IVp-1

IDENTIFICATION OF DNA SEGMENTS INVOLVED IN REGULATION BY BLUE LIGHT AND MYCELIAL REPRESSION IN THE PROMOTER OF GENE CON-10 OF NEUROSPORA CRASSA

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The gene con-10 of Neurospora grassa is expressed during conidiation and following illumination of vegetative mycelia with light. The promoter of con-10 is composed of DNA segments involved in repression in the dark and during mycelial growth, and DNA segments required for the activation during conidial development. Dark repression sites have been located at positions –1559 to –779 (from the transcription start site) and –353 to –265. A mycelial repression site has been located at position –778 to –353. Two conidiation activation sites have been located at positions –353 to -265 and –236 to –191. We are using a series of fusions between segments of the con-10 promoter and the lacZ gene to investigate the DNA sequences involved in regulating the expression of con-10. We have used plasmid vector (pDE3) that allowed the targeted integration of all the con-10/lacZ fusions to the his-3 locus to reduce the variability in gene expression associated with genome position. The transformants were selected by their ability to grow in minimal agar, purified after three vegetative cycles, and tested for at single integration event by Southern hybridization. Two strains have been selected for each con-10/lacZ fusion. The strains were grown in the dark for 48 h before applying light. Beta-galactosidase activity in cell extracts was a measure of the con-10 promoter activity. The complete con-10 promoter (-1559) fused to the lacZ gene behaved as the original con-10 gene. Beta galactosidase activity was induced about ten fold from the activity found in the dark cultures. Other con-10 promoter fusions only contained sequences to –913, to –839, and to -517. Their light-dependent activity will locate the position of the first dark repression site and the mycelial repression site. To confirm the presence of the second dark repression site (-353 to –265) and the mycelial repression site (-517 to –354) we have fused each segment to a con-10 minimal promoter (-191) that shows no response to light or conidiation. Additional fusions have been created to confirm the location of the dark repression site: from –353 to –282, and from –337 to –265. These and other con-10/lacZ fusions will allow us to locate the sequences involved in the regulation of con-10 transcription.

IVp-2

IDENTIFICATION AND CHARACTERISATION OF A PUTATIVE ASPERGILLUS NIDULANS TOR SIGNALLING PATHWAY WITH RESPECT TO NITROGEN METABOLISM.

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Under nitrogen limiting conditions in the filamentous fungus Aspergillus nidulans, the GATA transcription factor AREA up-regulates the expression of numerous genes involved in nitrogen metabolism allowing the organism to grow on a wide range of nitrogen sources. The Saccharomyces cerevisiae homologue of AREA, GLN3p, activates nitrogen metabolising genes in response to nitrogen limitation. GLN3p is translocated between the cytoplasm and the nucleus, depending on the nitrogen source available to the organism. Exposure of S. cerevisiae to the anti-fungal macrolide rapamycin causes G1 cell cycle arrest and mimics nitrogen-limiting conditions by causing nuclear accumulation of GLN3. Rapamycin, complexed with FKBP12 binds with high affinity to the Target of Rapamycin (TOR) proteins 1 and 2. The TOR proteins mediate the expression of the nitrogen metabolic pathway genes in S. cerevisiae via a phosphorylation pathway involving a URE2/GLN3 transcription complex. Our findings have shown that there are homologues of the yeast TOR pathway genes TOR1, FRB1, TIP41, SIT4 and TAP42 in A. nidulans. These homologues have been named torA, frbA, tipA, sitA and tapA. Additionally, rapamycin sensitivity in A. nidulans was demonstrated by reduced growth rate and little to no conidiation. We have selected rapamycin resistant strains, containing mutations in three separate genes, torA, frbA and tipA. Putative protein sequences for the A. nidulans TOR pathway genes have been elucidated from the cDNA sequences. The A. nidulans tipA mutants (tipA5 and tipA10) have been studied in detail. They demonstrate both a rapamycin resistant phenotype and reduced utilisation of poor nitrogen sources such as 2-pyrrolidone. Northern blot analysis has confirmed that the expression levels of lamA and lamB are significantly lower in these mutants compared to the wild type. Currently, further genetic tests are being conducted to elucidate the role of tipA with respect to nitrogen metabolism.

