



VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in *Aspergillus carbonarius*



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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, teratogenic and immunotoxic properties which represents a serious risk for human and animal health. *Aspergillus carbonarius* is considered the main OTA-producing species in grapes and products such as raisins, wine or juices, although it has also been isolated from coffee, cocoa and cereals. Till now not much information is available about regulatory mechanisms of OTA production by *A. carbonarius*. A better understanding of how environmental factors influence OTA production and which genes are involved in its regulation could help us design new control strategies. In this study, we have evaluated the role of VeA and LaeA transcriptional factors, which have been shown to regulate secondary metabolism in response to light in *A. carbonarius*. To this aim, *veA* and *laeA* genes were deleted in an ochratoxigenic *A. carbonarius* strain by targeted gene replacement using *Agrobacterium tumefaciens*-mediated transformation. Loss of *veA* and *laeA* in *A. carbonarius* yields to an organism with slight differences in vegetative growth but a strong reduction in conidial production. A drastic decrease of OTA production that ranged from 68.5 to 99.4% in ΔveA and $\Delta laeA$ null mutants was also observed, which was correlated with a downregulation of a nonribosomal peptide synthetase involved in OTA biosynthesis. These findings suggest that VeA and LaeA have an important role regulating conidiation and OTA biosynthesis in response to light in *A. carbonarius* in a similar way to other fungi where functions of VeA and LaeA have been previously described. This is the first report of a transcriptional factor governing the production of OTA by *A. carbonarius*.

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1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by some species of *Aspergillus* and *Penicillium* genera that may pose a serious health hazard due to its nephrotoxic, teratogenic and immunotoxic properties (Creppy, 1999; Kuiper-Goodman and Scott, 1989; Petzinger and Ziegler, 2000; Pfohl-Leschowicz and Manderville, 2007). OTA has been classified as a possible human renal carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1993). *Aspergillus carbonarius* is considered the main OTA producer species in grapes and products such as raisins (Battilani et al., 2006; Cabañes et al., 2002; Perrone et al., 2007). Additionally, *A. carbonarius* has been found in coffee (Joosten et al., 2001; Taniwaki et al., 2003), cocoa (Mounjouenpou et al., 2008), peanuts (Magnoli et al., 2007) and maize (Shah et al., 2010).

Frequently, genes responsible for mycotoxin biosynthesis are clustered on a chromosome and co-regulated by transcriptional factors, as has been described for sterigmatocystin in *Aspergillus nidulans* (Brown et al., 1996), aflatoxins in *Aspergillus parasiticus* (Yu et al., 2004), fumonisins in *Gibberella moniliformis* (Proctor et al., 2003; Seo et al., 2001) or tricothecenes in *Fusarium* (Brown et al., 2004). Till now not much information is available about the OTA biosynthetic

pathway in *A. carbonarius*, since only the implication of a nonribosomal peptide synthetase and a polyketide synthase has been reported (Gallo et al., 2009, 2012). Clusters often include a gene encoding a transcriptional factor that regulates the mycotoxin biosynthesis, such as aflR, an in-cluster pathway regulator which encodes a Zn(2)-Cys(6) that activates transcription by binding to DNA sequences in the promoters of aflatoxin and sterigmatocystin biosynthesis genes (Ehrlich et al., 1999; Fernandes et al., 1998). However, mycotoxin production is also responsive to environmental factors that could be regulated by transcription factors located outside the mycotoxin gene cluster. It has been reported that OTA production in *A. carbonarius* is influenced by carbon and nitrogen sources (Abbas et al., 2009; Ferreira and Pitout, 1969; Medina et al., 2008) and pH (Esteban et al., 2005; O'Callaghan et al., 2006). Carbon signaling may be mediated by the transcription factor CreA (Dowzer and Kelly, 1989), while nitrogen signaling could be regulated by AreA (Hynes, 1975) and pH signaling by PacC (Tilburn et al., 1995).

Recently, the heterotrimeric velvet complex VelB/VeA/LaeA that couples in *A. nidulans* secondary metabolism with fungal development, including asexual and sexual growth, in response to light was identified (Bayram et al., 2008a). VeA is a global regulator which is transported from cytoplasm to nucleus in response to illumination. VeA interacts with LaeA in the nucleus regulating production of secondary metabolites and with VelB inducing sexual development (Palmer et al., 2013). Studies of diverse filamentous fungi have identified several structural

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homologous of *veA* and *laeA*. These two global regulators modulate the sporulation capacity, sclerotia and mycotoxin production in *Aspergillus fumigatus*, *Aspergillus flavus*, *A. nidulans*, and *A. parasiticus* (Bok and Keller, 2004; Calvo et al., 2004; Duran et al., 2007; Kato et al., 2003; Krappmann et al., 2005). Therefore, null mutants of *veA* and *laeA* (ΔveA and $\Delta laeA$) have lower gliotoxin production in *A. fumigatus*, sterigmatocystin in *A. nidulans* as well as aflatoxin and cyclopiazonic acid in *A. flavus* and *A. parasiticus*. Similar results have been reported for other secondary metabolites such as cephalosporin C in *Acremonium chrysogenum* (Dreyer et al., 2007), penicillin in *Penicillium chrysogenum* (Hoff et al., 2010), deoxynivalenol in *Fusarium graminearum* (Jiang et al., 2011) and fumonisin in *Fusarium fujikuroi* (Wiemann et al., 2010). Additionally, velvet complex *VelB/VeA/LaeA* regulates virulence in the phytopathogenic fungi *Botrytis cinerea* and *Fusarium oxysporum* (López-Berges et al., 2013; Yang et al., 2013).

In this study the possible role of *veA* and *laeA* as regulators of the OTA biosynthesis pathway in *A. carbonarius* has been investigated. For that purpose we have deleted *veA* and *laeA* genes in the ochratoxigenic *A. carbonarius* UdL-TA 3.83 strain by targeted gene replacement using *Agrobacterium tumefaciens*-mediated transformation.

2. Materials and methods

2.1. Strains and growth conditions

The *A. carbonarius* ochratoxigenic strain used in this study was UdL-TA 3.83, previously isolated from Spanish grapes and deposited in the culture collection of the Food Technology Department of Lleida University. *A. tumefaciens* AGL-1 strain was kindly provided by L. Peña (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain). *A. carbonarius* was sub-cultured on Malt Extract Agar (MEA) plates in the dark at 28 °C for 6 days to enable production of conidia. The fungal strain was stored as conidial suspension at –80 °C with 40% glycerol.

