

Identification of a Splicing Coactivator Gene that Affects the Production of Ochratoxin A in *Aspergillus carbonarius*

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ABSTRACT

Ochratoxin A is a mycotoxin produced by some fungi species. Among them, Aspergillus carbonarius is considered a powerful producer. Genes involved in the ochratoxin A biosynthesis pathway have been identified in some producer species. However, there are few studies that purpose to identify these genes in A. carbonarius. The use of insertion mutants to identify genes associated with certain properties has been increased in the literature. In this work, the region of T-DNA integration was investigated in one A. carbonarius ochratoxin-defective mutant previously obtained by Agrobacterium tumefaciens-mediated transformation, in order to find an association between interrupted gene and the biosynthesis of ochratoxin A. The integration occurred in a gene that possibly encodes a splicing coactivator protein. The analysis of the relative expression of the splicing coactivator gene from A. carbonarius wild type strain in four different media showed high correlation between the transcript levels and the ochratoxin A production.

Key words: ochratoxigenic fungi, mycotoxin, real time PCR, splicing coactivator protein

INTRODUCTION

Ochratoxin A (OTA) is a toxin produced by some fungal species, known to have nephrotoxic effects and carcinogenic potential. This mycotoxin has been found in grapes and grape products such as juice, wine, and dried vine fruit (Zimmerli and Dick, 1996; Macdonald et al., 1999; Majerus et al., 2000; Da Rocha Rosa et al., 2002; Valero et al., 2005). Due to the potential for human and animal health hazard, the European Union (EU) has

introduced maximum limits for OTA in dried vine fruits ($10 \mu\text{g kg}^{-1}$) and wine ($2 \mu\text{g kg}^{-1}$). OTA has also been found in market samples of roasted coffee (Fujii et al., 2007). By the same token, the EU (European Commission, 2005) established limit of OTA in roasted ($5 \mu\text{g kg}^{-1}$) and soluble ($10 \mu\text{g kg}^{-1}$) coffees. The results of several years of investigation to recognize whose fungi were responsible for the OTA production in coffee and grapes showed that *Aspergillus carbonarius* is one

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of the most important species (Taniwaki et al., 2003).

OTA is a polyketide-derived secondary metabolite consisting of chlorinated isocoumarin derivative linked to L-phenylalanine (Moss, 1996). O'Callaghan and Dobson (2006a) reviewed the biosynthesis of this mycotoxin and results suggest it is a process consisting of four main steps: (1) biosynthesis of the pentaketide 7-methyl-mellein by a PKS (polyketide synthase); (2) oxidation by cytochrome P450 to produce the intermediate 7-carboxy-mellein; (3) chlorination, probably by a chloroperoxidase producing 7-carboxy-5-chloro-mellein, and (4) the condensation of this intermediary with phenylalanine by the OTA synthase, probably a non-ribosomal peptide synthase. Some structural genes were already identified as related to OTA biosynthesis in *Aspergillus ochraceus* and *Penicillium* species (O'Callaghan et al., 2003; Geisen et al., 2004; Karolewicz and Geisen, 2005; Atoui et al., 2006; Bogs et al., 2006). More recently, a structural gene (a *pks* gene) possibly involved in the OTA biosynthesis in *A. carbonarius* was reported (Gallo et al., 2009). However, as far as we know, genes involved in the regulation of the structural ochratoxin gene were not described until now for none of the ochratoxigenic species.

The T-DNA insertional mutagenesis approach is a powerful tool for detecting genes involved in a particular function. This strategy is based on the capability of *Agrobacterium tumefaciens* to transform host genomes by transferring its T-DNA to the host nuclei. The exogenous sequence is able to insert itself at random within chromosomes (Azpiroz-Leehan and Feldmann, 1997), and therefore can be used as a mutagen to create loss-of-function mutations in several organisms. The T-DNA not only disrupts the gene where it is inserted, but also acts as a marker for subsequently identifying the mutation. This approach has been used for cloning genes, promoters, and regulatory sequences (Radhamony et al., 2005). Bundock et al. (1995) firstly described the protocols for the transference of T-DNA into fungi cells. Only recently, the *Agrobacterium*-mediated transformation (AMT) protocol was adapted to transform *A. carbonarius* and used for generating insertional mutants (Morioka et al., 2006; Lunardi et al., 2006).

