Scientific paper

Progesterone-induced Gene Expression Profile of the Filamentous Fungus *Cochliobolus lunatus*

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Received: 03-01-2008

Abstract

Suppression subtractive hybridization (SSH) was employed to study differential gene expression upon progesterone treatment of the filamentous fungus *Cochliobolus lunatus*, a plant and opportunistic human pathogen. The transcription profile of progesterone-induced vs. non-induced *C. lunatus* revealed changes in the number of genes involved in facilitated and vesicle mediated transport, amino acid and derivative metabolism, protein biosynthesis, cell wall biogenesis, lipid metabolism, carbohydrate metabolism, and generation of precursor metabolites and energy. These results suggest that progesterone induces a global adaptive stress response in the organism. Such a response is not surprising, as the steroidal ring structure is similar to certain antifungal plant defense compounds. In *C. lunatus*, the conversion of such molecules to hydroxylated and less-toxic substances is mediated by enzymes of the cytochrome P450 superfamily, however little is known of the genes encoding them. We identified several putative cytochrome P450 cDNA sequences and quantitatively analyzed their relative mRNA levels upon progesterone induction using Real-time RT-PCR. None of the selected cytochromes P450 showed significant up-regulation (more than 2 fold induction). As an additional inevitable consequence of the large-scale sequencing of cDNA clones, valuable insight into the genome of this non-model organism was obtained.

Keywords: Progesterone; cytochrome P450; SSH; gene expression; filamentous fungi; *Cochliobolus lunatus*.

1. Introduction

Among secondary metabolism bioconversions carried out by fungal cytochromes P450 (CYPs), the bioconversion of steroids holds valuable biotechnological potential.[1] The exploitation of this potential has been limited due to the complexity and low catalytic activity of microbial CYPs with known functions.[2] In most cases, however, the P450 monooxygenase (gene) involved in a specific bioconversion has not even been identified.[3]

The filamentous fungus *Cochliobolus lunatus* has progesterone hydroxylating activity at position 11β, as well as 7α and 14α, and oxidizes the 11β-hydroxy group to the ketone.[4–7] The gene encoding steroid 11β-hydroxylase, the cytochrome P450 responsible for this conversion is presently still unknown, mainly because of the poor amino acid sequence similarity between mammalian 11β-hydroxylases and putative fungal CYPs, capable of such a conversion.

Fungal cytochromes P450 have been shown to be induced by their substrates. In the filamentous ascomycete *Aspergillus niger* and basidiomycete *Phanerochaete chrysosporium* for example, benzoate para-hydroxylase (CYP53) gene expression was induced with benzoic acid.[8,9] Steroid-hydroxylating activity was shown to be induced in *C. lunatus* by progesterone, as well as plant defense compounds.[10–12] In fungi, exogenous steroids induce not only the enzymes directly involved in their bioconversion, but also elicit a global response of the organism affecting cell growth, morphogenesis, and virulence.[13] The zygomycete *Rhizopus nigricans* has evolved defense mechanisms which include heat shock protein synthesis, 11-α hydroxylase expression and plasma membrane progesterone receptor activation.[14–16]

To gain further insight into the secondary metabolism molecular mechanisms that respond to the addition of steroids in *C. lunatus*, and with the specific aim to identify up-regulated progesterone converting cytochrome P450 genes, we have used Suppression Subtractive Hybridization (SSH). Not only is this screening technique useful in studying differential gene expression during specific biological processes, it is also valuable as it provides a glimpse into the genome of a non-model organism.[17] As somewhat of an alternative to the numerous fungal genome sequencing projects completed in recent years, our study provides an insight into the genome of *C. lunatus*. Its conidial anamorph *Curvularia lunata* is a phytopathogen invading cereal crops (maize, rice, milo) and grasses.[18,19] In rare cases, *Curvularia* also infects humans, causing diseases of the respiratory tract, skin, endocardium, central nervous system and cornea.[20–22]

2. Experimental

2. 1. Organism and Culture Conditions

The strain obtained from the strain collection of the Friedrich Schiller University of Jena, Germany, was listed as teleomorph *Cochliobolus lunatus* m118. It is also deposited at the Mycothèque de L'Université Catholique de Lovain, Belgium, as MUCL 38696, and designated as non-compatible anamorph *Curvularia lunata* var. *lunata*.[23] The fungus was grown as described previously.[24]

