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Specific PCR primers for the detection of isolates of *Aspergillus carbonarius* producing ochratoxin A on grapevine

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Abstract

Aspergillus carbonarius is the main fungus responsible of ochratoxin A (OTA) production on grapes and wine. Fungal polyketide synthases are involved in OTA biosynthesis in *Aspergillus* species. The ketosynthase (KS) domain of a polyketide synthase (*pks*) gene of *A. carbonarius* was isolated, cloned and sequenced. The nucleotide sequence showed high similarity to the KS domain of other *Aspergillus* species. The sequence was used to design a new set of primers in order to identify isolates of *A. carbonarius* potential producers of OTA. The primers specifically amplified all the *A. carbonarius* strains tested and did not amplify other species of *Aspergillus* and *Penicillium*, normally found on grapes or involved in OTA biosynthesis. Further, the gene expression was related to the OTA production. Both the gene transcription and the mycotoxin were higher when *A. carbonarius* was grown on YES at 15°C and 30°C, whereas a low transcription and mycotoxin presence were observed when the fungus was grown on PDB at the same temperatures. No transcription and OTA production

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were observed when the fungus was grown on YES or PDB at 10°C. The primers will be useful for the detection of ochratoxigenic strains of *A. carbonarius* both in vineyard and during wine production.

Keywords: *Aspergillus carbonarius***;** HPLC; RT-PCR; ochratoxin A; polyketide synthase; vineyard.

Introduction

Ochratoxin A (OTA) has been reported as a dangerous nephrotoxic and carcinogenic mycotoxin, produced by various species of *Aspergillus* and *Penicillium* (Petzinger and Ziegler, 2000). The presence of OTA in wine was reported for the first time by Zimmerli and Dick (1996). Since then, several surveys on wines have shown a considerable level of contamination, with higher toxin concentrations and incidence rate in red wines originating from southern and warmer regions of Europe (Pietri et al., 2001) and lower contamination levels in northern and cooler regions (Spadaro et al. 2010a). OTA has also been detected in other grape-derived products (Battilani et al., 2004). OTA contamination on grapes takes place in the field and is produced mainly by black aspergilli. *Aspergillus carbonarius* is the most important OTA-producing species.

To prevent OTA contamination in grapes or in any other foodstuffs, it is necessary to have a rapid and specific method to detect OTA producing fungi in early stages of infection. Detection of these fungi, in the case of grapes, is particularly critical around harvest time, when contamination levels and OTA production are considered high. Usual identification and quantification methods of food-borne fungi require multiple steps. They are time-consuming and often, mycological expertise is necessary. Different molecular techniques can be used to identify the presence of *A. carbonarius* in vineyard, including sequencing of amplified products or PCR-RFLP (Gullino et al., 2008). Nowadays, many studies have been reported based on PCR methods to produce more specific, sensitive, and rapid detection of the target organism. One of the most important factors in the development of such methods is the reliability of the primer set designed and the targeted DNA sequence of interest organism.

Chemically, OTA is composed of an isocoumarin polyketide and the (modified) amino acid phenylalanine, which are linked via peptide bond. Fungal polyketide synthases (PKSs) have recently been demonstrated to be involved in OTA biosynthesis in both *Penicillium* and *Aspergillus* species

(O'Callaghan et al., 2003; Geisen et al., 2004). PKSs are multifunctional proteins with several domains encoded by one gene. The nucleotide sequences of the domains are homologous between different fungal species (Bingle et al., 1999). In the present study, we designed a set of specific primers, AcPKS-F1 (forward) and AcPKS-R1 (reverse) on the DNA sequence of a putative polyketide synthase (*pks*) gene of *A. carbonarius* strain AC06. This primer set was tested for primer specificity by PCR on different species of *Aspergillus* and *Penicillium*, normally present in vineyard or able to produce ochratoxin A. Further, reverse transcription studies were performed to confirm the involvement of the *pks* gene amplified during OTA biosynthesis. The *pks* gene was expressed only under OTA permissive conditions.