In this contribution, we verified the integration of T-DNA in one *A. carbonarius* ochratoxin-defective mutant obtained by AMT, and identified

a gene which was probably involved in the regulation of OTA biosynthesis.

MATERIALS AND METHODS

Strains

Aspergillus carbonarius wild type (ITAL187) used in the present study was obtained from coffee beans and kindly provided by Taniwaki et al. (2003). The mutant T188 was obtained by using *Agrobacterium*-mediated transformation approach by Lunardi et al. (2006). This mutant produces significantly lower amounts of OTA than the wild type strain after growing for four days on YES medium.

DNA and RNA extraction

Genomic DNA of *A. carbonarius* T188 mutant and wild type strain ITAL187 was extracted according to Azevedo et al. (2000) and quantified by a fluorimetric method (Dyna Quant, Pharmacia). Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions.

Southern blot analysis

DNA digestion was performed using *Sst*I that cuts the expression cassette once, outside of the *hph* gene. Standard procedures described in Sambrook and Russell (2001) was used for restriction endonuclease digestion, agarose-electrophoresis, and transference onto nylon membranes. Approximately 5 µg of digested genomic DNA were submitted to electrophoresis in each track. As a probe, a fragment of the *hph* gene (0.6 kb) was used. This fragment was obtained by PCR using the primer pair *hph*1 (5'-TTCGATGTAGGAGGGCGTGGAT) and *hph*2 (5'-CGCGTCTGCTGCTCCATACAAG). DNA probe labeling and hybridization were performed using a digoxigenin (DIG) hybridization system (Roche) according to the manufacturer's recommendations.

Identification of DNA sequences flanking T-DNA insertions

The T-DNA flanking region was extended by using an adaptation of the PCR-walking methodology as described by Cottage et al. (2001). Following the manufacturer's instructions, genomic DNA (100 ng) was partially digested for 3 h at 37°C with 5 U *Ssp*I, a blunt-cutting

restriction enzyme (New England Biolabs) in a final volume of 25 μ L. Adapters were prepared by mixing equimolar amounts of the oligonucleotides 5'-ACTCGATTCTCAACCCGAAAGTATAGATCCCA and 5'-TGGGATCTATACTT (with 3' amino group), which were ligated to the blunt-ended fragments for 3 h at 17°C. The reaction was performed by adding an equal volume of a ligation solution i.e. 30 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 1.0 mM ATP, 1 μ M adapters, and 1.2 units of T4 DNA ligase (New England Biolabs). The resulting products were diluted with ultra pure water and used as templates in the amplification reaction. A primer specific to the adaptor (5'-ACTCGATTCTCAACCCGAAAG) and nested primers specific to T-DNA (RB1, RB2, RB3 or LB1, LB2, LB3) (Combiar et al., 2003) were used to amplify the flanking region. The adapter sequence does not contain a primer-binding site, which is created only by the PCR extension from the walking-primer (Fig. 1). In order to obtain greater specificity, PCR products were submitted to two semi-nested-PCRs using internal primers i.e. RB2 and RB3 or LB2 and LB3 combined with the primer specific to the adapter. The final PCR products were resolved on agarose gels, purified (QIA quick[®] Kit, Qiagen) and cloned (TOPO TA Cloning for Sequencing Kit, Invitrogen). Cloned inserts were sequenced with DYEnamic[™] ET dye Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech, Inc.) for MegaBace 1000. By using the BLASTX and BLASTN algorithm (Altschul et al., 1997), the resulting sequences were searched against *Aspergillus niger* genome (<http://genome.jgi-psf.org/Aspni1/Aspni1.home.html>) and others (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Characterization of the T-DNA integration by PCR