2.2. Genomic DNA extraction

Cultures were grown for 2 days at 28 °C on 500 µL of Malt Extract broth (2% w/v malt extract, 0.1% w/v peptone, 2% w/v glucose). Mycelium was recovered after 10 min of centrifugation at 17,500 ×g and 300 µL of DNA extraction buffer (200 mM Tris–HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added. The mycelium suspension was vortexed with five 2.8 mm stainless steel beads (Precellys, Bertin Technologies, France) for 10 min. After centrifugation at 17,500 ×g for 10 min, 150 µL of 3 M sodium acetate (pH 5.2) were added to the supernatant. Then, the supernatant was stored at –20 °C for 10 min and centrifuged (17,500 ×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 min of incubation at room temperature the DNA suspension was centrifuged (17,500 ×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. Construction of *veA* and *laeA* deletion strains

The identification of *VeA* and *LaeA* was achieved by homology search of the *A. carbonarius* genome sequence produced by the US Department of Energy Joint Genome Institute (<http://www.jgi.doe.gov>) in collaboration with the user community. A Blastp algorithm with *VeA* amino acid sequence from *Aspergillus niger* and *A. flavus* (Accession numbers XP_001392627 and DQ296645.1) and *LaeA* from *A. nidulans* and *A. fumigatus* (Accession numbers AAQ95166 and EAL90797) as queries was performed.

To construct the *veA* and *laeA* gene replacement plasmids, 1.5 kb upstream and downstream fragments from the promoter and terminator regions were cloned into the plasmid vector pRF-HU2 (Frandsen et al.,

2008), a binary vector designed to be used with the USER friendly cloning technique (New England Biolabs, USA), as described previously (Crespo-Sempere et al., 2011). The specific primers used for amplifying the promoter and terminator regions (VA, VB, VC and VD for *veA* and LA, LB, LC and LD for *laeA*, Table 1, Fig. 1B) including vector-specific 9 bp long overhangs containing a single 2-deoxyuridine nucleoside in the 5' end, which ensured directionality in the cloning reaction. Upstream and downstream fragments were amplified by PCR from genomic DNA of *A. carbonarius* (UdL-TA 3.83) with DFS-Taq DNA Polymerase (Bioron, Germany). Cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 60 °C for 1.5 min and 72 °C for 3 min and a final elongation step at 72 °C for 10 min. Both DNA inserts and the digested vector were mixed together and treated with the USER (uracil-specific excision reagent) enzyme (New England Biolabs, USA) to obtain plasmids pRFHU2-VEA and pRFHU2-LAEA (Fig. 1A). An aliquot of the mixture was used directly in chemical transformation of *Escherichia coli* DH5α cells without prior ligation. Kanamycin resistant transformants were screened by PCR. Proper fusion was confirmed by DNA sequencing. Then, plasmids pRFHU2-VEA and pRFHU2-LAEA were introduced into chemically competent *A. tumefaciens* AGL-1 cells.

Transformation of *A. carbonarius* was done as described previously (Crespo-Sempere et al., 2011) using *A. tumefaciens* AGL-1 cells carrying the plasmids pRFHU2-VEA and pRFHU2-LAEA. Equal volumes of IMAS-induced bacterial culture (De Groot et al., 1998) and conidial suspension of *A. carbonarius* (10⁶ 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 Potato Dextrose Agar (PDA) 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. Hygromycin resistant colonies appeared after 3 to 4 days of incubation at 28 °C.

Disruption of *veA* and *laeA* was confirmed by PCR analyses of the transformants (Fig. 1B). The insertion of the selection marker was checked with the primer pair VE–VF and LE–LF for *veA* and *laeA* respectively (Table 1). Additionally, deletion of *veA* and *laeA* was corroborated with a primer pair designed within the sequence of the gene, VI–VJ and LI–LJ (Table 1). Real-time genomic PCR analyses were carried out in order to determinate the number of T-DNA molecules that have been

Table 1

List of primers used in this study.

Primer name	Primer sequence (5' → 3')
LA	GGTCTTAUACCGGCCGACCTTTGCTGC
LB	GGCATTAAUUCTATTGCGGTCCCATTTGCTCACTG
LC	GGCATTAAUCCGAAGCATCGAGGTCGTTCACTC
LD	GGGTTTAAUCCGAGCCACGGCTCTCTGGTC
LE	GGACATTATGCCATCACGGAC
LF	TGATTTACCTGTGCTGCGAGA
LG	CATAGGTGCTCCAGGCAGAACTC
LH	CTCATTGCGGTCCCATTTGCTCACT
LI	CACCTATACAACCTCCGAACCAC
IJ	GGTTCGGCCAACCGACGACGCTG
VA	GGTCTTAUCCGAGGTCCAAGATGGCAGGAC
VB	GGCATTAAUACATTGATCGCGGGGATCGATG
VC	GGACTTAAUACAGATTGACCGACTCCGAACGAC
VD	GGGTTTAAUUGGTCCAGCTCTTCGGCGTCATC
VE	CGCCAATGTACGCATCGATCC
VF	CTCCTTGCCTCAAGTCCGACA
VG	GAGTACACCGGCTGGTGGTTAGGA
VH	TCTTTCCATCGCGGGTATTGCGGT
VI	TCCCGTTCTCACAGGCGTA
VJ	GCTGTCTTGGTCTCTCGTA
NRP_F	CTCCACCATCTCTCCCGTTC
NRP_R	AATCCATGTCCTCACCATCCG
BT_HKF	CGCATGACGCTCACTTCAACGAG
BT_HKR	AGTTGTTACCAGACCGGACT

