An eternal clock? - monitoring the *Neurospora crassa* circadian clock in chemostat culture

Tewes Tralau*, Karin Lanthaler, Geoff Robson, Susan Crosthwaite

Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, U.K.

Neurospora crassa is a filamentous fungus and a model organism for the study of the molecular basis of circadian clocks. Rhythmic sporulation of this organism is maintained for prolonged periods in the dark with a periodicity of approximately 22 h. Three genes; *frequency, white collar-1 and white collar-2,* and their associated kinases and phosphatases, are key components of the circadian clock that drives this switch between vegetative hyphal growth and asexual development. To date regulation of these genes and the interactions of their RNA and protein products has been investigated using tissue from batch cultures of *Neurospora*. The use of batch cultures sets a time limit on observations of the molecular clockwork and culture conditions change throughout the experiment as the organism utilizes the carbon source and excretes by-products of metabolism. Here we report conditions for chemostat cultures of *Neurospora*, that overcome the afore mentioned problems associated with batch cultures.

Following exposure to light and attainment of steady-state conditions, *Neurospora* was grown in constant conditions of darkness at 25 °C for 8 days at a dilution rate of D = 0.074 /h. Following the establishment of steady state in the chemostat, tissue samples were taken every 4 h for the extraction of RNA and protein. Additionally, the time on the clock was monitored every 4 h by inoculating race tubes with samples of *Neurospora* from the chemostat. Our results show that the clock continued to run in liquid culture throughout the 8 days of the experiment. This sets a new record for the length of time *Neurospora* has been grown in a chemostat and opens up the possibility of studying the response of *Neurospora* to a range of stimuli in highly defined culturing conditions that are an essential prerequisit for genome wide gene expression studies.

Vp-2

Conserved mechanisms for light-sensing in the fungal kingdom

Alexander Idnurm[1*], Julio Rodríguez-Romero[2], Luis M. Corrochano[2], Catalina Sanz[3], Enrique A. Iturriaga[3], Arturo P. Eslava[3], Joseph Heitman[1]

[1] *Duke University Medical Center, USA*
[2] *University of Seville, Spain*
[3] *University of Salamanca, Spain*

Light is a major environmental signal perceived by fungi to regulate diverse aspects of their biology. The best characterized photosensory system is the White Collar (WC-1/WC-2) blue light receptor and transcription factor complex of the ascomycete *Neurospora crassa*. We describe the discovery of a similar set of genes in the basidiomycete *Cryptococcus neoformans* (*BWC1* and *BWC2*) and the zygomycete *Phycomyces blakesleeanus* (*madA*) that function in the response to blue/UV wavelengths. While the effects of light on these three model fungi are mediated by a conserved sensory system and can cause similar respones, not all effects of light are conserved. To gain an understanding of the downstream targets of the White collar genes in *C. neoformans*, a microarray experiment was undertaken to discover light regulated transcripts. These experiments provide insight into the photobiology of *C. neoformans*, and all fungi.

Early steps of pheromone signaling in *Ustilago maydis*

Doris Greilinger*, Regine Kahmann

Department of Organismic Interactions, Max Planck Institute for Terrestrial Microbiology, 35043 Marburg, Germany

In *U. maydis* pathogenic development is initiated by fusion of two compatible strains. Fusion is regulated by a pheromone-receptor system, and pheromone signaling involves the activation of a conserved cAMP pathway as well as of a MAP kinase module. Heterotrimeric G-proteins are considered as the initial transmitters of the pheromone signal during mating partner recognition in filamentous fungi and yeasts. *U. maydis* encodes four G-alpha-subunits (*gpa*1−4). Since the only G-alpha-subunit knockout (gpa3) which is defective in cell fusion can be "reverted" by cAMP supplementation, we hypothesized that there might be redundancy of G-alpha-subunits. To test this hypothesis we generated a dominant-negative version of *gpa*1 (*gpa*1^{GA}) and constructed triple (*gpa*1,2,4) as well as quadruple (*gpa*1−4) mutants. In the mutant expressing gpa1^{GA} the protein was expressed at wild type level and membrane associated. However, when this mutant was tested for its pheromone response, it responded like wild type. The *gpa*1,2,4 triple mutant was indistinguishable from wild type in its pheromone response while the *gpa*1−4 mutant behaved like the *gpa*3 mutant. These results argue against redundancy in G-alpha-subunit function and suggest the presence of yet unidentified signal transmitters in the early steps of pheromone signaling.

Vp-4

The osmo-signalling cascade of *Botrytis cinerea* is also involved in development, fongicide action and virulence.

Weiwei LIU[1*], Muriel VIAUD[2], Jai Santosh Polepalli[3], Laurent LEGENDRE[3], Pierre LEROUX[1], Sabine FILLINGER[1]

[1] UPMC INRA Versailles, Route de Saint-Cyr, 78026 Versailles, France
[2] PMDV INRA Versailles, Route de Saint-Cyr, 78026 Versailles, France
[3] Centre for Horticulture and Plant Sciences, University of Western Sydney, Australia

Botrytis cinerea is the causal agent of grey mould in more than 200 crops. Chemical control remains the principal means to reduce the incidence of grey mould on the economically important crop grapevine. Among the botryticides used intensively figure dicarboximides. Strains resistant to this class of fungicides (ImiR) have rapidly emerged in the vineyards. Interestingly, laboratory isolated ImiR strains show cross-resistance to phenylpyrroles and osmosensitivity. Since such ImiR strains have never been identified in fields that have been treated with both fungicides we were interested in testing their fitness and aggressiveness. We therefore disrupted the histidine kinase encoding *bos1* gene where most of the ImiR mutations map. The Δ*bos1* transformants do not sporulate under all conditions tested. Microscopic observations revealed that conidiophores formed normally in the null mutants but failed to differentiate to conidia, suggesting that BOS1 is not involved in the initiation of conidiation, but required in the conidia differentiation. The mutants *Δbos1* are osmosensitive, resistant to three classes of fungicides (dicarboximides, phenylpyrroles and aromatic hydrocarbons) and cross-resistant to menadione. But they are not sensitive to other stress conditions. We demonstrate that fungicides and osmotic stress induce intracellular glycerol accumulation via BOS1 signalization cascade in *B. cinerea*. Moreover our pathogenicity tests show that the mutants *Δbos1* are severely impaired for virulence. The Bos1 histidine kinase therefore constitutes a major pathogenicity factor of *B. cinerea*.

BOS1 presumably regulates a signal-transduction cascade involved in osmosensing comparable to the HOG (high-osmolarity glycerol) pathway of *Saccharomyces cerevisiae*. In contrast to the yeast Sln1 sensor histidine kinase, BOS1 does not have a detectable transmembrane domain. The histidine-kinases of the OS1 type are conserved among filamentous fungi where they regulate the response to osmotic stress and development. Our results suggest that they may also regulate the virulence of fungal plant pathogens. Other components of this BOS1 cascade are under investigation. Results of gene inactivation and epistasy analyses will be presented.

Vp-5

Role of the TOR signalling pathway in the control of the development of the phytopathogenic fungus *Botrytis cinerea*

Heber Gamboa Meléndez*, Geneviève Billon-Grand, Géraldine Mey

Laboratoire de Pathogénie des Champignons Nécrotrophes, Université Claude Bernard, Lyon 1, UMR CNRS-INSA-UCBL 5122, 10 rue Dubois, Bât Lwoff, 69622 Villeurbanne Cedex, France. Phone: (+33) 4 72 44 85 43, Fax: (+33) 4 72 43 11 81, E-mail: geraldine.mey@univ-lyon1.fr

Plant fungal pathogens use nutrients drawn from the plant cells to support their parasitic growth. The enzymatic maceration of plant tissues by the necrotrophic fungi ensures the release of compounds which can be directly assimilated by the fungus and may act as signal molecules. In yeast like in humans, nutrient availability is perceived by a conserved signalling pathway, compraising the kinase protein TOR (Target of Rapamycin). In yeast and high eukaryotes, TOR activity is postively regulated by the GTPase RHEB (Ras Homologue Enriched in Brain) and repressed by the complex formed by the propyl-isomerase FKBP12 (FK506 Binding Protein of 12 kDa) and rapamycin, a natural macrolide with immunosupressive and antifungal properties. Our research project is focused on the TOR-dependant signalisation cascade and its role in the control of the metabolism of amino acids by the grey mould fungus *Botrytis cinerea*. *FKBP12* silenced mutants were generated by homologous recombination and the *RHEB* gene was inactivated by RNA interference. The impact of both mutations in the amino acids uptake and their metabolism is currently being evaluated. The synthesis of extracellular proteases was followed using enzymatic assays together with SDS-PAGE and Western blot analyses. In a wild-type strain, the production of proteolytic enzymes is induced in the presence of polymers (casein, gelatine, sunflower leaves) and repressed by compounds such as glucose, ammonium and sulphate. Whether these relgulations are disturbed in the mutants is being studied. Taken together, our results may contribute to a better understanding of the TOR pathway role in the control of the synthesis of both secreted and intracellular enzymes ensuring amino acids incorporation and the development of *Botrytis cinerea*.

Vp-6

Importance and role of communication molecules in zygomycetes

Mareike Richter[1*], Doreen Schachtschabel[1], Regina Stieber[1], Christine Schimek[2], Johannes Wöstemeyer[2], Wilhelm Boland[1]

[1] Department of Bioorganic Chemistry, Max-Planck-Institute of Chemical Ecology, Hans-Knöll-Strasse 8, D-07745 Jena, Germany
[2] General Microbiology and Microbial Genetics, FSU Jena, Neugasse 24, D-07743 Jena, Germany

Zygomycetes are ubiquitous soil fungi and use asexual as well as sexual processes during their life cycles. During sexual interaction, zygomycete fungi communicate via carotene-derived compounds, namely trisporic acid and its cross-exchanged early precursors. Many trisporoids stimulate sexual differentiation. The first sexually determined structures formed after stimulation in both mating types of *Mucor mucedo* are specialized aerial hyphae termed zygophores.
These sexual hyphae show a uniform distribution of proteins and nuclei. Carotenoids, in contrast, accumulate in the hyphal tip, as shown by 2D-Raman-Spectroscopy [1]. The same technique also gives evidence for the presence of trisporoids in the zygophore tips.
The formation of zygophores is an important parameter to assess the bioactivity of trisporoid isomers and analogues. Various derivates of trisporoid compounds exhibit a graded influence on zygophore formation in *Mucor mucedo*.
Besides zygophore induction, synthetic trisporin, a (-) mating type-specific pheromone and trisporic acid precursor, increases the formation of β-carotene in *Mucor mucedo* (+) in the same way as a complementary mating partner after confrontation. Carotene biosynthesis and trisporoid production are linked via a feed back-loop in mating reactions. By correlation of morphological responses with chromatographically determined β -carotene concentrations, we are analyzing the structure-function relationships in trisporoid actions [2]. The detailed regulation mechanisms of the chemical dialogue using trisporoids realized in different zygomycetes remains to be established.
In addition, Real-Time-PCR is used to quantify gene transcription after stimulation with certain trisporoids. Stimulation with pure substrates allows the analysis of the regulation sequence occurring in nature as reaction to contact with a complementary mating partner. The impact of different trisporoids on the HMG-CoA reductase and phytoene desaturase gene transcription in *Mucor mucedo* is studied.

[1] J. Popp, U. Neugebauer, Institute of Physical Chemistry, FSU Jena
[2] D. Schachtschabel, C. Schimek, *et al.* (2005). "Biological activity of trisporoids and trisporoid analogues in *Mucor mucedo* (-)." PHYTOCHEMISTRY 66(11): 1358-1365

Analysis of the *bem46* gene from *Ascobolus immersus* and *Neurospora crassa*

Moritz Mercker, Nancy Weiland, Silke Alves, Frank Kempken*

Abteilung für botanische Genetik und Molekularbiologie, Botanisches Institut und Botanischer Garten, Christian-Albrechts-Universität Kiel, Am Botanischen Garten 1-9, 24118 Kiel, Germany; fkempken@bot.uni-kiel.de

We present data regarding the expression and function of the *Neurospora gene bem46*. The deduced amino acid sequence of this gene appears to be conserved among eukaryotes. It was first identified in *Schizosaccharomyces pombe* (Ref. 1), where it is involved in cell polarity and signal transduction. Recently the Arabidopsis *bem46* homolog was found to have a function in root growth (Ref. 2). We identified a homologous sequence some years ago in the filamentous fungus *Ascobolus immersus*, which was located near a truncated transposable element (Ref. 3).