Based on the two regions flanking the T-DNA (left and right borders), a primer-pair was designed to characterize the supposed deletion caused by the T-DNA integration. One primer sequence recognized the F-actin gene (5'-CGATGAAGGAGGATGCGAGT), and the other one recognized the splicing coactivator gene (5'-CACACCCTGATGAACCG). This primer pair

was used to amplify DNA from the T188 mutant and the wild type strain ITAL187. The PCR was performed in a total volume of 100 μ L containing DNA 5 ng, 1.5 mM MgCl₂, 0.2 mM dNTP, PCR buffer 1x (Invitrogen), 0.5 μ M primer, 1 U Taq DNA polymerase (Invitrogen). The amplification conditions used were 5 min 94°C, 30 cycles (45 s 94°C, 1 min 62°C, 1 min and 15 s 72°C) and 5 min 72°C. An aliquot of PCR products was separated by electrophoresis in 0.8% agarose gels and visualized by ethidium bromide staining. The remaining reaction mixture was purified and submitted to sequencing on MegaBace 1000 as described above.

Kinetic study of ochratoxin A production

For a kinetic study of the OTA production by the ITAL 187 strain, conidia were inoculated at a density of 10⁶ mL⁻¹ into 500-mL flasks containing 100 mL of permissive liquid medium (CY). The flasks were incubated in the dark at 25°C for up to 10 days. After 2, 4, 6, 8, and 10 days, the OTA content in culture broths was measured by high performance liquid chromatography (HPLC). OTA extraction and purification was done according to OCHRAPREF[®]-Quantitative Detection of Ochratoxin A (R-Biopharm Ltd) kit instruction. A LC-10VP HPLC system (Shimadzu) was used with fluorescence detection set at 333 nm excitation and 443 nm emissions. A Spherisorb ODS2 (5 μ m x 25 cm x 5 mm) column was employed. The mobile phase was an acetonitrile-water-acetic acid (51/47/2, v/v/v) mixture and the flow rate was 1 mL min⁻¹. An OTA standard (Sigma) was used for the construction of a calibration curve, peak area versus concentration (μ g⁻¹). The OTA concentration in sample extract was determined by the interpolation of peak area results from the calibration graph.

RT-real time PCR analysis

Firstly, the ITAL 187 strain was grown for sporulation at 25°C on potato dextrose agar for 5 days. Conidia (approximately 10⁶ mL⁻¹) were inoculated into 100 mL of four different media: CY, YES, PD, and EMP. The pH of the media was adjusted to 5.0 by adding acetic acid. After growing in the dark for four days, the RNA was extracted using Trizol reagent (Invitrogen).