2. 2. Progesterone Induction of C. lunatus

Seven-day-old mycelium from malt extract medium (MEM) agar slants was inoculated in 100 ml MEM, pH 5.5 and incubated for 20–24 h on a rotary shaker at 180 rpm and 28 °C. Fungal pre-culture (10 ml) was transferred into fresh medium (100 ml) and cultivated for 24 h, under the same conditions. For the induction experiment, mycelium (700 mg wet weight) was suspended in 10 ml phosphate buffer saline (PBS) (0.75 mM Na_3PO_4, 0.21 mM EDTA, 0.04 mM reduced glutathione; pH 5.5) supplemented with progesterone (100 μg/ml final concentration) and incubated for 3 h on a rotary shaker at 110 rpm and 28 °C. For the control experiment, mycelium was transferred into PBS buffer supplemented with solvent (dimethylformamide) alone.

2. 3. Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from progesterone-induced and non-induced *C. lunatus* following the SV Total RNA Isolation System technical manual No.048 (Promega, USA) protocol. The purity, integrity and concentration of isolated RNA were determined on Agilent 2100 bioanalyzer (Agilent Technologies, USA). For differential SSH analysis, the cDNA libraries (CLI – progesterone-induced; CLN – non-induced) were constructed from total RNA using the BD Smart PCR cDNA Synthesis Kit (BD Biosciences Clontech, USA). This kit was also used to generate the progesterone-induced cDNA library. Briefly, 1 μg of total RNA was reverse transcribed using the CDS primer and PowerScript reverse transcriptase to produce the first-strand cDNA. Double-stranded cDNA was synthesized with the SMART II-A oligo in 17 cycles of PCR amplification using an Advantage 2 DNA polymerase (BD Biosciences Clontech, USA). cDNA used in Real-time RT-PCR experiments was synthesized with SuperScript II Reverse Transcriptase (Invitrogen, USA).

2. 4. Suppression Subtractive Hybridization

Suppression subtractive hybridization (SSH)[25] was used to compare gene expression profiles of progesterone-induced (CLI) and non-induced (CLN) *C. lunatus*. The SSH procedure was performed with the Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech, USA) as directed by the manufacturer. Briefly, the CLI cDNA pool, from which progesterone up-regulated (target) sequences were sought, was used as tester, and the CLN cDNA population for comparison was utilized as the driver. For the determination of down-regulated sequences the CLN cDNAs were employed as tester, and CLI cDNA population was used as driver. Both tester and driver cDNA populations were digested with *Rsa*I. The tester cDNA was divided into two equal portions, and each was ligated with a different adaptor (Adaptor 1 and Adaptor 2R, respectively). An excess of driver cDNA was added to each tester sub-population and hybridized for 8 h at 68 °C, allowing equalization of high and low abundance cDNAs. For the second hybridization, excess driver cDNA was added to the pooled mixture of the primary hybridizations and incubated at 68 °C for 18 h. After an extension reaction to fill in sticky ends, PCR primer 1 that anneals to each of the adaptors was used to PCR amplify only those molecules with both adaptors (allowing exponential amplification). In the second PCR amplification, nested primers 1 and 2R were utilized to enrich differentially expressed sequences and to further reduce background.

2. 5. Cloning of Subtracted cDNA Libraries, Plasmid Isolation and Sequencing

Following the manufacturer's protocol, the subtracted CLI and CLN cDNA libraries were ligated into pCR II vector using the TA Cloning Kit Dual Promoter (Invitrogen, USA) and transformed into TOP10F' competent cells (Invitrogen, USA). After blue-white screening for inserts, all white colonies were picked for plasmid isolation. From colonies grown overnight in LBA medium recombinant plasmids were isolated with the Nucleospin® Multi-96 Plus Plasmid Kit (Macherey-Nagel, Germany). DNA

sequencing of plasmid DNA was performed by Macrogen (Korea).