When the *Neurospora crassa* genome sequence became available, we continued working on the *bem46* gene of *N. crassa*. Mutations were introduced using the RIP technique. However, ascospores of strong RIP mutants of *bem46* do not germinate or terminate germination at an early stage. We therefore established strains over-expressing *bem46*, a *bem46:gfp* fusion, and a RNAi construct down-regulating *bem46* expression. Results obtained with these strains will be presented.

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2. Mochizuki*et al.* (2005) *Plant Cell* 17:537-547
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Vp-8

A broad spectrum high-copy suppressor of calcofluor hypersensitivity in *Aspergillus nidulans*

Darlene M Loprete*, Terry W Hill, Caroline V Sartain

Rhodes College, Departments of Biology and Chemistry

In filamentous fungi, as in yeasts, hypersensitivity to the chitin-binding agent Calcofluor White (CFW) has been correlated with defects in cell wall integrity. We have identified several mutant strains of *Aspergillus nidulans*, which demonstrate the CFW-hypersensitive phenotype and show further evidences (such as Echinocandin- or temperature-inducible changes in spore or hypha morphology) to support the conclusion that a wall defect is present. During attempts to clone the respective genes of four of these strains (*calC*, *calD*, *calF*, and *calH*) through complementation with *A. nidulans* genomic DNA libraries, and have repeatedly recovered complementing DNA sequences which contain a wild type *A. nidulans* gene, which we designate GP-1. We have cloned the sequence and confirmed its complementing ability by re-transformation of each mutant. However none of these strains contains a mutation in the ORF for GP-1 or within 300 base pairs upstream, leading to the conclusion that the gene acts in each case as a high copy extragenic suppressor. The hypothetical translated product of GP-1 is an ST-rich protein (42% S/T) of 27.4 kDa mass (unprocessed), with a cleavable N-terminal ER-targeting domain and a probable internal membrane anchor. A complementation-competent GFP- GP-1 hybrid localizes to the plasma membrane of vegetative hyphae and is especially enriched in septa. Secondary localization in vacuoles probably represents steps in degradation of misfolded proteins. No known homologies have been identified in the databases. Taking into consideration the protein's cell surface location and influence on a wide range of cell surface defects, we hypothesize a role in signal transduction as part of a cell wall integrity pathway.

VeA subcellular localization is dependent on light in the filamentous fungus *Aspergillus nidulans*

Suzanne M Stinnett[1], Laura Cobeño[2], Eduardo Espeso[2], Ana M Calvo[1]

[1] *Department of Biological Sciences, Northern Illinois University, Dekalb, Illinois, USA*
[2] *Centro de Investigaciones Biológicas (C.S.I.C.), Microbiologia Molecular, Madrid, Spain*

The *veA* gene is a light-dependent regulator that governs development and secondary metabolism in *Aspergillus nidulans.* We have previously reported a putative bipartite NLS motif in the *A. nidulans* VeA deduced amino acid sequence and demonstrated that this NLS is functional when expressed in a yeast system. Furthermore, we showed that the migration of VeA to the nucleus is dependent on an alpha-importin protein. We have recently demonstrated the functionality of this bipartite NLS when VeA is expressed in *A. nidulans*. Interestingly, we found that VeA migration to the nucleus is light-dependent in *A. nidulans*. While in the dark VeA is located mainly in the nuclei, under the light VeA is found abundantly in the cytoplasm. On the other hand, the VeA1 mutant protein form (lacking the first 37 amino acids at the N-terminal region) was found predominantly in the cytoplasm independent of the illumination regimen, indicating that the truncated bipartite NLS in VeA1 is not functional and fails to respond to light. These results explain the lack of the morphological light-dependent response in strains carrying the *veA1* allele. We also evaluated the effect of light on the production of the mycotoxin sterigmatocystin in a *veA* wild-type strain and compared it with that of a *veA1* mutant strain. In our studies we saw that the highest amount of toxin was produced by the *veA+* strain growing in the dark, a condition that favored the accumulation of VeA protein in the nucleus.

Vp-10

Light- and inducer-dependent modulation of cellulase gene expression by the Gα protein Gna3 of *Hypocrea jecorina (Trichoderma reesei)*

Roberto Nascimento Silva[1*], Christian P. Kubicek[2], Monika Schmoll[2]

[1] Laboratório de Enzimologia, Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74.001-940, Goiânia, Go, Brazil
[2] Research Area Gene Technology and Applied Biochemistry, Institute for Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, Vienna, 1060, Austria

An adenylate cyclase activating group III Gα protein-encoding gene, *gna3,* was cloned from *Hypocrea jecorina* (*Trichoderma reesei*) and investigated for its effect on the signalling of cellulose to cellulase (*cbh1* and *cbh2*) gene expression. Thereby *gna3* was found to be located in the genome in the vicinity of two further signalling genes, i. e. the MAP kinase *tmk3*, which is related to *S. cerevisiae* hog1p and the glycogen phosphorylase *gph1*. The mutant strain (*gna3*QL) bearing a constitutively activated copy of *gna3* exhibited the same growth rate as the parent strain but decreased sporulation, and as expected for a constitutive activation of *gna3*, intracellular cAMP levels are elevated. The *gna3*QL mutant showed a strongly increased cellulase induction by cellulose only in the presence of light, the dark-levels of transcript abundance remaining similar to the wild-type, thus reflecting that Gna3 modulates the previously reported influence of light/darkness (Schmoll *et al.* 2005. Eukaryotic Cell, 4(12):1998-2007) on cellulase induction. However, this constitutive activation of Gna3 did not lead to inducer-independent cellulase gene expression.

The gna3QL mutant showed unaltered response of the light regulatory genes *blr1*, *blr2* and *env1* to light and darkness. Our data show, that Gna3 is involved in cellulase gene expression on cellulose in light and acts downstream of the light input pathway.

Characterization of the *Aspergillus nidulans* putative blue light receptor gene *cryA* encoding a cryptochrome

Özgür Bayram*, Christoph Biesemann, Sven Krappmann, Gerhard H. Braus

Institute of Microbiology & Genetics, Department of Molecular Microbiology & Genetics, Georg-August-University Grisebachstr.8, 37077 Goettingen, Germany obayram@gwdg.de Tel:++49 551 39 3821 Fax:++49 551 39 3770

Cryptochromes have important roles in the blue light response and circadian rthyms of filamentous fungi, plants, flies, or mammals. The *Aspergillus nidulans* genome revealed one copy of a putative cryptochrome-encoding gene, conclusively named *cryA*. The deduced amino acid sequence from this locus shows 29 % identity to the *Arabidopsis thaliana cry1* gene product. The *cryA* coding sequence is 1.7 kb in length and consists of two exons interrupted by one short intronic region with the capacity to express a 567 amino acid protein that comprises highly conserved domains such as a photolyase domain as well as a FAD binging domain. For construction of a deletion cassette we made use of a recently described ligation-mediated PCR approach, followed by targeted deletion of the *cryA* locus in a wild-type genetic background. The resulting null mutant forms hülle cells in submerged culture and has a purple colour after prolonged incubation. This effect is rescued with the genomic fragment of the *cryA* gene. Additionally, double knock-out strains were created lacking established regulators of fruit body formation. Conditional growth and expression profiling experiments will reveal any influence of the putative *cryA*-encoded blue light receptor within the life cycle of the *A. nidulans*. The current state of the project will be presented.

Vp-12

The small GTPase Rac and the PAK-kinase Cla4 have severe impact on hyphal morphology and pathogenesis in *Claviceps purpurea*

Yvonne Rolke*, Paul Tudzynski

Institute of Botany, Schlossgarten 3, 48149 Muenster, Germany

An interesting model to study directed growth of fungal hyphae in a pathogen host interaction is represented by the biothrophic ascomycete *Claviceps purpurea* on rye. Besides its wide host range (200 species of grasses) it has a very specific infection pattern. *C. purpurea* grows down the style following the pollen tube path through the transmitting tissue, establishes a stable host-pathogen interface by tapping the vascular bundles and finally colonizes the entire ovary. Mutants with impaired growth can be identified in an in vitro system for cultivation and infection of rye ovaries. We are interested in the signalling mechanisms which guide the pathogen on it`s specific infection path.

Therefore the highly conserved Rho-GTPases Cdc42 and Rac and a downstream acting PAK-kinase which are well known to be involved in cell polarity are analyzed. It was shown by Jan Scheffer that Cpcdc42 is a prerequisite for directed growth of *C. purpurea* in the infection process[1]. The hypersporulating Cdc42 deletion mutant is able to invade stigmatic hairs, but stops infectious growth before reaching the transmitting tissue.

The deletion of the gene encoding the small GTPase Rac revealed a striking phenotype. Rac deletion mutants grow in a three dimensional, corral like shape with shortened, blistered cells. The PAK-kinase Cla4 seems to be activated by Rac because the Cla4 deletion mutant showed a very similar phenotype as the Rac deletion. Both mutants are non-sporulating and apathogenic whereas the complementations showed wildtype phenotype and restored pathogenicity.

To further investigate the role of these signal chain components, a constitutively active Cla4 protein was generated by deletion of the CDC42/RAC Interactive Binding Domain (CRIB) which autoinhibits the kinase domain in the endogenous protein. Transformation of the construct in wildtype background leads to a hypersporulating phenotype with abberant cell morphology. After single spore isolation the newly formed spores were not able to germinate under selective conditions. That indicates that the CRIB-deletion effect is dose dependent and probably letal.

Dominant active, dominant negative and overexpression mutants of RAC are generated in the moment and will hopefully contribute to the understanding of the relationships between RAC, CDC42 and Cla4 and their role in *C. purpurea* development.

[1]Scheffer *et al.* 2005. A CDC42 Homologue in *C. purpurea* is Involved in Vegetative Differentiation and is Essential for Pathogenicity. Eukaryotic Cell **4**:1228-1238

The role of heterotrimeric G-protein mediated signalling cascades of *Botrytis cinerea* in the interaction with plants

Julia Schumacher*, Christian Schulze Gronover, Bettina Tudzynski

Institut für Botanik, Westfälische Wilhelms-Universität Münster, Schlossgarten 3, D-48149 Münster, Germany

The α-subunit BCG1 of a heterotrimeric G-protein plays an important role during the infection of host plants by the gray mold fungus *Botrytis cinerea*. Δ*bcg1*-mutants are able to conidiate, to penetrate host tissue, and to produce small primary lesions. However, in contrast to the wildtype, the invasion of plant tissue stops at this stage. Thus, BCG1 triggers the transition to secondary lesion formation (1).

SSH was used to identify genes whose *in planta* expression is specifically affected in Δ*bcg1*-mutants. Among the differentially expressed genes we found those encoding proteases, enzymes involved in secondary metabolism, and cell wall-degrading enzymes. Unexpectedly, most of the BCG1-controlled genes are still expressed in the adenylate cyclase-mutant *in planta* (2), suggesting that BCG1 is involved at least in one additional signalling cascade beside the cAMP-depending pathway (3; see abstract: Viaud *et al.*).

These findings were supported by GUS reporter gene assays, comparing the activities of a xylanase-promotor (cAMP-pathway) and a metalloprotease-promotor (yet unknown pathway) in the wildtype- and Δ*bcg1*-background, respectively. Promotor mutation studies were performed in order to identify binding motifs for the corresponding transcription factors of both signalling pathways.

To find out whether the Gβγ-dimer of the G-protein controls the unknown signalling pathway, we cloned the Gβ-subunit encoding gene *bcgb1*. The characterization of Δ*bcgb1*- and Δ*bcg1*Δ*bcgb1*-mutants will show if BCGB1 acts in concert with BCG1 via the cAMP-PKA-pathway and if there exists also a BCG1-independent pathway, e.g. a MAP-kinase cascade which is controlled only by BCGB1.

