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ARTICLE

Post-genomic approaches to understanding interactions between fungi and their environment

Ronald P. de Vries[1,2], Isabelle Benoit[2], Gunther Doehlemann[3], Tetsuo Kobayashi[4], Jon K. Magnuson[5], Ellen A. Panisko[5], Scott E. Baker[5], and Marc-Henri Lebrun[6]

[1]Fungal Physiology, CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands; corresponding author e-mail: r.devries@cbs.knaw.nl

[2]Microbiology & Kluyver Centre for Genomics of Industrial Fermentation, Utrecht University, Padualaan 8, 3584 C Utrecht, The Netherlands

[3]MPI for Terrestrial Microbiology, Department of Organismic Interactions, Karl von Frisch Str. 10, 35043 Marburg, Germany

[4]Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan

[5]Fungal Biotechnology Team, Pacific Northwest National Laboratory, Richland, WA 99352, USA

[6]BIOGER-INRA, Grignon, Av Lucien Brétignières, 78 850 Thiverval, Grignon, France

Abstract: Fungi inhabit every natural and anthropogenic environment on Earth. They have highly varied life-styles including saprobes (using only dead biomass as a nutrient source), pathogens (feeding on living biomass), and symbionts (co-existing with other organisms). These distinctions are not absolute as many species employ several life styles (e.g. saprobe and opportunistic pathogen, saprobe and mycorrhiza). To efficiently survive in these different and often changing environments, fungi need to be able to modify their physiology and in some cases will even modify their local environment. Understanding the interaction between fungi and their environments has been a topic of study for many decades. However, recently these studies have reached a new dimension. The availability of fungal genomes and development of post-genomic technologies for fungi, such as transcriptomics, proteomics and metabolomics, have enabled more detailed studies into this topic resulting in new insights. Based on a Special Interest Group session held during IMC9, this paper provides examples of the recent advances in using (post-)genomic approaches to better understand fungal interactions with their environments.

Key words:
(post-)genomics
Aspergillus oryzae
Aspergillus niger
Phycomyces blakesleeanus
Thielavia terrestris
Ustilago maydis

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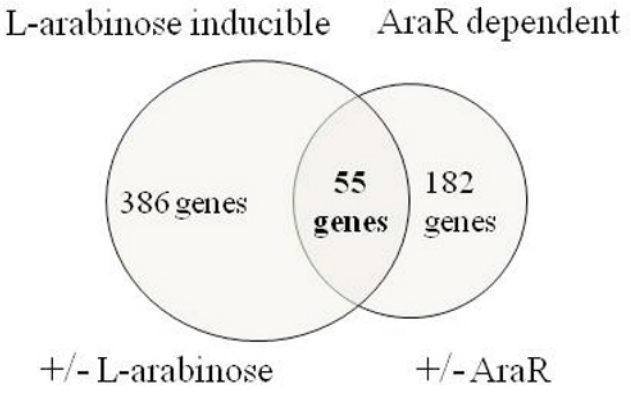
INTRODUCTION

The interaction between fungi and their environment is of major importance for saprobes, symbionts and pathogens and has been a topic of study for many decades. Currently, genome sequences are available for many fungi, including saprobes (Espagne *et al.* 2008, Galagan *et al.* 2003, 2005, Jeffries *et al.* 2007, Machida *et al.* 2005, Martinez *et al.* 2004, 2008, Ohm *et al.* 2010, Pel *et al.* 2007), plant pathogens (Cuomo *et al.* 2007, Dean *et al.* 2005, Kämper *et al.* 2006), human pathogens (Nierman *et al.* 2005) and mycorrhizae (Martin *et al.* 2008, 2010). Sequencing of additional fungal genomes is occurring at an increasing rate in many centres all over the world, but the two largest fungal sequencing programs are running in the US at the Joint Genome Institute of the Department of Energy (http://genome.jgi-psf.org/programs/fungi/index.jsf) and the Broad Institute (http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/fungal-genome-initiative). These initiatives are providing genome resources for a representative subset of the fungi, enabling full-genome comparison of fungal biodiversity.

The availability of fungal genome sequences was followed by the development of post-genomic resources, of which transcriptomics was the first. Initially transcriptomics was mainly available for fungal species which were studied by large consortia of scientists, due to the high costs involved in developing micro-arrays. Most papers on fungal transcriptomics have therefore addressed species such as *Saccharomyces cerevisiae*, various *Aspergillus* species, *Candida albicans*, *Neurospora crassa* and *Magnaporthe grisea* (Andersen *et al.* 2008, Bhadauria *et al.* 2007, de Groot *et al.* 2007, Gasser *et al.* 2007, Gowda *et al*, 2006, Hauser *et al.* 2009, Kasuga *et al.* 2005, Lashkari *et al.* 1997, Mogensen *et al.* 2006, Rossouw *et al.* 2008). However, with the development of RNA (cDNA) sequencing, transcriptomics