2. 6. Acquiring Cytochrome P450 Gene Fragments

The *C. lunatus* genomic library, created with the ZAP Express Predigested Vector Kit (Stratagene, USA) according to manufacturer's recommendations, was used to obtain cytochrome P450 sequences. DNA probes were generated using gene specific primer sets (gs1, gs2) constructed on the basis of putative P450 gene sequences from SSH cDNA clones. Probes were labeled with dioxygenin (DIG), using the DIG System (Roche Molecular Biochemicals, USA), either by random priming or PCR. Plaque hybridization was performed as described previously.[26] Positive clones were plaque purified. The pBK-CMV plasmids were excised according to the Stratagene protocols using the ExAssist helper phage (Stratagene, USA) and sequenced by Sequiserve (Germany). Alternatively, elongated or full-length P450 gene fragments were PCR amplified with a gene specific primer (gs1 or gs2) and either 3' SMART CDS Primer IIA or SMART IIA Oligo (Table 1). In this case, the PCR reaction was performed using the progesterone-induced cDNA library of *C. lunatus*, KOD polymerase (Novagen, USA) and the following conditions: 75 °C for 5 min, 95 °C for 1 min; and 25–30 cycles of 95 °C for 30 sec, 68 °C for 2 min.

2. 7. Real-time RT-PCR

PCR primers and TaqMan MGB probes, FAM dye-labelled, were constructed by the Assay-by-design Service (Applied Biosystems, USA) for five cytochrome P450 genes identified in the SSH analysis. The reaction was performed on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, USA) using the TaqMan Universal PCR Master Mix (Applied Biosystems, USA) according to manufacturer's instructions. Eukaryotic 18S rRNA (Applied Biosystems, USA) was selected as an endogenous control to correct for potential variations in RNA loading. The relative expression of specific cytochrome P450 to that of ribosomal 18S RNA was determined using Sequence Detection System software. The level of gene expression in progesterone-induced mycelia relative to non-induced mycelia was determined using the $2^{-\Delta\Delta Ct}$ method.[27] All experiments were performed in triplicates.

2. 8. Annotation of cDNA Clones

SSH cDNA sequences were trimmed for vector and adaptor contamination using ContigExpress software from the Vector NTI Advance 9.0 package (Invitrogen, USA). The non-redundant protein database at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was searched with the obtained DNA sequences using blastx.[28] To search for homologous sequences multi-fungi blast was also performed against fungal genomic sequences, gene predictions, and predicted protein sequences for the organisms hosted at the Fungal Genome Initiative (FGI), Broad Institute (http://www.broad.mit.edu/annotation/fgi/). Since many sequences produced hits to hypothetical or predicted proteins with unknown function, the closest relative of *C. lunatus* whose genome was sequenced and annotated, *Stagonospora nodorum*, was screened with the blastx algorithm to identify conserved protein domains. To further functionally categorize cDNA clones the well-studied model fungus *Saccharomyces cerevisiae* was searched for homologues at MIPS database (http:// mips.gsf.de/genre/proj/yeast/index.jsp).

3. Results and Discussion

Besides mammalian steroid converting systems, enzymes catalyzing steroid transformations were also found in fungi and different strains of bacteria.[29] In the plant pathogen *Cochliobolus lunatus* steroid bioconversi-

Table 1: Oligonucleotide primers and adaptors (5'> 3')

	name	Sequence	name	sequence
A	0-G11-gs1	CATAGTTGAGGAGCATGTGTGC	2-A12-gs2	GGTGTCGATTCGGCTGCCAAGG
	0-G11-gs2	CATGATTGCAGTCCCGATGG	2-B4-gs1	CAATGTCCCTGTTACCTCCAAT
	1-A5-gs1	CACGGCCCGAGGAGCTGTATAG	2-B4-gs2	CAGTTCCCGAGCACGAAATC
	1-A5-gs2	GCCCTGGATCTCTACCTTGTATTCG	2-H3-gs1	TGGATGCAGTCGGCGTGTTGAG
	2-A12-gs1	CCAGCATAGCAAGGGTTTGACC	2-H3-gs2	GCATTATCCGCACCAGGTTCCAG
B	CDS primer*	AAGCAGTGGTAACAACGCAGAGTACT(30)(A/C/G/T)(A/G/C		
	SMART II-A oligo*	AAGCAGTGGTAACAACGCAGAGTACGCGGG		
	Adaptor 1	CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT		
	Adaptor 2R	CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT		
	PCR primer 1	CTAATACGACTCACTATAGGGC		
	Nested PCR primer 1	TCGAGCGGCCGCCCGGGCAGGT		
	Nested PCR primer 2R	AGCGTGGTCGCGGCCGAGGT		

