European Journal of Plant Pathology **110:** 661–669, 2004.

Analysis of differentially-expressed ochratoxin A biosynthesis genes of *Penicillium nordicum*

Paul Färber and Rolf Geisen*
*Federal Research Center for Nutrition and Food, Institute of Hygiene and Toxicology, Haid-und-Neu-Str. 9, 76131 Karlsruhe, Germany; *Author for correspondence (Fax: +49-7216625453; E-mail: rolf.geisen@bfe.uni-karlsruhe.de)*

Key words: biosynthetic pathway, DDRT-PCR, differentially expressed genes, ochratoxin A, *Penicillium nordicum*, *Penicillium verrucosum*

Abstract

Analysis of differentially expressed genes under conditions which favour ochratoxin A production by *Penicillium nordicum* was carried out by Differential Display Reverse Transcriptase-PCR (DDRT-PCR). The differential conditions were achieved by growth on different minimal media which either support or inhibit ochratoxin A production. Thirty differentially expressed genes were identified. The DDRT-PCR fragments were cloned and sequenced. The sequences obtained were analysed for homology at the protein level by GeneBank comparison. This analysis revealed several fragments with homology to enzymes which are potentially involved in the biosynthesis of ochratoxin A, in particular a polyketide synthase, a non-ribosomal peptide synthetase, a halogenase, a phenylalanine t-RNA synthetase, a methylase and a fragment with homology to ABC transporter genes. All other induced genes either showed no homology to known sequences or are genes whose induction can be explained by growth in different minimal media.

Introduction

Ochratoxin A is an important mycotoxin which can occur in different food commodities such as cereals, coffee, wine, beer and spices (Höhler, 1998). Ochratoxin A is nephrotoxic with additional teratogenic and immunogenic effects (Petzinger and Ziegler, 2000). It is classified as a carcinogen and for that reason the occurrence of ochratoxin A in plant products produced for human nutrition has to be minimised. In regions with a temperate climate *Penicillium* species are mainly responsible for the production of ochratoxin A in cereals and cereal products. In warmer regions, *Aspergillus* species such as *A. ochraceus*, *A. carbonarius* or *A. niger* are responsible for the presence of ochratoxin A in products such as coffee (Bucheli et al., 2000; Joosten et al., 2001), spices (Thirumala-Devi et al., 2002) and wine (Pietri et al., 2001; Cabanes et al., 2002; Da Rocha Rosa et al., 2002). Until recently, *P. verrucosum* was the only species of the genus *Penicillium* which had been shown to produce ochratoxin A. Detailed analyses at the biochemical (Larsen et al., 2001) and genetic levels (Castella et al., 2002), however, revealed that two closely related but distinct species, namely *P. verrucosum* and *P. nordicum,* were able to produce ochratoxin A. *Penicillium verrucosum* occurs mainly on cereals and is responsible for the production of ochratoxin A in this food commodity, whereas *P. nordicum* is a contaminant of protein-rich foods such as cheeses and meat products.

Ochratoxin is a composite mycotoxin, consisting of the amino acid phenylalanine and the pentaketide dehydroisocoumarin (Figure 1). A biosynthetic pathway for ochratoxin A was outlined by Huff and Hamilton (1979). However, recent results demonstrate that there are ambiguities in the pathway, because mellein, a precursor suggested by Huff and Hamilton (1979) does not play a role in

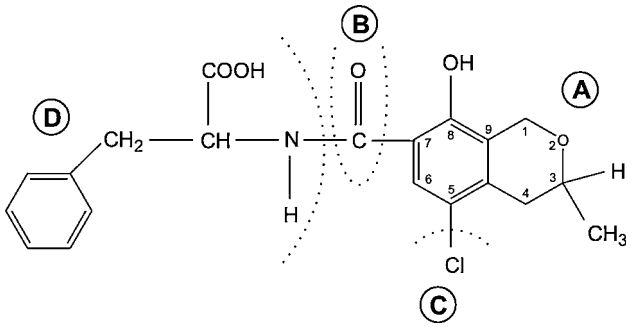

Figure 1. Structural formula of ochratoxin A demonstrating the metabolic origin of the structural parts of the molecule. (A) The polyketide dihydro-isocoumarin; (B) The carboxyl group, derived after oxidation of the methylated polyketide; (C) The chlorine introduced by halogenation of ochratoxin B; (D) The amino acid phenylalanine coming out of the shikimi acid pathway.