GH family	(Glycoside Hydrolase)	16
	α-arabionofuranosidase	4
	β-galactosidase	3
	α-galactosidase	1
	α-rhamnosidase	1
	α-amylase	1
	others	6
Metabolism		12
Transporter		7
Unknown		20

Fig. 1. Transcriptomics of *Aspergillus oryzae* during growth on L-arabinose. Left panel: Comparison of genes that are induced on L-arabinose to genes that are dependent on AraR. Right panel: functional annotation of the 55 genes that are induced by L-arabinose and regulated by AraR.

has become available for any species that has a sequenced genome and transcriptomic studies are now often included in genome papers. Proteomics was soon to follow and many studies on fungal proteomics have been reported in the last eight years (Acero *et al.* 2011, Grinyer *et al.* 2004, Ho *et al.* 2002, Kim *et al.* 2003, 2004, Lim *et al.* 2001, Matis *et al.* 2005).

These post-genomic studies have been aimed at many aspects of fungal biology. In this paper we present examples aimed at understanding how fungi interact with their environment. They were based on presentations at a Special Interest Group meeting during IMC9 in Edinburgh in August 2010.

CONTRIBUTIONS

Transcriptional regulation of genes involved in hemicellulose and cellulose utilization in *Aspergillus oryzae*

XlnR is a fungal transcription factor that regulates xylanolytic and cellulolytic enzymes in *Aspergillus*. *Aspergillus oryzae* XlnR was phosphorylated at low level in the absence of D-xylose, and was hyper-phosphorylated in its presence. Removal of D-xylose caused dephosphorylation of the hyper-phosphorylated forms leading to accumulation of less phosphorylated forms. XlnR activity is probably regulated by reversible phosphorylation (Noguchi *et al.* 2011).

While *Aspergillus niger* and *Aspergillus nidulans* have two XlnR homologs, AraR and GalA, *A. oryzae* has only AraR involved in L-arabinose catabolism. DNA microarray analysis for determining XlnR targets was performed by expression profiles of the XlnR overproducer and the XlnR deletion mutant after exposure to D-xylose for 30 min. The analysis revealed 75 genes as the possible targets of XlnR, including 32 glycoside hydrolases and three esterases for degradation of xylan and cellulose, seven transporters, and three genes for D-xylose catabolism (Noguchi *et al.* 2009). For AraR, two types of DNA microarray analysis were performed. One compared expression profiles of L-arabinose induced and un-induced conditions, and the other compared those in the wild type and the AraR deletion mutant under L-arabinose induced conditions. By combination of the results, 55 genes were identified as possible AraR targets (Fig. 1), including 16 glycoside hydrolases, seven transporters, and several candidate genes for L-arabinose catabolism. The candidate genes for L-arabinose catabolism were expressed as His-tagged proteins in *Escherichia coli*. Analysis of substrate specificity of the recombinant enzymes led to identification of L-arabinose reductase and L-xylulose reductase in *A. oryzae*. The DNA microarray analysis contributed substantially to an in depth understanding of the degradation and utilization of hemicellulose by *A. oryzae*.

Zonal differentiation in sugar beet grown colonies of *Aspergillus niger*

Fungal colonies do not behave as uniform entities, but show differentiation for many aspects of physiology. The first report of this phenomenon described that protein secretion and growth only took place the periphery of colonies of *Aspergillus niger* grown on maltose (Wösten *et al.* 1991). To study this phenomenon in more detail, a specific growth system was developed called the ring-plate system (Levin *et al.* 2007b). This system consists of a round polycarbonate plate with concentric channels that are filled with liquid medium and a perforated polycarbonate membrane (Fig. 2). This membrane allows transport of nutrients and proteins, but the pores are too small to allow passage of fungal hyphae. Using this system it was shown that not only protein secretion and growth, but also overall gene expression is highly differentiated in colonies of *A. niger* grown on maltose or D-xylose (Levin *et al.* 2007a). Part of this differentiation could be attributed to depletion of the carbon source in the zones towards the centre of the colony. However, this was not the case for all gene systems. For instance, genes involved in nitrate utilisation were only expressed in the periphery of the colony, even though there was no significant difference in the nitrate concentration in the zones (Levin *et al.* 2007a).