SIGNALING pH IN TRICHODERMA HARZIANUM

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Trichoderma harzianum is a filamentous fungus used in biocontrol, mainly against plant pathogen fungi. One of the mechanisms employed by Trichoderma during antagonism is the production of a complex group of cell wall degrading enzymes (CWDE), such as chitinases, glucanases and proteases.
pH, together with carbon and nitrogen sources, are the key factors that fungal cells have to adapt to. Trichoderma exerts a strict control of external pH, ensuring optimal values to the secreted enzymes. We have checked that, when growing in glucose and ammonium containing cultures, pH value decreases below 3. However, when Trichoderma grows with not easily assimilable carbon sources such chitin, the main component of fungal cell walls, pH is maintained around initial pH values (5.5), and chitinase activity is maximal around pH 5, the optimal pH of most chitinases. Proteases PapA and Prb1 activities seem also to be pH controlled, showing maximal values at acidic and alkaline pHs, respectively**.** Data obtained from activity correlate with mRNA levels, suggesting a pH-dependent transcriptional control to respond to different pH values with different proteases, each with an appropriate optimal value. Not only CWDE genes and their proteins, but also other genes involved in metabolism, seem to be strictly pH controlled, such as the glucose transporter gtt1 and a putative L-amino oxidase pao1. T. harzianum is thetefore able to modify ambient pH and to adapt its metabolism to best growth condition.
pacC gene from T. harzianum CECT 2413 has been isolated. PacC is a transcription activator of alkaline-expressed genes and a repressor of acid-expressed genes. This PacC dependent pH-regulatory system has been best described in Aspergillus nidulans, but it has been also described in other filamentous fungi including Aspergillus niger, Penicillium chrysogenum, Acremonium chrysogenum, Sclerotinia sclerotiurum, Fusarium oxysporum and Fusarium verticillioides. As expected, pacC expression increases when pH also increases from 4 to 7. pacC null mutants (acidic-mimicking phenotype) and strains carrying dominant active allele (alkaline-mimicking phenotype) will allow to study how pacC controls CWDE expression and its involvement in ambient pH modifications.

EFFECTS OF OVEREXPRESSION OF IREA ON THE UNFOLDED PROTEIN RESPONSE IN A. NIGER.

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In eukaryotic cells, accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum (ER) constitutes a fundamental threat to the cells. To deal with this stress, eukaryotic cells can respond to it via three different mechanisms: Transcriptional induction, translational attenuation, and degradation. We focus on the transcriptional induction in relation to ER stress. When unfolded proteins accumulate in the ER the cell reacts by upregulating the synthesis of ER resident protein-chaperones and foldases, like BiP, protein disulphide isomerase (PDI), and peptidyl prolyl cis-trans isomerases (PPIases), thereby increasing the folding capacity in the ER.
The response, which involves a signal transduction cascade from the ER to the nucleus, is often referred to as the Unfolded Protein Response (UPR). The unfolded proteins are sensed by IREA, a transmembrane serine-threonine kinase with endonuclease activity. The mRNA encoding the transcriptional activator of the UPR, HACA, undergoes non-conventional splicing upon accumulation of unfolded proteins within the ER. This splicing event, mediated by IREA, removes a 20 nt intron from the hacA mRNA. Moreover, the 5'-end of the hacA mRNA is shortened by 230 nt upon a UPR. This processing of the hacA mRNA results in efficient translation of hacA, and activation of the UPR.
Constitutive overexpression of the gene encoding IREA in A. niger led to the splicing of the 20 nt non-conventional intron from the hacA mRNA. Overexpression however, did not lead to the shortening of the 5´-end of the hacA mRNA by 230 nt, suggesting that an additional mechanism is responsible for the removal of the 5'-end.
Furthermore, only a minor upregulation of the ER resident foldases and chaperones was observed, indicating that also truncation of the 5'-end is necessary to fully activate the UPR.

IVp-5

A PROTEOMICS BASED APPROACH TO THE ANALYSIS OF PH REGULATION IN ASPERGILLUS NIDULANS

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Fungi must maintain pH homeostasis within the mycelium as they adapt to changing external pH. This is necessary in environments that include pathogenic infections of plants and animals, where genes and proteins that are differentially regulated by pH are of major interest. These include many proteases, phosphatases and phosphodiesterases that contribute to the disease process, suggesting that the anion phosphate, as well as pH, may be important. Regulation of this response in the model filamentous fungus Aspergillus nidulans, which can grow across the range pH 2.5 to pH 9.0, involves changes in gene expression regulated by the pacC/pal system. The Pal signalling pathway is responsible for transduction of an alkaline pH signal leading to activation of the PacC transcription factor. This results in repression of genes expressed under acidic conditions and activation of genes whose products are required under alkaline conditions. In acidic growth conditions, PacC is inactive. A second transcription factor, PalcA, regulates gene expression in response to phosphate. We are applying a proteomics-based approach to identify proteins in A. nidulans whose expression is subject to pH and/or phosphate control, using strains of A. nidulans that contain mutations in components of the pacC/pal system, or palcA. Following two-dimensional polyacrylamide gel electrophoretic separation, the gels are analysed to reveal proteins that are differentially expressed. These proteins are identified using a combination of mass spectrometry (ESI-QUAD) and the published genome sequences. Several proteins have been identified so far, including proteins implicated in translation and a histidine kinase.