Materials and methods

Fungal strain and culture conditions

Aspergillus carbonarius strain AC06 (Agroinnova culture collection centre, University of Turin, Italy), an ochratoxin A producing strain isolated from vineyard in Liguria region of Italy, was used throughout this study. identified by using a polyphasic approach (Oliveri et al., 2008) based on ITS sequencing, calmodulin sequencing, and PCR-RFLP (Spadaro et al., 2009). The ITS sequences of the strain was deposited at the National Centre for Biotechnology Information (NCBI, GenBank, New York, USA) with the following accession number: GQ468224 (Spadaro et al. 2010b). AC06 was routinely grown at 25°C on potato dextrose agar (PDA) (Merck) for 5 days. Conidial suspensions ($1x10^6$ spores ml^{-1}) were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of YES (30g l^{-1} yeast extract, 200 g l^{-1} sucrose, 0.5 g l^{-1} magnesium sulphate) and PDB [(24 g l^{-1}) Merck, Darmstadt, Germany] and incubated at 15°C and 27°C for 9 days. After incubation, the mycelium was harvested by filtration, frozen in

liquid nitrogen and stored at -80°C for nucleic acid extraction. The filtered PDB and YES liquid broth were analysed for OTA content.

The other strains, tested for primer specificity, were several strains of *A. carbonarius* isolated from vineyards in Northern Italy, or purchased at the Centralbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. The strains purchased at the CBS were *A. carbonarius* CBS127.49, *Aspergillus ellipticus* CBS707.79, *Aspergillus tubingensis* CBS119556, *Aspergillus niger* CBS119557, *Aspergillus aculeatus* CBS116.80, *Aspergillus japonicus* CBS119560, *Aspergillus brasiliensis* CBS121619, *Aspergillus ochraceus* CBS132.52, *Penicillium nordicum* CBS110769, and *Penicillium verrucosum* CBS226.71.

DNA extraction, PCR, cloning and sequencing

The frozen fungal mycelium was ground to fine powder, and 100 mg powder were taken to extract the DNA, by using the NucleoSpin Plant DNA kit (Macherey-Nagel, Iuren, Germany) according to the manufacturer's instructions. PCR was performed on the genomic DNA to amplify the ketosynthase domain of polyketide synthase using the KS1 (GGRTCNCCIARYTGIGTICCIGTICCRTGIG) and KS2 (MGIGARGCIYTIGCIATGGAYCCICARCA) primers. The amplified product was excised from the gel and purified using the S.N.A.P. Gel purification system (Invitrogen Life Technologies, Corlsbad, CA, USA). The resulting DNA was cloned onto pCR2.1 TOPO plasmid vector (Invitrogen Life Technologies, USA). The recombinant plasmid was purified using the rapid plasmid system and the insert was sequenced by BMR genomics DNA sequencing service Centre (Padova, Italy) with the ABI PRISM 3730 XL DNA sequencer. Sequencing data were used to design the primer pair AcPKS-F1 (AGCATCTATGCTGGCCAATC) and AcPKS-R1 (AATGTACTCTCGCGGGCTAA) from two highly conserved regions. PCR conditions were standardised and tested for specificity on the DNA of different species of *Aspergillus* and *Penicillium*. PCR consisted of 100-200 ng DNA, 50 mM KCl, 10