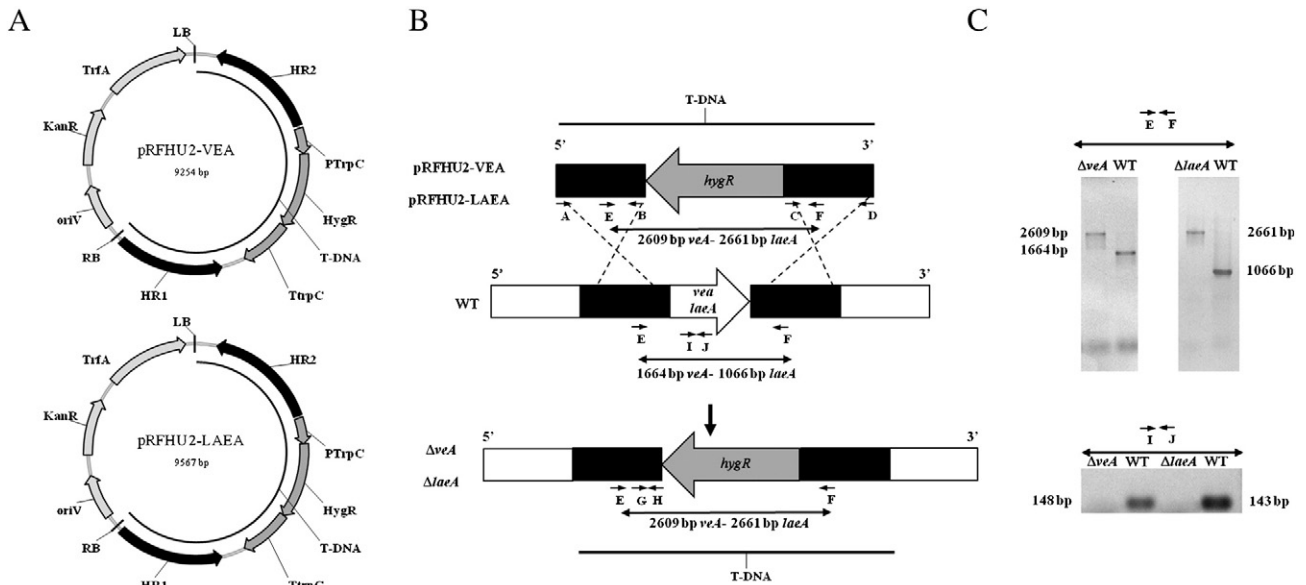


Fig. 1. Deletion of the *veA* and *laeA* genes in *A. carbonarius*. (A) Physical map of pRFHU2-VEA and pRFHU2-LAEA plasmids. LB = Left Border, HR2 = Homologous flanking region 2, PTrpC = Tryptophan promoter from *Aspergillus nidulans*, HygR = hygromycin phosphotransferase, TrpC = Tryptophan terminator 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 *veA* and *laeA* replacement with the *hygR* selectable marker from pRFHU2-VEA and pRFHU2-LAEA by homologous recombination to generate the ΔveA and $\Delta laeA$ null mutants. (C) Expected amplification band patterns with E–F and I–J pairs of primers for the wild-type, ΔveA and $\Delta laeA$ null mutants.

integrated in the genome of transformants, following basically the procedure established by Solomon et al. (2008). Two primer pairs, VG–VH and LG–LH for *veA* and *laeA* respectively (Fig. 1B, Table 1), were designed within the T-DNA in the promoter region of the target genes, close to the selection marker. qPCR reactions were performed in a final volume of 10 μ L, containing 1 \times of SsoAdvanced™ SYBR® Green Supermix (BIO-RAD, USA), 250 nM of each primer and 1 μ L of template DNA. All amplifications were performed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA). The standard protocol included one cycle at 98 °C for 2 min, followed by 40 cycles at 98 °C for 5 s and 56 °C for 30 s. Reactions were done in triplicate for each knockout mutant candidate, checking the PCR reaction quality by analyzing the dissociation and amplification curves. qPCR efficiency (E) for each pair of primers was calculated from the slopes of the standard curve (Lee et al., 2006). 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), which depends on E and the Crossing point (Cp) value of the 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 strain and transformant:

$$\text{copy number} = \frac{\left(E_{\text{target gene}}\right)^{\Delta C_{\text{p target gene (wild type-transformant)}}}}{\left(E_{\text{reference gene}}\right)^{\Delta C_{\text{p reference gene (wild type-transformant)}}}} \cdot$$

A nonribosomal peptide synthetase (Protein identification no. 132610) (*nrps*) gene was chosen as reference gene, using NRP_F and NRP_R primers (Table 1). All primers were designed using the OLIGO Primer Analysis Software V.7.

2.4. Phenotypic studies of ΔveA and $\Delta laeA$ disrupted mutants

For growth assessment, PDA plates were inoculated centrally with 5 μ L of conidia suspensions (10^6 conidia/mL) of the wild-type strain of *A. carbonarius* and the ΔveA and $\Delta laeA$ knockout strains. Cultures were incubated at 28 °C under two different conditions, white light (Mazda, 23W CFT/827, 1485 lm) and darkness. Two perpendicular diameters

of the growing colonies were measured daily over four days. Additionally, production of conidia and OTA was measured in the 4 day old cultures. To this aim, three agar plugs (5 mm in diameter) were removed from the inner, middle and outer part of the colonies and shaken for 30 s in 500 μ L of methanol to release conidia and extract OTA. An aliquot of 50 μ L was used for counting conidia using a Thoma counting chamber. For OTA quantification, the remaining 450 μ L of extracts were incubated for 60 min at room temperature. Then, vials were shaken and filtered (Millex-HV 0.45 μ m, 25 mm, Millipore Corporation, USA) into another vial. OTA extracts were stored at –20 °C until HPLC analysis. Separation, detection and quantification of OTA was performed by injecting 100 μ L of each extract into an HPLC system consisting of a Waters 265 Alliance Separations Module connected to a 2475 Waters Multi λ Fluorescence Detector, using a C18 column (5 μ m Waters Spherisorb, 4.6 \times 250 mm ODS2). For chromatographic separation of OTA, the mobile phase was acetonitrile: water: acetic acid, (57:41:2 v/v/v) under isocratic elution during 15 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. OTA standard was dissolved in methanol at a concentration of 25 mg/mL and stored at 4 °C in a sealed vial until use. The concentration in the stock solutions was checked by UV spectroscopy according to the AOAC Official Methods of Analysis, chapter 49 (Horwitz and Latimer, 2006). Working standard solutions (2, 1, 0.5, 0.1, 0.05, 0.01 and 0.005 μ g/mL) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the chromatographic system. OTA values were expressed per mm² of colony. The detection limit of the analysis was about 0.0005 ng OTA/mm², based on a signal-to-noise ratio of 3:1.

Assays were performed with independent biological triplicates and technical triplicates. 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 $p < 0.05$.

2.5. Gene expression analysis

PDA Petri dishes were inoculated with 100 μ L of a conidial suspension (10^6 conidia/mL) homogeneously spread and sub-cultured at 28 °C

under two different conditions, light and darkness. After 48 h, mycelium was collected, frozen in liquid nitrogen and stored at -80°C before nucleic acid extraction. RNA was extracted 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 (65°C) 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% (v/v) β -mercaptoethanol and 5 mL of Tris-equilibrated phenol. 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 $3900 \times g$ 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). RNA was precipitated during 3 h at -20°C by adding 3.3 mL of 12 M LiCl. After centrifugation at $27,200 \times g$ for 60 min, the pellet was washed with 500 μL of 70% ethanol. The resultant pellet was re-extracted with 250 μL of 3 M sodium acetate (pH 6.0) to remove residual polysaccharides. Then, RNA was washed again with 500 μL of 70% ethanol, 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 5 μg of total RNA using SuperScript III reverse transcription kit and an oligo(dT), according to the manufacturer's instructions (Invitrogen, USA).

Gene-specific primer sets, VI–VJ and LI–LJ, were designed with OLIGO Primer Analysis Software V.7 for gene expression analysis of *veA* and *laeA* respectively (Table 1). Additionally, the gene expression of the nonribosomal peptide synthetase (Protein identification no. 132610) (*nrps*) involved in OTA biosynthesis (Gallo et al., 2012) was also studied with primers NRP_F and NRP_R. Real-time RT-PCR reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA) to monitor cDNA amplification. The primer pair BT_HKF and BT_HKR (Table 1) was designed within the beta tubulin gene for using it as a reference gene. The standard protocol included one cycle at 98°C for 2 min, followed by 40 cycles at 98°C for 5 s and 58°C for 30 s. The expression of *veA*, *laeA* and *nrps* genes was calculated using the absolute quantification method but normalized with the absolute quantification of the beta tubulin gene expression in order to avoid inaccuracies due to variations in the retrotranscription efficiency among samples. Gene expression measures were derived from biological triplicates.