A – primers designed for cytochrome P450 elongation and DIG-labeled probe construction; B – primers and adaptors used in SSH library construction * – primers used for cytochrome P450 elongation

ons carried out by microsomal cytochromes P450 were also reported. Since steroid hydroxylases represented only a minor proportion of the CYP microsomal membrane constituent population,[30] the Suppression Subtractive Hybridization (SSH) technique, that equalizes high and low-abundance transcripts, was employed to examine gene expression during exposure of *C. lunatus* to progesterone (100 µg/ml; 3h).

3. 1. Characterization of *C. lunatus* SSH Libraries

Nucleotide sequences of partial cDNA clones (ranging from 150 to 850 bp) from the progesterone-induced (CLI) and non-induced (CLN) SSH libraries of *C. lunatus* were used in the blastx search against the non-redundant protein database at NCBI, and the *Stagonospora nodorum* and *Saccharomyces cerevisiae* genome databases at FGI, Broad Institute. From the 452 CLI cDNA clones and the 480 CLN cDNA clones sequenced (Supplement 1 and 2, respectively, deposited at http://acta.chem-soc.si/55/55-1-S1.xls, http://acta.chem-soc.si/55/55-1-S2.xls), 66% of progesterone-induced and 32% of non-induced transcripts produced hits with significant similarity (expected value (*E*-value) less than 1.0E-5) to known sequences (Figure 1). The blastx search identified 14% and 13% of hypothetical proteins with unknown function in CLI and CLN respectively. The significant proportion of cDNA fragments that did not match known protein entries in public databases (19% in CLI and 53% in CLN) is probably the result of the presence of low-complexity sequences un-likely to be derived from mRNA or chimeras generated as artifacts during the subtraction procedure.[31]

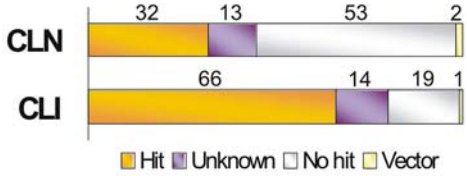

Figure 1: Distribution of progesterone-induced (CLI) and non-induced (CLN) cDNA clones from *C. lunatus* SSH libraries. Hit – transcripts with homology to known proteins; Unknown – transcripts with homology to predicted proteins with unknown function; No hit – transcripts with no significant similarity to sequences in NCBI and FGI databases; Vector – plasmids without inserts. Numbers above bars represent distribution of clones into listed categories in percent.

3. 2. Identification of Progesterone-Induced and Non-Induced *C. lunatus* Transcripts

In order to assign molecular function to *C. lunatus* cDNA clones, we compared them to homologous putative proteins in *S. nodorum,* the closest sequenced relative of *C. lunatus*. However, most of the automatically predicted proteins and annotated conserved protein domains in the *S. nodorum* database have no experimental validation. Therefore, gene function was designated based on information from homologous sequences in the established model organism *S. cerevisiae* at the MIPS Comprehensive Yeast Genome Database (CYGD). We constructed a list of putative protein-coding open reading frames (ORFs), corresponding to 142 and 94 homologues in the CLI and CLN libraries, respectively (Supplement 3, deposited at http://acta.chem-soc.si/55/55-1-S3.xls). By comparing sets of ORFs in the CLI and CLN gene list using Phyton 2.5.1 programming language (http://www.python.org/), we found only 14 ORFs common to both lists and excluded them from further analysis.

3. 3. Cytochrome P450-dependent Response to Progesterone

P450-mediated progesterone hydroxylation was reported in *C. lunatus*.[30] In an effort to identify the elusive gene, encoding the candidate P450 enzyme, the *C. lunatus* cDNA clones producing blastx hits to putative P450 monooxygenases were further investigated (Figure 2).