the biosynthetic pathway according to results of Harris and Mantle (2001). Much less is known about the genetic background of ochratoxin A biosynthesis. According to the proposed biosynthetic pathway, the minimal requirements at the genetic level are genes for a polyketide synthase, required for the synthesis of the polyketide dihydro-isocoumarin, a methylase for the formation of the carboxyl group, an ochratoxin A synthase (peptide synthetase) for ligation of the amino acid phenylalanine and the polyketide, and finally a chloroperoxidase or halogenase for the introduction of the chlorine atom. In a recent report, the putative polyketide synthase from *P. nordicum* has been partly characterised (Geisen et al., in press) and its expression was correlated to ochratoxin A production. O'Callaghan et al. (2003) also described part of a gene coding for a polyketide synthase involved in ochratoxin A biosynthesis in *Aspergillus ochraceus*.

The Differential Display Reverse Transcriptase-PCR (DDRT-PCR) approach was first described by Liang and Pardee (1992) for use in plants. In the meantime, this approach has also been adapted to identify differentially expressed genes in fungi. Benito et al. (1996) used this method to identify genes which are up-regulated during plant-fungus interactions. Melin et al. (1999) analysed changes in gene expression in *Aspergillus nidulans* due to the presence of the antibiotic bafilomycin. To use the DDRT-PCR approach for the analysis of genes differentially expressed during ochratoxin A biosynthesis, two different RNA populations were compared. One RNA population was isolated under conditions which did not support the production of ochratoxin A and the other RNA population was isolated under ochratoxin A-producing conditions. In this paper we report the identification of differentially expressed genes of *P. nordicum* by DDRT-PCR under conditions which support ochratoxin A production.

Materials and methods

Strains and culture conditions

Penicillium nordicum BFE487 was used throughout this study. This strain is a reliable ochratoxin A producer. Ochratoxin A production was determined on YES medium. Strain maintenance and routine growth experiments were performed on malt extract agar/medium (17 g l^{-1} malt extract, 5 g l^{-1} glucose, 15 g l^{-1} agar, pH 6.5). Incubation was carried out at 25 °C for 4–7 days. For differential ochratoxin A production, the strains were grown in a mineral salt minimal medium (3.8 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \times 7H_2O$, 0.1 g l^{-1} NaCl, 0.1 g l^{-1} $CaCl_2$, 0.7 g l^{-1} KOH, 15 g l^{-1} agar, pH 6.5) which supported ochratoxin A production (supplemented with 5.0 g l^{-1} glycerol and 2.5 g l^{-1} NH_4Cl) and the same minimal medium, supplemented with 5.0 g l^{-1} glucose and 1.2 g l^{-1} KNO_3, which suppressed ochratoxin A production.

Isolation of fungal DNA

DNA was isolated using a method modified from that of Yelton et al. (1984). Seventy-two to 96 h old mycelia were harvested from a submerged culture by filtration. The mycelia were transferred

to a mortar and frozen in liquid nitrogen. The frozen mycelia were ground to a powder and resuspended in lysis buffer (50 mM EDTA; 0.2 % SDS; pH 8.5). This suspension was heated to 68 °C for 15 min and centrifuged for 15 min at 15.000 × *g*. After centrifugation, 7 ml of the supernatant was transferred to a new centrifuge tube and 1 ml of 4 M sodium acetate added. This solution was placed on ice for 1 h and centrifuged for 15 min at 15.000 × *g*. After centrifugation 6 ml of the supernatant were transferred to a fresh tube. The solution was extracted with phenol and the isolated DNA was precipitated by the addition of 2.5 volumes of ethanol. The isolated DNA was assessed on an agarose gel and the concentrations were determined spectrophotometrically (Sambrook et al., 1989).

Isolation of total RNA from pure cultures

Penicillium nordicum was grown in the respective minimal medium for 4 days. The mycelium was harvested and 0.5 g was frozen in liquid nitrogen and ground to powder in a mortar. Two hundred mg of the powder was used for isolation of total fungal RNA. For that purpose the E.Z.N.A. Fungal RNA kit (PeqLab, Erlangen, Germany) was used. Eighty µl of the RNA preparation was treated with 2 µl DNase I (2.5 Kunitz units/µl, QIAGEN, Hilden, Germany) for degradation of traces of genomic DNA. The solution was incubated for 60 min at 37 °C and subsequently for 10 min at 65 °C to inactivate the DNase. An aliquot of the RNA was separated on an agarose gel, prepared as described by Sambrook et al. (1989), to check the integrity of the RNA.

cDNA synthesis

Eight µl of the total RNA treated with DNase I was used along with the Omniscript Reverse Transcription kit (QIAGEN, Hilden, Germany). The reaction mixture was incubated at 37 °C for 1 h. The cDNA was either directly used for DDRT-PCR experiments or stored at −20 °C.