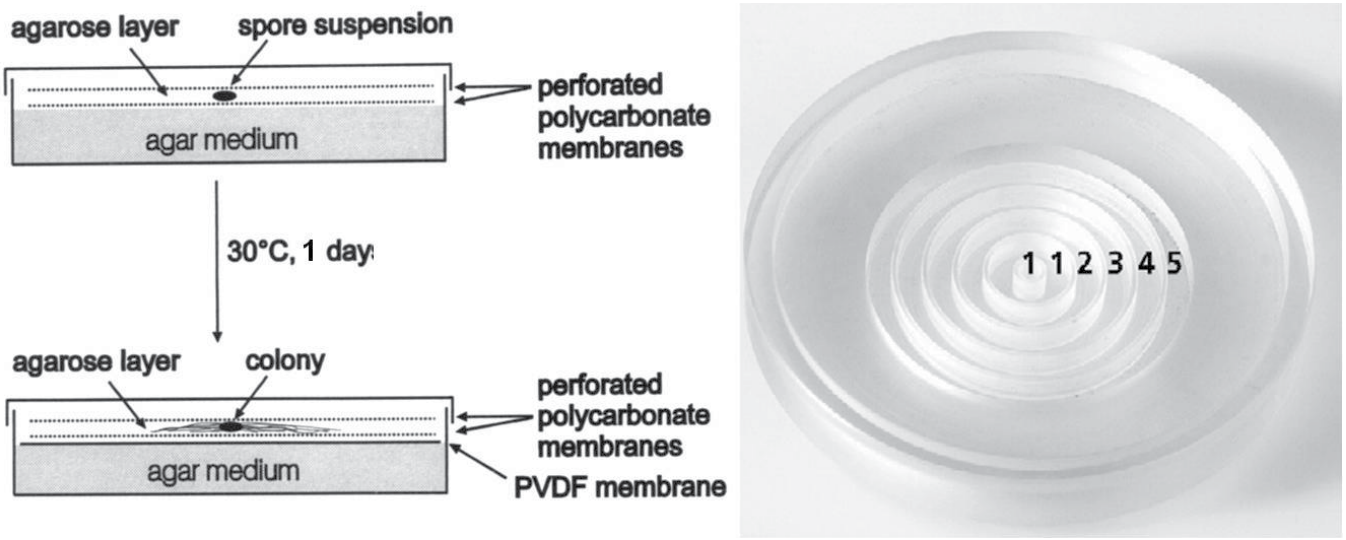


Fig. 2. Schematic presentation of the ring-plate system . Reproduced with permission from Levin *et al*. (2007b).

In nature, fungi do not grow on high levels of pure mono- or disaccharides, but rather on mixtures of polymeric compounds (e.g. plant biomass). To release the monomeric carbon sources that can be taken up by the fungal cell, fungi produce complex mixtures of extracellular enzymes. The complexity of these mixtures was illustrated by a review presenting an overview of *Aspergillus* enzymes involved in plant cell wall degradation (de Vries & Visser 2001). This indicates that in nature fungi aren't subjected to a gradual reduction of a single carbon source, but more likely to substrates with changing compositions and levels.

To study zonal differentiation on natural substrates, *A. niger* was grown on the ring-plate system using sugar beet pulp as a carbon source. Sugar beet pulp is a waste product of the sugar industry and consists mainly of cellulose, xyloglucan and pectin. Growth on this substrate requires enzymatic hydrolysis of the polysaccharides.

Transcriptomic analysis using whole genome micro arrays for *A. niger* demonstrated that differentiation occurred to a lesser extent than on D-xylose and maltose (Benoit & de Vries, unpubl.). In addition, growth and protein secretion were observed throughout the colony, rather than only at the periphery (Benoit & de Vries, unpubl.).

Secretomes: Proteomic clues to fungal life-style choice

Many filamentous fungi have evolved to fill saprobic niches. This life-style, breaking down and utilizing complex biopolymers, requires a variety of hydrolytic enzymes secreted into the immediate environment of the fungus. Examination of the variations in the secretome of a fungus when confronted with different complex or simple substrates can reveal what the fungus is capable of utilizing and which secreted enzymes in its genome are expressed under particular conditions. We grew the zygomycete *Phycomyces blakesleeanus* and the ascomycete *Thielavia terrestris* on four complex carbon sources and glucose as a control using a defined medium of inorganic nutrients as a base. The complex carbon sources were alder sawdust, pine sawdust, wheat bran and soybean hulls representing hardwoods, softwoods, grasses and dicotyledonous herbaceous plants respectively. The cultures were incubated at 30 °C (*P. blakesleeanus*) or 40 °C (*T. terrestris*) for four days and the fungal and plant solid material was removed by filtration through miracloth. The resulting liquid constituted the secretomes of the fungi. The proteins were concentrated on 10 kDa membranes, reduced and carboxamidomethylated, then digested with trypsin. The peptide solution was cleaned on disposable reverse phase (C18) columns and analyzed by high throughput liquid chromatography mass spectrometry (LC-MS) proteomics. The resulting MS and MS/MS spectra (molecular ion and fragmentation patterns, respectively) were analyzed against the protein databases of these two fungi using the SEQUEST program (Yates *et al.* 1998). The proteomics results for the zygomycete *P. blakesleeanus* suggest a bias towards scavenging protein with many proteases expressed under the different conditions (Table 1), but relatively few glycoside hydrolases. These proteomics observations are consistent with the paucity of glycoside hydrolase genes in this zygomycete's genome relative to saprobic ascomycetes. The ascomycete *T. terrestris* expressed a wide array of glycoside hydrolases, and some proteases, on the various substrates. The cellulose related CAZymes are shown as an example (Table 2). These proteomics results are consistent with the rich diversity of CAZymes found in saprobic ascomycete genomes (e.g. Martinez *et al*. 2008). Proteomics is a powerful technique for investigating which proteins are actually expressed and secreted by fungi when they are presented with different substrates in their environment.

