Identification of Genes Differentially Expressed Between Ochratoxin-Producing and Non-Producing Strains of *Aspergillus westerdijkiae*

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Abstract Approximately 70 % of *Aspergillus westerdijkiae* strains are able to produce ochratoxin A (OTA), a nephrotoxic and carcinogenic mycotoxin which have been found in cereal and food commodities. Despite of its importance there is, up to now, no information available about which genes are differentially expressed between *A. westerdijkiae* ochratoxin-producing and non-producing strains. Using cDNA RDA approach we successfully sequenced 231 raw ESTs expected to be enriched in the ochratoxin-producing strain. BLASTX searches against the public databases showed that of these, 205 ESTs (79 %) exhibited significant similarities with proteins of known functions, 28 ESTs (11 %) had matches to hypothetical proteins, and the remaining 27 ESTs (10 %) had no significant hits. EST alignment resulted in a total of 14 non-redundant consensus sequences. Three putative genes encoding oxidoreductases were validated as up-expressed in the OTA producer strain using RT-qPCR approach. The expression of the putative genes encoding a cytochrome P450 family protein, 3-hydroxyphenylacetate-6-hydroxylase, and endoplasmic reticulum oxidoreductin were higher (32-, 2.8- and 20-fold respectively) in the OTA producer strain compared to the non-producer strain.

Keywords Ochratoxin · *Aspergillus westerdijkiae* · RDA · Oxidoreductases

Introduction

Aspergillus westerdijkiae is a filamentous fungus that was dismembered from the *A. ochraceus* taxa [1]. Several strains

of *A. westerdijkiae*, about 70 %, are able to produce ochratoxin A (OTA), a nephrotoxic and carcinogenic mycotoxin that have been found in cereal and food commodities.

Interesting, the cause of the variation of this capability is unknown until now. OTA is a polyketide-derived secondary metabolite consisting of a chlorinated isocoumarin derivative linked to L-phenylalanine. Polyketides are a large class of secondary metabolites assembled by the activity of polyketide synthases (PKS) via a common mechanism that involves the sequential condensation of small carboxylic acids. Some important functional groups are added to polyketide skeletons in post-PKS tailoring steps to complete the final molecules [2]. Despite of its importance, only a small number of genes were designed to be involved in the biosynthetic pathway of OTA in *Aspergillus* species. These include P450 monooxygenase, non-ribosomal peptide synthetase, chloroperoxidase and polyketide synthase genes [3–6].

Subtractive cDNA library has been used to identify specific differentially expressed genes in various fungal species [7–10].

In the present study, our aim was to identify differences in gene expression between *A. westerdijkiae* OTA-producing and non-producing strains grown under permissive conditions for OTA production.

Materials and Methods

Strains and Culture Conditions

The *A. westerdijkiae* strains UEL91 (OTA producer) and ITAL163 (OTA non-producer) were used for the construction of the RDA library. For the construction of the RDA library, conidia were inoculated (a density of approximately 10^7 mL⁻¹) into 250 mL Erlenmeyer flasks containing 100 mL of YES medium (20 g L⁻¹ yeast extract, 200 g L⁻¹

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sucrose). The strains were grown at 25 °C for 96 h in darkness without shaking.

RAPD Analysis

For RAPD analysis, total fungal DNA extraction was performed according to [11]. Randomly amplified polymorphic DNA (RAPD) analysis was performed as described by Fungaro et al. [12].

cDNA Subtracted Library

Based on previous results of the kinetics of OTA production by UEL91 strain on YES medium, 96 h was chosen as the time point for extracting total RNA for library construction. Mycelium of both strain were used for isolation of total RNA using Trizol reagent (Invitrogen). cDNA was prepared from total RNA using the SMART PCR Synthesis Kit (Clontech Laboratories) according to the manufacturer's instructions. Briefly, cDNA from an OTA producing strain (UEL91) was used as the tester and cDNA from OTA non-producing strain (ITAL163) was used as the driver. A double-stranded cDNA sample of each strain was digested with *Mbo*I (Invitrogen Life Technologies), but only the tester cDNA was ligated to the JBam24/12 adapters (Table 1). The first differential product (DP1) was obtained by hybridization (18 h at 67 °C) of the tester and driver cDNAs mixed at a 1:10 ratio, followed by PCR amplification using JBam24 primer. To generate the second (DP2) differential product, RBam24/12 adapters were ligated to the tester and the hybridization was performed at a 1:100 tester/driver ratio.

Cloning and Sequencing of RDA Products

The final RDA products (DP2) were cloned using the TOPO TA Cloning for Sequencing Kit (Invitrogen Life Technologies) according to the manufacturer's instructions. Cloned inserts were sequenced by a standard protocol using BigDye terminator (Applied Biosystems, Foster City, CA). The resulting sequences were compared to the Genbank database using the BLASTX and BLASTN algorithms at the National Center for Biotechnology Information (NCBI). Sequences returning matches with an *E*-value $\leq 10^{-5}$ were annotated and classified based on their putative molecular function and/or biological process.