3. Results

3.1. Alignment of *A. carbonarius* VeA and LaeA homologs

Blast analysis against the *A. carbonarius* genome, provided by the Department of Energy Joint Genome Institute (<http://www.jgi.doe.gov>), allowed identification of VeA and LaeA homologs (Protein identification no. 202676 and 5941, respectively). VeA and LaeA amino acid sequences were used for Blastp search against NCBI database to analyze the similarity with identified VeA and LaeA from other fungal species. The Blastp search returned hits significant with e-values of 0.0 (Table 2), which indicate that VeA and LaeA are highly conserved proteins in fungi. The

fungal genome deposited in public databases with greatest similarity to *A. carbonarius* is the *A. niger* genome because of the phylogenetic closeness between both fungi. However, whereas *A. carbonarius* VeA shares 79% identity with *A. niger* VeA, *A. carbonarius* LaeA shares 79% identity with *A. parasiticus* LaeA. Nevertheless, *A. carbonarius* LaeA shares 76% identity with *A. niger* LaeA which is also a high degree of similarity. Additionally, the *A. carbonarius* VeA N-terminal region contains the conserved bipartite nuclear localization signal found and characterized in *A. nidulans* VeA (Stinnett et al., 2007), which putatively allows VeA to enter to the cell nucleus. On the contrary, no nuclear export signal (NES) can be predicted by in silico analysis for VeA (NES, <http://www.cbs.dtu.dk/services/NetNES/>).

3.2. Disruption of *veA* and *laeA* genes

To investigate whether *veA* and *laeA* are involved in OTA regulation, two null mutants were constructed (ΔveA and $\Delta laeA$). Gene replacement plasmids, pRFHU2-VEA and pRFHU2-LAEA, detailed in Fig. 1A, were obtained by a USER Friendly cloning system (Frandsen et al., 2008). Transformation of the fungus was mediated by *A. tumefaciens* (AGL-1), employing target gene replacement strategy. Fig. 1B shows a diagram of the procedure followed to delete *veA* and *laeA* genes. Cocultivation of *A. tumefaciens* cells carrying pRFHU2-VEA and pRFHU2-LAEA with the conidia of *A. carbonarius* led to the appearance of hygromycin B-resistant colonies approximately 4 days after transfer to the selective PDA plates. The T-DNA integration was confirmed in the hygromycin-resistant transformants by PCR analysis based on expected genomic patterns (Fig. 1B) with location specific primers, VE–VF for *veA* and LE–LF for *laeA*, detailed in Table 1. While PCR fragments of 1664 and 1066 bp were amplified using VE–VF and LE–LF in the wild-type strain, the PCR fragment in deletion mutants had 2609 and 2661 bp for *veA* and *laeA*, respectively (Fig. 1C). Additionally, no amplification could be found with primers VI–VJ and LI–LJ in the ΔveA and $\Delta laeA$ disrupted mutants. The gene replacement efficiency by homologous recombination obtained was 8.3% for *laeA* and 7.7% for *veA*. Additionally, it was found by qPCR that null mutants from *veA* and *laeA* genes only had one T-DNA copy integrated within its genome using VG–VH and LG–LH primers (Fig. 1B). Thus, no T-DNA ectopic insertions may affect to ΔveA and $\Delta laeA$ phenotypes.

3.3. Analysis of ΔveA and $\Delta laeA$ phenotypes

Some differences in growth and colony morphology were observed in ΔveA and $\Delta laeA$ transformants when compared with the wild-type strain on non-selective media (PDA plates). In the case of ΔveA and $\Delta laeA$ mutants, colonies consisted of a compact white basal felt covered by a layer of dark-brown to black conidial heads showing marked radial grooves that were not present in the wild-type strain (Fig. 2). Another interesting finding was the concomitant presence of exudates when $\Delta laeA$ was incubated in darkness. Except from the latter, no other differences were identified between strains incubated under dark and light.

Growth was also estimated as colony diameter observing small, but significant, differences between ΔveA and $\Delta laeA$ transformants and the wild-type strain (Fig. 3A and B). After two days of incubation, the

Table 2

Amino acid similarities between VeA and LaeA proteins from *A. carbonarius* and amino acid sequences deposited on the NCBI database using the Blastp algorithm.

Prot ID 202676 VeA <i>A. carbonarius</i>				Prot ID 5941 LaeA <i>A. carbonarius</i>			
Organism	Accession No.	E value	Identity	Organism	Accession No.	E value	Identity
<i>A. niger</i>	XP_001392627.1	0.0	79%	<i>A. parasiticus</i>	AAX68414.1	0.0	79%
<i>Aspergillus kawachii</i>	GAA88482.1	0.0	79%	<i>Aspergillus oryzae</i>	XP_001819665.2	0.0	79%
<i>Aspergillus clavatus</i>	XP_001269004.1	4e-150	65%	<i>A. nidulans</i>	AAQ95166.1	0.0	78%
<i>A. oryzae</i>	ABF61220.1	8e-146	66%	<i>A. kawachii</i>	GAA86709.1	0.0	76%
<i>A. flavus</i>	ADF30049.1	5e-145	66%	<i>A. niger</i>	XP_001389674.2	0.0	76%
<i>A. parasiticus</i>	AAS07022.1	5e-145	66%	<i>A. fumigatus</i>	XP_752835.1	0.0	74%

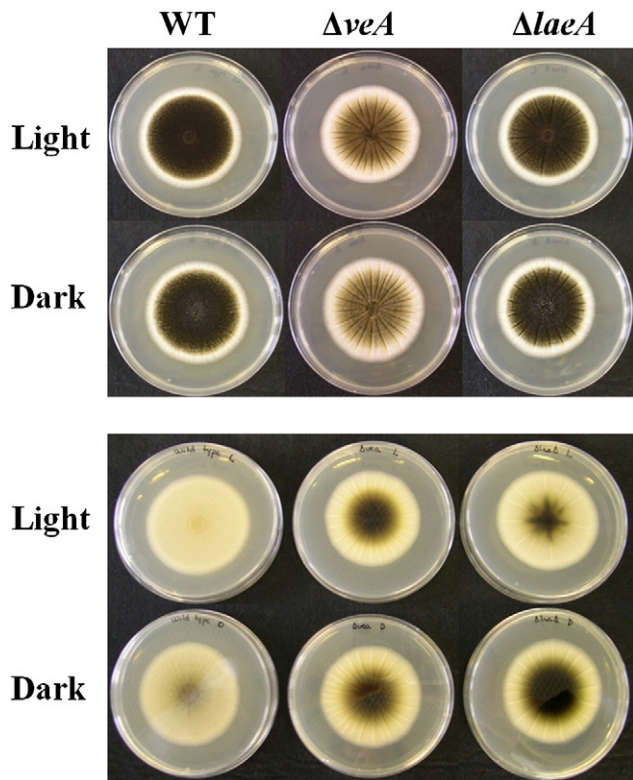


Fig. 2. Front (top) and reverse (bottom) colony view of the wild-type, ΔveA and $\Delta laeA$ *A. carbonarius* strains point inoculated on PDA plates incubated for 4 days at 28 °C under dark and light conditions.

growth of the ΔveA transformant was slightly slower compared to the wild-type strain, both in the light and in the dark. The 4-day colony diameter of the ΔveA null mutant was between 1.2 and 2.75 mm smaller than the wild-type colony diameter. In contrast, it was found that when $\Delta laeA$ transformant was incubated under light conditions it grew slightly faster than the wild-type strain until the third day of incubation, with differences around 1 mm per day.