DDRT-PCR experiments

A CLONTECH Delta™ Differential Display Kit (PT1173-1, CLONTECH Laboratories Inc., Palo Alto, USA) was used. The displays were completed with negative as well as positive control reactions. PCRs were performed in the presence of [α-^{33}P]dATP (25 µCi) to label the DDRT-PCR fragments. Electrophoresis of DDRT-PCR products was performed by the use of a temperature-controlled electrophoresis system (Sequi-Gen GT, Biorad, Hercules, USA) for maximum resolution. Products of DDRT-PCR reactions were separated on 6% polyacrylamide/8 M urea gels (2 mm) in 0.5X TBE buffer (1200 V). The gels were pre-run for 30 min. After electrophoresis the gel was transferred on to a Whatman filter paper and dried at 75 °C for 1 h.

Autoradiography

The dried gel was exposed to a Kodak BioMax MR (875 6447, Kodak, Rochester, New York, USA) scientific imaging film for 12–14 h at −70 °C. The position of the X-ray film on the dried gel was marked to ensure the precise excision of differentially expressed bands.

Isolation of the DDRT-PCR fragments

The developed X-ray film was aligned on the dried polyacrylamide gel. The gel regions below the autoradiographic signals were cut out with a scalpel. The gel blocks were transferred into a fresh reaction tube with 40 µl of Tricine-EDTA buffer (10 mM Tricine (pH 9.5), 0.2 mM EDTA). A few drops of mineral oil were used to overlay and to protect the solution against evaporation during the following heating step at 100 °C for 5 min.

Reamplification

All isolated differentially expressed bands were reamplified and assessed on an agarose gel for their size and purity (data not shown).

Cloning and sequencing of the DDRT-PCR fragments

All isolated DDRT-PCR fragments were cloned into the Invitrogen TOPO TA-cloning vector (K4595-40, Invitrogen, Carlsbad, California, USA). The cloned DDRT-PCR fragments were sequenced using standard techniques (Sambrook et al., 1989).

Standard nucleotide and protein BLAST analysis

Standard BLAST analyses were performed at the nucleotide and protein level (http://www.ncbi.nlm.nih.gov/BLAST/). For homology searches at the protein level, possible continuous open reading frames were determined in both directions and translated to the amino acid sequences.

Results

Ochratoxin A production by Penicillium nordicum *on different media*

To identify growth conditions which either support or inhibit ochratoxin A production by *P. nordicum* without influencing other pathways or genes, a minimal medium which contained the basic nutrients was varied in its composition. In experiments with media in which only one of the basic nutrients was exchanged, KNO_3 and glucose inhibited toxin production, whereas NH_4Cl and glycerol supported ochratoxin A biosynthesis (data not shown). For the experiments described, the inhibitory or supporting nutrients were used in combination to enhance this effect. As a result, it was possible to adjust supporting and inhibiting conditions by just changing two basic nutrients, in particular the carbon and nitrogen sources. The minimal medium which contained glucose and KNO_3 proved to be inhibitory for ochratoxin A production, whereas the same minimal medium supplemented with glycerol and NH_4Cl supported ochratoxin A production. This was clearly demonstrated by thin layer chromatography (Figure 2). The growth rates were nearly unaffected in both media compared to the routinely used complex media (Figure 3), indicating that the change in the medium composition specifically influences ochratoxin A biosynthesis and not general growth behaviour.

Analysis of differentially expressed genes under ochratoxin A production conditions by DDRT-PCR

Cultures of *P. nordicum* were grown for 4 days under shaking conditions and the mycelium was harvested. The total RNA was isolated, reverse transcribed and subjected to DDRT-PCR with different sets of anchored and random primers. An example of the results is shown in Figure 4. Genes which are up-regulated under ochratoxin A production conditions were highlighted by this method (Figure 4, arrow). In total, 30 upregulated genes were identified under ochratoxin A-produc-