IVp-6

HETEROTRIMERIC G PROTEINS TRANSMIT SIGNALS FOR REGULATING DEVELOPMENT, PATHOGENECITY AND SECONDARY METABOLISM IN FUSARIUM GRAMINEARUM

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The homothallic ascomycetes Fusarium graminearum (teleomorph=Gibberella zeae) is a major pathogen which causes head blight disease on small grains, including barley, wheat, and rice, and stalk and ear rot on corn. It also produces mycotoxins such as trichothecene and zearalenone threatening human and animal health as well as plant. Despite the agricultural and food toxicological importance, the mechanism and regulation of fundamental biology such as growth and development and pathogenecity are largely unknown. To understand upstream signaling for virulence and mycotoxin production as well as growth and development, heterotrimeric G protein system that is highly conserved in all eukaryotic system was speculated. Take advantage of full genome sequence database of F. graminearum, we isolated and characterized three putative Galpha subunits, designated as FgGPA1, FgGPA2 and FgGPA3 respectively, and a Gbeta subunit, named FgGPB1. In addition to G protein subunits, at least eight putative G protein coupled receptors (GPCRs), four regulators of G protein signaling (RGS), and conserved protein kinase A (PKA) catalytic and regulatory subunits were also isolated. Like many other pathogenic fungi, deletion of each Galpha subunit affects pathogenecity, sexual development, and secondary metabolism including mycotoxin production and pigmentation. Deletion of FgGPA1 which is homologous to the fadA gene, which governs main growth signal in model fungus Aspergillus nidulans, and magB in Magnaporthe grisea showed infertile phenotype which can make no fruiting bodies. Unlike FgGPA1, deletion of FgGPA3, which encodes homolog of GanB that is involved in germination and carbon sensing of A. nidulans, resulted in normal fruiting body formation but is defective in pigmentation. Furthermore, FgGPA3 deletion mutant failed to cause disease on head of barley, indicating FgGPA3 plays important role for pathogenecity. Taken together, these results highly suggest that the G protein signal transduction is responsible for mating, virulence, and secondary metabolism of F. graminearum.

PROMOTER ANALYSIS OF THE afp GENE ENCODING THE ANTIFUNGAL PROTEIN OF ASPERGILLUS GIGANTEUS IN THE MODEL ORGANISM ASPERGILLUS NIDULANS

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Aspergillus giganteus is of considerable interest because of its ability to secrete an antifungal protein (AFP) which inhibits growth of a variety of filamentous fungi without affecting the growth of bacteria and yeast. The application of this protein holds promise in plant protection, as AFP is highly effective against diverse plant-pathogenic fungi (e.g. Fusarium spp.). As the host range of AFP includes opportunistic human pathogens (e.g. A. fumigatus, A. niger), the protein is also attractive for use in clinical applications. Knowledge of signals regulating AFP expression and secretion could offer ways to increase AFP production and facilitate such applications.
We have shown recently that transcription of the corresponding afp gene is modulated by various environmental cues, e.g. changes in ambient pH and phosphate concentration. afp transcription is highest under alkaline pH conditions and two putative PacC binding sites within the afp promoter are recognized in vitro by the A. nidulans PacC transcription factor. Excess phosphate leads to delayed induction of afp transcription and to a decreased AFP titre in the culture medium. In addition, afp transcription occurs only in the vegetative mycelium and induction coincides with colony competence for conidiophore development. This study aims to characterize further the regulation of afp expression in response to environmental conditions. We use the model organism Aspergillus nidulans, for which convenient mutant strains and homologous integration systems are available. A reporter gene (lacZ) was put under the control of a 1.1 kb afp promoter fragment and some mutated derivatives. Analysis of beta-galactosidase activities in different genetic backgrounds of A. nidulans are under investigation and should reveal which regulatory systems are involved in modulation of afp promoter activity.