In order to identify and to compare (more) *in planta* target genes of BCG1, BCGB1 and other components of the cAMP signalling pathway by a cDNA-macroarray approach, deletion mutants of the different subunits of the protein kinase A are created.

(1) Schulze Gronover *et al.* (2001): *MPMI* 14:1293-1302.
(2) Klimpel *et al.* (2002): *Mol. Plant Pathol.* 3:439-450.(3) Schulze Gronover *et al.* (2004): *MPMI* 17:537-546.

Vp-14

The Gα subunit BCG1 and the Ca2+/calmodulin-dependent calcineurin phosphatase act in concert to regulate gene expression in the gray mold *Botrytis cinerea*

Muriel Viaud[1], Julia Schumacher[2*], Adeline Simon[1], Bettina Tudzynski[2]

[1] Unité de Phytopathologie et Méthodologies de la Détection, Institut National de la Recherche Agronomique, Route de Saint-Cyr, F-78086 Versailles, France
[2] Institut für Botanik, Westfälische Wilhelms-Universität Münster, Schlossgarten 3, D-48149 Münster, Germany

Botrytis cinerea, the causal agent of gray mold diseases of plants, is a typical necrotroph: its infection strategy includes killing the host cells, e.g. by unspecific phytotoxins such as botrydial and botcinolides.

Recently, the botrydial biosynthesis gene *bcbot1* has been identified by a gene replacement approach. The gene is part of a physical cluster of calmodulin/calcineurin-dependent genes (CND) the expression of which is affected by cyclosporin A (2). In addition, the expression of botrydial-biosynthesis genes is under control of BCG1, an α-subunit of a heterotrimeric G-protein which is essential for full virulence on bean plants (1; see abstract: Schumacher *et al.*).

In order to prove whether this coregulation of genes by BCG1 and calcineurin is a common feature, a cDNA macroarray approach was performed. The expression pattern of about 5000 genes were compared between the wild-type and the Δ*bcg1*-mutant incubated with or without the calcineurin-inhibitor cyclosporin A. This screening led to the identification of a set of genes whose expression is regulated either by both BCG1 and calcineurin, or only by one of them.

Three of the genes which are under control of both, BCG1 and calcineurin, were shown to be located in a second physical gene cluster that could be responsible for the biosynthesis of a yet unknown (probably phytotoxic) secondary metabolite. The identified cluster consists of six genes including the polyketide synthase BcPKS6 encoding gene. Two of them were previously identified by SSH as *in planta*-induced target genes of BCG1 (3), indicating a potential role of the yet unknown polyketide compound in the fungus-plant interaction.

Based on the expression data, a hypothetical model for the interconnection between cAMP- and calcineurin dependent signalling pathways in *B. cinerea*, mediated by the the Gα-subunit BCG1, was developed in which BCG1 acts possibly upstream of the calcineurin phosphatase in regulation of gene expression.

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(2) Viaud *et al.* (2003): *Mol. Microbiol.* 50:1451-1465.
(3) Schulze Gronover *et al.* (2004): *MPMI* 17:537-546.
(4) Siewers *et al.* (2005): *MPMI* 18:602-612.

Cytosolic progesterone receptors from the fungus *Rhizopus nigricans*: some molecular characteristics and partial purification

Tomaz Makovec, Helena Lenasi*, Katja Breskvar

Institute of Biochemistry, Faculty of Medicine, University of Ljubljana

Mammalian steroid hormones, especially progesterone, are toxic for a saprophytic fungus *Rhizopus nigricans* (class Zygomycetes, order Mucorales).When cultivated *in vitro* the fungus expresses defense mechanisms containing cytochrome P450 which converts hydrophobic progesterone into water soluble less toxic product 11α-hydroxyprogesterone. The enzyme P450 could be induced by progesterone and some other steroid inducers (1); the induction process flows, most likely, via cytosolic progesterone receptors (2). In the presented study we determined some molecular characteristics of fungal progesterone receptors and partially purified the receptors.

Molecular mass (S values) of cytosolic progesterone receptors was determined by centrifugation in sucrose gradient under different experimental conditions: in low salt buffer, in the presence of stabilizing agent molybdate and in high ionic strength buffer (activation conditions). The activation of receptors was followed by their binding to DNA-cellulose and herewith their biological role estimated.

With the aim to get additional information on the functional and evolutional characteristics of steroid receptors, we purified receptors. DEAE/Trisacryl and MONO-P HR5/20 FPLC ion exchange column and DNA-cellulose column was used; purification grade was assesed by SDS-PAGE.

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Vp-16

cAMP signalling is not a prerequisite for colonisation of perennial ryegrass by the fungal symbiont *Epichloë festucae* Fl1

Christine R Voisey*, Mike J Christensen, Charlotte N Gaborit, Richard D Johnson, John P Koolaard, Gregory T Bryan

AgResearch Grasslands, Forage Biotechnology, Tennent Drive, Private Bag 11008, Palmerston North, New Zealand

In fungal pathogenesis the cAMP signalling cascade is usually essential for the synthesis of pathogenicity factors, and deletion of the adenylate cyclase gene often results in an avirulent phenotype. The aim of this study is to determine the signalling mechanisms responsible both for the colonisation of perennial ryegrass and for regulation of secondary metabolites by the fungal symbiont *Epichloë festucae* Fl1.

Cool season grasses are often found in association with fungal endophytes of the genera *Neotyphodium* and *Epichloë*. These ancient symbioses have evolved mechanisms to tightly coordinate growth of the endophyte with that of their host. Hyphal growth is confined to intercellular spaces, and is synchronised with extension of the leaf; fungal growth ceases when the blade reaches maturity. The production by endophytes of secondary metabolites in plants is also tightly regulated and responsive to both plant and environmental factors

We have identified genes from several signalling networks, and here report on the outcomes of targeted disruption of *E. festucae* adenylate cyclase (AC), the enzyme responsible for synthesis of the ubiquitous second messenger, cAMP.

The cAMP signalling network in *E. festucae* Fl1 appears unique in that, unlike other fungal colonisers of plants and animals, the endophyte is not dependent on cAMP for colonisation and survival in perennial ryegrass. Targeted disruption of the adenylate cyclase gene (*Nlac*) from *E. festucae* Fl1 resulted in a reduced rate of radial growth in culture as reported for other species of filamentous fungi. However, when the culture was artificially inoculated into perennial ryegrass seedlings, it was capable of growing at the same rate as the plant, and the symbiotum appeared phenotypically normal. We will present results concerning observations of *E. festucae* Fl1 *ΔNlac* growing both in culture and *in planta*, plus the effects of AC disruption on production of conidia and secondary metabolites. Although the components of signalling cascades are highly conserved, fungal species have co-opted them to perform different functions. The implications of this discovery are discussed.

An experimental setup for the identification of differentially expressed genes during polarized and non-polarized growth in *Aspergillus niger*

Mark Arentshorst*, Cees AMJJ van den Hondel, Arthur FJ Ram

Leiden University, Institute of Biology Leiden, Fungal Genetics Research Group, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

The highly polarized growth of filamentous fungi requires a continuous transport of secretion vesicles to the hyphal tips. Fusion of the vesicles with the plasma membrane occurs in a highly polarized manner, at the very tip of the apex. The transport and fusion of secretion vesicles is mediated by the actin cytoskeleton and various actin interacting proteins. Actin organization and dynamics in all eukaryotic cells is controlled by Rho-related GTPases (Rho, Rac and Cdc42). Analysis of the function of RacA in *A. niger* has implicated a role for RacA during polarized growth. Deletion of the *racA* gene results in abnormal branching at the hyphal tip. Overexpression of the dominant active form of RacA ($RacA^{G12V}$) has a dramatic effect on fungal morphology and results in loss of polarized cell growth. The wild-type strain and the strain overexpressing the wild-type RacA form long hyphae, which are characteristic for filamentous fungi. However, cells expressing $RacA^{G12V}$ grow isotropically, resulting in round cells.

Our aim is to identify genes that are differentially expressed during polarized and isotropic growth by transcriptomic analysis. Therefore, a strain containing the wild-type Rac protein expressed from the glucoamylase promoter (GlaA-RacA) and a strain overexpressing the dominant active form of RacA (GlaA-$RacA^{G12V}$) were grown in shake flasks as well as under controlled conditions in a BioFlo 3000 reactor. Under non-induced conditions (xylose as a carbon source), the growth of the two strains was identical, indicating that there is no expression of $racA^{G12V}$ from GlaA promoter on xylose. Also the morphology of the two strains was identical during growth on xylose. Just before the xylose in the bioreactor was consumed, maltose was added to the growth medium to induce expression of the glucoamylase promoter. Northern analysis confirmed induction of *racA* expression in both strains. Examination of the morphology revealed that overexpression of the *racA* wild-type gene has no effect on polarized cell growth, whereas overexpression of the dominant active form of *racA* induces isotropic growth. RNA samples from different time points have been collected and analyzed using microarrays. The first results indicate that many of the induced genes in the $RacA^{G12V}$ strain are involved in cell wall reinforcement, suggesting that the switch from polar to non-polar cell growth results in activation of the cell wall integrity pathway (Damveld *et al.*, 2005a, b).

References:
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Vp-18

RNA silencing in *Mucor circinelloides*: Functional characterization of two *dicer*-homologous genes

Rosa M. Ruiz-Vazquez*, Francisco E. Nicolas, Juan P. de Haro, Santiago Torres-Martinez

Department of Genetics and Microbiology, Faculty of Biology University of Murcia, Murcia 30071, Spain

RNA silencing is a complex regulatory mechanism that involves suppression of gene expression through the cleavage or translational repression of the target mRNA. This mechanism is triggered by double-stranded RNA molecules (dsRNA), which are processed into small RNAs (siRNAs or miRNAs) to prevent the expression of exogenous nucleic acids (viral genomes, transposons and transgenes) or to regulate endogenous pathways, respectively. Both gene silencing pathways share a common protein set (the "RNAi machinery") devoted to produce and amplify these small RNAs. Within this machinery, the Dicer protein is the RNaseIII enzyme that processes dsRNA molecules into the small size RNAs. These are later incorporated into the RISC complex, which specifically degrades or prevents the translation of all mRNA sharing sequence identity with the small RNAs.

Our research group has demonstrated the existence of a transgene-induced RNA silencing mechanism in the fungus *M. circinelloides* (Nicolás *et al.*, 2003). The easy manipulation of this organism and some specific characteristics of its silencing mechanism make *M. circinelloides* a model organism to study some unresolved questions in the silencing mechanism. Gene silencing in *M. circinelloides* is associated with two size classes of siRNA, 21-nt and 25-nt long. These two classes of siRNAs are differentially accumulated through the vegetative growth of the silenced strains, which is the most outstanding characteristic of gene silencing in *M. circinelloides*. The two classes of siRNA, 21-nt and 25-nt long, could be produced by two different Dicer enzymes, showing different expression patterns and/or acting in different cellular compartments. We have cloned two *M. circinelloides dicer*-like genes (*dcl-1* and *dcl-2*) by using degenerated oligonucleotides derived from conserved regions of known Dicer proteins. The corresponding protein products contain all the structural domains found in Dicer enzymes, including two RNaseIII domains and an RNA/helicase domain. Phenotypic analysis of null *dcl-1* mutants has demonstrated that the *dcl-1* gene is not essential for transgene-induced gene silencing, since *dcl-1* mutants are able to silence gene expression and to produce the two classes of siRNA, 21 and 25 nt long, with their characteristic accumulation patterns. Instead, the phenotype shown by *dcl-1* mutants suggests a role for this gene in a putative miRNA pathway. Nevertheless, we cannot discard that *dcl-1* participates in the siRNA pathway, having a redundant function with the second *dicer* gene identified in *M. circinelloides*. The role of *dcl-2* gene in the silencing mechanism, as deduced from the phenotype of *dcl-2* mutants, will be discussed.

(1) Nicolás *et al.*, (2003) Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes. EMBO J., 22: 3983-3991.

Effects of two antifungal proteins from *Aspergillus giganteus* and *Aspergillus niger* on the morphology and physiology of sensitive *Aspergillus sp*.