Loss of *veA* and *laeA* genes resulted in a reduction of conidial production under light and dark conditions (Fig. 3C). When the strains were cultivated in the dark, the reduction was 87.5 and 38% for ΔveA and $\Delta laeA$, respectively, compared to the wild-type strain. In the light, the ΔveA transformant produced 89% less conidia than the wild-type strain, while a 37% reduction of conidial production was observed for the $\Delta laeA$ mutant. Interestingly, it was observed a remarkable positive effect of light on the conidiation in the wild-type strain. It was found that the wild-type strain produces 25% more conidia when it is incubated under the light rather than in darkness. Noteworthy is the absence of sclerotia for the wild-type, ΔveA and $\Delta laeA$ strains either in cultures under light or in darkness for 21 days.

The effect of *veA* and *laeA* deletion was also evaluated on OTA biosynthesis by *A. carbonarius*. Table 3 shows that OTA production was drastically reduced in ΔveA and $\Delta laeA$ transformants. In the dark the production of OTA in ΔveA and $\Delta laeA$ transformants decreased a 99.4 and 97% compared to the wild-type strain. Similarly, the reduction in the light compared to the wild-type strain was 89 and 68.5% for ΔveA and $\Delta laeA$ transformants, respectively. It is interesting to mention that when *A. carbonarius* wild-type strain is incubated under light, it produces around 90% less OTA compared to dark conditions.

To further elucidate the function of *veA* and *laeA* as regulators of OTA biosynthesis, the expression of a nonribosomal peptide synthetase involved in the OTA production pathway (Gallo et al., 2012) by quantitative real-time PCR (qRT-PCR) was assayed. As shown in Table 4, *veA* and *laeA* transcripts were completely absent in their respective deletion

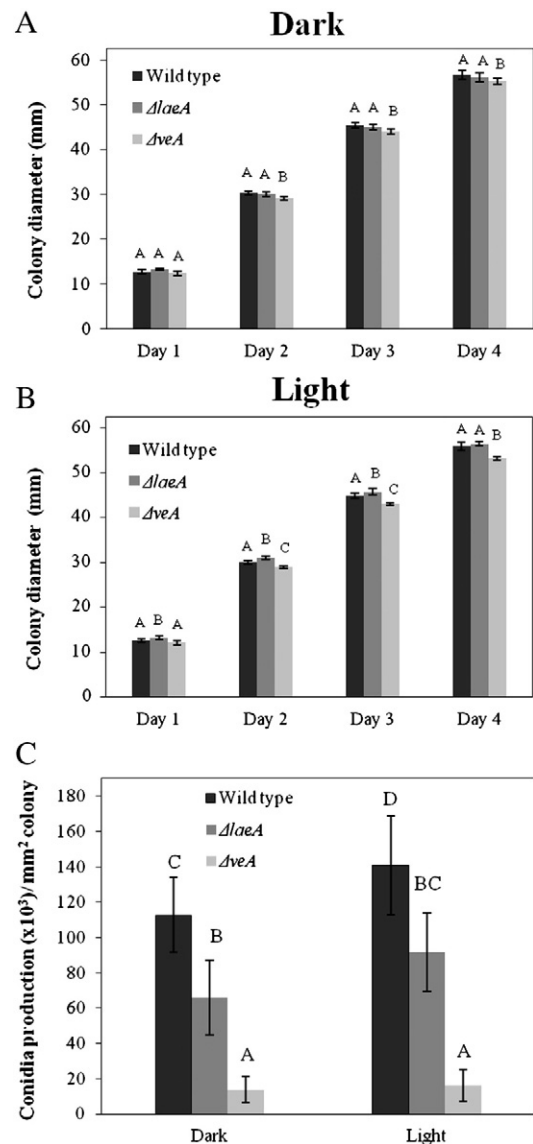


Fig. 3. Phenotypic analysis of ΔveA and $\Delta laeA$ *A. carbonarius* strains. Mycelial growth of ΔveA , $\Delta laeA$ and wild-type strains of *A. carbonarius* (UdL-TA 3.83) inoculated in PDA plates without selection marker and incubated for 4 days at 28 °C under dark (A) and light (B) conditions. (C) Conidia production per mm² of colony in the ΔveA , $\Delta laeA$ and wild-type strains inoculated in PDA plates without selection marker and incubated for 4 days at 28 °C under dark and light conditions. Error bars indicate standard errors. Letters indicate homogeneous groups within the same day (ANOVA, $p < 0.05$).

mutants, confirming the loss of these genes. It was also observed that transcription of *veA*, *laeA* and *nrrs* in the wild-type strain is relatively similar under light and dark conditions. In addition, the amount of *nrrs* transcripts in ΔveA and $\Delta laeA$ transformants was drastically reduced in all conditions tested. These data are correlated with the fact

Table 3

OTA production in ΔveA , $\Delta laeA$ and wild-type strains of *A. carbonarius* (UdL-TA 3.83) inoculated in PDA plates without selection marker and incubated for 4 days at 28 °C under dark and light conditions. Letters indicate homogeneous groups (ANOVA, $p < 0.05$).

	OTA production (ng/mm ² colony)	
	Dark	Light
Wild type	1.932 ± 0.470 (e)	0.178 ± 0.045 (d)
ΔveA	0.011 ± 0.003 (a) (99.4%) ^a	0.019 ± 0.007 (ab) (89%)
$\Delta laeA$	0.059 ± 0.025 (c) (97%)	0.056 ± 0.024 (bc) (68.5%)

^a Indicates the percentage of OTA reduction compared to the wild type.

Table 4
Absolute expression of *veA*, *laeA* and *nrps* genes normalized with beta tubulin gene expression in the ΔveA , $\Delta laeA$ and wild-type strains of *A. carbonarius* (UdL-TA 3.83) inoculated in PDA plates without selection marker and incubated for 48 h at 28 °C under dark and light conditions. Letters indicate homogeneous groups (ANOVA, $p < 0.05$).