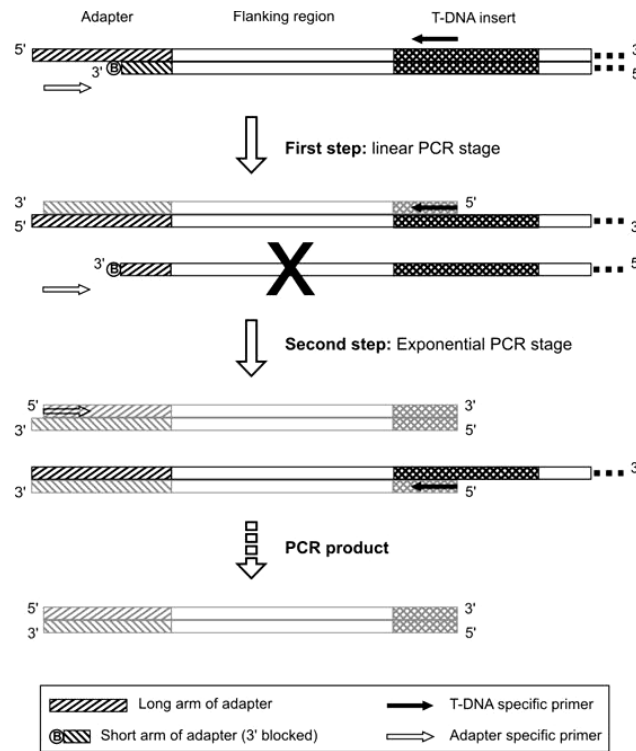


Figure 1 - Amplification scheme of PCR-walking method for identification of T-DNA flanking sequences. DNA was digested with a restriction enzyme and an adapter was ligated to the ends of the restriction fragments. This adapter has the 3' end of the short strand blocked by the presence of an amino group that prevents the extension with Taq DNA polymerase. The amplification of T-DNA flanking sequences involved a two-step PCR procedure. Although both T-DNA and adapter primers are in the PCR, a first step was carried out as a linear (asymmetric) PCR using only the specific primer to T-DNA (RB1 or LB1) because in this step the binding site to the adapter primer is not present. The adapter-binding site is generated after the extension from T-DNA primer that prevents amplification from adapter to adapter. In the second step, an exponential amplification was carried out using both T-DNA and adapter primers.

First-strand cDNA synthesis was performed with reverse transcriptase (RT M-MLV, Invitrogen) according to the manufacturer's protocol from 500 µg of total RNA. Real time PCRs were performed in a PTC 200 DNA Engine Cycler using a Chromo4 Detection System (MJ Research). The primers, 5'-CACCACCCCTGATGAACCG and 5'-GGGCTTCTTCGGTGTTCGGA, respectively, were used as forward and reverse for expression analysis of the splicing coactivator gene by using RT-real time PCR. Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen) was used as reaction mixture, adding 0.4 µM of each primer and 2 µL of template cDNA, in a final volume of 25 µL. The PCR thermal cycling conditions were as follows: an initial step at 95°C for 5 min, and 40 cycles at 95°C for 20 s, 62°C for 15 s, and 72°C for

20 s, followed by 95°C for 10 s and 40°C for 1 min. A melting curve analysis was persistently performed at the end of the reaction to check for primer-dimer artifacts and contamination. In addition, in all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination. All experiments were performed with two replications. The values were normalized against the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (G3PDH). The primers for amplification of cDNA for *A. carbonarius* G3PDH were g3pdh-bf (5'-TCGTCAACGGCAAGAAGATT) and g3pdh-br (5'-TAGCAAGGGGAGCAAGGCAGT), as described by O'Callaghan et al. (2006b). The analysis of relative expression results in real time

PCR was performed using the REST-MCS[®] software (www.gene-quantification.info) (Pfaffl et al., 2002).

RESULTS AND DISCUSSION

The mutant denoted T188 that produces significantly less OTA (1.6 %) than the wild type strain (100 %) (ITAL 187) was investigated by using Southern analysis to detect the T-DNA copy

number integrated into the genome. As shown in Figure 2, a single foreign DNA copy was found integrated in the T188 mutant genome, meaning that it is appropriate to look for associations between the gene disrupted by insertional mutagenesis and its phenotype. After PCR-based amplification of DNA fragments spanning insertion site borders and sequencing, the BLASTN and BLASTX algorithms was used to characterize the insertion of T-DNA within the host genome.



Figure 2 - Southern blot analysis of the *Aspergillus carbonarius* T188 mutant. Genomic DNA was digested with *Sst*I and electrophoresed on a 0.8% agarose gel; for hybridization, a 0.6 kb-fragment of the *hph* gene labeled with digoxigenin was used. M: molecular marker (Kb); A: Untransformed wild type strain (ITAL187); B: T188 mutant.