Ulrike Binder[1*], Mogens T. Hansen[2], Florentine Marx[1]

[1] Biocenter, Division of Molecular Biology, Innsbruck Medical University, Fritz-Pregl Strasse 3, A-6020 Innsbruck, Austria;
[2] Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark

Two new antifungal proteins from *Aspergillus giganteus* (AFP NN5353) and from *A. niger* (ANAFP C40-1), which belong to a new group of proteins with a low molecular mass (50-55 amino acids), a basic character (high content of basic aa) and a high content of cysteine residues (Marx *et al.* 2004), revealed growth inhibitory activity against filamentous Ascomycetes. Indirect immunofluorescence studies proved the internalization of these proteins by the sensitive model organism *A. nidulans*, whereby the uptake was shown to be energy dependent and thus to resemble an endocytotic mechanism. A better understanding of the activity of these antifungal peptides will be a prerequisite to consider them for the development of novel antimycotic drugs which might be applied in medical treatment, in agriculture or in food preservation. To this end we performed further experiments addressing the impact of these peptides on changes in morphology and physiology of *A. nidulans* and *A. niger*. We show by the application of distinct fluorescent dyes the induction of morphological changes, impairment of the plasma membrane, generation of reactive oxygen species and metabolic inactivity by AFP NN5353 and ANAFP C40-1. Marx F. (2004), Appl. Microbiol. Biotechnol. 65: 133-142. This work is supported by the Förderungsbeitrag X8 and X34 from the University of Innsbruck, the Austrian National Bank (OENB 9861) and the Austrian Science Foundation (FWF 15261) to F.M. and by Novozymes A/S.

Vp-20

The *Penicillium chrysogenum* antifungal protein PAF induces an apoptosis-like phenotype in the sensitive *Aspergillus nidulans*

Florentine Marx[1*], Éva Leiter[2], Christoph Oberparleiter[1], Tamás Emri[2], Willibald Salvenmoser[3], István Pócsi[2]

[1] Biocenter, Division of Molecular Biology, Innsbruck Medical University, Austria
[2] Department of Microbiology and Biotechnology, Faculty of Science, University of Debrecen, Hungary
[3] Institute of Zoology, Division of Ultrastructure and Evolutionary Biology, University of Innsbruck, Austria

The small, basic and cysteine-rich protein PAF is abundantly secreted from the beta-lactam producer *Penicillium chrysogenum* and exhibits growth inhibitory properties against numerous important plant and zoopathogenic filamentous ascomycetes (1). We report here that growth inhibition is accompanied by the generation of intracellular reactive oxygen species (ROS), e.g. peroxides and superoxide. The accumulation of intracellular ROS can have severe impact on cells, resulting in the oxidation of biopolymers and consequently in the destruction of cellular membranes and organelles. In fact, the abnormal cellular ultrastructure of PAF-treated *A. nidulans* hyphae analyzed by transmission electron microscopy suggested that ROS-elicited membrane damage and the disintegration of mitochondria played a major role in the cytotoxicity of PAF. When the ROS burden of the cell reaches a critical level, basic physiological functions are impaired and programmed cell death (PCD) might occur. We present data of an apoptosis-like phenotype in *A. nidulans* in response to PAF. Two PCD-related phenomena, namely the exposure of phosphatidylserine on the surface of PAF-treated protoplasts and DNA strand breaks, were proved by Annexin V staining and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL), respectively (2). These results underline the occurrance of a PCD-like mechanism in filamentous fungi, which was confined to multicellular higher eukaryotes in the past. Finally, the antifungal protein PAF represents a promising tool for further characterization of apoptotic-like events in filamentous fungi.

(1) Marx (2004), Appl Microbiol Biotechnol 65:133-142, Review.
(2) Leiter *et al.* (2005), Antimicrob Agents Chemother 49: 2445-2453.

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Vp-21

Studies of an *Aspergillus niger* Ca-ATP-ase deletion mutant using a novel recombinant pH-probe

Tanja Bagar[1], Kirsten Altenbach[2], Nick D Read[2], Mojca Bencina[1*]

[1] Laboratory of Biotechnology, National Institute of Chemistry Slovenia, Hajdrihova 19, SI-1000 Ljubljana
[2] Fungal Cell Biology Group, Institute of Cell Biology, University of Edinburgh, Edinburgh EH9 3JH

Accurate measurement of intracellular pH in unperturbed cells is fraught with difficulties. We have developed a recombinant pH-probe, called RaVe_C, from a class of pH-sensitive GFP's. RaVe_C has a ratiometric dual excitation and displays reversible emission ratio changes in the range from pH 5.5 to pH 7.8. The emission peak (fluorescence intensity at 508 nm) upon excitation at 395 nm increases whereas the one upon 475 nm excitation decreases with an increase in pH. With a pK_a of 7.0 RaVe_C is ideal for monitoring pH-changes in the cytosol. *In vivo* calibration using nigericin proved that the RaVe_C probe is highly pH-sensitive in living fungal hyphae, therefore RaVe_C can successfully be used as a non-invasive genetically encoded intracellular sensor. We have analysed the performance of this probe *in vivo* using confocal laser scanning microscopy and addressed the following questions: (1) Does the pH homeostat respond to changes in extracellular pH and pharmacological treatments? (2) Does the deletion of a vacuolar Ca-ATP-ase influence the pH homeostasis?

Vp-22

Distinct *white collar-1* genes control specific light responses in *Mucor circinelloides*

Fátima Silva*, Santiago Torres-Martínez, Victoriano Garre

Departamento de Genética y Microbiología. Facultad de Biología. Universidad de Murcia. 30071 Murcia. Spain

Light regulates developmental and physiological processes in a wide range of organisms, including filamentous fungi. In recent years, considerable effort has been dedicated to study the light perception mechanisms as well as the components of the signal transduction pathways in fungal models. In the present study, three *white collar-1* genes (*mwc-1a*, *mwc-1b* and *mwc-1c*) coding for proteins showing similarity to the WC-1 photoreceptor of *Neurospora crassa* have been identified in the fungus *Mucor circinelloides*. The protein sequences encoded by the three genes present a LOV domain, similar to those of fungal and plant blue light receptors. Knockout mutants for each *mwc-1* gene were generated by gene replacement to characterize their function in light regulation. Only the *mwc-1c* mutants were impaired in the light induction of carotene biosynthesis, the only light response that has so far been characterized in this fungus. The carotenogenic defect shown by the *mwc-1c* mutants was associated with a low level of photoinduced gene expression of structural carotenogenic genes. This indicates that *mwc-1c* is a key element in the light transduction pathway that control *M. circinelloides* carotenogenesis. In addition, we have observed that light produces a positive phototropism in *M. circinelloides* sporangiophores. This positive phototropism is controlled by a different *white collar-1* gene, the *mwc-1a*, since the *mwc-1a* mutant sporangiophores are defective in this response. This results suggest that *M. circinelloides* shows distinct signal transduction pathways to control the different responses to light.

A novel ELAV-like RNA-binding protein determines polarity in the pathogen *Ustilago maydis*

Philip Becht, Julian Koenig*, Michael Feldbruegge

MPI for terrestrial Microbiology, Department for Organismic Interactions, Karl-von-Frisch-Strasse, 35043 Marburg

Cell polarity is commonly achieved by localisation of mRNA to defined subcellular regions via active transport of ribonucleoprotein (RNP) particles along cytoskeletal tracks. In the plant pathogen *Ustilago maydis* the formation of a polar-growing filament is essential for infection. Here we demonstrate that loss of Rrm4 causes polarity defects. This novel RNA-binding protein contains three RRMs with characteristic spacing known from ELAV-like proteins and a PABC domain functioning in protein interaction. Rrm4 assembles into particles that shuttle bi-directionally along cytoplasmic microtubules involving conventional kinesin. Its RNA-binding is substantially increased during polar growth and mutations in the RNA-binding domain lead to loss of function. Rrm4 does not accumulate at the poles and its RRMs are dispensable for particle formation. Thus, Rrm4 is not hitchhiking but constitutes an intrinsic component of shuttling RNP particles responsible to recruit RNA. Since the PABC domain is essential for formation of shuttling particles, its unique domain architecture enables this ELAV-like protein to assemble in particles. We provide evidence that a developmentally regulated ELAV-like protein functions as RNA transporter to determine polarity during infection. This is the first example of a role for RNP transport during plant disease and indicates that microtubule-dependent RNP transport is evolutionarily ancient.

Vp-24

Biochemical evidence for the presence of a His-Asp phosphorelay in filamentous fungi

Nobuhiro Azuma, Kyoko Kanamaru, Akinori Matsushika, Takashi Yamashino, Takeshi Mizuno, Masashi Kato*, Tetsuo Kobayashi

Graduate School of Bioagricultural Sciences, Nagoya University

His-Asp phosphorelays are the widespred signal transduction mechanisms in both prokaryotes and eukaryotes. The system consists of three types of common signal transducers: a sensor with histidine kinase activity, a response regulator containing a phospho-accepting receiver and often a histidine containing phosho-transmitter (HPt). The filamentous fungus *Aspergillus nidulans* is a model organism with which extensive studies on the transcriptional regulation and signal transduction. A computer-aided similarity search has identified the 15 ORFs as a sensory histidine kinase, one ORF as a Hpt, and four ORFs as a response regulator in the genome of *A. nidulans*. In eukaryotic microorganisms, *Saccharomyces cerevisiae* and *Shizosaccharomyces pombe*, the His-Asp phosphorelay systems are well characterized. These organisms have only a small number of the common signal transducers: one histidine kinase, one HPt, and two response regulators in *S. cervisiae*, while three histidine kinase, one HPt and two response regulators in *S. pombe*. Therefore, the His-Asp phoshorelay systems in *A. nidulans* could respond more complicate environmental stimuli. At the same time, a simple question whether all the histidine kinases can transfer the phosphate group to the HPt has arisen. To address the question, we have attempted to examine the in vitro phospho-transfer with the all recombinant transducers. In this time, as a first step to construct the in vitro phospho-transfer network with all the components of *A. nidulans*, we purified the recombinant HPt and a response regulator SrrA and subjected to the in vitro phospho-transfer system using an *Escherichia coli* histidine kinase ArcB as a phosphate-donor. We found the phospho-transfer from ArcB to *A. nidulans* HPt and the subsequent transfer from HPt to SrrA, which is the first direct biochemical evidence for the presence of phospho-transfer system in filamentous fungi.

Exploring appressorial formation in the cereal biotroph powdery mildew

Sally R Gilbert[1*], Bart Fraaije[1], Andy Bailey[2], Hans Cools[1], John Lucas[1]

[1] Plant-Pathogen Interactions Department, Rothamsted Research, Harpenden, Herts AL5 2JQ UK
[2] School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG UK

Wheat and barley powdery mildews (*Blumeria graminis* f.sp *tritici (Bgt)* or *hordei (Bgh))* are obligate, fungal biotrophs which invade the host with an infection structure, termed an appressorium. There is considerable interest in understanding the sequence of events that leads to appressorial formation and ultimately successful penetration of the host plant. A greater understanding of the signal transduction pathway leading to appressorial formation, has and continues to facilitate the development of novel fungicides that target specific stages in this pathway.

The aim of this project is to use real-time RT-PCR to identify potential target-encoding gene(s) of a novel fungicide known to inhibit appressorial formation. Eight genes that play a role in appressorial formation were chosen. These encode an integral membrane protein (*PTH11*), a G protein α subunit (*Bgene*), a GTPase activating protein (*GAP*), calmodulin (*CAM*), a mitogen-activated Protein Kinase (*MAP1*), Protein Kinase C (*PKC*), a cAMP-dependent Protein Kinase A (*CPKA*) and an adenylate cyclase (*BAC1*). In addition, a Catalase encoding gene *(CATB*) was chosen as a indicator of successful penetration, as it is involved in scavenging activated oxygen species generated during the plant/pathogen interaction. TaqMan® probes have been designed, based on sequence homology between *Bgh* and *Bgt* genes, and used to determine changes in expression of these genes in the presence of fungicides as compared to the untreated control.