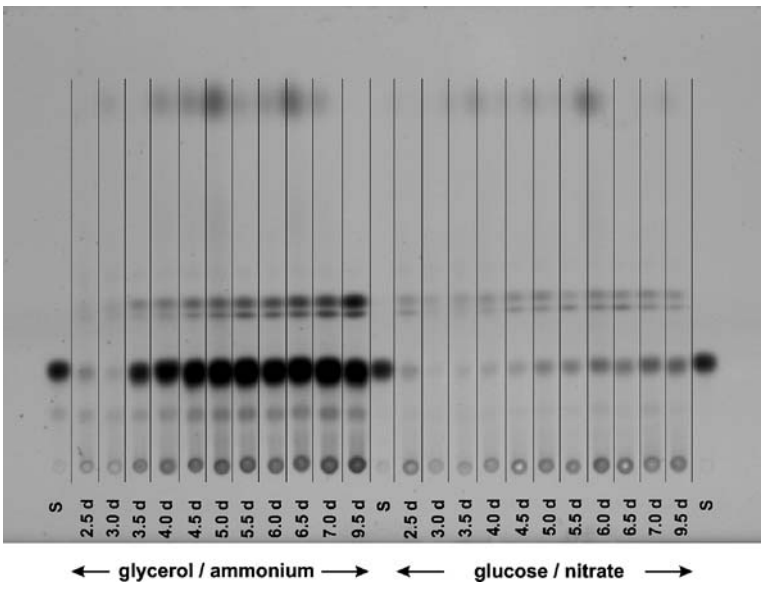

Figure 2. Differential production of ochratoxin A after growth in different media monitored by thin layer chromatography. Both minimal media had the same basal composition of nutrients, except for the carbon and nitrogen sources, as indicated.

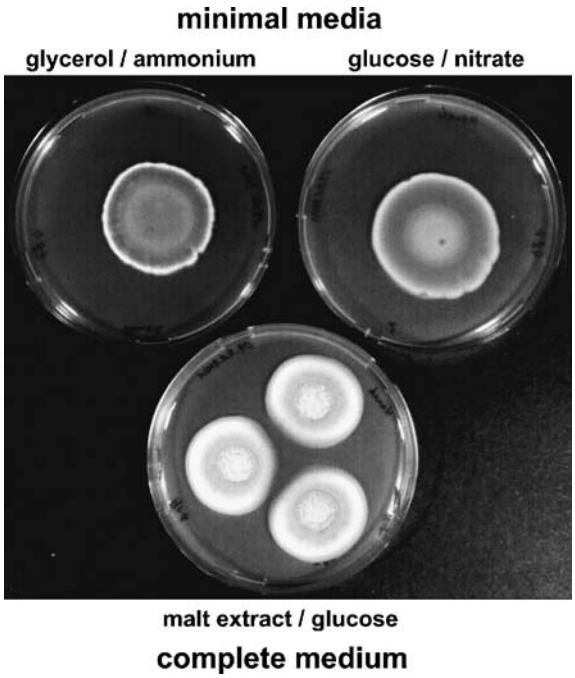

Figure 3. Comparison of mycelial growth on both minimal media and on complete medium.

ing conditions. All 30 fragments were excised from the gel, cloned into a TA cloning vector and sequenced. The sequences of the DDRT-PCR fragments were translated into the protein sequence and compared to published sequences in the GeneBank database by BLAST searches. The homologies found are listed in Table 1. Several of the identified fragments had homologies to genes postulated for the ochratoxin A biosynthetic pathway, in particular a polyketide synthase (3 fragments), a non-ribosomal peptide synthase (2 fragments), an phenylalanine-tRNA synthetase (1 fragment), a methyl transferase (1 fragment), a halogenase (1 fragment) and ABC transporter genes related to secondary metabolism (1 fragment). Other upregulated genes are house-keeping genes and appear to be induced by growth in different media, in the same way as the up-regulation of membrane channel proteins, enzymes of the citrate cycle, putative transcription factors, translation elongation factor, genes of ammonium metabolism and several ribosomal proteins. The results obtained by this DDRT-PCR approach are conclusive under these growth conditions.