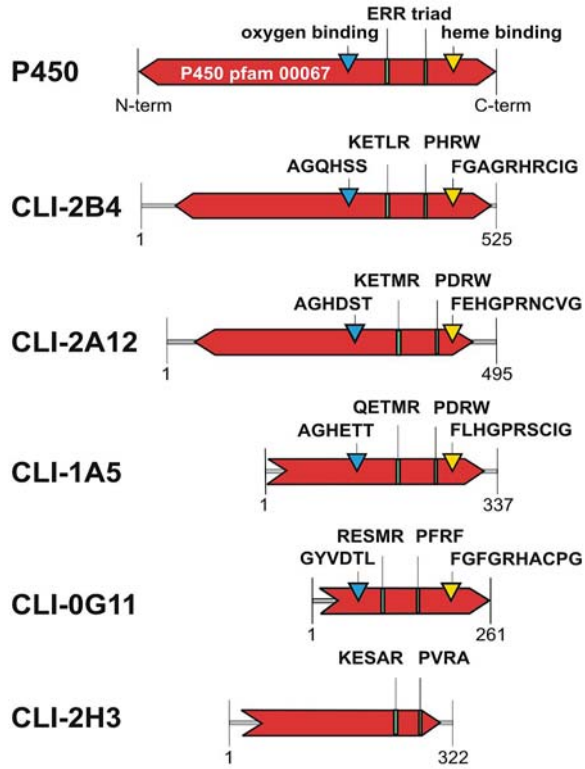

Figure 2: Schematic representation of putative cytochrome P450 sequences obtained from the progesterone-induced *C. lunatus* SSH library. Top: Cytochrome P450 conserved protein domains. Below: aligned cDNA fragments with P450 characteristic domains. Sequence data is deposited in Supplement 1, at http://acta.chem-soc.si/55/55-1-S1.xls, under the depicted cDNA clone name.

Some sequences produced hits to known and functionally characterized cytochromes P450. The translated cDNA clone CLI-2B4 (525 aa) showed high similarity (86% identity) to sterol 14-α demethylase (ERG11) from *Leptosphaeria maculans*, an essential enzyme for biosynthesis of ergosterol, an important constituent of fungal membranes, and main target for azole antifungal compounds. The amino acid sequence (495 aa) encoded by clone CLI-2A12 was homologous to sterigmatocystin biosynthesis P450 monooxygenase from *Aspergillus nidulans* (42% identity). The secondary metabolite sterigmatocystin, a precursor of aflatoxins, is an acutely toxic and carcinogenic mycotoxin acting as an inhibitor of DNA synthesis.[32,33] Among highly similar hits (52% identity) to translated clone CLI-1A5 (340 aa) was the *Aspergillus fumigatus* putative cytochrome P450, FUM15. This enzyme was identified as a constituent of the fumonisin biosynthetic gene cluster comprised of 15 genes in *Giberella moniliformis*.[34] Fumonisins are structurally similar to sphingolipid intermediates which can disrupt sphingolipid metabolism.[35] cDNA clones CLI-0G11 and CLI-2H3 were identical to putative cytochromes P450 with unknown function from *Chaetomium globosum* (49% identity) and *Stagonospora nodorum* (42% identity), respectively. P450 sequences were also found in the non-induced SSH library. The translated sequence of clone CLN-3B10 (165 aa) produced a hit to linoleate diol synthase from *Cercospora zeae-maydis* (50% identity). CYP alkane hydroxylase from *Aspergillus fumigatus* was homologous (44% identity) to the translated sequence of fragment CLN-3C6 (238 aa). Another putative cytochrome P450 sequence in the non-induced library was the CLN-4A12 clone with 39% identity to *Aspergillus fumigatus* putative cytochrome P450.

With the aim to gain information on cytochrome P450 components involved in the response to treatment with progesterone, we also performed a quantitative expression analysis of five cytochrome P450 genes with Real-time RT-PCR. Relative CYP mRNA levels of CLI-0G11 and CLI-1A5 were increased 1.5 fold and 1.3 fold, respectively, in the progesterone-induced *C. lunatus*. Expression of CLI-2B4 was 1.5 fold decreased, whereas expression levels of the other two genes (CLI-2A12 and CLI-2H3) did not differ among the induced and non-induced samples. These results were in accordance with findings of Vitas et al.[30] who observed no evidence of increased microsomal P450 content upon induction with progesterone in *C. lunatus*, but instead detected higher steroid hydroxylase activity, as did Jänig et al.[4] It is also possible that the 3 h incubation time of *C. lunatus* mycelia in progesterone-containing media is too long for monitoring changes at the transcriptome level. Banerjee et al.[36] in their study performed on commercial and custom-made yeast microarrays reported that the steroid response was fast and transient, peaking at 30 min and noted that levels of induction decreased with time.