ISOLATION OF NEUROSPORA CRASSA MUTANTS ALTERED IN BLUE LIGHT ADAPTATION

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The gene con-10 of Neurospora is expressed during conidiation and following illumination of vegetative mycelia with light. The photoactivation of con-10 disappears after two hours of illumination (light adaptation). To investigate the molecular nature of light adaptation in Neurospora, we have designed a method to isolate mutants altered in the adaptation of con-10 photoactivation. We are using a strain of Neurospora with a fusion of the con-10 promoter to the gene conferring resistance to hygromycin. This strain is sensitive to the drug when the promoter is inactive, i.e. during vegetative growth either in the dark or under continuous light. We have isolated four mutants (SN1 to SN4) that grow in the presence of hygromycin under continuous light but not in the dark. Presumably this is due to a defect in the mechanism controlling light adaptation. The mutant strains were illuminated for several periods of time, from 30 min to 5 hours, to assess the adaptation of con-10 photoactivation. All the mutants showed and enhanced accumulation of the con-10/hygromycin fusion gene compared to the parental strain. The increased accumulation of the con-10/hygromycin gene fusion was observed after five hours of light confirming that the mutants are altered in the adaptation of con-10 photoactivation. All the strains carry two copies of the con-10 promoter, the one in the naturally occurring con-10 gene and an additional one fused to the hygromycin gene. The mutations in strains SN1 and SN2 are specific for the con-10/hygromicin fusion. The photoactivation in the original con-10 gene is similar to that in the parental strain. These mutants are likely to carry mutations in the con-10 promoter fused to the hygromycin gene and will identify sequence elements required for the appropriate regulation of con-10 photoactivation. On the contrary, the mutations in strains SN3 and SN4 have also altered the photoactivation of the original con-10. They should carry mutations in genes responsible for proteins regulating con-10 photoactivation. The isolation and the characterization of light-adaptation mutants in Neurospora may help to unveil the molecular mechanisms regulating gene activation by blue light.

IVp-9

REGULATION BY BLUE LIGHT OF THE GENE ENCODING THE HEAT-SHOCK PROTEIN HSP100 IN PHYCOMYCES BLAKESLEEANUS

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The Zygomycete Phycomyces blakesleeanus develops two types of fruiting bodies of very different size, macrophores and microphores. Blue light stimulates macrophorogenesis and inhibits microphorogenesis. In addition, blue light regulates the direction of macrophore growth (phototropism) and activates beta-carotene biosynthesis (photocarotenogenesis). To investigate the role of differential gene expression during photophorogenesis in Phycomyces we have adapted a method based on the polymerase chain reaction with arbitrary primers. With this method we have isolated a cDNA segment for the heat-shock protein HSP100 that is induced by blue-light at the onset of sporangiophore development. The gene for HSP100 (hspA) is induced by blue-light and heat-shock. The expression of hspA is induced eleven fold after 30 minutes of blue light but longer exposure times reduced its expression. The mRNA for hspA was not detected in mycelia illuminated during two hours and several hours in the dark were required before hspA could be photactivated again. Illuminations with red light didn't activate hspA gene transcription. A short illumination (10 seconds) induced hspA but a period of incubation in the dark was necessary to detect the mRNA. The threshold for blue-light activation of hspA is 1 J/m^2, much higher than the threshold for the cellular photoresponses (around 10^{-4} J/m^2). Phycomyces cultures are usually grown at 22 degrees. A heat-shock, 34 degrees during 30 min, induced the hspA gene about 100 fold but incubations at higher temperatures reduced its expression. Our results suggest that light and heat can activate the expression of hspA to different levels. Several mutants altered in the Phycomyces light responses are available. Mutations in genes madA and madB impair all photoresponses, including the photoactivation of hspA. Mutations in gene madC only affect phototropism. Accordingly, madC mutants behave as the wild type for photophorogenesis and hspA photoactivation. The activation by heat-shock of hspA was not affected in the mad mutants. Our results suggest an independent regulation of hspA activation by environmental signals and give the first clues to the molecular events occurring in the Phycomyces mycelium at the onset of sporangiophore development.

IVp-10

YPXL/I IS A PROTEIN INTERACTION MOTIF RECOGNIZED BY PROTEINS OF THE PALA/RIM20/AIP1/ALIX FAMILY

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Regulation of gene expression by ambient pH in filamentous fungi and yeast is mediated by the PacC/Rim101 family of transcription factors. Analysis of this regulatory system in the model organism Aspergillus nidulans has shown that the zinc-finger transcription factor PacC undergoes two-step proteolytic activation in response to alkaline ambient pH. PalA and its yeast homologue Rim20 are components of the fungal ambient pH signal transduction pathway. A PalA/Rim20 homologue in mammals, AIP1/Alix, interacts with apoptosis-linked protein ALG-2. We show that PalA, Rim20 and AIP1/Alix recognize a short YPXL/I amino acid sequence motif, where Tyr, Pro and Leu/Ile are crucial for its interactive properties. PacC contains two such motifs flanking the signaling protease cleavage site, which are completely conserved in all members of the PacC/Rim101 family and which are required for the signaling cleavage of PacC. PalA can bind PacC in a closed conformation, suggesting that PalA binding constitutes the first stage in the two-step proteolytic cascade, recruiting or facilitating access of the signalling protease, presumably PalB. In addition to recognizing YPXL/I motifs, PalA, Rim20 and AIP1/Alix interact with Vps32, a member of a protein complex involved in the early steps of the multivesicular body pathway, suggesting that this interaction is an additional feature of proteins of the PalA/Rim20/AIP1/Alix family.

