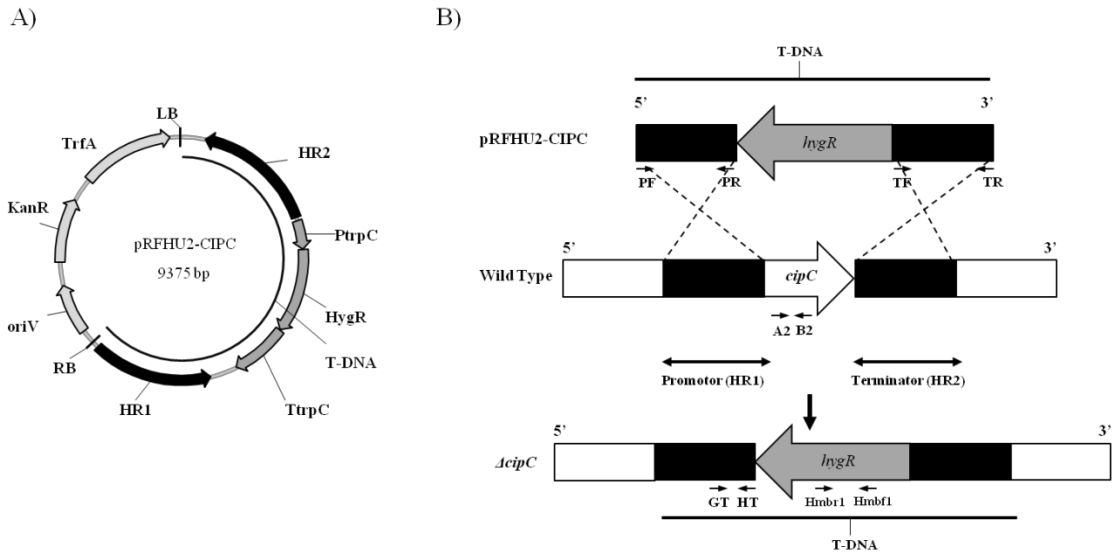

Figure 1. A) Physical map of plasmid pRFHU2-CIPC. LB= Left Border, HR2 = Homologous flanking region 2, pTrpC = Tryptophan promoter from *Aspergillus nidulans*, HygR = hygromycin phosphotransferase, TtrpC = Tryptophan terminator from *A. nidulans*, HR1 = Homologous flanking region 1, RB=Right Border, oriV = origin of replication in *E. coli*, KanR = kanamycin resistance, TrfA = replication initiation gene (broad-host-range). B) Diagram of the *cipC* replacement with the *hygR* selectable marker from pRFHU2-CIPC by homologous recombination to generate the Δ*cipC* mutant.

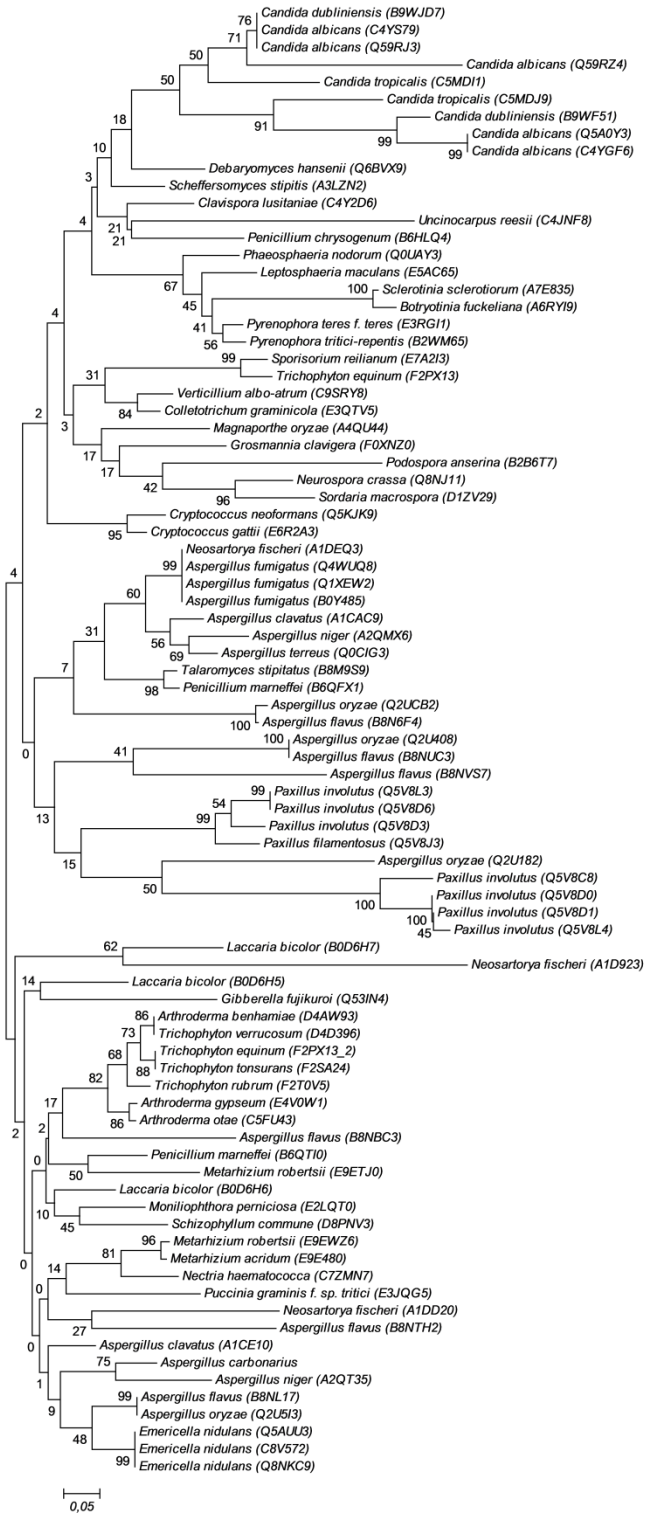

Figure 2. Phylogenetic tree derived from the alignment of 85 proteins that contain the PFAM-DUF3759 domain. The tree was constructed using neighbor joining. The numbers at the nodes give bootstrap values (%) of 1000 iterations. The proteins are designated by their TrEMBL ID number and the species name. Down left: scale of the phylogenetic distances.