106 mM Tris-HCl, 80 μM each dNTP, 1 μM each primer, 2 mM $MgCl_2$, and 1 U DNA polymerase. A *T-*
107 *gradient*® thermal cycler (Biometra, Germany) was programmed as follows: an initial step at 94°C for 4
108 min, followed by 35 cycles at 94°C for 40 s, 55°C for 40 s, 72°C for 40 s, and a final elongation step of
109 72°C for 10 min.
110
111 **RNA extraction and cDNA synthesis**
112 Mycelia were harvested from liquid culture, frozen immediately at -80°C, and then ground in liquid
113 nitrogen with sterilized pre-cooled mortar and pestle. Total RNA was extracted from the pulverised
114 mycelium using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The
115 extracted RNA was treated with RNAse-free DnaseI (Promega, Madison, WI) to eliminate traces of
116 contaminating DNA. The cDNA was synthesised using about 1.5 μg of total RNA, oligo $(dT)_{18}$ primer,
117 and the SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) according to the
118 manufacturer's protocol. The cDNA was stored at 4°C.
119
120 **RT-PCR**
121 For the analysis of *AcPks* gene expression in two different media with permissive and restrictive
122 conditions, aliquots of cDNA were used as templates for RT-PCR amplification with the primers
123 AcPKS-F1 and AcPKS-R1. The number of cycles used was set to 30 cycles, to avoid amplification
124 reaching a plateau and to allow a better normalization of the template amounts. RT-PCR conditions
125 were 94ºC for 5 min, 30 cycles at 94ºC for 30 s, 55ºC for 50 s, 72ºC for 50 s, and a final extension step
126 at 72ºC for 5 min.
127
128 **Extraction of OTA from culture broth**

For the detection of OTA levels in the media, the culture filtrate (3 ml) was centrifuged at 10,000 rpm for 10 min. The samples were diluted ten times with distilled water, and OTA was extracted from the culture filtrate using and quantified through HPLC by following the method of Bragulat et al. (2001). Samples were analyzed in a HPLC Agilent series 1100 formed by a degasser, an autosampler, a quaternary pump, a thermostated column and a fluorimeter. An analytical column RP-18 (150 mm x 4.6 mm i.d., 5 μm) with a pre-column was used. The mobile phase, eluting at 1 ml min^{-1}, consisted of an isocratic mixture of acetonitrile:water:acetic acid (45:45:10) for 18 min. Samples of 100 μl were injected onto the HPLC column and the retention time of OTA was 6.15 min. The amount of OTA in the final solution was determined by using a calibration graph of concentration versus peak area and expressed as ng ml^{-1}, achieved by injection onto the HPLC column of 100 μl of standard solutions of OTA (Sigma Chemical Co.). The standard solutions had concentrations of 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, and 100.0 μl l^{-1}. The recovery was determined on a blank YES broth spiked at three concentrations of OTA (0.1, 2.0 and 10.0 ng ml^{-1}). Recovery was performed in four replicates and ranged from 90.8% to 92.1%. Repeatability ranged from 2.64 to 2.71 % per replicate. The detection limit was 0.01 ng OTA ml^{-1} YES medium.

Results and Discussions

Primer specificity

The PCR performed with the primers KS1 and KS2 on the genomic DNA of *A. carbonarius* AC06 permitted to amplify the ketosynthase domain of a putative polyketide synthase, called *AcPks* and deposited in GenBank with the accessions number EU926157. An open reading frame (ORF) of 569 bp encoding a 190 amino acid protein was characterized (GenBank: ACH47947.1). Protein BLAST revealed that the several sequences had a query coverage of 99 or 100%. The putative protein sequence

belongs to the ketoacylsynthase superfamily, and in particular to the polyketide syhnthases. Specifically, *Acpks* displayed nucleotide identity (99%) to the PKS sequence of a *A. carbonarius* (Accession no. AAS98197.1), and lower level of nucleotide identity (84%) to a protein of *Aspergillus niger* strain CBS 513.88 (XP001394930.1) and to a putative PKS of *Aspergillus clavatus* strain NRRL 1 (XP001271445.1). The alignment showed high homology with two main conserved regions in the ketosynthase domain of *Aspergillus* spp.. A new set of primers (AcPKS-F1/AcPKS-R1) was designed on the conserved regions and the primers amplified a PCR product with an expected size of 186 bp (Fig. 1). The primers were tested for their specificity against several strains of *A. carbonarius* isolated from Italian vineyards and another strain of *A. carbonarius* from the CBS. All the *A. carbonarius* isolates amplified a band of the expected size (Fig. 1). Further, the primers were tested for their specificity on the genomic DNA of different species of *Aspergillus* and *Penicillium* isolated from Italian vineyards or coming from the CBS. The species tested as negative control include species of *Aspergillus* known from literature to be ochratoxigenic (*A. ochraceus*, belonging to the section *Circumdati* group) or belonging to the section *Nigri*. The number of reports dealing with the production of OTA by members of section *Nigri* has been increasing since the first description of OTA production by *Aspergillus niger* var. *niger* (Abarca et al. 1994). *Aspergillus* section *Nigri* includes uniseriate species (*A. aculeatus* and *A. japonicus*), the species *A. carbonarius*, species belonging to *A. niger* aggregate (*A. tubingensis, A. niger, A. brasiliensis*) and uncommon species, such as *A. ellipticus*. Some isolates of the species *A. niger, A. carbonarius* (Horie, 1995), *A. japonicus* (Dalcero et al., 2002), *A. aculeatus* (Battilani et al., 2003), and *A. tubingensis* (Perrone et al., 2007) can be ochratoxin A producers. Moreover, also two species of *Penicillium*, known to produce ochratoxin A, were included: *P. verrucosum* occurs predominantly in stored cereals in temperate regions, while *P. nordicum* can contaminate protein-rich substrates, mainly meat (Larsen et al. 2001). The PCR results showed that a unique PCR product was amplified only from the genomic DNA of *A. carbonarius* isolates. No