3. 4. Global Changes in the Expression Profile of *C. Lunatus* Upon Progesterone Exposure

Several recent studies report that exogenous progesterone is perceived as cellular stress in fungi, eliciting a global response of the organism observed at the trancriptome[13,36] and proteome levels[37]. A stress response involving similar secondary metabolism molecular mechanisms and components was reported for the filamentous fungus *Aspergillus fumigatus* during antifungal drug exposure.[38] Since we could not directly associate a particular cytochrome P450 enzyme with conversion of the xenobiotic progesterone to a less toxic metabolite(s), we suspected that other mechanisms could be involved in detoxification. The lists of candidate *Saccharomyces cerevisiae* ORFs, corresponding to homologous CLI and CLN genes, were subjected to Genecodis,[39] by selecting biological process and molecular function GOSlim co-occurrence annotation (http://genecodis.dacya.ucm.es/). The major categories of progesterone-induced (CLI) genes (Figure 3A) involved in particular biological processes include facilitated (23%) and vesicle-mediated (4%) transport, lipid metabolism (8%), amino acid metabolism and protein biosynthesis (6% and 7%, respectively), protein modification (7%), carbohydrate metabolism and generation of precursor metabolites and energy (3% and 6%, respectively), cell wall organization and biogenesis (4%), cell cycle and DNA metabolism (3% and 3%, respectively), and response to stress (3%). The genes in the non-induced CLN library fell into the following categories (Figure 3B): transport (9%) and vesicle-mediated transport (4%), amino acid metabolism and protein biosynthesis (11% and 15%, respectively), protein modification (5%), organelle organization and biogenesis (6%), generation of precursor metabolites and energy (5%), DNA metabolism, signal transduction, and response to stress (4% each).

Major molecular function categories of genes in the CLI library were transporter activity (16%), oxidoreductase activity (13%), metabolic enzyme activity (including transferases (10%), hydrolases (7%), lyases (5%), ligases (3%), and peptidases (3%), structural molecule activity (8%), protein binding (4%), signal transduction (including protein kinases (4%) and phosphoprotein phosphatases (2%), transcription regulators, DNA binding proteins and helicase activity (3%, 2%, and 2%, respectively). The genes in the CLN library were functionally classified into transporter activity (8%), oxidoreductase activity (10%), metabolic enzyme activity (including transferases (11%), and hydrolases (8%)), structural molecule activity (11%), protein binding (13%), DNA and RNA binding protein (4%, and 5%, respectively) categories.

In *C. lunatus*, exogenous progesterone induces changes in transcription levels of transport facilitation genes involved in drug and xenobiotic efflux, including several