Gene expression studies of wild-type isolate 23D5 revealed low levels of transcription for all genes during appressorial development in the absence of fungicide. In the presence of either quinoxyfen or proquinazid, differences in the expression of genes encoding *CPKA, GAP* and *Bgene* were observed when comparing fungicide treatments and the untreated control.

Vp-26

Induction of conidiation by mycelial injury in *Trichoderma atroviride*

Alfredo Herrera-Estrella*, Miguel Hernández-Oñate

Laboratorio Nacional de Genómica para la Biodiversidad, Cinvestav-Campus Guanajuato Km. 9.6 Libramiento Norte Carretera Irapuato-León A.P. 629 C.P. 36500 Irapuato, Gto. Mexico.

As ubiquitous and often predominant components of the mycoflora in numerous soils, in all climates, fungi belonging to the genus *Trichoderma* play an important role in ecosystem health. The main mechanism for survival and dispersal of *Trichoderma* is through the production of conidia. Conidiation of *Trichoderma* can be triggered by several environmental factors such as light and nutrient availability, and by different types of stress, including desiccation and space limitation. Recently, we found that *Trichoderma atroviride* conidiates in response to mycelial injury. All eukaryotes respond to injury in diverse manners to protect themselves or to preserve their life. To the best of our knowledge, *Trichoderma* is the only fungus where the phenomenon of induction of asexual reproduction by injury has been observed. Light microscopy observations have revealed that aerial hyphae are produced at the site of the injury, which later develop conidiophores. This response is blocked by primary nitrogen sources and strongly influenced by the type of carbon source available. Because the exogenous application cAMP promotes sporulation, and atropine, a compound known to inhibit adenylyl cyclase in *Neurospora*, prevents sporulation, we decided to test the possible role of cAMP dependent protein kinases in this response. For this purpose, we cloned a gene encoding the protein kinase A regulatory subunit (*tpk-r*) from *T. atroviride* and generated transformants that express an antisense version of the gene. The transformants showed elevated levels of PKA activity and did not respond to mycelial injury. Suggesting that PKA activity is involved in the negative control of the response. Additionally, preliminary data indicate that the presence of reducing compounds in the media inhibit the response, suggesting the participation of oxidative stress. Finally, microarray analysis using a collection of above 2000 *T. atroviride* unigenes has allowed us to identify genes that are either induced or repressed upon injury of the mycelium.

Light stimulation of growth and conidiation in *Hypocrea atroviridis* is carbon source dependent

Martina Friedl*, Monika Schmoll, Christian P Kubicek, Irina S Druzhinina

Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria

In fungi, light is primarily known to influence general morphogenesis and both sexual and asexual sporulation. Some authors also report that blue light influences several metabolic processes. To investigate this possibility on a global metabolic scale, we applied Phenotype MicroArray technique to test the effect of light on the utilization of 95 carbon sources by *Hypocrea atroviridis (*anamorph*Trichoderma atroviride)* and compared the results to those obtained with two mutant strains in which one of the two genes encoding the blue light receptor *blr1* and *blr2* had been deleted, respectively. The light-enhanced growth (1.5 – 2-folds) was detected on some carbohydrates (mono-, di- and polysaccharides), sugar acids, sugar amines and polyols, but not that on such carbon sources as amino acids, aliphatic acids. The increase in the growth rate was not observed in the *blr1* and *blr2* delta strains. Light also triggered conidiation of *H. atroviridis* on some carbon sources, most of them were the same as those whose growth was stimulated by light. In order to test whether the specificity of light on selected carbon sources involves signalling via cyclic AMP, experiments were also performed in which dibutyryl-cAMP and the phosphodiesterase inhibitor IBMX were added to the wild-type and the *blr1* and *blr2* mutants and incubated in the dark on the Phenotype MicroArrays. These results will be discussed with respect to a possible cross-talk between light and cyclic AMP in light-stimulation of growth and sporulation in *Hypocrea/Trichoderma*.

Vp-28

Characterisation of cAMP signal transduction in *Aspergillus fumigatus*

Alexander Gehrke, Niklas Möhle, Thorsten Heinekamp*, Axel A. Brakhage

Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology - Hans-Knoell-Institute (HKI), and Friedrich-Schiller-University, Jena, Germany

Infections with mould pathogens have emerged as an increasing risk faced by patients under sustained immunosuppression. Species of the *Aspergillus* family account for most of these infections and in particular *Aspergillus fumigatus* can be regarded as the most important airborne-pathogenic fungus. One of the important questions concerning *A. fumigatus* is the identification of pathogenicity determinants. It is becoming more and more obvious that the fungus has certain physiological characteristics that enables it to avoid or suppress the residual immune system in immunocompromised patients, making it an aggressive opportunistic pathogen. In particular, we have been interested in the cAMP signalling network of *A. fumigatus* and its meaning for pathogenicity. In previous studies several elements of the cAMP signal transduction pathway were characterised by our group and the data indicate that this pathway contributes to pathogenicity of *A. fumigatus*. Here, we present the analysis of additional components of G protein mediated signalling and their relation to cAMP signal transduction.

The MAP kinase MpkC of *Aspergillus fumigatus* is required for utilization of sorbitol

Guadalupe Reyes, Angela Romans, C. Kim Nguyen, Gregory S. May*

Division of Pathology and Laboratory Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA

Mitogen-activated protein kinases (MAPKs) play a central role in regulating fungal cell physiology in response to nutritional status and environmental stresses (e.g. hypertonic shock, heat shock, oxidative stress, and reactive nitrogen species). MAPKs also function in sexual development, asexual development, and fungal cell morphogenesis. Thus defects in MAPK signaling pathways could decrease the virulence of the fungus in a host. A number of studies of plant and animal fungal pathogens have shown that mutants defective in MAPK signaling have reduced virulence The genome of *Aspergillus fumigatus* has four genes that encode mitogen-activated protein kinases, *sakA*/*hogA*, *mpkA*, *mpkB*, and *mpkC*. The functions of the MpkB and MpkC MAPKs are unknown in *A. fumigatus* or the closely related and genetically amenable species *Aspergillus nidulans*. *mpkC* deletion mutants were made in *A. fumigatus* and their phenotypes characterized. The *mpkC* deletion mutants were viable, had normal conidial germination and hyphal growth on minimal or complete media. This is in contrast to deletion mutants for the closely related MAPK gene *sakA*/*hogA* that we previously reported had a nitrogen source dependent germination phenotype. Similarly, growth of the *mpkC* deletion mutants was wild type on high osmolarity medium. Consistent with these two MAP kinase genes regulating different cellular responses, we determined that the *mpkC* deletion mutants were unable to grow on minimal medium with sorbitol or mannitol as sole carbon source. This result implicates MpkC signaling in carbon source utilization. We investigated changes in messenger RNA (mRNA) levels by Northern blotting in wild type, and *sakA* and *mpkC* deletion mutants in response to hyperosmotic stress, exposure to hydrogen peroxide and a shift in carbon source from dextrose to sorbitol. The *mpkC* mRNA is not detectable on Northern blots but is by reverse transcription and PCR, suggesting that *mpkC* mRNA levels are normally very low. We also found that mpkC transcripts fall into 4 size classes based on sequences of cDNA clones. This analysis demonstrated that SakA and MpkC dependent patterns of change in mRNA abundance are distinct and have minimal overlap.

Vp-30

Affinity purification and mass spectrometric analysis identifies two novel nuclear pore complex (NPC) proteins required to maintain the mitotic core the *Aspergillus* NPC

Hui-Lin Liu*, Stephen A. Osmani

Department of Molecular Genetics. The Ohio State University, Columbus, OH 43210-1292

The nuclear pore complex (NPC) is a huge structure providing a regulated conduit between the nucleoplasm and cytoplasm. During human open mitosis the NPC is completely disassembled whereas during yeast closed mitoses the NPC remains intact. In spite of this dramatic difference some 30 functionally conserved proteins interact to form the NPC in all eukaryotes studied. We are interested in the mitotic regulation of the *Aspergillus nidulans* NPC as it undergoes partial disassembly with over one third of its 30 identified components reversibly dispersing during mitosis whilst a conserved minimal core structure containing An-Nup96 remains in the nuclear envelope. How disassembly and reassembly of NPCs is regulated is not clear. Studying the structural components of the NPC during *A. nidulans* partial open mitosis may provide keys to understanding the mechanism of this regulation.

After endogenous S-tagging, the An-Nup96 complex was affinity purified to homogeneity. Mass spectrometry analysis of purified bands confirmed An-Nup96 purification and additionally identified stoichiometric amounts of An-Nup120, An-Nup85 and lower amounts of An-Sec13. In addition to these conserved components of the Nup96 complex, two novel proteins, termed NupA and NupB, were also identified. Both new proteins are conserved in filamentous fungi but are lacking in *Saccharomyces cerevisiae*. NupA and NupB were subsequently endogenously tagged and affinity purified. In these pull downs all components of the Nup96 sub complex were identified in similar stoichiometry to the An-Nup96 purifications. Confirming NupA and NupB to be new components of the Nup96 sub complex, endogenously GFP-tagged versions were found to locate specifically to the NPC throughout mitosis as do the other components of this complex.

Gene deletion analysis demonstrates *nupA* and *nupB* are not essential. However, their deletions show synthetic lethality with deletions of components in the Nup96 sub complex. Moreover, nupA+nupB double deleted cells are temperature sensitive at 42 degrees. Most interestingly, we observed that in *nupA* deleted cells, An-Nup96 and An-Nup133 no longer stay at the NPCs during mitosis. Instead, An-Nup96 and An-Nup133 become partially disassembled from the NPC from prophase until telophase.

In conclusion, we have identified two new fungal specific NPC proteins that are part of the conserved Nup96 sub complex. Both are required for normal NPC function and play a role in maintaining the core structure of the NPC during mitosis. This study suggests that NupA and NupB represent evolutionarily specialized NPC proteins involved in maintaining the core structure of the NPC during fungal mitosis.

The FVVEA gene regulates filamentous growth and conidiation pattern in *Fusarium verticillioides*

Shaojie Li[1], Kyung Myung[1], Diana Guse[1], Brett Donkin[1], Robert H Proctor[2], Scott Grayburn[1], Ana M Calvo[1*]

[1] *Department of Biological Sciences, Northern Illinois University, Dekalb, Illinois, USA*
[2] *USDA-ARS-NCAUR, Peoria, Illinois, USA*

The velvet gene *veA* is known to coordinate asexual and sexual sporulation in the homothallic fungal species *Aspergillus nidulans*. In addition, it regulates sclerotial production in the aflatoxin producer *Aspergillus parasiticus*. Whether *veA* has the same role in morphogenesis in other fungal genera has not been investigated. In this work, we study the role of the *veA* homolog, FVVEA, in the heterothallic fungus *Fusarium verticillioides.* Deletion of FVVEA suppresses filamentous growth and markedly stimulated conidiation in submerged cultures. Moreover, the *fvveA* deletion mutants (of both mating types) exhibited yeast-like budding growth, which was promoted in shaken liquid cultures where aeration is increased. This suggests that FVVEA controls a transition between filamentous growth and conidiation in *F. verticillioides***.** Remarkably, deletion of FVVEA also dramatically increased the ratio of macroconidia to microconidia. Supplementation of osmotic stabilizers restored the wild-type phenotype in the deletion mutants.