177 amplification was observed from the genomic DNA of the other species of *Aspergillus* or *Penicillium*
178 tested (Fig. 1). The results showed that only the strains belonging to *A. carbonarius* species produced a
179 PCR amplification. A single PCR product observed from each DNA of the ochratoxigenic isolates of *A.*
180 *carbonarius* confirmed the specificity of the primers.
181 The primer sets were selected from the most conserved regions of the ketosynthase domain of a
182 polyketide synthase, a key gene required for OTA production. Probably, these DNA conserved regions
183 were not present in the other fungal species tested, although many of them are known as producers of
184 ochratoxins or other polyketides. The PCR amplification is often used for the detection of pathogenic
185 or toxigenic microorganisms in various substrates, including foods (Dao et al., 2005). Two PCR assays
186 were developed to detect *A. carbonarius* and *A. ochraceus* with primers designed on the internal
187 transcribed spacers of rDNA units (Patiño et al., 2005). Several reports demonstrated that the primers
188 for the detection of mycotoxin producing fungi in foods should be designed on the conserved domains
189 of genes involved in the biosynthetic pathway of mycotoxins (O'Callaghan et al., 2003; Dao et al.,
190 2005). When the primers are designed on other genes, such as calmodulin, not involved in mycotoxin
191 biosynthesis, the PCR amplification can help to differentiate the species (Perrone et al., 2004) but it
192 cannot be used to identify mycotoxigenic isolates (Mulè et al., 2006). Polyketosynthases (PKSs) are
193 multifunctional enzymes which are encoded by a single gene and typically possess up to eight types of
194 functional domains (Bingle et al., 1999). The cloning and molecular characterization of many genes
195 encoding fungal PKSs has been greatly facilitated by the conserved nature of these functional domains,
196 which has allowed to design gene probes and degenerated primers used to isolate the gene fragments
197 encoding the PKS domains in several fungal species (Cox et al., 2004; Nicholson et al., 2001). This
198 approach has been successfully employed to characterize five KS domains belonging to putative PKSs
199 in *A. carbonarius* (Atoui et al., 2006). Previous PCR approaches for the quantification of *A.*
200 *carbonarius* isolates were developed on PKS domains: a SYBR-Green I approach targeted to the