Transcriptome profiling of the *Ustilago maydis* – maize interaction

Infection of maize by the fungal biotroph *Ustilago maydis* leads to formation of tumors in basically all aerial parts of the plant. Transcriptome profiling of *U. maydis* during pathogenic development was performed using a custom Affymetrix Gene-

Table 1. Proteases found in the secretomes of *Phycomyces blakesleeanus*. The Rhizopuspepsins represent a large family of A1A type proteases found in zygomycetes with six genes in *P. blakesleeanus*.

Glucose	Alder Sawdust	Pine Sawdust	Soybean Hulls	Wheat Bran	Hit Description	MEROPS Family
51	13	28	26	189	Rhizopuspepsin	A1A
		1	1	26	Rhizopuspepsin	A1A
2		4	29	20	Rhizopuspepsin	A1A
				15	Serine carboxypeptidase	S10
				14	Rhizopuspepsin	A1A
5					Aspartic protease	A1A
			5		Rhizopuspepsin	A1A

Table 2. CAZymes related to cellulose degradation found in the secretomes of *Thielavia terrestris* on various biomass sources (note that cellobiose dehydrogenase is not a glycoside hydrolase but is included as an enzyme important in the breakdown of cellulose).

Glucose	Alder Sawdust	Pine Sawdust	Soybean Hulls	Wheat Bran	Hit Description	GH Family
0	0	0	2	30	β-glucosidase	3
11	0	0	0	0	β-glucosidase	3
0	27	4	34	74	Endoglucanase	5
0	22	0	0	7	Endoglucanase	5
0	0	0	7	0	Endoglucanase	5
0	5	0	0	0	Endoglucanase	5
0	4	2	12	23	Exoglucanase	6
0	19	0	0	0	Exoglucanase	6
0	18	0	70	34	Endoglucanase	7
0	2	0	30	0	Exoglucanase	7
0	0	0	8	5	Exoglucanase	7
0	23	0	1	0	Exoglucanase	7
0	12	6	9	3	Endoglucanase	45
0	2	0	9	1	Endoglucanase	?
0	4	0	0	0	Cellobiose dehydrogenase	na

chip® microarray. This approach, together with sequencing of the *U. maydis* genome, identified 12 gene clusters encoding secreted effectors that are transcriptionally upregulated specifically during biotrophic interaction (Kämper *et al.* 2006). Deletion mutants for five of these gene clusters were altered in tumor formation, demonstrating the potential of transcript profiling for identifying virulence factors (Kämper *et al*. 2006)

A major step in understanding the impact of *U. maydis* infection on maize gene expression was achieved by transcriptome profiling of maize seedling leaves at different stages of infection (Doehlemann *et al.* 2008). Use of the Affymetrix maize genome array® identified a broad reprogramming of the maize primary- and secondary metabolism, particularly modulation of hormone signaling pathways as well as a shutdown of photosynthesis (Doehlemann *et al*. 2008). A major finding of this study was the attenuation of plant defences as soon as biotrophy has been established (Doehlemann *et al*. 2008).

Obviously there are fundamental differences between the various maize organs that *U. maydis* transforms into tumors. Therefore, transcriptome profiling was applied to study whether the distinctive developmental changes necessary for converting maize primordia to tumors would require organ-specific gene expression. To enable simultaneous transcript profiling of host and pathogen, a two organism microarray was designed using the Agilent® platform (Skibbe *et al.* 2010). Expression data from infected seedling leaf, adult leaf, and tassel revealed organ-specific expression programs of both interaction partners. In particular *U. maydis* genes encoding secreted effector proteins appeared to underlie organ-specific regulation (Skibbe *et al*. 2010). Moreover, *U. maydis* mutants deleted for clusters of secreted effectors (Kämper *et al*. 2006) showed significant differences in virulence depending on the infected maize organ (Skibbe e*t al*. 2010). Together, these results show that tumor formation requires organ-specific gene expression by both partners. This finding

of organ-specific activity of pathogen effectors set a new paradigm in plant pathology and further demonstrates the power of transcriptome profiling in understanding of complex organismic interactions.

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