The nucleotide sequence flanking one side of the insertion site (left border, 1251 nt) showed significant similarity with the initial part of the locus An11g05460 from *A. niger* strain CBS 513.88 (E value = $2e^{-50}$), which is annotated as encoding an F-actin subunit alpha protein. The nucleotide sequence flanking the other side of the insertion site (right border, 228 nt) showed high similarity with locus An11g05450 from the same *A. niger* strain CBS 513.88 (E value = $5e^{-47}$), which is annotated as encoding a hypothetical protein (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). This same sequence was also searched for in the genome of the *A. niger* strain ATCC 1015 (<http://genome.jgi-psf.org/Aspni1/Aspni1.home.html>). Equally significant nucleotide similarities were found ($2e^{-39}$), but in this case the gene is annotated as

encoding an unnamed protein of 647 aa (ID 39109) with KOG description as a splicing coactivator SRm160/300, subunit SRm300, i.e. a protein involved in RNA processing and modification. When an exact joining of T-DNA and fungus genomic DNA takes place, sequences of the same gene are expected for both sides of the integrated T-DNA. Our results mean that a non-exact joining of T-DNA and fungus genomic DNA occurred during the integration event. Information on the pattern of T-DNA integration in fungi was very limited, with only a few T-DNA insertion sites having been characterized in several species transformed using *A. tumefaciens* (Bundock et al., 1995; Mullins et al., 2001; Leclerque et al., 2004; Choi et al., 2007; Li et al., 2007; Meng et al., 2007). The most significant contribution for understanding the mechanisms and characteristics

of T-DNA integration has been given from analysis of *Magnaporthe oryzae* transformants. Choi et al. (2007) conducted a broad analysis of T-DNA insertion sites in transformants of *M. oryzae* and showed that exact joining (without deletion, addition, and microhomology) of T-DNA and genomic DNA occurred in only 14.1% of the integration events. Non-exact joining of T-DNA and fungus genomic DNA occurred very frequently. The most frequent non-exact joining was that represented by a deletion of genomic DNA at the insertion site (78.3%). Deletions of genomic DNA ranged from 1 to 1950 bp, most of them being less than 35 bp (77.8%), while those larger than 100 bp comprised only 9.8%. In addition, small duplications (1-11 bp) and chromosomal translocations were also observed in 7.6% of integration events. Also studying *M. oryzae*, Li et al. (2007) showed that deletions due to T-DNA integration are found frequently in

genomic target sites. Although small deletions (1-80 bp in size) were more frequent, a deletion of 505 p in size was found in one transformant. Equally, Meng et al. (2007) examined the junctions between chromosomal DNA and the left and right T-DNA borders in several *M. oryzae* transformants for possible deletions surrounding the target site. Among 36 informative T-DNA insertions, 34 exhibited a genomic deletion at the target site. Five of them were deletions larger than 1 kb.

In order to confirm the deletion caused by T-DNA integration, we amplified the region between a portion of F-actin gene and a portion of splicing coactivator gene by using DNA from the wild type strain ITAL 187 and the mutant strain T188. As shown in Figure 3, the amplification products obtained were of 6040 bp and 970 bp from the insertional mutant (T188) and the wild type strain (ITAL 187), respectively.

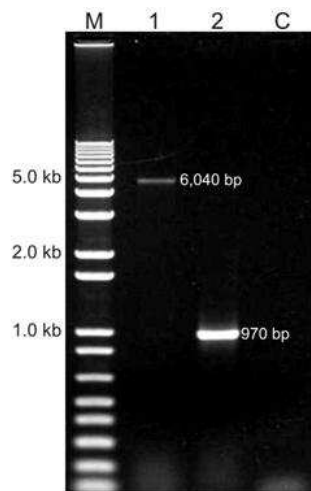


Figure 3 - Amplification of the region between splicing coactivator gene and F-actin gene in *Aspergillus carbonarius* wild type and mutant. M: 1kb molecular marker; Lane, 1: *A. carbonarius* T188 mutant; Lane, 2: *A. carbonarius* ITAL187 wild type; C: negative control.

The sequence analysis in fact showed that T-DNA integration into the mutant T188 provoked a deletion of 727 bp in length. The deletion includes

a final portion of the splicing coactivator gene and a non-coding region between this gene and the F-actin gene (Fig. 4).