Discussion

Very little is known about the genetics of ochratoxin A biosynthesis in *Aspergillus* or *Penicillium*. Recently a polyketide synthase was characterised and it was expressed in relation to ochratoxin A biosynthesis (Geisen et al., in press). In the described DDRT-PCR analysis, fragments with homology to polyketide synthase (*pks*) genes were identified, supporting the observation that a *pks* gene is induced under conditions where ochratoxin

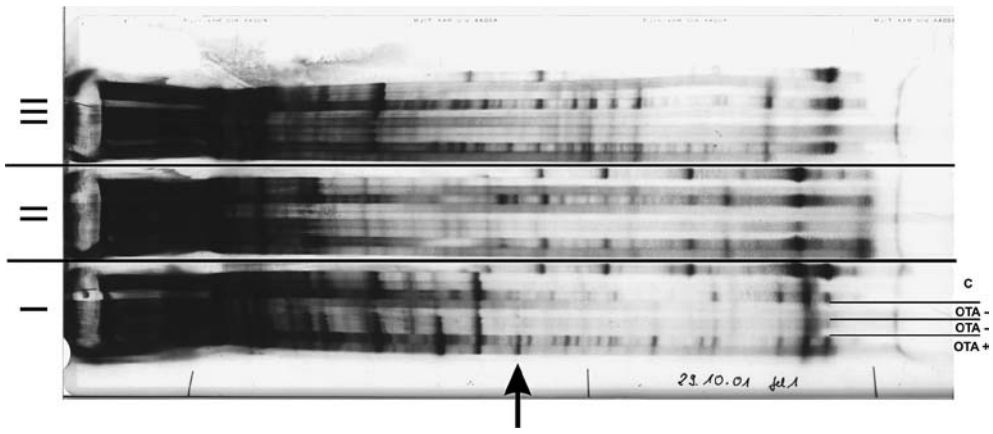

Figure 4. Autoradiography of a differential display of amplificates derived from three different DDRT-PCR reactions using three different primer combinations (I, II, III). Differentially expressed cDNA fragments (indicated by an arrow) are produced under permissive conditions for ochratoxin A biosynthesis (OTA^+). They are not visible under restrictive conditions (OTA^-) or in control reactions (C).

Table 1. Homology of sequenced DDRT-PCR fragments at the protein level

DDRT-PCR	Direction of DNA sequence		Putative function:	Class:*
fragment no:	forward:	reverse complement:		
T-01/A	no sequence homology detected	non-ribosomal peptide synthetase [*Hypocrea virens*]	- secondary metabolism - OTA biosynthesis	A
T-02/B	no sequence homology detected	phenylalanyl-tRNA synthetase [*Caenorhabditis elegans*]	- primary metabolism - precursor for OTA biosynthesis	B
T-03/B	ammonium transporter AmtC [*Dictyostelium discoideum*]	polyketide synthase [*Cordyceps brongniartii*]	- transport	C
T-04/B	no sequence homology detected	putative non-ribosomal peptide synthetase [*Actinomadura* sp.]	- secondary metabolism - OTA biosynthesis	A
T-05/B	no sequence homology detected	K^+ channel [*Arabidopsis thaliana*]	- transport	C
T-06/B	acetyl-CoA synthetase [*Emericella nidulans*]	no sequence homology detected	- primary metabolism - precursor for OTA biosynthesis	B
T-07/A	putative DNA-binding protein [*Dendrobium grex*]	no sequence homology detected	- general regulatory protein - OTA specific regulatory protein	A / C
T-08a/B	tryptophan halogenase [*Caulobacter crescentus*]	no sequence homology detected	- secondary metabolism - OTA biosynthesis	A
T-08b/B	methyltransferase [*Bacillus anthracis*]	no sequence homology detected	- primary metabolism - OTA biosynthesis	A / C
T-08c/B	no sequence homology detected	translation elongation factor 1-alpha (EF-1-alpha) [*Aspergillus oryzae*]	- general gene expression	C
T-09a/A	non-ribosomal peptide synthetase [*Pseudomonas syringae*]	no sequence homology detected	- secondary metabolism - OTA biosynthesis	A
T09c/A	acetyl-CoA carboxylase [*Arabidopsis thaliana*]	no sequence homology detected	- primary metabolism	C
T-10/A	no sequence homology detected	decarboxylase [*Streptomyces* sp.]	- primary metabolism	C
T-11/A	no sequence homology detected	no sequence homology detected	- - -	- - -
T-12/A	no sequence homology detected	ammonia monooxygenase [uncultured soil bacterium]	- primary metabolism	C
T-13/B	no sequence homology detected	ABC-type transmembrane transport protein [*Campylobacter jejuni*]	- transport of secondary metabolites	A / C
T-14/B	NADH dehydrogenase, subunit F [*Chromolepsis heterophylla*]	no sequence homology detected	- primary metabolism	C
T-15/B	no sequence homology detected	no sequence homology detected	- - -	- - -
T-16/B	no sequence homology detected	no sequence homology detected	- - -	- - -
T-17/A	peptide ABC transporter [*Vibrio cholerae*]	no sequence homology detected	- transport	C
T-18/A	no sequence homology detected	no sequence homology detected	- - -	- - -
T-19/A	polyketide synthase, type I [*Streptomyces avermitilis*]	no sequence homology detected	- secondary metabolism - OTA biosynthesis	A