Reverse Transcriptase Quantitative Real Time PCR for Gene Expression Comparisons Between OTA Producer and Non-Producer Strains After Grown in Permissive Medium

For reverse transcriptase quantitative real time PCR (RT-qPCR), RNA from the strains UEL91 and ITAL163, grown

for 96 h at 25 °C on YES medium, was extracted using Trizol reagent (Invitrogen). First-strand cDNA synthesis was performed with reverse transcriptase (RT M-MLV, Invitrogen) using 1 µg of total RNA as the template as according to the manufacturer's protocol.

Real time qPCR reactions were performed in a PTC 200 DNA Engine Cycler using a Chromo4 Detection System (MJ Research). The oligonucleotides utilized in these experiments are listed in Table 1. The Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen Life Technologies) was used as the reaction mixture to which 0.4 µM of each primer and 2 µL of template cDNA was added to make a final volume of 25 µL. The data were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNAs amplified in each set of PCRs experiments. The relative expression data were obtained using the $2^{-\Delta\Delta CT}$ method. All the experiments were performed with two independent cultures, and each cDNA sample was analyzed in two technical replicates for each primer pair.

Results and Discussion

Here we describe the application of cDNA RDA to identify genes differentially expressed between OTA producer and non-producer strains of *A. westerdijkiae* after growing in YES medium. The strains UEL91 and ITAL163 were selected for the purpose of this research because they showed expressive level of genetic similarity as revealed by RAPD profiles, but contrasted for their ability to produce OTA. This tactic was chosen as an alternative to that adopted by other authors [5, 13], who have made comparisons of gene expression in a single strain growing in two different media (permissive and restrictive medium for OTA production) in order to minimize the detection of transcripts not related to OTA biosynthesis.

Approximately 231 raw ESTs expected to be enriched in the UEL91 strain were successfully sequenced. BLASTX searches against the public databases showed that of these, 205 ESTs (79 %) exhibited significant similarities with proteins of known functions, 28 ESTs (11 %) had matches to hypothetical proteins, and the remaining 27 ESTs (10 %) had no significant hits. EST alignment resulted in a total of 14 non-redundant consensus sequences, which were compared to the Genbank database using the BLASTX algorithm. Only the sequences returning matches with an *E*-value $\leq 10^{-5}$ were annotated and classified based on their putative molecular function and/or biological process using the Gene Ontology Classification System. Table 2 lists the identity of the sequences isolated.

We did not identify any gene sequences previously substantiate to be involved in the biosynthetic pathway of OTA in *A. westerdijkiae* (= *A. ochraceus*) [3, 6, 13]. However among

Table 1 List of primers used in this study

Name	Sequence 5'–3'	Annealing temperature (°C)
JBam-12	GATCCGTTTCATG	–
JBam-24	ACCGACGTCGACTATCCATGAACG	–
Rbam-12	GATCCTCGGTGA	–
Rbam-24	AGCACTCTCCAGCCTCTCTCACCGAG	–
P450-EST4-F	TTGTAGGGTTGCTGCGATT	60
P450-EST4-R	GCCAACAACAAGGAAAGCC	60
P450-EST5-F	GTTGCTCTATCCACCGTTCT	60
P450-EST5-R	CTTTACAGTTCTTCGCCTCG	60
Oxi-1-EST9-F	CCAAACTGTGCGAAATGCGG	60
Oxi-1-EST9-R	GACGAGACGAAGAACGGCGA	60
G3PDH-F	CGGCTTCGGTCGTATTGG	60
G3PDH-R	TGGAGGAGGGGATGATGTT	60

all of the ESTs here identified, three of them (EST4, EST5 and EST9) particularly attracted our attention. The EST4 and EST5 encode cytochrome P450 enzymes (CYPs) and EST9 encodes an oxidoreductin.

As revised by Cresnar and Petric [14], CYPs have been described as implicated in many cellular processes and playing diverse roles. These enzymes catalyze the conversion of hydrophobic intermediates of primary and secondary metabolic pathways, detoxify natural and environmental pollutants and allow fungi to grow under different conditions [15]. Regarding to mycotoxins, the genes associated with their biosynthesis are often found physically linked or clustered within a giving region of a genome. Many studies have been done on deciphering gene clusters encoding enzymes involved in mycotoxins production [16–18]. Exhaustive studies on aflatoxin B₁ (AFB₁) biosynthesis revealed that six CYPs are involved [18]. As mentioned, it has also been suggested that two putative p450-type monooxygenase genes, p450-H11 and p450-B03, may be involved in OTA biosynthesis in *A. ochraceus* [6]. On the other hand, cytochrome P450 may be accumulated in response to mycotoxins because chemical detoxifications are important mechanisms to protect fungal cells against self-toxicity [19].