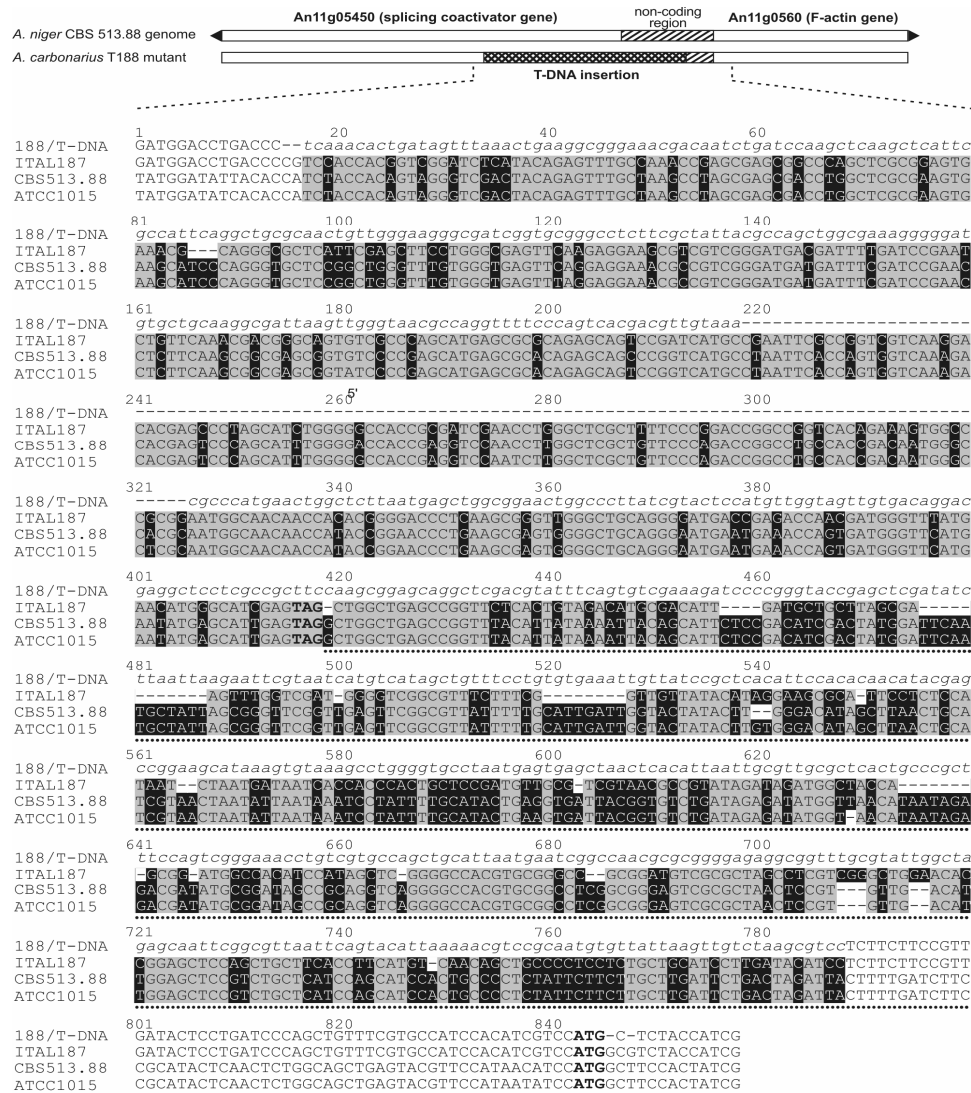


Figure 4 - Alignment of the nucleotide sequences of *Aspergillus niger* (CBS 513.88 and ATCC 1015 strains), *A. carbonarius* wild type (ITAL187), and the mutant (T188/T-DNA). The T-DNA integration into the T188 mutant caused a deletion of 727 bp which includes the end of the splicing coactivator gene and a non-coding region (shaded region, the conserved nucleotides are in gray). The non-coding region is represented by ••••. The F-actin gene was not affected by integration. The T-DNA sequence is in italic. The stop codon of splicing coactivator gene (TAG) and start codon of F-actin gene (ATG) in *A. niger* are in bold.