IVp-11

IDENTIFICATION OF AN RGS PROTEIN OF THE CHESTNUT BLIGHT PATHOGEN CRYPHONECTRIA PARASITICA THAT REGULATES ACTIVITY OF G-ALPHA SUBUNIT CPG-1.

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The chestnut blight fungus Cryphonectria parasitica expresses at least three G-alpha subunits. G-alpha subunit CPG-1 has been shown to be essential in regulating growth, pigmentation, sporulation and virulence. RGS proteins play an important role in regulating G-protein signaling through interactions with the G-alpha subunits, and enhancing their intrinsic GTPase activity. Deletion of RGS results in prolonged activity of its target G-alpha subunit.

An RGS-encoding gene, cprgs-1, was isolated from C. parasitica using degenerate PCR. Cprgs-1 gene deletion mutants showed reduced growth, sparse aerial mycelium, and lacked pigmentation, sporulation and virulence. Cprgs-1 gene deletion also caused severe post-translational reduction in accumulation of CPG-1 and G-beta subunit CPGB-1 protein, and severely reduced expression of the hydrophobin-encoding gene cryparin. The changes in phenotype, cryparin expression, and G-beta protein accumulation caused by cprgs-1-gene deletion were also observed in a strain containing a mutationally activated copy of CPG-1 (CPG1-QL).

In comparison, mutational activation of a second G-alpha subunit, cpg-2, resulted in colonies with reduced growth, but slightly increased pigmentation and sporulation whereas strains expressing an activated allele of G-alpha subunit cpg-3 grew faster than wildtype colonies but were unable to produce pigments or conidia. Cryparin gene expression was slightly upregulated in both mutant strains, and CPG-1 or CPGB-1 protein levels were essentially unaltered. Furthermore, cprgs-1 transcript levels were increased in the activated cpg-1 strain, but unaltered in activated cpg-2 and cpg-3 strains. The results strongly suggest that CPRGS-1 is involved in regulation of G-alpha subunit CPG-1 activity, and is essential for correct CPG-1-mediated signaling.

IVp-12

THE *Aspergillus nidulans alcA* PROMOTER PERMITS IDENTIFICATION OF ESSENTIAL GENES IN *Aspergillus fumigatus*

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Aspergillus fumigatus causes invasive aspergillosis, an usually fatal mycosis in immunocompromised patients. The availability of the genomic sequence of this fungus permits functional analysis aiming the identification and validation of novel therapeutic targets in this human pathogen. Nevertheless, there is still a need for appropriate molecular genetic tools to facilitate functional genomics.

In this work we analysed the capacity of the tightly regulated *alcA* promoter (*alcA*^{p}) of *Aspergillus nidulans* for driving conditional gene expression permitting identification of essential genes in *A. fumigatus*.

Expression of the *A. fumigatus nudC* gene, a nuclear migration gene with a terminal phenotype, was put under control of the *A.nidulans alcA*^{p} using the promoter replacement technique. Microscopic analyses showed that under repressing conditions, 3% glucose MM or YEPD medium, the *A. fumigatus alcA*^{p}-*nudC* strain displayed a *nudC* phenotype with 4-8 nuclei inside the growth-arrested hyper-swelling spores. Inducing conditions, 0.1M theronine MM, or non-repressing conditions, 0.1 M glycerol MM, allowed normal germination and growth, as occurs when the *nudC* gene is expressed in wild-type cells.

Northern analysis demostrated that the conditional gene expression driven by the *A.nidulans alcA*^{p} was transcriptionally regulated in a similar manner in *A. fumigatus* and *A. nidulans* (Romero et al. 2003).

Reference:
- Romero B, Turner G, Olivas I, Laborda F, De Lucas JR (2003). Fungal Genetics & Biology 40: 103-114.