Vp-32

Expression and functional analysis of genes induced during appressorium formation by environmental signals and cyclic AMP in *Magnaporthe grisea*

YeonYee Oh[1], Nicole Donofrio[1], Helen Pan[1], Sean Coughlan[2], Thomas K Mitchell[1], Ralph A Dean[1*]

[1] Fungal Genomics Lab, Center for Integrated Fungal Research, North Carolina State University, Raleigh NC.
[2] Agilent Technologies, Inc., Wilmington DE

Magnaporthe grisea, the casual agent of rice blast disease, develops an appressorium, a specialized infection structure to gain access into its host. To identify the core set of genes induced during appressorium formation, we compared global gene expression patterns in appressoria induced by cAMP and a physical cue. Microarray experiments were performed using the *M. grisea* 22K element oligonucleotide array created in collaboration with Agilent Technology. The microarray contains 13,666 *M. grisea* and 7,124 rice elements. RNA was extracted from spores germinated on either appressorium-inductive (hydrophobic) or non-inductive (hydrophilic) surfaces as well as from spores germinated on hydrophilic surfaces in the presence of cAMP. At the time of RNA extraction, under appressoria inducing conditions, germ tube tips had ceased polar growth and were beginning to develop melanized appressoria. Spores germinated on the non-inductive surface elaborated long germ tubes but formed no appressoria. Approximately 3 % of the predicted genes in *M. grisea* were differentially expressed during appressorium formation in response to both a hydrophobic surface and exogenous cAMP, compared to spores germinated under non-inducing conditions. Analysis of differentially expressed genes suggest that during appressorium induction, amino acid metabolism, protein degradation and secondary metabolism are activated while overall protein biosynthesis is repressed.

Vp-33

Functional analysis of the *ATG8* homologue *Aoatg8*, and role of autophagy in differentiation and germination in *Aspergillus oryzae*

Takashi Kikuma*, Mamoru Ohenada, Manabu Arioka, Katsuhiko Kitamoto

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Phone: +81-3-5841-5161. Fax: +81-3-5841-8033. E-mail: tkikuma@hotmail.com

Autophagy is a protein degradation system conserved in eukaryotic cells, and is used to recycle macromolecules and aid cell survival under nutritional starvation conditions. Recently, it was reported that autophagy is involved in the turnover of cellular components, development, differentiation, immune responses, protection against pathogens and cell death in various eukaryotes including yeasts, plants, mammals and so on. When autophagy is induced, bulk cytoplasm and/or organelles are sequestered within autophagosomes. The contents of autophagosome then are delivered into the lumen of the vacuoles and degraded by vacuolar hydrolases. *ATG8* is an autophagy-related gene found in *Saccharomyces cerevisiae* that plays an important role in the formation of autophagosomes. Atg8 is localized in the membrane of pre-autophagosomal structures (PAS), autophagosomes and autophagic bodies, and has therefore been used as a marker of these organelles. In filamentous fungi, the processes of autophagy have been observed and studied in *Podospora anserina*. In this species, autophagy is induced during cell death by incompatibility, which occurs when cells of different genotypes fuse. Null mutants of the *idi-7*/Pa*ATG8* gene (the orthologue of *S. cerevisiae ATG8*) form fewer aerial hyphae and no protoperithecia. Furthermore, a null mutant of Pa*ATG1* (the orthologue of *S. cerevisiae ATG1*) shows the same defects as the Δ*idi-7*/Pa*ATG8* mutants.

The deuteromycete filamentous fungus *Aspergillus oryzae* is an important microorganism in Japanese fermentative industries, as it plays a role in the production of sake, miso and soy sauce. More recently, *A. oryzae* has been described as an excellent host for the production of homologous and heterologous enzymes In this study, we isolated the *ATG8* gene homologue *Aoatg8* from *A. oryzae* and visualized autophagy by the expression of DsRed2-AoAtg8 and EGFP-AoAtg8 fusion proteins in this fungus. The fusion proteins were localized in PAS, isolation membranes and autophagosomes, and were found within vacuoles under starvation conditions or in the presence of rapamycin. DsRed2 expressed in the cytoplasm was also taken up into vacuoles under starvation conditions or during the differentiation of conidiophores and conidial germination. Deletion mutants of *Aoatg8* did not formed aerial hyphae and conidia, and exhibited defect in autophagy. This phenotype was restored by expressing of *Aoatg8* under the control of the thiamine-regulatable *thiA* promoter. Furthermore, the *Aoatg8* conditional mutants showed a delay in conidial germination in the absence of nitrogen source. These results suggest that autophagy functions in both the differentiation of aerial hyphae and in conidial germination in *A. oryzae*.

Vp-34

Spectrin family protein represented in *Neurospora crassa* by alpha-actinin-like protein

Marta Cotado Sampayo[1*], Mukti Ojha[1], Ruben Dario Ortega Perez[1], Marie-Louise Chappuis[1], Carol Seum[2], François Barja[1]

[1] *Department of Botany and Plant Biology, University of Geneva, Jussy-Geneva, Switzerland*
[2] *Department of Zoology, University of Geneva, Geneva, Switzerland*

The filamentous fungus, *Neurospora crassa* provides a simple, genetically tractable model for studies of the function of cytoskeletal proteins in cellular morphogenesis. The major proteins of the fungal cytoskeleton, actin and tubulin, have been well characterized but there is not much information about associated proteins. The spectrin superfamily proteins comprised of spectrin, -actinin and dystrophin/utrophin are involved in the organization of actin cytoskeleton.

The presence of a spectrin superfamily protein was examined in crude extracts from exponentially growing *N. crassa* using polyclonal antibodies against α/β-spectrin. Analysis of SDS-PAGE gels showed the presence of a single band of about 100 kDa. The immunofluorescence and immunogold labeling of this protein in the germ tube and hyphae showed its predominance in the tip region and along the plasma membrane. There is no evidence of the classical spectrin gene in fungi. We have found a gene, NCU06429.2, closely related to α -actinin, in the *N. crassa* genomic data base. This gene codes for a protein (theoretical Mr of 110 kDa) containing two calponin homology domains, a rod domain composed of two spectrin repeats and an EF-hand domain. We have cloned this gene and purified the recombinant GST-NCU06429.2 corresponding to a peptide of Mr of 140 kDa. Deducting the GST tag sequence (aprox. 26 kDa), the Mr of the expressed protein corresponds to what can be expected.

The results on the spatial localization of NCU06429.2 in the growing *N. crassa* hyphae will be presented and compared to that revealed by α/β-spectrin specific antibodies.

On the basis of fungal database analysis and our results, the presence of a homologous to higher eukaryote spectrin in *Neurospora* could not be established.

Localization of chitin synthases in living hyphae of *Neurospora crassa*

Meritxell Riquelme[1*], Juan Manuel Gonzalez-Prieto[2], Eddy Sanchez-Leon[1], Michael Freitag[3], Salomon Bartnicki-Garcia[1]

[1] Department of Microbiology. Center for Scientific Research and Higher Education of Ensenada CICESE. 22860 Ensenada, Baja California, Mexico.
[2] Center for Genomic Biotechnology. Laboratory of Plant Biotechnology. 88730 Ciudad Reynosa, Tamaulipas, Mexico.
[3] Institute of Molecular Biology. University of Oregon. Eugene, OR 97403-1229. USA

Chitin, the main structural component of the cell wall of most fungi, is a linear homopolymer composed of ß-1,4 linked N-acetylglucosamine subunits, whose biosynthesis is catalyzed by chitin synthases (CHS). Bioinformatic analysis of the genomic sequence of the fungus *Neurospora crassa* shows seven open reading frames with homology to described *chs* genes. Only four of these, *chs-1, chs-2*, *chs-3* and *chs-4* belonging to classes III, II, I and IV, respectively, have been previously described in *N. crassa*. Despite the vast amount of knowledge on the role of different CHS in fungi, mainly from yeast, we lack information on trafficking of these enzymes to their sites of action on the cell surface in regions of active cell wall growth in filamentous fungi.

As part of an ongoing project to characterize the organization of the secretory pathway in filamentous fungi, we tagged several key proteins of the secretory network with fluorescent proteins (FPs). We used high-resolution bioimaging to discern CHS vesicle traffic from synthesis sites to the plasma membrane in growing hyphae of *N. crassa*. We have labeled two CHS (CHS-3 and CHS-6) with GFP to analyze the localization and traffic of chitosomes, the microvesicular carriers of chitin synthase. We observed similar distribution patterns along the hyphae in both CHS-3-GFP and CHS-6-GFP strains. In the distal subapical region (beyond 45 µm from the tip), CHS-GFP is found mainly in a highly stained network of large endomembranous compartments many of them spherical; closer to the hyphal tip, the label becomes dispersed into numerous vesicles or groups of vesicles that moved predominantly forward at 0.4 µm/sec until reaching the proximal subapical region (15-20 µm from the tip); significantly, this is the same speed of hyphal elongation. At the tip, the fluorescence congregates into a conspicuous single body. By using FM4-64 to label the Spitzenkörper (Spk), we observed that CHS-GFP was localized in the inner core of the Spk, the same region in which microvesicles are detected by transmission electron microscopy. Seemingly, these morphologically diverse fluorescent compartments constitute the secretory path traveled by CHS in the hyphal cell. We are presently investigating the nature and dynamics of the endomembranous compartments involved in this secretory route.

Vp-36

Ectomycorrhizal interactions of *Tricholoma* species

Katrin Krause, Ines Schlunk, Felicia Gherghel, Erika Kothe

Friedrich-Schiller-University, Dept. Microbiology, Microbial Phytopathology, Neugasse 25, 07743 Jena, Germany

Mycorrhizal fungi play an important role in plant nutrition and plant health, especially under stress conditions. However, the molecular mechanisms are poorly understood. Differential display was carried out to identify genes showing differential expression in ectomycorrhiza between the basidiomycete *Tricholoma vaccinum* and its compatible host, the spruce *Picea abies,* using ectomycorrhizal roots of different stages, pure roots and cultures of the fungus. Of 133 PCR fragments were verified and the clone's origin and expression pattern were checked. Sequence analyses identified genes with function in plant pathogen response, signal transduction, nutrient exchange, growth *in planta* and stress answer.

Four genes were studied inmore detail: an aldehyde dehydrogenase (expression pattern), a retrotransposons (occurrence within the genus *Tricholoma*), a MATE transporter protein and an APS kinase (expression in oak mycorrhizas from polluted and non-polluted soils).

To investigate the function of genes in the root-*Tricholoma* interaction a transformation technique using *Agrobacterium tumefaciens* T-DNA transfer was developed. The transformants carry the gene for the resistance against hygromycin B (*hph*) and the enhanced green fluorescent protein gene (EGFP). Putative transformants were checked for the presence of *hph* and EGFP by PCR. Southern blot analysis and ectomycorrhiza forming properties of the transformants are presented.

A mutation in a protein kinase C homologue causes Calcofluor hypersensitivity in *Aspergillus nidulans*

Annette G Teepe, Darlene M Loprete, Terry W Hill*

Rhodes College, Departments of Biology and Chemistry

We have identified a set of mutant strains in *Aspergillus nidulans*, which show elevated sensitivity to the wall compromising agent Calcofluor White (CFW), indicating a probable defect in cell wall integrity. One of these, designated *calC*, shows several secondary phenotypes also frequently observed in wall mutants, including sensitivities to SDS, Caspofungin acetate, and Congo Red. In addition, Nikkomycin, Tunicamycin and Caspofungin induce excessive spore swelling in the *calC*, strain during spore germination at 30C. Sporulation is depressed at 42C, but can be osmotically remediated. We have complemented CFW hypersensitivity from an *A. nidulans*, plasmid genomic library, leading to the identification of AN0106.2 (identified in the Broad Institute database as "KPC1_ASPNG Protein kinase C-like")as the complementing sequence. This sequence is located in the same region of chromosome VIII to which the *calC* mutation has been mapped. The *calC*, PKC contains a predicted glycine-to-arginine substitution at position 564. The cloned wild type gene reduces sensitivity of the *calC* strain to CFW, SDS, Caspofungin, and Congo red, while reducing temperature-induced suppression of sporulation and drug-induced swelling of spores. Resistance of the *calC* strain to CFW was slightly improved by the PKC activator phorbol 12,13-diacetate. Interestingly, CFW resistance was even more strongly improved by the protein kinase inhibitors chelerythrine chloride and bisindolylmaleimide I HCl. These drugs had no effect on the CFW resistance/sensitivity of the wild type. This suggests that the possibility that multiple protein kinases participate in controlling cell wall integrity in *A. nidulans*.