Because the mutant T188 has a deletion of the final portion of the splicing coactivator gene and it produces significantly smaller amounts of OTA than the wild type strain, we investigated the possible association between the amount of OTA and transcription levels of the gene being studied. Based on the kinetics of OTA production (Fig. 5),

the 4th day was chosen as the point for extracting total RNA and OTA quantification. The wild type strain (ITAL 187) was grown in four different media, CY, YES, PD, and EMP. The highest amount of OTA was detected in CY medium (272 ng mL⁻¹). After growing on both YES and PD media, the production of OTA was significantly

lower than that observed in CY, 135 and 118 ng mL⁻¹, respectively. As expected, production of OTA after growing on EMP medium was very low (4.7 ng mL⁻¹). The relative expression of the splicing coactivator gene in the four different media was analyzed and an association between the amount of OTA and the transcript levels was observed (Fig. 6). A high Pearson correlation coefficient was found (0.96), showing a supposed involvement of this gene in the production of OTA.

In general the genes involved in the biosynthesis of secondary metabolites are clustered, as already described for aflatoxin/sterigmatocystin (Brown et al., 1996), lovastatin (Kennedy et al., 1999), trichothecenes (Brown et al., 2001), and for gibberellins (Tudzynski and Holter, 1998). In most cases, transcriptional regulation is governed by a

pathway-specific DNA-binding protein encoded by a gene located within the cluster (Keller et al., 2005). Analysis of the genomic context of *A. niger* near the splicing coactivator gene revealed that it was not located within a cluster expected for OTA biosynthesis. No genes assumed to be necessary for OTA production, i.e. encoding polyketide synthases, chloroperoxidase, reductase, esterase, and dehydratase, were found near the splicing coactivator gene. However, some genes that are not located in the gene cluster have also been described as affecting the biosynthesis of some mycotoxins. A typical example is four *Fusarium verticillioides* genes that are located outside the fumonisin gene cluster but affect fumonisin biosynthesis (Shim and Woloshuk, 2001; Flaherty et al., 2003; Flaherty and Woloshuk, 2004; Bluhm and Woloshuk, 2006).

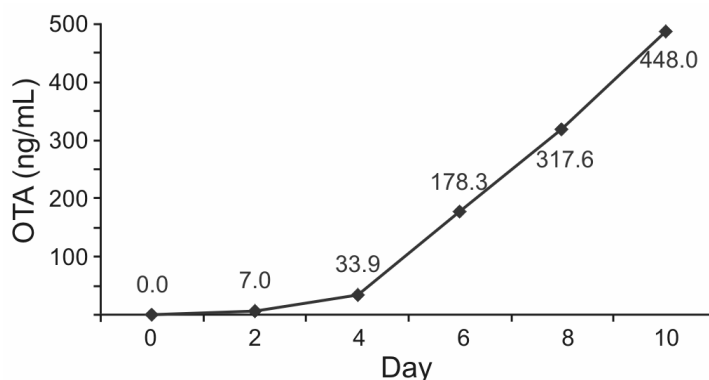


Figure 5 - Kinetic of ochratoxin A production by *Aspergillus carbonarius* ITAL187 wild type in CY medium.