201 acyltransferase domain of a PKS (Atoui et al., 2007). Also Selma et al. (2008) developed a real-time
202 procedure based on two specific primers directed to the ketosynthase domain of a polyketide synthase
203 for the detection of *A. carbonarius* in wine grapes, but they did not confirm the correlation of the gene
204 expression with the OTA production.
205
206 **Transcription of *pks* gene and ochratoxin A production**
207 In order to determine if a positive correlation existed between the cloned KS domain of the *pks* gene
208 identified in this study and OTA production, the strain AC06 of *A. carbonarius* was grown in two
209 media incubated at different temperatures known to affect OTA biosynthesis (Spadaro et al., 2010b).
210 The transcription of the *pks* gene was monitored using a reverse transcription (RT)-PCR based
211 approach, and contemporarily OTA production was measured through HPLC analysis. When the strain
212 AC06 was grown in YES (permissive) medium for 9 days, it showed higher production of OTA (2.9 μg
213 l^{-1}) at 30ºC, while OTA level was lower when the strain was incubated at 15ºC (1.8 μg l^{-1}). Similarly,
214 when grown in PDB (restrictive) medium for 9 days, AC06 produced higher amounts of OTA at 30ºC
215 (0.5 μg l^{-1}) than at 15ºC (0.3 μg l^{-1}), but the level of OTA produced was significantly lower on PDB
216 then on YES. Less production of OTA by *A. ochraceus* on PDB was reported also by O'Callaghan et
217 al. (2006). No OTA production was observed when the strain was incubated at 10ºC either in YES or
218 PDB (Fig. 2). Several researchers reported that the optimum temperature for growth and OTA
219 production of *A. carbonarius* strains is from 25ºC to 35ºC (Mitchell et al., 2004; Battilani, et al., 2004;
220 Bellí et al., 2004a,b; Esteban et al., 2004). Analysis of the transcript level clearly indicated that the
221 *AcPks* gene was expressed differentially in different environmental conditions (media and temperature
222 of growth) and its transcription was related to the level of OTA production (Fig. 3). Temperature of
223 10ºC did not affect significantly the expression level in both media.

A similar approach was employed to characterize five KS domains belonging to putative *pks* in *A. carbonarius* (Atuoi et al., 2006). More recently, also Gallo et al. (2009) reported that a *AcPks* gene from *A. carbonarius* was differentially expressed on permissive and restrictive media and it was related to the level of OTA production. The same authors reported that *AcPks* gene was differentially expressed according to the pH of the medium. Higher pH values were accompanied by a reduction of the *pks* gene transcript accumulation and by a reduced level of OTA production (Gallo et al., 2009). Geisen (2004) reported that the expression of *otapksPN* in *Penicillium nordicum* was down regulated under acidic conditions which accompanied the decrease in OTA biosynthesis.

The results obtained in the current study revealed that the PCR primers developed will be useful to detect specifically OTA producing strains of *A. carbonarius* both in vineyard or in wine industries.

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Figure legends

Fig. 1. PCR products obtained from genomic DNA of various *Aspergillus* and *Penicillium* species using primers AcPKS-F1/ AcPKS-R1. Amplification was observed only for *A. carbonarius* strains and not for the other species. Lane 1: *A. ellipticus* CBS 707.79; lane 2: *A. tubingensis* CBS 119556; lane 3: *A. niger* CBS 119557; lane 4: *A. aculeatus* CBS 116.80; lane 5: *A. japonicus* CBS 119560; lane 6: *A. brasiliensis* CBS 121619; Lane 7: *A. ochraceus* CBS 132.52; Lane 8: *P. nordicum* CBS 110769; Lane 9: *P. verrucosum* CBS 226.71E; Lane 10: *A. carbonarius* CBS 127.49; Lane 11: *A. carbonarius* AC06 (Agroinnova culture collection centre); lanes 12 to 18: *A. carbonarius* isolates from Italian vineyards; Lane M: 100 bp plus molecular weight marker (Gelpilot, Qiagen).

Fig. 2. Ochratoxin A production in fungal mycelium by *A. carbonarius* strain AC06 on two different media (PDB and YES) incubated at different temperatures. OTA accumulation was determined by HPLC. Error bars denote the standard error of the mean of three replicates from independent cultures.

Fig. 3. RT-PCR analysis of *AcPks* gene in *A. carbonarius* strain AC06, performed on the cDNA of 9 day-old mycelium grown in two different media (PDB and YES) incubated at different temperatures. Lanes 1, 2 and 3: mycelium grown on PDB at 10°C, 15°C and 30°C; Lanes 4 and 5: mycelium grown on YES at 15°C and 30°C; M: 50 bp molecular weight marker (Gelpilot, Qiagen).