Table 1. (Continued)

DDRT-PCR	Direction of DNA sequence		Putative function:	Class:*
fragment no:	forward:	reverse complement:		
T-20/B	putative DNA-binding protein [*Dendrobium grex*]	no sequence homology detected	- general regulatory protein - OTA specific regulatory protein	A / C
T-21/A	no sequence homology detected	no sequence homology detected	- - -	- - -
T-22/B	no sequence homology detected	acyl-CoA synthetase [*Plasmodium falciparum*]	- primary metabolism - precursor for OTA biosynthesis	B
T-23/A	no sequence homology detected	no sequence homology detected	- - -	- - -
T-24/A	no sequence homology detected	ABC transporter, ATP-binding protein [*Vibrio cholerae*]	- transport of secondary metabolites	A / C
T-25/B	no sequence homology detected	succinyl-CoA synthetase [*Plasmodium falciparum*]	- primary metabolism	C
T-26/A	no sequence homology detected	polyketide synthase [*Mycobacterium tuberculosis*]	- secondary metabolism - OTA biosynthesis	A
T-27/B	no sequence homology detected	ammonium transporter [*Bacillus halodurans*]	- transport	C

*A: secondary metabolism, postulated genes of the ochratoxin biosynthetic pathway; B: primary metabolism, generation of precursors for ochratoxin biosynthesis; C: general cell function.

A is produced. Homologues of genes of many assumed enzymes for ochratoxin A biosynthesis were identified. A non-ribosomal peptide synthase was induced under ochratoxin A production conditions. This might be the postulated ochratoxin A synthetase (Huff and Hamilton, 1979) which completes the peptide coupling of the amino acid phenylalanine to the polyketide dihydro-isocoumarin. In addition, the induction of a methyl transferase was observed. Ochratoxin A is a methylated compound (Harris and Mantle, 2001). According to the suggested pathway (Huff and Hamilton, 1979) the polyketide is methylated and the methyl group is further oxidised to the carboxyl group, which is involved in the peptide bond to the amino acid phenylalanine (Figure 1). The methylation inhibitor ethionine completely inhibits the production of ochratoxin A, indicating the importance of these enzymatic activities (Yamazaki et al., 1971). A gene with homology to an halogenase is also induced during ochratoxin A biosynthesis. Kirner et al. (1998) described an halogenase responsible for the chlorination of L-tryptophane during the biosynthesis of pyrrolnitrin, a secondary metabolite with antifungal activity produced by *Pseudomonas fluorescens*. The halogenase homologue identified in this study might be responsible for the chlorination of ochratoxin B to ochratoxin A (Huff and Hamilton, 1979).

During ochratoxin A biosynthesis, the metabolic flux of phenylalanine is re-routed from protein biosynthesis to ochratoxin A biosynthesis. The analysed strain of *P. nordicum* is a very strong ochratoxin A-producing organism. If this strain grows under ochratoxin A production conditions, a large part of the celluar phenylalanine might apparently be shifted to the ochratoxin A biosynthetic pathway. In the present study, we observed induction of a phenylalanine-tRNA synthetase. According to these results, it can be assumed that the organism tries to ensure that enough phenylalanine-tRNA is available for protein biosynthesis under ochratoxin A biosynthesis conditions, by upregulating the phenylalanine-tRNA synthetase gene.

ABC transporter genes are also induced during ochratoxin A production. It was discussed by Antrade et al. (2000) that these ABC transporters are needed for the export of secondary metabolites,

including mycotoxins. The observed upregulation of genes for ABC transporters supports this view.

A putative DNA binding protein is up-regulated under ochratoxin A production conditions. Mycotoxin biosynthetic pathways are usually controlled by transcription factors, like the *afl*R protein in the case of aflatoxin (Ehrlich and Cary, 1995) or the *tri*6 and *tri*10 genes in the case of the trichothecenes (Peplow et al., 2003). For this reason, the identified DNA binding protein might be involved in the induction of the genes for ochratoxin A biosynthesis. However, further experiments are needed to show whether the identified DNA binding protein is indeed involved in ochratoxin A biosynthesis or is up-regulated because of the presence of different carbon and nitrogen sources.