	Dark			Light		
	Wild type	ΔveA	$\Delta laeA$	Wild type	ΔveA	$\Delta laeA$
<i>veA</i>	6.247 \pm 1.3988 (de)	0	0.858 \pm 0.2272 (ab)	5.962 \pm 2.4982 (de)	0	0.2585 \pm 0.0316 (ab)
<i>laeA</i>	4.430 \pm 1.7519 (cd)	2.323 \pm 1.9229 (abc)	0	5.988 \pm 3.7992 (de)	3.035 \pm 1.0821 (bc)	0
<i>nrps</i>	7.625 \pm 2.6309 (ef)	0.0008 \pm 0.0003 (a)	0.0008 \pm 0.0004 (a)	6.118 \pm 0.20 (de)	0.0015 \pm 0.0012 (a)	0.0747 \pm 0.024 (ab)

that the OTA production in ΔveA and $\Delta laeA$ transformants decreased by a percentage between the 68.5 and 99.4% compared to the wild-type strain, suggesting the involvement of *veA* and *laeA* in regulation of OTA production.

4. Discussion

Although *A. carbonarius* is an important OTA producing fungus, little is known about the transcriptional factors involved in OTA regulation. Understanding how external cues such as light may affect OTA biosynthesis and what the mechanisms of regulation are could help us to identify target sites for controlling OTA formation. Here, we have studied the possible role of *veA* and *laeA* homologs as regulators of OTA biosynthesis in *A. carbonarius*.

Loss of *veA* and *laeA* in *A. carbonarius* yields an organism with slight differences in vegetative growth but a strong reduction in conidial production. The small decrease in hyphal growth observed in ΔveA *A. carbonarius* was showed previously in *A. parasiticus* by Calvo et al. (2004), but with higher differences in colony diameter. In the light, the reduction of colony diameter in *A. parasiticus* was 25% while in the dark the reduction was approximately 50%. ΔveA in *F. oxysporum* also showed a decrease in hyphal growth (López-Berges et al., 2013). In contrast, a slight increase in vegetative growth, particularly in the dark, was found in ΔveA transformants in *A. flavus* (Duran et al., 2007). Additionally, $\Delta laeA$ transformants from both *A. fumigatus* and *F. oxysporum* had an increase in hyphal growth (Bok and Keller, 2004; López-Berges et al., 2013), as has been observed when *A. carbonarius* was incubated under light.

Several studies have reported that *LaeA* and *VeA* have a role in regulating sexual and asexual development in fungi (Bayram et al., 2008a; Calvo, 2008; Sarikaya Bayram et al., 2010). Kim et al. (2002) showed that *veA* was required for forming sexual structures such as Hülle cells and cleistothecia in *A. nidulans*. Large numbers of these sexual structures and much reduced number of conidial heads were formed when *veA* was over-expressed. So, *veA* in *A. nidulans* activates sexual development and inhibits asexual development. Similarly, in other fungal species the loss of *veA* produces an increase of conidial production, as is the case of *Neurospora crassa* and *F. graminearum* (Bayram et al., 2008b; Jiang et al., 2011). Contrariwise, *veA* regulates conidial production activating asexual reproduction in several fungal species such as *A. fumigatus*, *A. parasiticus*, *B. cinerea*, *F. oxysporum*, *P. chrysogenum*, and *F. fujikuroi* as well as in *A. carbonarius*, as abovementioned, where the loss of *veA* led to a reduction of almost 90% of conidia (Calvo et al., 2004; Hoff et al., 2010; Krappmann et al., 2005; López-Berges et al., 2013; Wiemann et al., 2010; Yang et al., 2013). Loss of *laeA* yields a reduction in the production of conidia which is variable between fungal species. Whereas the decrease of conidiation in *A. carbonarius* $\Delta laeA$ strain observed in our study was approximately 40%, $\Delta laeA$ strains of *A. nidulans* showed little difference in conidia production, $\Delta laeA$ transformants of *P. chrysogenum* showed drastically reduced conidiation and about a 30% reduction was observed for $\Delta laeA$ mutants of *F. fujikuroi* (Bok and Keller, 2004; Hoff et al., 2010; Wiemann et al., 2010).

Only one-third of the described species of *Aspergillus* have a known sexual stage, although genome analysis suggests that most of them should be able to reproduce sexually (Geiser, 2009; Krijgheld et al., 2012). *A. nidulans*, *Neosartorya fischeri*, *A. flavus*, *A. parasiticus*, *A. fumigatus*

and *Aspergillus nomius* are known to have sexual reproduction, but until now a sexual stage for *A. carbonarius* has not been described. In addition, it has been indicated that *veA* gene is required for sclerotial production in *A. parasiticus* and *A. flavus* (Calvo et al., 2004; Duran et al., 2007). *A. carbonarius* occasionally produce sclerotia (Samson et al., 2004) but this was not the case for the isolate used in this study. Thus, we could not test the effect of *veA* deletion on sclerotial production.

In this study, a reduction of OTA production in ΔveA and $\Delta laeA$ knock out deletion mutants was observed, which was correlated with a downregulation of a nonribosomal peptide synthetase involved in OTA biosynthesis. Interestingly, we found that light, as an external signal, affects ochratoxin A and conidial production in *A. carbonarius*. In the wild type, light causes a 90% reduction of OTA and a 25% increase of conidia. These findings might be explained according to the role described for *VeA* and *LaeA* for other fungal species. It has been reported that the complex formed by *VeB*, *VeA* and *LaeA* proteins connect light-response with developmental secondary metabolism (Bayram et al., 2008a). In light conditions *VeA* is found mainly in the cytoplasm because *VeA* transport to nucleus is inhibited by light (Stinnett et al., 2007). *VeA* contains nuclear localization signal motifs, which are recognized by the carrier protein *KapA*, transporting *VeA* to the nucleus under dark conditions. Once in the nucleus, *VeA* interacts with *LaeA* that controls secondary metabolite production by chromatin remodeling (Shwab et al., 2007). Therefore, it might be suggested that *VeA* transport to the nucleus could be also inhibited by light in *A. carbonarius*, not interacting with *LaeA*. This would lead to the 90% reduction of OTA and a 25% increase of conidiation mentioned above. Schmidt-Heydt et al. (2011) also found that OTA biosynthesis was reduced when ochratoxigenic fungi such as *A. carbonarius*, *A. niger*, *Penicillium verrucosum* and *Penicillium nordicum* were incubated under light conditions. Additionally, they reported that *A. carbonarius* produced high amounts of an ochratoxin β derivative under certain light conditions, shifting the mycotoxin production from OTA to the ochratoxin β derivative. As the enzyme *nrps* could be responsible for both OTA and ochratoxin β biosynthesis, this shift from OTA to ochratoxin β production would explain why in our study, the *nrps* showed the same expression level in the dark and the light, while OTA production was highly reduced in light.