IVp-13

TARGETED IDENTIFICATION OF CELL SIGNALLING GENES FROM A FUNGAL SYMBIONT OF PERENNIAL RYEGRASS

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One of New Zealand's most important pasture species, perennial ryegrass (Lolium perenne), forms a symbiotic relationship with a biotrophic endophytic fungus, Neotyphodium lolii. The relationship improves the fitness of the plant by conferring resistance against stresses, and in return the fungus relies entirely on the host for nutrients and propagation. In other plant/ fungal associations, most notably interactions between pathogenic fungi and plants, components of fungal signal transduction pathways have proven to be essential for initiating and maintaining successful infections. Since N. lolii is closely related to endophytic fungi that have a pathogenic stage in their lifecycle, we have initiated a study to identify cell signalling genes from N. lolii based on their similarity to cell signalling genes from pathogenic fungi. Degenerate PCR and screening of a genomic DNA library were two successful approaches used to isolate cell signalling gene orthologues from N. lolii. Functional analyses will be used to determine whether these genes are important for symbiosis between N. lolii and grasses. Here we discuss the identification of genes from the MAP kinase and cAMP signalling pathways of N. lolii.

IVp-14

ANALYSIS OF HIGH-OSMOLARITY RESPONSE MAPK PATHWAY OF ASPERGILLUS NIDULANS

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In Saccharomyces cerevisiae, external high-osmolarity activates the HOG (high-osmolarity glycerol) MAPK pathway (ScHOG), which consists of two upstream branches (Sln1 and Sho1) and common downstream elements including the Pbs2p MAPKK and the Hog1p MAPK. Activation of the pathway results in the induction of genes responsible for osmotic adaptation. Although Aspergillus nidulans has all counterparts of the ScHOG pathway (AnHOG) in its genome (AnHOG), the A. nidulans hogA null mutant is not sensitive to high-osmolarity and the null mutant of tcsB that is the counterpart of yeast SLN1 does not exhibit a detectable phenotypes. These results suggest that A. nidulans has more complex and robust osmoregulatory systems than ScHOG pathway and has unknown high-osmolarity response pathways. In the present study, we investigated the gene expression regulated by AnHOG pathway. We cloned A. nidulans-the pbsB gene encoding a homologue of yeast Pbs2pPBS2 MAPKK. Expression of the pbsB suppressed the sensitivenessity of yeast pbs2 mutant toin a high-osmolarity medium. Then, we constructed a constitutively active mutant of pbsB (pbsB(DD)) and its overexpressioned the mutant in the wild type A. nidulans. Overexpression of the pbsB(DD) in A. nidulans wild-type cell led to a defect of hyphal development in the high-salinity medium., However, but this defect was suppressed by deletion of the hogA gene. These results suggest that the HogA MAPK of the wild-type, in whichthat overexpresses the pbsB(DD) is overexpressed, is constitutively activated regardless of increased external osmolarity , and whichhence that the strain would beisis responsible for the sensitiveness of the pbs(DD) expressed strainsensitive to the high-salinity medium. Transcriptome analysis of the strain using DNA microarray will be also discussed.

Role of the TOR signalling pathway in the necrotrophic development of the phytopathogenic fungus Botrytis cinerea.

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Necrotrophic fungi use the nutriments available in the plant as well as the degradation products released by the enzymatic degradation of the plant tissues to support their growth within the host. The compounds surrounding the fungal cells may thus play a role as both nutrients and signal molecules. In yeast like in humans, nutrient sensing is transmitted via a kinase protein named TOR (Target of Rapamycin). The complex formed by the propyl-isomerase FKBP12 (FK506 Binding Protein of 12 kDa) and rapamycin, a natural macrolide with antifungal properties, specifically binds and inhibits TOR activity. Our research project is focused on the TOR-dependant signalisation cascade and its role in the control of the synthesis of lytic enzymes, during the parasitic growth of the grey mould fungus Botrytis cinerea.
Protease assays together with SDS-PAGE and Western blot analyses showed that the synthesis of some extracellular proteases is regulated, being induced in the presence of polymers (casein, gelatine, sunflower leaves) and repressed by readily metabolisable components such as glucose, ammonium and sulphates. Whether this regulation is disturbed by rapamycin, and thus mediated by a TOR-like kinase is currently being studied.
FKBP12 coding sequence was amplified by PCR and used to screen a genomic library of B. cinerea. The FKBP12 gene, which owns five introns, codes for a 117 amino-acid long protein. The corresponding cDNA was obtained by RT-PCR and used to construct a translational fusion FKBP12-$(His)_6$. The fusion protein produced by Escherichia coli was purified and used to raise antibodies against the propyl isomerase. SDS-PAGE and Western blot analyses were undertaken to follow the synthesis of the enzyme during plant infection.
A replacement vector carrying the 5' and 3' parts of the FKBP12 gene was elaborated and used to transform a B. cinerea wild type strain using Agrobacterium tumefaciens. Among the transformants hygromycin-resistant obtained, a deltaFKBP12 mutant was identified by PCR and further characterized. The effect of the inactivation of the FKBP12 gene on the synthesis of proteases and on the pathogenic behaviour of B. cinerea will be presented.