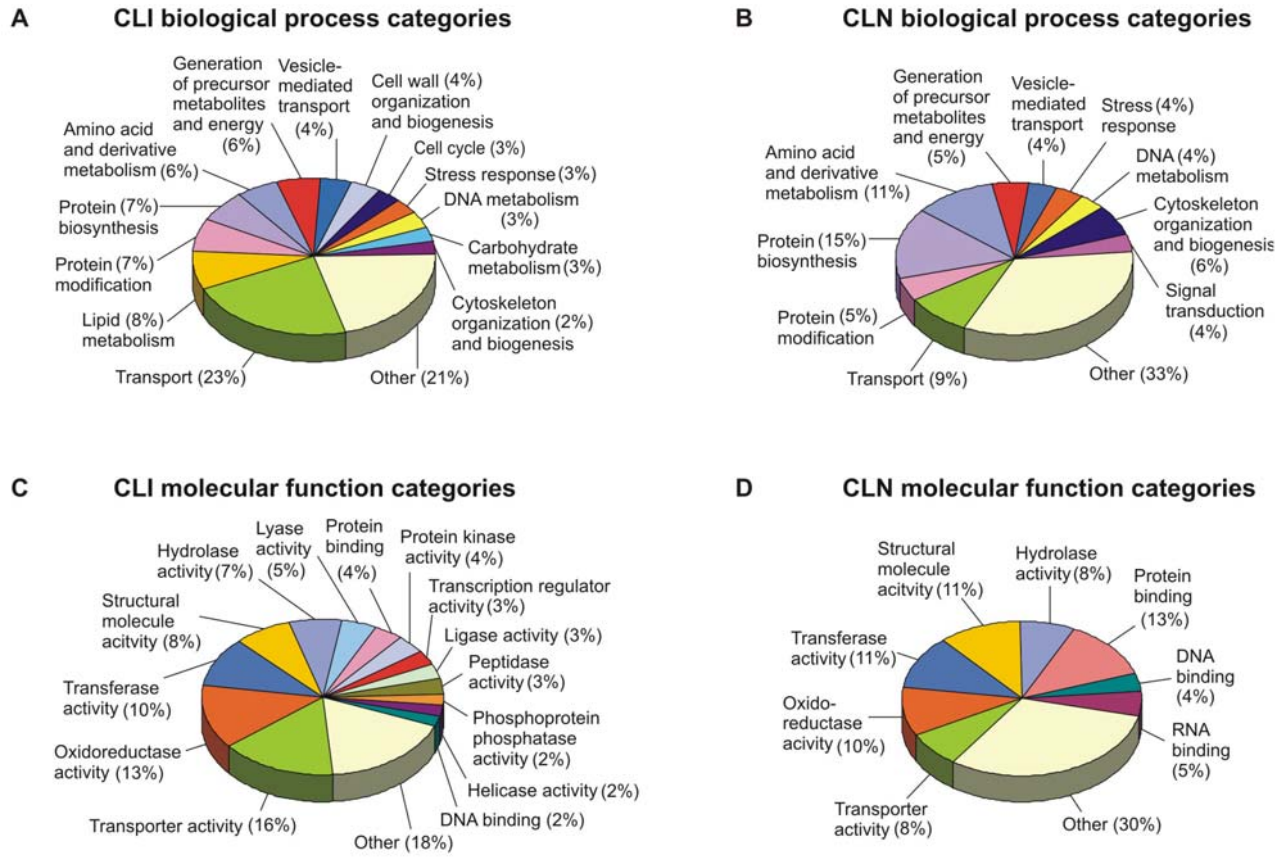

Figure 3: Biological process (A, B) and Molecular function (C, D) categories of S. cerevisiae ORFs homologous to genes found in progesterone-induced (CLI) and non-induced (CLN) *C. lunatus* SSH libraries, as predicted by Genecodis[39] GOSlim Process and GOSlim Function co-occurrence annotation, respectively (http://genecodis. dacya.ucm.es/).

ATP-binding cassette (ABC) transporter genes and other genes of the Major Facilitator Superfamily (MFS) transporters. This phenomenon was observed in other fungi as well.[40,41] The most recent study on multidrug resistance transcriptional networks in yeast, reports of two pathways responsible for pleiotropic drug resistance; the early-adaptation PDR pathway and late-response ergosterol biosynthesis pathway.[42,36] The first pathway, most efficiently induced by progesterone, acts through increased transcriptional activation of ABC transporters, membrane phospholipid metabolism and transport proteins. This response is followed by the induction of genes involved in ergosterol biosynthesis, including ERG11, also found in the progesterone-induced SSH cDNA library. Additionally, the most commonly observed resistance mechanism in azole resistant yeast strains involves the up-regulation of transporter genes of the ABC transporter family.[43] Moreover, ABC transporters and MFS transporters in fungal plant pathogens are involved mycotoxin secretion during host invasion.[40,44]

Facilitated transport requires a significant amount of ATP and other energy-rich molecules, which in turn demand increased carbohydrate utilization, the generation of precursor metabolites and energy, and increased oxidoreductase activity. Progesterone treatment also influenced cell wall biogenesis and lipid composition of plasma membrane. Genes involved in the cell cycle control and DNA metabolism were affected as well. In progesterone-induced *C. lunatus*, protein biosynthesis and amino acid derivative metabolism categories were half of that observed in the CLN library. Our combined results agree with microarray experiments performed on yeast exposed to progesterone, where up-regulation of transport facilitation genes, general oxidoreductases/ dehydrogenases, proteins involved in carbohydrate utilization, regulation and transport, cell wall biogenesis, cell cycle control and mitosis was reported. Genes involved in glycogen and trehalose metabolism, purine biosynthesis and ribosomal protein synthesis were down-regulated.[13]