Interestingly, it was also observed that transcription of *veA* and *laeA* in the *A. carbonarius* wild-type strain is relatively similar under light and dark conditions. These results were congruent with findings in *A. nidulans* which showed that expression levels of *veA* and *laeA* were almost constant over all stages and under both light and dark conditions (Bayram et al., 2008a; Kim et al., 2002). Stinnett et al. (2007) found that, in the dark, *VeA* is mainly in the cell nucleus while under light the level of *VeA* accumulation in the nucleus was lower compared with that observed in the dark. It could be proposed that to regulate OTA production in response to light, the important point is not the transcription level of *veA* or *laeA* but the transport to nucleus and the subcellular localization of *VeA*. Therefore, *veA* and *laeA* regulation process may possess multiple levels of regulation such as RNA splicing, translational and post-translational modifications. These layers of regulatory control could be responsible for the gene expression interdependence of *veA* and *laeA* observed in their null mutants as $\Delta laeA$ showed a reduced expression of *veA* and ΔveA a decreased *laeA* expression, although in the latter case the difference was not statistically significant.

Results obtained in this study revealed that *A. carbonarius* increases OTA production under dark conditions, light activates conidiation and the loss of *veA* and *laeA* leads to a drastic reduction of OTA and conidial production. Moreover, the N-terminal region of VeA in *A. carbonarius* contains a nuclear localization signal which putatively allows VeA to enter to the cell nucleus. These findings suggest that in *A. carbonarius* VeA and *LaeA* have an important role regulating conidiation and OTA biosynthesis in response to light, which is similar to other fungi where functions of VeA and *LaeA* have been described previously.

This is the first report of a transcriptional factor governing the production of OTA by *A. carbonarius*. VeA and *LaeA* could be used as target sites for designing new control strategies over OTA formation.

Acknowledgments

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References

- Abbas, A., Valez, H., Dobson, A.D.W., 2009. Analysis of the effect of nutritional factors on OTA and OTB biosynthesis and polyketide synthase gene expression in *Aspergillus ochraceus*. *International Journal of Food Microbiology* 135, 22–27.
- Battilani, P., Magan, N., Logrieco, A., 2006. European research on ochratoxin A in grapes and wine. *International Journal of Food Microbiology* 111 (Suppl. 1), S2–S4.
- Bayram, Ö., Krappmann, S., Ni, M., Bok, J.W., Helmstaedt, K., Valerius, O., Braus-Stromeyer, S., Kwon, N.-J., Keller, N.P., Yu, J.-H., Braus, G.H., 2008a. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* 320, 1504–1506.
- Bayram, Ö., Krappmann, S., Seiler, S., Vogt, N., Braus, G.H., 2008b. *Neurospora crassa* ve-1 affects asexual conidiation. *Fungal Genetics and Biology* 45, 127–138.
- Bok, J.W., Keller, N.P., 2004. *LaeA*, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryotic Cell* 3, 527–535.
- Brown, D.W., Yu, J.H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., Adams, T.H., Leonard, T.J., 1996. Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences* 93, 1418–1422.
- Brown, D.W., Dyer, R.B., McCormick, S.P., Kendra, D.F., Plattner, R.D., 2004. Functional demarcation of the *Fusarium* core trichothecene gene cluster. *Fungal Genetics and Biology* 41, 454–462.
- Cabañes, F.J., Accensi, F., Bragulat, M.R., Abarca, M.L., Castella, G., Minguez, S., Pons, A., 2002. What is the source of ochratoxin A in wine? *International Journal of Food Microbiology* 79, 213–215.
- Calvo, A.M., 2008. The VeA regulatory system and its role in morphological and chemical development in fungi. *Fungal Genetics and Biology* 45, 1053–1061.
- Calvo, A.M., Bok, J., Brooks, W., Keller, N.P., 2004. *veA* is required for toxin and sclerotial production in *Aspergillus parasiticus*. *Applied and Environmental Microbiology* 70, 4733–4739.
- Creppy, E.E., 1999. Human ochratoxicosis. *Journal of Toxicology: Toxin Reviews* 18, 273–293.
- Crespo-Sempere, A., López-Pérez, M., Martínez-Culebras, P.V., González-Candelas, L., 2011. Development of a green fluorescent tagged strain of *Aspergillus carbonarius* to monitor fungal colonization in grapes. *International Journal of Food Microbiology* 148, 135–140.
- De Groot, M.J.A., Bundock, P., Hooykaas, P.J.J., Beijersbergen, A.G.M., 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology* 16, 839–842.
- Dowzer, C.E.A., Kelly, J.M., 1989. Cloning of the *creA* gene from *Aspergillus nidulans*: a gene involved in carbon catabolite repression. *Current Genetics* 15, 457–459.
- Dreyer, J., Eichhorn, J., Friedlin, E., Kürnsteiner, H., Kück, U., 2007. A homologue of the *Aspergillus* velvet gene regulates both cephalosporin C biosynthesis and hyphal fragmentation in *Acremonium chrysogenum*. *Applied and Environmental Microbiology* 73, 3412–3422.
- Duran, R.M., Cary, J.W., Calvo, A.M., 2007. Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotial formation. *Applied Microbiology and Biotechnology* 73, 1158–1168.
- Ehrlich, K.C., Montalbano, B.G., Cary, J.W., 1999. Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. *Gene* 230, 249–257.
- Esteban, A., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2005. Influence of pH and incubation time on ochratoxin A production by *Aspergillus carbonarius* in culture media. *Journal of Food Protection* 68, 1435–1440.
- Fernandes, M., Keller, N.P., Adams, T.H., 1998. Sequence-specific binding by *Aspergillus nidulans* AfIR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Molecular Microbiology* 28, 1355–1365.
- Ferreira, N.P., Pitout, M.J., 1969. The biosynthesis of ochratoxin. *Journal of the South African Chemical Institute* 22, S1.
- Frandsen, R., Andersson, J., Kristensen, M., Giese, H., 2008. Efficient four fragment cloning for the construction of vectors for targeted gene replacement in filamentous fungi. *BMC Molecular Biology* 9, 70.
- Gallo, A., Perrone, G., Solfrizzo, M., Epifani, F., Abbas, A., Dobson, A.D.W., Mulè, G., 2009. Characterisation of a *pks* gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. *International Journal of Food Microbiology* 129, 8–15.
- Gallo, A., Bruno, K.S., Solfrizzo, M., Perrone, G., Mulè, G., Visconti, A., Baker, S.E., 2012. New insight into the ochratoxin A biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. *Applied and Environmental Microbiology* 78, 8208–8218.
- Geiser, D.M., 2009. Sexual structures in *Aspergillus*: morphology, importance and genomics. *Medical Mycology* 47, S21–S26.
- Hoff, B., Kamerewerd, J., Sigl, C., Mitterbauer, R., Zadra, I., Kürnsteiner, H., Kück, U., 2010. Two components of a velvet-like complex control hyphal morphogenesis, conidiophore development, and penicillin biosynthesis in *Penicillium chrysogenum*. *Eukaryotic Cell* 9, 1236–1250.
- Horwitz, W., Latimer, G.W., 2006. Official Methods of Analysis of AOAC International, Chap. 49, Natural Toxins, 18th ed. AOAC, Gaithersburg, Maryland.
- Hynes, M.J., 1975. Studies on the role of the *areA* gene in the regulation of nitrogen catabolism in *Aspergillus nidulans*. *Australian Journal of Biological Sciences* 28, 301–313.
- IARC, 1993. Ochratoxin A. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 56, 489–521.
- Jiang, J., Liu, X., Yin, Y., Ma, Z., 2011. Involvement of a velvet protein FgVeA in the regulation of asexual development, lipid and secondary metabolites and virulence in *Fusarium graminearum*. *PLoS One* 6, e28291.
- Joosten, H.M.L.J., Goetz, J., Pittet, A., Schellenberg, M., Bucheli, P., 2001. Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. *International Journal of Food Microbiology* 65, 39–44.
- Kato, N., Brooks, W., Calvo, A.M., 2003. The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by *veA*, a gene required for sexual development. *Eukaryotic Cell* 2, 1178–1186.
- Kim, H.-S., Han, K.-Y., Kim, K.-J., Han, D.-M., Jahng, K.-Y., Chae, K.-S., 2002. The *veA* gene activates sexual development in *Aspergillus nidulans*. *Fungal Genetics and Biology* 37, 72–80.
- Krappmann, S., Bayram, Ö., Braus, G.H., 2005. Deletion and allelic exchange of the *Aspergillus fumigatus* *veA* locus via a novel recyclable marker module. *Eukaryotic Cell* 4, 1298–1307.
- Krijgheld, P., Bleichrodt, R., van Veluw, G.J., Wang, F., Müller, W.H., Dijksterhuis, J., Wösten, H.A.B., 2012. Development in *Aspergillus*. *Studies in Mycology* 74, 1–29.
- Kuiper-Goodman, T., Scott, P.M., 1989. Risk assessment of the mycotoxin ochratoxin A. *Biomedical and Environmental Sciences* 2, 179–248.
- Lee, C., Kim, J., Shin, S.G., Hwang, S., 2006. Absolute and relative qPCR quantification of plasmid copy number in *Escherichia coli*. *Journal of Biotechnology* 123, 273–280.
- López-Berges, M.S., Hera, C., Sulyok, M., Schäfer, K., Capilla, J., Guarro, J., Di Pietro, A., 2013. The velvet complex governs mycotoxin production and virulence of *Fusarium oxysporum* on plant and mammalian hosts. *Molecular Microbiology* 87, 49–65.
- Magnoli, C., Astoreca, A., Ponsone, M.L., Fernández-Juri, M.G., Barberis, C., Dalcero, A.M., 2007. Ochratoxin A and *Aspergillus* section *Nigri* in peanut seeds at different months of storage in Córdoba, Argentina. *International Journal of Food Microbiology* 119, 213–218.
- Medina, Á., Mateo, E.M., Valle-Algarra, F.M., Mateo, F., Mateo, R., Jiménez, M., 2008. Influence of nitrogen and carbon sources on the production of ochratoxin A by ochratoxigenic strains of *Aspergillus* spp. isolated from grapes. *International Journal of Food Microbiology* 122, 93–99.
- Mounjouenpou, P., Gueule, D., Fontana-Tachon, A., Guyot, B., Tondje, P.R., Guiraud, J.-P., 2008. Filamentous fungi producing ochratoxin A during cocoa processing in Cameroon. *International Journal of Food Microbiology* 121, 234–241.
- O'Callaghan, J., Stapleton, P.C., Dobson, A.D.W., 2006. Ochratoxin A biosynthetic genes in *Aspergillus ochraceus* are differentially regulated by pH and nutritional stimuli. *Fungal Genetics and Biology* 43, 213–221.
- Palmer, J.M., Theisen, J.M., Duran, R.M., Grayburn, W.S., Calvo, A.M., Keller, N.P., 2013. Secondary metabolism and development is mediated by LlmF control of VeA subcellular localization in *Aspergillus nidulans*. *PLoS Genetics* 9, e1003193.
- Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakarnchanakul, W., Samson, R.A., 2007. Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology* 59, 53–66.
- Petzinger, E., Ziegler, K., 2000. Ochratoxin A from a toxicological perspective. *Journal of Veterinary Pharmacology and Therapeutics* 23, 91–98.
- Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29, E45.
- Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E., 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genetics and Biology* 38, 237–249.
- Rasmussen, R., 2001. Quantification on the LightCycler instrument. In: Meuer, S., Wittwer, C., Nakagawara, K. (Eds.), *Rapid Cycle Real-time PCR: Methods and Applications*. Springer, Heidelberg, pp. 21–34.
- Sarikaya Bayram, Ö., Bayram, Ö., Valerius, O., Park, H.S., Irniger, S., Gerke, J., Ni, M., Han, K.-H., Yu, J.-H., Braus, G.H., 2010. *LaeA* control of velvet family regulatory proteins for light-dependent development and fungal cell-type specificity. *PLoS Genetics* 6, e1001226.
- Schmidt-Heydt, M., Rüfer, C., Raupp, F., Bruchmann, A., Perrone, G., Geisen, R., 2011. Influence of light on food relevant fungi with emphasis on ochratoxin producing species. *International Journal of Food Microbiology* 145, 229–237.
- Seo, J.-A., Proctor, R.H., Plattner, R.D., 2001. Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal Genetics and Biology* 34, 155–165.