CLONING OF THE Blakeslea trispora GENE HOMOLOGOUS TO THE PHOTOCAROTENOGENIC REPRESSOR crgA OF Mucor circinelloides

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The biosynthesis of carotenes is induced by light in M. circinelloides, but is repressed in the closely related Zygomycete B. trispora. Gene crgA of M. circinelloides represses the expression in the dark of carotenogenic structural genes and other light-regulated genes. Isolation of the crgA-homologous gene of B. trispora could provide a useful tool for understanding the molecular basis that underlies the differences in the light-regulation of carotenogenesis between both fungi.
The crgA-homologous gene of B. trispora, named chgA gene, has been cloned by heterologous hybridization using the crgA gene of Mucor circinelloides as a probe. The deduced amino acid sequence of chgA is highly similar to that of crgA, both proteins sharing the same domain architecture: one degenerated and one consensus RING-finger zinc-binding domain, a LON domain disrupted by glutamine-rich sequences and a putative isoprenylation site. These proteins might be the prototype of a new group of evolutionarily conserved eukaryotic proteins, since proteins of unknown function deduced from genes of mammals, insects, plants and fungi show a similar domain organization.
The chgA gene is probably the ortholog of crgA, since it was able to restore the wild-type phenotype for carotenogenesis of a null crgA mutant of M. circinelloides. Analysis of the accumulation of chgA transcripts in B. trispora indicated that the expression of this gene is light-induced and photoadapted, as is the case for the crgA gene of M. circinelloides. Light induction and photoadaptation was also observed when the expression of the chgA gene, controlled by its native promoter, was analyzed in the heterologous host M. circinelloides, which suggests that some of the mechanisms involved in light regulation are conserved between these filamentous fungi.

IVp-17

SIGNALLING IN THE INTERACTION CLAVICEPS PURPUREA AND RYE

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Claviceps purpurea, the ergot fungus, is a common phytopathogenic ascomycete which colonizes only grass florets. For infection the fungus invades the ovary following the pollen tube path. After tapping the vascular bundles it colonizes and replaces the plant ovarian tissue. This interaction represents an interesting fungal model for oriented growth comparable e.g. to the guidance of axons to neural synapses in animals. The identification of signalling cascade components involved in directed growth during infection is our main interest (see also poster of Rolke et al.).
A strong influence on growth behavior and pathogenicity could be shown by deleting cppk1, a gene for a Ser/Thr protein kinase homologous to COT1, which is responsible for hyphal elongation in Neurospora crassa. In vitro infection assays revealed that deletion mutants are blocked in a very early step of colonization.
Another important gene supposed to be involved in directed and/or oriented growth is cpcdc42. The influence on growth behavior was demonstrated by using heterologous dominant active and dominant negative forms of this small GTPase. Further characterization by gene knock out is underway.
Using deletion mutants of two MAP kinases, cpmk1 and cpmk2, homologues to mps1 and pmk1 in M. grisea, which have strong impact on pathogenicity as shown by Mey et al. (2002a, 2002b)[1], an in planta SSH library was constructed to identify further main signal chain components.

[1]Mey G, Held K, Scheffer J, Tenberge KB, Tudzynski P (2002) CPMK2, am SLT2-homologous mitogen-activated protein (MAP) kinase, is essential for pathogenesis of Claviceps purpurea on rye: evidence for a second conserved pathogenesis-related MAP kinase cascade in phytopathogenic fungi. Molecular Microbiology 46(2), 305-318

[1]Mey G, Oeser B, Lebrun M H, Tudzynski P (2002) The Biotrophic, Non-Apressorium-Forming Grass Pathogen Claviceps purpurea Needs a Fus3/Pmk1 Homologous Mitogen-Activated Protein Kinase for Colonization of Rye Ovarian Tissue. MPMI 15(4),303-312

IVp-18

HETEROLOGOUS EXPRESSION AND ROLE OF gpa3 FROM Mucor circinelloides.