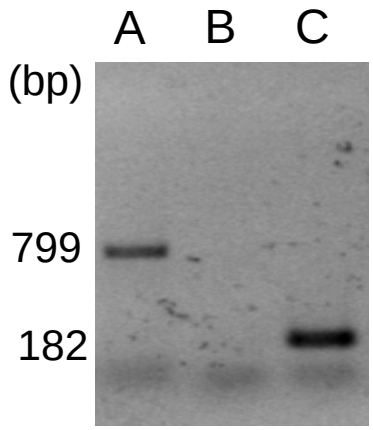

Figure 3. PCR amplification of the hygromycin resistant cassette in the Δ*cipC* knockout mutant with primers Hmbr1 and Hmbf1 (lane A) and amplification of *cipC* gene with primers CIPC.A2 and CIPC.B2 in the Δ*cipC* knockout mutant and the wild type strain (lane B and C respectively).

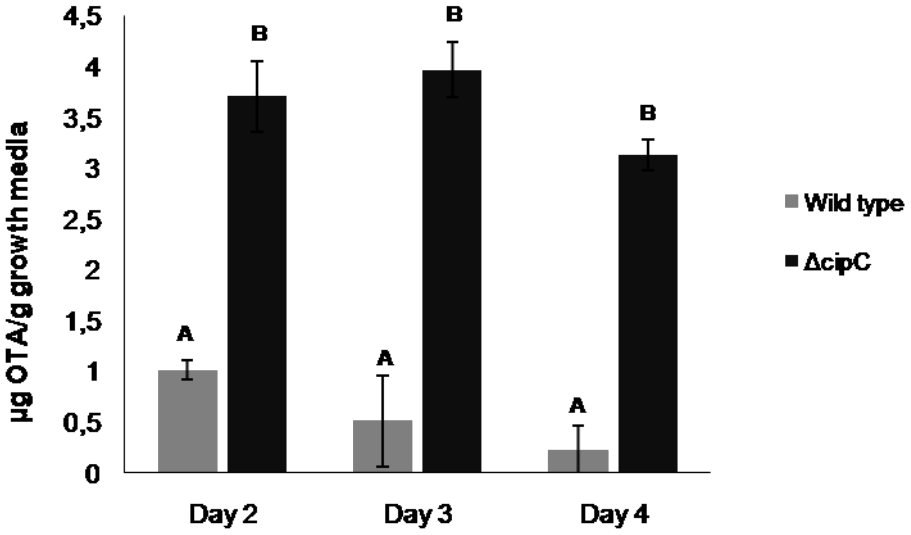

Figure 4. OTA production in the Δ*cipC* mutant and wild type strain of *A. carbonarius* (W04-40) inoculated in CYA plates. Error bars indicate standard errors. Letters indicate homogeneous groups (ANOVA, $p < 0.05$).

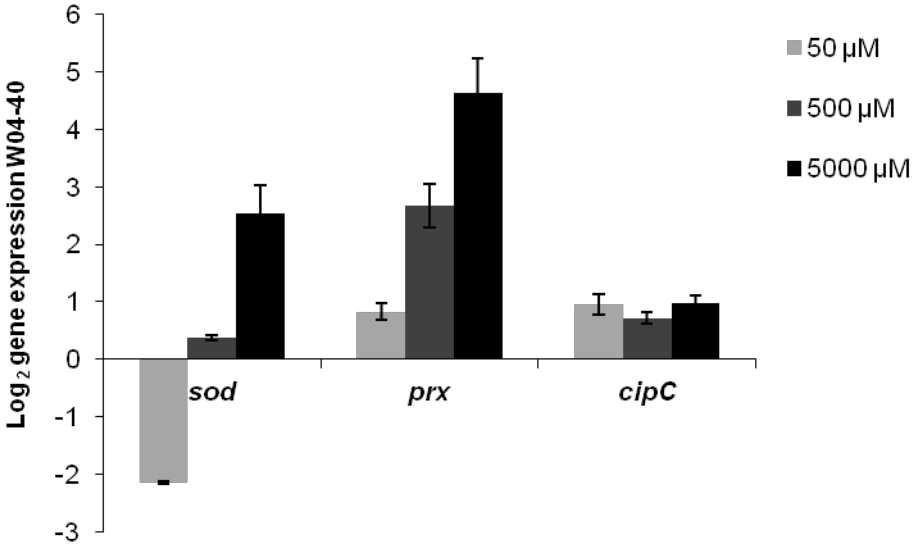

Figure 5. Relative expression of *sod, prx* and *cipC* in *A. carbonarius* W04-40 grown in the presence of different concentrations of hydrogen peroxide with respect to expression level in the same medium without hydrogen peroxide. Error bars indicate standard errors.