Vp-38

Whole genome analysis of *Magnaporthe grisea* gene expression during infection and colonization

Nicole Donofrio[1], Helen Pan[1], Sean Coughlan[2], Thomas K Mitchell[1], Ralph A Dean[1*]

[1] Fungal Genomics Laboratory, Center for Integrated Fungal Research, North Carolina State University, Raleigh, NC, USA
[2] Agilent Technologies Inc., Little Falls, DE USA

Magnaporthe grisea causes the devastating blast disease on rice, as well as on other members of the grass family, such as barley. We exploited this particular host-fungal interaction to gain a genome-wide perspective of fungal genes expressed during infection. We analyzed transcript levels during three time-points representing pre-symptom (48 hours post-inoculation), chlorotic lesion (72 hours) and necrotic lesion (96 hours) stages of disease. Several hundred genes showed significant, detectable expression during infection. A group of genes common among the 96 and 72 hour time-points included genes potentially involved in protein biosynthesis and stress response, as determined by Gene Ontology. Expression of four of these genes was independently confirmed using RT-PCR. Of the genes expressed at the 96 hour time-point, 22% are likely involved in stress responses, comprising the largest functional category. This group includes two superoxide dismutases as well as one catalase-peroxidase, which also had significant expression at the 72 hour time-point. One gene found to be common to all three time-points is a member of the 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily, sharing 65% and 45% amino acid similarity with such genes from *Aspergillus fumigatus* and *Streptomyces hygroscopicus*, respectively. Together, our results suggest that *M. grisea* could be mounting an oxidative response to kill cells for its own growth and development, and/or expressing genes (such as the superoxide dismutase and catalase-peroxidase) involved in detoxifying oxidative compounds produced during the host's defense response.

Studies on the subcellular localisation of PalC, the second amongst the pH signalling pathway proteins that contains a Bro1 domain

Antonio Galindo[1*], Joan Tilburn[2], Juan C. Sanchez-Ferrero[1], E. Reoyo[1], América Hervás-Aguilar[1], Olivier Vincent[3], Herbert N. Arst Jr.[2], Miguel A. Peñalva[1]

[1] Departamento de Microbiología Molecular. Centro de Investigaciones Biológicas CSIC, Ramiro de Maeztu 9, Madrid 28040, Spain.
[2] Department of Infectious Diseases, Imperial College, Du Cane Rd., London W12 0NN, United Kingdom.
[3] Unidad de Bioquímica y Genética de Levaduras. Instituto de Investigaciones Biomédicas "Alberto Sols", Arturo Duperier 4, Madrid 28029, Spain

PalC is one of the six proteins of the ambient pH signalling pathway which, under alkaline conditions, mediates the first cleavage in the two-step proteolytic activation of the transcription factor PacC. Although PalC is present in both ascomycetes and basidiomycetes, it appears to be restricted to the proteomes of filamentous fungi and *Yarrowia lipolytica*, as it is absent from yeasts. PalC is notable in that it does not appear to have a mammalian orthologue, which might be of applied interest because an intact pH signalling pathway is required for fungal pathogenicity to plants and mammals(1). Sequence characterisation of PalC gave no clue as to its precise molecular function. A genetic approach combined with bioinformatic analyses of databases using Hidden Markov models (HMM) revealed that PalC contains a highly conserved region showing convincing similarity to the ~160 residue PFAM Bro1 domain, that we denoted the 'PalC Bro1 Homology Domain' (PCBROH) because this region is considerably larger than the PFAM Bro1 domain(2). However, in agreement with our genetic/bioinformatic analyses, simultaneous determination of the crystal structure of the Bro1 domain in yeast Bro1 by Kim *et al.*(3), revealed that the Bro1 domain is indeed 367 residues long, thereby extending considerably beyond the 160 residue PFAM domain. Refined sequence alignments and detailed consideration of the Bro1 3D structure revealed that PalC actually contains a Bro1 domain covering most of its sequence apart from the C-terminal di-aromatic motif which is universally conserved in PalC homologues and plays a functional role, although not an essential one. The presence of a Bro1 domain in PalC strongly suggests that, like PalA, is connected to the ESCRT-III oligomeric complexes (endosomal sorting complexes required for transport III) at the membrane of endosomes(4). A PalC-GFP fusion protein is cytosolic and shows a punctuate pattern of localisation which is lost when single-residue substitutions resulting in loss of PalC function are introduced in the reporter fusion protein.

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Vp-40

Isolation of an *Aspergillus* specific nucleolar protein as a copy number suppressor of *nimA1*

Leena Ukil*, Stephen A Osmani

Department of Molecular Genetics, The Ohio State University, Columbus, OH 43210-1292

Regulation of the cell cycle is critical for normal development of multicellular organisms and an understanding of this process is crucial for studying cell proliferation and cancer. A number of cell cycle dependent protein kinases have been identified that specifically control mitotic progression and chromosome segregation. The *nim*A gene in *Aspergillus nidulans* encodes one such protein kinase that is both required and sufficient for chromosome condensation, mitotic spindle formation and disassembly of the nuclear pore complex to allow tubulin and regulators to enter nuclei during mitosis. There exist protein kinases structurally similar to NimA in other organisms, including humans. Given the crucial role played by NimA in mitosis, we set up a screen to identify copy number suppressors of the *nimA*1 mutant to identify new mitotic regulators. The screen identified a novel *Aspergillus* specific gene termed *mcn*A.

Endogenously GFP-tagged McnA appears as a single dot in the nucleus. Co-localization studies of McnA with nucleolar markers show McnA-GFP locates to the nucleolus and to have a unique pattern of segregation during mitosis. During G2, McnA-GFP is located in the vicinity of nucleolar markers in a sub domain of the nucleolus. At mitosis, McnA-GFP locates in a focal point in the cytoplasm whereas some other nucleolar proteins are dispersed throughout the cell. When mitosis is completed McnA-GFP remains as a single dot outside newly formed daughter nuclei as the dispersed nucleolar proteins re-locate to the daughter nucleoli. It is only later in G1 that McnA-GFP begins to appear within new daughter nucleoli with intermediate dots appearing in the cytoplasm during this process. In lower eukaryotes that have completely closed mitosis, the nucleolus is known to divide within nuclei. In higher eukaryotic open mitosis the nucleolus disassembles and then undergoes a stepwise reassembly as daughter nuclei reform. Our studies of nucleolar McnA-GFP and other endogenously tagged nucleolar proteins, indicate that *A. nidulans* also undergoes disassembly then reassembly of its nucleolus during mitosis.

Affinity purification of S-tagged McnA and SDS PAGE indicates that McnA migrates as several specific molecular weights species. Using mass spectrometric analysis these different forms of McnA have been determined to be modified by phosphorylation.

In conclusion, this study has identified a novel phosphoprotein specific to the Aspergilli which interacts with the NimA kinase as a copy number suppressor of *nimA1*. The data further show *A. nidulans* nucleoli undergo mitotic segregation in a manner similar to higher eukaryotic nucleoli.

Isolation of a potential cell cycle regulated transcription factor as a copy number suppressor of *nimA1*

Leena Ukil*, Stephen A Osmani

Department of Molecular Genetics, The Ohio State University, Columbus, OH 43210-1292

NimA is a cell cycle regulated protein kinase required for initiation of mitosis in *Aspergillus nidulans*. It has been previously shown that the levels of *nim*A mRNA become elevated as cells enter mitosis and drop sharply as cells progress through mitosis. While much is known about the role played by NimA during mitosis to regulate chromosome condensation, spindle formation and disassembly of the nuclear pore complex, less is know about its regulators or its transcription factors.

In an attempt to identify possible interacting genes of *nim*A, we performed a copy number suppressor screen of the *nim*A1 mutation. The *nim*A*1* mutation is temperature sensitive at 42°C and results in a reversible G2 arrest. The *nim*A1 mutant is a point mutation in the non-catalytic domain which restricts the protein to the cytoplasm instead of letting it accumulate in the nucleus at mitosis. On the other hand, the *nim*A5 mutation is in the catalytic domain and inactivates the kinase at restrictive temperatures. There are therefore two potential ways of suppressing the *nim*A1 mutation, one by making more of the protein so that there is enough that diffuses into the nucleus or secondly by changing the transport properties of the nuclear envelope so that NimA1 can enter the nucleus. The screen identified *mcn*B which can suppress *nim*A1 but not *nim*A5 when expressed from the high copy number plasmid AMA1. Elevated expression of *mcn*B causes up-regulation of the amount of NimA1 protein. The protein sequence of *mcn*B has homology to fork head domain containing transcription factors. An endogenously GFP-tagged version of McnB accumulates in nuclei in late G2 and disappears from the nucleus at the onset of mitosis before re-accumulating in the next G2. This demonstrates that McnB is regulated through the cell cycle. Since *mcn*B expression up-regulates NimA, it is possible that it is a unique cell cycle specific *nim*A transcription factor.

In conclusion, we have identified a potential cell cycle specific transcription factor for *nimA* which can drive increased expression of NimA and is itself regulated in a cell cycle specific manner.

Vp-41

Insights into the two-step proteolytic processing of PacC

América Hervás-Aguilar[1*], Olga Rodríguez-Galán[1], José María Rodríguez-Rodríguez[1], Elena Reoyo[1], Herbert N Jr Arst[2], Joan Tilburn[2], Miguel A Peñalva[1]

[1] Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas del CSIC, Ramiro de Maetzu 9, Madrid 28040, Spain
[2] Department of Infectious Diseases, Imperial College London, Ducane Road, London, W12 0NN, UK

pH regulation in fungi/yeasts is mediated by the zinc finger transcription factor PacC/Rim101. In *A. nidulans*, PacC is synthesised as a largely inactive primary translation product, PacC72. Ambient alkaline pH activates the pal signal transduction pathway, comprised of PalA, B, C, F, H and I, resulting in the two-step proteolytic activation of PacC72. PacC72 is first converted to PacC53 by the action of a signalling (ambient alkaline pH-sensitive) protease. PacC53 is a committed intermediate which is converted into PacC27 by a second (ambient pH insensitive) 'processing' protease. PalB is likely to be the signalling protease. In agreement, PalB is not involved in processing PacC53 to PacC27 and its action takes place downstream of the 'plasma membrane complex' (1). PalB levels do not change and PalB would not appear to undergo post-translational modification in a pH/pal-dependent manner. We hypothesise that the mechanism underlying PalB regulation involves its subcellular localisation. Yeast PalB orthologue Rim13 is a two-hybrid interactor of ESCRT-III Vps32, strongly suggesting that Rim13/PalB forms, with PalA (2) and PalC (poster by Galindo *et al.*) an 'endosomal membrane pH signalling complex'. Indirect immunofluorescence revealed that HA-PalB shows a punctuate distribution and that 40-50% of PalB associates to P13 membrane fractions. However, PalB and Vps32 do not interact in two-hybrid assays, suggesting that either the yeast Rim13-Vps32 interaction is indirect, involving another 'bridging' ESCRT-III component or that Rim13 and PalB use different ESCRT-III components for their recruitment to endosomes. The ~pH-independent processing protease, conserved in yeast, cleaves PacC at residues 252-254 but specificity determinants for this reaction are remote from the processing site(3). A major determinant of processing efficiency lies within residues 266-407 (4). One possible candidate for the processing protease would be the proteasome, which mediates regulation by proteolytic processing of the likely PacC homologue *Drosophila Cubitus interruptus*(5). Targeting proteins to the proteasome involves ubiquitin -amino group(s) of one orεligase-mediated substrate polyubiquitination at the more Lys. We hypothesised that the proteolytic efficiency determinant might contain a docking site for a ubiquitin ligase and/or key ubiquitinylable Lys residues and used gene replacements to introduce Arg substitutions in candidate Lys residues. A PacC protein carrying four such substitutions is markedly affected in PacC processing and these substitutions strongly impair processing to PacC27of a truncated protein approximating PacC53. These data would agree, amongst other possibilities, with proteasomal involvement in PacC processing. Notably phenotypic analyses provided convincing evidence that, in addition to PacC27[6], PacC53 can play a functional role in pH regulation.