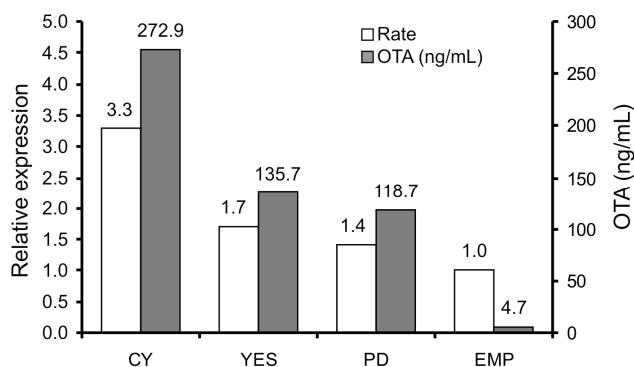


Figure 6 - Association between the transcription levels of the splicing coactivator gene and the amount of OTA produced by *Aspergillus carbonarius* wild type (ITAL187) in four different media, CY, YES, PD, and EMP.

An analysis of the genes within the *F. verticillioides* fumonisin cluster showed the presence of alternative splicing forms (ASFs) for eight genes, including *FUM1*, *FUM11*, and *FUM12*, that code for polyketide synthase, tricarboxylate carrier and cytochrome P450 monooxygenase, respectively (Brown et al., 2005), and the gene *FUM21* which has a Zn(II)₂Cys₆ DNA-binding motif, supposedly involved in the transcriptional regulation of the genes in the fumonisin cluster (Brown et al., 2007). The differential expression of the ASFs of *FUM* genes suggests that splicing regulation can play a role in fumonisin biosynthesis (Brown et al., 2005). Some proteins that participate in the splicing mechanism show serine- and arginine-rich domains, known as RS domains. These proteins play a dual role in the splicing process, affecting both constitutive and alternative splicing and they therefore have a key role in genic expression at the RNA processing level (Fu, 1995). In mammals, the SRm160/300 protein is related to spliceosome formation and plays a part in the recognition of exonic splicing enhancers (ESEs). Both subunits SRm300 and SRm160 have RS domains, but do not show RNA recognition motifs (RRMs) (Blencowe et al., 2000). The splicing coactivator gene, identified in this work, displayed similarities to gene An11g05450 of *A. niger* CBS 513.88, which amino acid-derived sequence has no RRM, but does have a serine-rich domain (annotation KEGG at <http://www.uniprot.org>).

In conclusion, although the splicing coactivator gene identified in this study is not found in an ochratoxin gene cluster, it is in fact associated with the production of OTA.

ACKNOWLEDGMENTS

Our study was supported (grants and fellowships) by the following Brazilian institutions: Fundação Araucária, Conselho Nacional de Pesquisa (CNPq), Financiadora de Estudos e Projetos (FINEP), and Coordenadoria de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES). Rede Integrada de Biotecnologia Aplicada ao Desenvolvimento Sustentável da Agricultura Familiar no contexto do Agro-negócio Paranaense (BIOAGROPAR) and Laboratório de Apoio à Pesquisa Agropecuária (LAPA).

RESUMO

A ocratoxina A é uma micotoxina frequentemente encontrada em uma grande variedade de produtos alimentares e apresenta efeitos nefrotóxicos e potencial carcinogênico para animais e humanos. É naturalmente produzida por algumas espécies fúngicas, como *Aspergillus carbonarius*, que é considerado um potente produtor. Apesar disso, o número de estudos que visam identificar genes que são essenciais para a biossíntese de ocratoxina em *A. carbonarius* é ainda reduzido. Um mutante de *A. carbonarius* com baixa produção de ocratoxina A previamente obtido por transformação mediada por *Agrobacterium tumefaciens* foi investigado com o objetivo de encontrar uma associação entre o gene interrompido e a biossíntese desta micotoxina. Os resultados mostraram a ocorrência de uma junção não exata entre o T-DNA e o DNA genômico do fungo durante o evento de integração. A integração do T-DNA no genoma do mutante T188 provocou deleção de 727 nucleotídeos. Esta deleção inclui uma porção final do gene coativador de splicing e uma região não-codificante entre este gene e o gene F-actina. A expressão relativa do gene coativador de *splicing* na linhagem selvagem cultivada em quatro diferentes meios mostrou associação entre a quantidade de ocratoxina A e os níveis dos transcritos.

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