Other genes which have been identified using this approach are apparently up-regulated due to the growth in different media. For both media the same basal composition of nutrients were used, only the nitrogen and carbon sources were exchanged. The non-inducing medium contained potassium nitrate and glucose and the inducing medium contained ammonium chloride and glycerol. It is noteworthy that two genes of ammonium metabolism, a gene with homology to an ammonium monooxygenase and a gene with homology to an ammonium transporter, were up-regulated under ochratoxin A inducing conditions. In particular the homologue of the former gene is present in nitrifying bacteria and is responsible for the oxidation of ammonia to hydroxylamine. Fungi assimilate NH_4^+ *via* glutamic acid and usually do not oxidize ammonium. Thus the role of this putative gene is currently not known. Surprisingly, the sequence from this DDRT-PCR fragment seems to be specific for ochratoxinogenic Penicillia. It has also been possible to develop a primer pair from this sequence, which gave a positive PCR signal with ochratoxinogenic Penicillia, such as *P. nordicum* and *P. verrucosum*, but not with other food related filamentous fungi including the ochratoxinogenic Aspergilli (data not shown).

The other genes which are apparently up-regulated because of the differences in the carbon source, are acetyl-CoA synthetase, NADH dehydrogenase and succinyl-CoA synthetase. The upregluation of the acetyl-CoA synthetase might also be correlated to ochratoxin A biosynthesis, as acetyl-CoA serves as a precursor for polyketide biosynthesis.

Inhibition of ochratoxin A by glucose contrasts with the biosynthesis of aflatoxin by Aspergilli. Luchese and Harrigan (1993) reported that glucose and glycerol are excellent carbon sources for growth and aflatoxin production by *A. parasiticus*. In the case of ochratoxin A, glycerol supports, but glucose inhibits, toxin production. Inhibition of synthesis of penicillin by glucose was also observed with *A. nidulans* (Brakhage et al., 1992), indicating that glucose indeed has an influence on secondary metabolite biosynthesis. On the other hand, regulation by the nitrogen source seems to be similar for aflatoxin and ochratoxin A biosynthesis. Aflatoxin production by *A. flavus* and *A. parasiticus* is also reduced after growth in nitrate-based medium and increased in ammonium salt-based medium (Ehrlich and Cotty, 2002). Feng and Leonhard (1998) suggested that the reduced levels of aflatoxin were caused by nitrate suppression of *afl*R, the regulatory gene of the aflatoxin biosynthetic pathway. We also observed that a putative DNA-binding protein was down-regulated when nitrate was present and up-regulated after growth in ammonium containing medium. This might be a further indication that this putative regulatory protein is involved in ochratoxin A biosynthesis.

In the present study, the induction of genes assumed to be involved in ochratoxin A biosynthesis has been monitored. It was shown that the DDRT-PCR approach is feasible for generating an overall picture of up-regulated genes during ochratoxin A production. Moreover the isolated DDRT-PCR fragments can serve as gene probes for the isolation of the whole genes. Further detailed analysis of these genes, which is now possible, should provide more detailed insights into the genetic regulation of ochratoxin A biosynthesis in Penicillia.

Acknowledgements

This work was supported by the EU Project QLK1-CT-1998-01380 (European Commission, Quality of Life and Management of Living Resources Programme (QOL), Key Action 1 on Food, Nutrition and Health, Contract No. QLK1-CT-1999-01380).

References

Antrade AC, Van Nistelrooy JGM, Peery RB, Skatrud PL and De Waard MA (2000) The role of ABC transporters from

Aspergillus nidulans in protection against cytotoxic agents and in antibiotic production. Molecular and General Genetics 263: 966–977.

Benito EP, Prins T and van Kan JAL (1996) Application of differential display RT-PCR to the analysis of gene expression in a plant–fungus interaction. Plant Molecular Biology 32: 947–957.

Brakhage AA, Browne P and Turner G (1992) Regulation of *Aspergillus nidulans* penicillin biosynthesis genes *acvA* and *ipnA* by glucose. Journal of Applied Bacteriology 174: 3789–3799.

Bucheli P, Kanchanomai C, Meyer I and Pittet A (2000) Development of Ochratoxin A during Robusta (*Coffea canephora*) coffee cherry drying. Journal of Agricultural and Food Chemistry 48: 1358–1362.