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The ambient pH-responsive signalling proteolysis of *Aspergillus nidulans* PacC

Maria M Peñas[1*], Joan Tilburn[2], Miguel A Peñalva[3], Herb N Arst[2]

[1] Department of Infectious Diseases and Immunity, Faculty of Medicine, Imperial College London, W12 ONN, UK. Departamento de Produccion Agraria. Universidad Pública de Navarra. Campus de Arrosadia, s/n. Pamplona, 31006. Spain
[2] Department of Infectious Diseases and Immunity, Faculty of Medicine, Imperial College London. London W12 ONN, UK.
[3] Centro de Investigaciones Biológicas, CSIC. Madrid 28040. Spain

The activation of the *A. nidulans* PacC transcription factor requires two sequential proteolytic steps. In the first, signalling proteolysis step, the PacC translation product (PacC72) is converted to an intermediate (PacC53) by a signalling protease in response to alkaline ambient pH signal transduction. In the second, pH-independent, processing proteolysis step, the intermediate is converted to the processed product (PacC27) containing the ~250 N-terminal residues, by a yet unidentified processing protease. The processing protease reaction is apparently independent of the amino acid sequence at the cleavage site whereas the signalling proteolysis was thought to require a conserved 24-residue "signalling protease box" sequence (or at least a portion of it).

Although the approximate site of PacC cleavage by the signalling protease has been determined (Diez *et al.,* 2002) and PalB is the obvious candidate for the signalling protease (Arst and Peñalva, 2003), neither the specificity requirements for signalling proteolysis nor the precise identity of the signalling protease has been determined.

We have carried out pacC gene replacements with alanine residues substituting different amino acids within the 24-residue conserved 'signalling protease box', focusing particularly on residues which are highly conserved in interspecies comparisons. In spite of conservation of most of the residues, only three of these substitutions, in addition to the classical mutations Leu498Ser or Phe, pacC$^{+/-}$209 or pacC$^{+/-}$210, respectively, have been found impair function. These results suggest that primary sequence requirements for signalling proteolysis are not very stringent.

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Vp-43

The mitogen-activated protein kinase Tmk1 is involved in *Trichoderma atroviride* mycoparasitism

Barbara Reithner[1*], Rainer Schuhmacher[2], Susanne Zeilinger[1]

[1] Technical University of Vienna, Institute for Chemical Engineering, Group Molecular Biochemistry of Fungi, Getreidemarkt 9/1665, 1060 Wien, Austria
[2] IFA Tulln, University of Natural Resources and Applied Life Sciences Vienna, Center for Analytical Chemistry, Department for Agrobiotechnology, Konrad Lorenz Str. 20, 3430 Tulln, Austria

Mycoparasitic *Trichoderma* strains were shown to act against a number of plant pathogenic fungi and are therefore commercially applied as biological control agents as an alternative to chemical fungicides. The host-specific mycoparasitic action of *Trichoderma* involves morphological changes and secretion of hydrolytic enzymes and antifungal metabolites. As the mechanisms of pathogenicity are highly conserved throughout pathogenic fungi and signal transduction via heterotrimeric G-proteins has a significant impact on the regulation of mycoparasitism-related functions in *T. atroviride*, we studied additional signalling components of this fungus such as MAP-kinases. In particular, the *tmk1* gene of *T. atroviride* P1 coding for a MAP-kinase closely related to Pmk1 of *Magnaporthe grisea* and TmkA of *T. virens* was isolated and characterized.

Tmk1 knockout mutants showed slightly reduced radial growth rates and sporulated even in the dark when compared to the wild type. Expression of mycoparasitism-related genes such as the extracellular chitinases Nag1 (a 73 kDa N-acetyl-glucosaminidase) and Ech42 (a 42 kDa endochitinase) is increased in all *Δtmk1* strains when replaced to colloidal chitin as sole carbon source. Plate confrontation assays with *Rhizoctonia solani* showed reduced mycoparasitism of the *tmk1*-negative strains compared to the wild type both in overgrowth and lysis of the respective host fungi. The over all antibiotic activity of the knockout mutants against *R. solani* and *Botrytis cinerea* is significantly improved when compared to the wild type, probably due to the overproduction of the antifungal metabolite 6-pentyl-α-pyrone. As a summary these data strongly suggest the involvement of Tmk1 in various aspects of mycoparasitism-related signal transduction.

Proteomic analysis of nitrogen regulation and signalling in *Aspergillus nidulans*

Igor Morozov*, Meriel G Jones, Huw H Rees, Mark X Caddick

School of Biological Sciences, University of Liverpool, Liverpool, L69 7ZB, UK

Nitrogen metabolism in *Aspergillus nidulans* is subject to regulation by the GATA transcription factor AreA, the activity of which is determined by the quality and quantity of the available nitrogen sources. Under conditions of nitrogen limitation, AreA activity increases and this results in the expression of a large, though as yet undefined, number of genes. Here we report a proteomic-based analysis of nitrogen metabolism and signalling. The response, within two minutes, to the addition of glutamine to a nitrogen-starved culture was monitored using 2D gel electrophoresis. The expression profile of three proteins was altered. They were identified by using MS-MS. One showed homology to proteins involved intracellular signalling through the presence of a PH domain. A second appeared in two forms with different apparent molecular weights. Under nitrogen limitation the lower molecular weight form predominated while the opposite occurs under derepressed conditions. The protein contains a CS domain, suggesting a role as a HSP90 co-chaperone. The third protein, which is only apparent in the presence of glutamine, contains the RanBP1 domain, characteristic of a role in regulation of receptor-mediated transport between the nucleus and cytoplasm. To monitor the AreA regulatory domain and function we have compared the proteome of 1) wild type and *areA*$^{-}$ strains, after two hours of starvation. 2) Wild type and *xprD1* (a mutation in *areA* leading to general derepression) after growth under nitrogen repressing conditions. Of the ~600 proteins being monitored, differential expression between was seen in about 5% (*xprD1*/WT) and 10% (*areA*$^{-}$/WT). Many of these proteins are involved in nitrogen or amino acid metabolism as well as intracellular protein transport and turnover. This study is being complemented by transcriptomic analysis. A number of genes identified through this analysis have been deleted and the respective strains are currently being characterised. The implication of these findings in relation to the regulation of nitrogen metabolism and nitrogen signalling will be discussed.

Vp-45

Development and gene expression in *Trichoderma virens*: insights into biocontrol-related signaling pathways

Ella Pardovitz-Kedmi[1*], Mala Mukherjee[2], Ruthi Hadar[1], Naomi Trushina[1], Ada Viterbo[3], Ilan Chet[3], Benjamin A Horwitz[1], Prasun K Mukherjee[2]

[1] *Department of Biology, Technion, Haifa, Israel*
[2] *Nuclear Agriculture and Biotechnology Division, BARC, Mumbai, India*
[3] *Weizmann Institute of Science, Rehovot, Israel*

Trichoderma virens IMI 304061 was isolated from soil as a mycoparasite on *Sclerotium rolfsii*, and is an aggressive mycoparasite on the sclerotia of *S. rolfsii* and on the hyphae and sclerotia of *Rhizoctonia solani*. *T. virens* strains produce secondary metabolites including the antifungal compounds gliovirin, viridin and viridiol. In addition, *T. virens* can induce systemic resistance in plants against invading pathogens. To understand the role of signaling pathways in mycoparasitism and development, we have isolated several signal transduction-related genes including three G-protein alpha subunit genes (TgaA, TgaB and TgaC) and a MAP kinase (TmkA). Mutants in these genes display developmental phenotypes, and in some cases, their biocontrol potential is altered in a host-specific manner. Furthermore, biocontrol ability depends on the *T. virens* strain. Suppression-subtractive hybridization (SSH) libraries were made to identify genes specific to hypersporulation in *tmkA* mutants, as well as to identify candidates for host-induced genes.

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Ras signaling in the basidiomycete *Schizophyllum commune*

Nicole Knabe, Daniela Schubert, Erika Kothe

Friedrich-Schiller-University Jena, Dept. of Microbiology, Jena, Germany

The small monomeric G-protein Ras has been shown to induce MAPK cascade signaling, cAMP-dependent pathways and cytoskeleton via cdc42 in different fungi. In the model basidiomycete *Schizophyllum commune*, cAMP signaling has been implicated in fruitbody development. The involvement of Ras in sexual development and cell morphogenesis was investigated by characterization of *ras1* and *gap1* genes in *S. commune*.

To analyse the function of Ras, amino acid mutations in the GTP-binding region and GTPase domains were introduced *in vitro*. The phenotypes of the resuting transformants carring the dominant negative allele Ras^{G15N} and the constitutive alleles Ras^{G12V} and Ras^{Q61L} were examined. Ectopic transformation of the three mutant alleles in wild-type *S. commune* resulted in transformants which are phenotypically characterized.

Disruption of *gap1* encoding a Ras-dependent GAP (GTPase activating protein) leads to accumulation of Ras in its activated, GTP-bound state and to constitutive Ras signaling. The phenotype of a *gap1* deletion strains shows disorientated growth pattern. This failure of maintenance of growth direction was especially obvious in dikaryons during clamp formation as hook cells failed to fuse with the peg beside them. Instead, the dikaryotic character of the hyphae was rescued by fusion of the hooks with nearby developing branches. *Dgap1*/*Dgap1* dikaryons formed increased numbers of fruit body primordia, whereas the amount of fruit bodies was not raised. Mature fruitbodies formed no or abnormal gills. No production of spores could be observed.

From these data a model for Ras signaling in different morphogenetic pathways in mushrooms like *Schizophyllum* is derived.

Vp-47

The nuclear migration protein NUDF associates with NUDC and BNFA in *Aspergillus nidulans*

Kerstin Helmstaedt[1*], Katja Voßkuhl[2], Silke Busch[1], Özgür Bayram[1], Gerhard H. Braus[1]

[1] Institut für Mikrobiologie und Genetik, Georg-August-Universität Göttingen, Grisebachstr. 8, D-37077 Göttingen, Germany
[2] present address: Abteilung Gastroenterologie, Hepatologie und Endokrinologie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30623 Hannover, Germany

In filamentous fungi, migration of nuclei is established through the interplay of the molecular motor dynein in combination with dynactin and microtubuli. Several proteins like NUDF, NUDE, and NUDC had been identified as regulators of dynein-mediated movement, which are not part of the dynein/dynactin motor complex itself (Xiang et al, 1995; Efimov & Morris, 2000). We intended to identifiy interaction partners of these regulatory proteins in *Aspergillus nidulans* in order to clarify the regulatory pathway leading to dynein activation. Applying tandem affinity purification, we isolated ten proteins binding to NUDF during vegetative growth. Among these was a protein unique to *A. nidulans*, which we named BNFA (binding of nudF) and characterized in more detail. We confirmed this interaction by yeast two-hybrid analysis and constructed an *A. nidulans* knock-out strain. We also analyzed the potential interaction between NUDF and NUDC by yeast two-hybrid analysis. Furthermore, the putative phosphorylation of NUDF was investigated to decipher its role in protein complex formation and localization of NUDF.

Vp-48

Analysis of the protein kinase ImeB in *Aspergillus nidulans*

Fatih Sari*, Özgür Bayram, Gerhard H. Braus, Stefan Irniger

Institut of Microbiology and Genetics, Georg August University Göttingen, Griesebachstr.8, D-37077 Göttingen, Germany

Protein kinases are central regulators of both the mitotic and the meiotic cell cycle. In the yeast, *Saccharomyces cerevisiae,* the *IME2* gene encoding a serin/threonin protein kinase is expressed only during meiosis. Ime2 is essential for meiotic cell cycle and it was shown that it can take over several functions of the cell cycle regulator Cdc28. A survey of the *Aspergillus nidulans* genome revealed a putative *IME2* homolog, conclusively named *imeB* showing 39% identities to the yeast *IME2.* Investigation of the 781 amino acid *imeB* protein sequence revealed a highly conserved protein kinase domain, suggesting a similar function to the yeast Ime2 in *Aspergillus nidulans*. To elucidate the role of *imeB* in *A. nidulans* a targeted deletion of the *imeB* locus in a wild-type genetic background was conducted. *ImeB* mutants show slower growth and form more sexual structures on plates. These results suggest that ImeB may be a negative regulator of sexual development in *A. nidulans*. We will discuss these unexpected findings, which indicate that ImeB and Ime2 have divergent roles in *A. nidulans* and yeast. The Current status of the ongoing project will be presented.