Two CYPs were identified in the present study as up-expressed in OTA production strain. The EST4, (GenBank accession number GU644491) which represents 8.6 % of RDA clones analyzed, showed high identity (71 %) to an *A. oryzae* cytochrome P450 family protein (CYPX) whose function is unknown. The EST5, (GenBank accession number GU644492) which represents 0.4 % of RDA clones analyzed, showed high identity (85 %) to an *A. oryzae* 3-hydroxyphenylacetate-6-hydroxylase (CYP504B1) which

is involved in the degradation of aromatic compounds. This enzyme converts 3-hydroxy- and 3,4 hydroxyphenylacetate to homogentisate and 2,3,5, trihydroxyphenylacetate, respectively [14]. To confirm that the two putative CYPs genes identified in the present study are differentially expressed in the OTA producer strain, we used quantitative real time RT-PCR (RT-qPCR), to compare transcription levels between the UEL91 and ITAL163 strains after growing in permissive YES liquid medium. The expression of the genes encoding EST4 and EST5 were higher (32.0- and 2.8-fold respectively) in the OTA producer strain compared to the non-producer strain.

Another gene transcript, the EST9 (GenBank accession number GU644490) enriched in the UEL91 strain also attracted our concentration. The EST9, which represents 0.4 % of RDA clones analyzed, showed high identity (97 %) to an *A. clavatus* endoplasmic reticulum oxidoreductin 1 (ERO1), which is implicated in protein folding. To confirm that the *ERO1* gene is differentially expressed in the OTA producer strain we used the RT-qPCR approach. The expression of the gene encoding EST9 were higher (20-fold) in the OTA producer strain compared to the non-producer strain.

Concluding, we report herein the results of the first effort to identify genes that are differentially expressed between OTA producer and non-producer strains of *A. westerdijkiae*. Three putative genes encoding oxidoreductases were validated as up-expressed in the OTA producer strain using the RT-qPCR approach. Information generated in our study provides substance for future works, mainly to reveal the functionalities of these gene products and their utility to monitor OTA production in agricultural commodities.

Table 2 Summary of computational analysis of sequenced inserts from the cDNA-RDA library, enriched with the OTA-producing strain of *Aspergillus westerdijkiae*

EST designation	Putative molecular function and/or biological process ^a	Description	Best hit/number access ^b	<i>E</i> value ^c	Identity (%)	Redundancy
EST1	Catabolism protein	Proteasome component Pre 6 putative	<i>Aspergillus nidulans</i> FGSC A4 CBF73792	3e ⁻⁴⁷	94	68
EST2	Metabolism	AMP-binding enzyme putative	<i>Aspergillus flavus</i> NRRL3357 XP002378810	1e ⁻¹⁶³	81	60
EST3	Transferase activity	<i>N</i> -glycosyl-transferase	<i>Aspergillus nidulans</i> FGSC A4 CBF77614	3e ⁻⁷⁴	93	23
EST4	Metabolism/carrier electron	Cytochrome P450 family protein putative	<i>Aspergillus oryzae</i> 3.042 EIT781661	3e ⁻¹⁰⁶	71	20
EST5	Metabolism/carrier electron	Cytochrome P450 phenylacetate hydroxylase putative	<i>Aspergillus oryzae</i> RIB40 XP001818548	2e ⁻¹⁰³	85	1
EST6	Intracell sinalizator	Cell division control protein Cdc25/Ras1 guanine nucleotide exchange factor putative	<i>Aspergillus oryzae</i> RIB40 XP001822457	3e ⁻⁶³	75	20
EST7	Glutamate metabolism	Glutamate decarboxylase I	<i>Aspergillus oryzae</i> RIB40 XP001826915	4e ⁻¹⁰³	94	7
EST8	Metabolism/carrier electron	Pyridine nucleotide-disulphide oxidoreductin family protein, putative	<i>Aspergillus fumigatus</i> CAE47920	1e ⁻¹³⁶	90	5
EST9	Protein folding/electron carrier activity	ER oxidoreductin	<i>Aspergillus clavatus</i> XP001276620	4e ⁻⁵²	97	1
EST10		Conserved hypothetical protein	<i>Aspergillus flavus</i> NRRL3357 XP002375216	4e ⁻⁰⁹	74	1
EST11		Conserved hypothetical protein	<i>Aspergillus fumigatus</i> XP752544	8e ⁻⁵³	65	16
EST12		Conserved hypothetical protein	<i>Aspergillus fumigatus</i> XP753856	7e ⁻⁴⁴	68	5
EST13		Conserved hypothetical protein	<i>Neosartorya fischeri</i> XP001265177	7e ⁻⁴⁹	83	2
EST14		Conserved hypothetical protein	<i>Aspergillus nidulans</i> XP661540	1e ⁻¹³	49	2

^a Putative molecular function and/or biological process^b Accession number of the gene products in the GenBank database^c *E* value according to information from BLASTX searches of the non redundant database at NCBI

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