4. Conclusions

Progesterone-induced gene expression was qualitatively evaluated with Suppression Subtractive Hybridization (SSH) in the filamentous fungus *Cochliobolus lunatus*. Our findings suggest that progesterone elicits a global stress response of the organism observed at the transcripto-

me level. A higher number of genes involved in the transport of xenobiotics (ABC transporters and others of the MFS superfamily), in the maintenance of appropriate plasma membrane lipid composition, cell wall biogenesis, carbohydrate utilization and generation of energy rich molecules was present in the progesterone-induced SSH library, while protein biosynthesis genes, amino acid and derivative metabolism genes were comparatively overrepresented in the non-induced SSH library. These results corroborated the quantitative microarray experiments where gene ontology analysis indicated that progesterone induces a fast and transient pleiotropic stress response in *Saccahromyces cerevisiae* and *Candida albicans*.[36]

Since antifungal secondary metabolites possess membranolytic, fungitoxic and antibiotic activities, fungi confront these compounds employing various strategies, including avoidance, tolerance and enzymatic degradation.[45] In the plant pathogen *C. lunatus*, the conversion of exogenous steroids to hydroxylated and less-toxic compounds is mediated by enzymes of the cytochrome P450 superfamily, however little is known of the genes encoding them, or their biological role. Few investigations have been conducted to reveal their probable role in the biotransformation of plant defense compounds such as steroidal alkaloids α-tomatine and tomatidine, which roughly resemble steroidal ring structure.[12,46] In the present study, the involvement of cytochromes P450 in detoxification of a steroid substrate could not be confirmed. Gene expression and functional characterization experiments of identified cytochromes P450 with unknown function are currently underway.

Last but not least, the importance of this large scale study lies not only in the identification of differentially expressed genes in *C. lunatus* during exposure to progesterone with SSH analysis, but serves to gain important insight into the genome of a non-model organism.[17]

5. Acknowledgement

Authors would like to thank Jelka Lenarčič and Dubravka Germ for excellent technical assistance. The *C. lunatus* genomic library was kindly provided by Tea Lanišnik Rižner. The work was supported by research project L4-4353 from the Slovenian Ministry for High Education, Science and Technology, by research program grant P1-0104 and by grant awarded to junior investigator Ljerka Lah, both provided by the Slovenian Research Agency (ARRS).

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Povzetek

S pomočjo zaviralne odvzemne hibridizacije (SSH – Suppression Subtractive Hybridization) smo raziskali diferencialno izražanje genov nitaste glive *Cochliobolus lunatus*, rastlinskega in priložnostnega človeškega patogena, po dodatku progesterona. Transkripcijski profil s progesteronom inducirane glive je, v primerjavi z neinducirano glivo, pokazal spremembe v številu genov, udeleženih pri olajšani difuziji in vezikularnemu transportu, presnovi aminokislin in njihovih derivatov, biosintezi proteinov, biogenezi celične stene, presnovi lipidov in ogljikovih hidratov ter nastanku prekurzorskih metabolitov in energije. Ti rezultatinakazujejo, da progesteron v glivi izzove globalni stresni odgovor. Tak odziv ni presenetljiv, saj je steroidni skelet strukturno podoben določenim rastlinskim protiglivnim obrambnim spojinam. V glivi *C. lunatus* poteka pretvorba takih molekul v hidroksilirane in manj toksične snovi s pomočjo encimov naddružine citokromov P450, vendar je o njihovih genskih zapisih le malo znanega. Identificirali smo nekaj zaporedij cDNA za domnevne citokrome P450 in z uporabo PCR v realnem času kvantitativno ovrednotili relativno raven njihove mRNA po indukciji s progesteronom, vendar nobeden izmed izbranih citokromov P450 ni izkazal značilnega zvišanja ravni izražanja (več kot 2-kratna indukcija). Hkrati z določanjem zaporedij velikemu številu klonov cDNA naša raziskava ponuja vpogled v doslej še neznani genom nemodelnega organizma *C. lunatus.*