Cabanes FJ, Accensi F, Bragulat MR, Abarca ML, Castella FA, Minguez S and Pons A (2002) What is the source of ochratoxin A in wine? International Journal of Food Microbiology 79: 213–215.

Castella G, Larsen TO, Cabanes J, Schmitt H, Alboresi A, Niessen L, Färber P, and Geisen R (2002) Molecular characterization of ochratoxin A producing strains of the genus *Penicillium*. Systematic and Applied Microbiology 25: 74–83.

Da Rocha Rosa CA, Palacios V, Combina ME, Fraga ME, De Oliveira Rekson CE, Magnoli CE and Dalcero AM (2002) Potential ochratoxin A producers from wine grapes in Argentina and Brazil. Food Additives and Contaminants 19: 408–414.

Ehrlich K and Cary JW (1995) An aflatoxin biosynthesis regulatory protein (AFLR) is a sequence-specific DNA binding protein. Fungal Genetic Newsletter 42A: 57.

Ehrlich KC and Cotty PJ (2002) Variability in nitrogen regulation of aflatoxin production by *Aspergillus flavus* strains. Applied Microbiology and Biotechnology 60: 174–178.

Feng GH and Leonhard TJ (1998) Culture conditions control expression of the genes for aflatoxin and sterigmatocystin biosynthesis in *Aspergillus parasiticus* and *A. nidulans*. Journal of Applied and Environmental Microbiology 64: 2275–2277.

Geisen R, Mayer Z, Färber P and Karolewiez A (in press) Development of a Real Time PCR system for detection of *Penicillium nordicum* and for monitoring ochratoxin A production by targeting the ochratoxin A polyketide synthase gene. Systematic and Applied Microbiology.

Harris JP and Mantle PG (2001) Biosynthesis of ochratoxins by *Aspergillus ochraceus*. Phytochemistry 58: 709–716.

Höhler D (1998) Ochratoxin A in food and feed: Occurrence, legislation and mode of action. Zeitschrift für Ernährungswissenschaften 37: 2–12.

Huff WE and Hamilton PB (1979) Mycotoxins – their biosynthesis in fungi: Ochratoxins – metabolites of combined pathways. Journal of Food Protection 42: 815–820.

Joosten HMLJ, Goetz J, Pittet A, Schellenberg M and Bucheli P (2001) Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. International Journal of Food Microbiology 65: 39–44.

Kirner S, Hammer PE, Hill DS, Altmann A, Fischer I, Weislo LJ, Lanahan M, van Pee KH and Ligon J (1998) Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens*. Journal of Bacteriology 180: 1939–1943.

Larsen TO, Svendsen A and Smedsgaard J (2001) Biochemical characterization of ochratoxin A-producing strains of the genus *Penicillium*. Journal of Applied and Environmental Microbiology 67: 3630–3635.

Liang P and Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967–971.

Luchese RH and Harrigan WF (1993) Biosynthesis of aflatoxin – the role of nutritional factors. Journal of Applied Bacteriology 74: 5–14.

Melin P, Schnürer J, Gerhart E and Wagner H (1999) Changes in *Aspergillus nidulans* gene expression induced by bafilomycin, a *Streptomyces*-produced antibiotic. Microbiology 145: 1115–1122.

O'Callaghan JO, Caddick MX and Dobson ADW (2003) A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. Microbiology 149: 3485–3491.

Peplow AW, Tag AG, Garifullina GF and Beremand MN (2003) Identification of new genes positively regulated by *Tri10* and a regulatory network for trichothecene mycotoxin production. Journal of Applied and Environmental Microbiology 69: 2731–2736.

Petzinger E and Ziegler K (2000) Ochratoxin A from a toxicological perspective. Journal of Veterinary Pharmacology and Therapy 23: 91–98.

Pietri A, Bertuzzi T, Pallaroni L and Piva G (2001) Occurrence of ochratoxin A in Italian wines. Food Additives and Contaminants 18: 647–654.

Sambrook J, Fritsch EF and Maniatis T (1889) Molecular Cloning. Cold Spring Harbour Laboratory Press, New York, USA.

Thirumala-Devi K, Mayo MA, Reddy G, Emmanuel KE, Larondelle Y and Reddy DVR (2002) Occurrence of ochratoxin A in black pepper, coriander, ginger and turmeric in India. Food Additives and Contaminants 18: 830–835.

Yamazaki M, Maebayashi Y and Miyaki K (1971) Biosynthesis of ochratoxin A. Applied Microbiology 22: 276–277.