- Shah, H.U., Simpson, T.J., Alam, S., Khattak, K.F., Perveen, S., 2010. Mould incidence and mycotoxin contamination in maize kernels from Swat Valley, North West Frontier Province of Pakistan. *Food and Chemical Toxicology* 48, 1111–1116.
- Shwab, E.K., Bok, J.W., Tribus, M., Galehr, J., Graessle, S., Keller, N.P., 2007. Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryotic Cell* 6, 1656–1664.
- Solomon, P.S., Ipcho, S.V.S., Hane, J.K., Tan, K.C., Oliver, R.P., 2008. A quantitative PCR approach to determine gene copy number. *Fungal Genetics Reports* 55, 5–8.
- Stinnett, S.M., Espeso, E.A., Coboño, L., Araújo-Bazán, L., Calvo, A.M., 2007. *Aspergillus nidulans* VeA subcellular localization is dependent on the importin α carrier and on light. *Molecular Microbiology* 63, 242–255.
- Taniwaki, M.H., Pitt, J.I., Teixeira, A.A., Iamanaka, B.T., 2003. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. *International Journal of Food Microbiology* 82, 173–179.
- Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., Penalva, M.A., Arst, H.N., 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *The EMBO Journal* 14, 779–790.
- Wiemann, P., Brown, D.W., Kleigrew, K., Bok, J.W., Keller, N.P., Humpf, H.-U., Tudzynski, B., 2010. FfVe1 and FfLae1, components of a velvet-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. *Molecular Microbiology* 77, 972–994.
- Yang, Q., Chen, Y., Ma, Z., 2013. Involvement of BcVeA and BcVeB in regulating conidiation, pigmentation and virulence in *Botrytis cinerea*. *Fungal Genetics and Biology* 50, 63–71.
- Yu, J., Bhatnagar, D., Cleveland, T.E., 2004. Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*. *FEBS Letters* 564, 126–130.