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Mucor circinelloides is a dimorfic fungus, and is able to grow as yeast or hyphal cells depending on the environmental or culture conditions (1). Almost all the biological phenomena involve an extracellular signal. Heterotrimeric guanine nucleotide-binding proteins (G-proteins) act as signal transducers that couple cell surface receptors to cytoplasmic effectors. These proteins are made up of abg). Subunit G? contains the sites for nucleotide binding and GTPase activity (2). Studies of G? subunits from several saprophytic and pathogenic fungi have revealed roles for these proteins in the regulation of virulence, differentiation processes, as well as in the sexual and asexual developments (3). Our group has cloned and sequenced a gene (gpa3) that codifies a Ga subunit of M. circinelloides that predicts a protein of 40 kDa of molecular weight. Its primary sequence shows highly conserved domains of all Ga proteins, and a potential site for ADP-ribosylation by cholera toxin (CTX). In this work, the *gpa*3 cDNA was cloned in pQE30, and expressed in E. coli XL10GOLD. The purified protein His-GPA3 was ADP-ribosylated by CTX. Also, this recombinant protein was recognized by heterologous commercial antibodies anti-$Ga_{i/o/t/z}$ (Santa Cruz). In addition, this protein blocked the binding of the antibody anti-Ga to mixed-membrane proteins obtained from germ cells of *Mucor*. On the other hand, to investigate the role of this gene, the antisense of gpa3 cDNA was introduced in Mucor using an homologous expression vector. Transformant cells that express an antisense version showed an hypersporulating phenotype.
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Envoy and minute, two novel genes influencing cellulase gene expression in Hypocrea jecorina

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Cellulase gene expression by the fungus Hypocrea jecorina (Trichoderma reesei) is known to be inducible but components involved in the signalling process are still unknown. Using a RaSH approach, we have recently been able to isolate a number of genes which are strongly upregulated on cellulose, but not in a non-inducible mutant, and which could be components of such a cascade (M. Schmoll, S. Zeilinger, R.L. Mach and C.P. Kubicek, ms submitted). One of these genes encodes Envoy, a small protein of the PAS/LOV protein superfamily, which in part displays similarity to Neurospora crassa VIVID, a protein involved in photoadaptation and sensing of different light intensities. *Envoy* is strongly upregulated under cellulase-inducing conditions, its expression under constitutive and induced conditions thereby occurring from different transcription start points. In collaboration with L. Franchi and G. Macino (Rome, I), we also found that complementation of a *vvd*-mutant strain with *envoy* failed to restore the function of *vvd.* Deletion of the PAS domain of *envoy* resulted in significantly increased induction kinetics of transcription of the cellulase gene *cbh1*. Another gene, *minute*, encodes a 5.8 kDa protein containing three identical helices separated by short spacers, and which bears no homology to proteins in other fungal or eukaryotic genome databases. Its pattern of expression differs from that of *envoy*. A *minute* knock-out mutant has no significant effect on the rate of *cbh1* transcript accumulation upon induction, but results in a loss of transcription of *envoy* under cellulase inducing conditions. *Envoy* and *minute* are therefore both components of a network negatively influencing cellulase induction by cellulose.

THE TGA3 G ALPHA SUBUNIT IS INVOLVED IN SIGNAL TRANSDUCTION RESULTING IN THE MYCOPARASITIC RESPONSE OF TRICHODERMA ATROVIRIDE

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The mycoparasite T. atroviride is commercially applied as biological control agent against a number of plant pathogenic fungi. The mycoparasitic interaction is host-specific and not merely a contact response. Although investigations on the mycoparasitic interaction revealed a major role for hydrolytic enzymes like chitinases in the attack of the host accompanied by secretion of antibiotic substances and morphological changes like coiling, elucidation of the underlying signal transduction pathways only recently started and supported the hypothesis that fungal pathogens (plant , human and animal pathogenic fungi, as well as mycoparasites) have developed conserved signalling cascades for regulating pathogenicity-related functions. Beside MAP kinases, G proteins play a major role in signal transduction, and are therefore a matter of particular interest. We isolated Tga3, an alpha subunit of a heterotrimeric G protein from T. atroviride, which is related to fungal G alpha subunits of subgroup 3.
Tga3 gene disruption in T. atroviride resulted in mutants with greatly reduced radial growth rates compared to the wild type and light-independent hypersporulation. Additionally, knockout of the tga3 gene resulted in a complete loss of formation of extracellular chitinases upon induction with N-acetylglucosamine or colloidal chitin, whereas transcription of nag1 (coding for N-acetylglucosaminidase) and ech42 (coding for endochitinase 42) was heavily increased. Furthermore, the knockout mutants completely lost their biocontrol activity in plate confrontation assays against Rhizoctonia solani as host. These data suggest involvement of the Tga3 G alpha subunit in mycoparasitism-related expression of chitinase-encoding genes, secretion of the corresponding enzymes, and in conidiation.

IVp-21

CLAVICEPS PURPUREA – SIGNALLING IN DIRECTED GROWTH

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An interesting model to study directed growth of fungal hyphae in a pathogen host interaction is represended by the phytopathogenic ascomycete Claviceps purpurea on rye. This biotrophic fungus infects more than two hundred species of grasses but besides its wide host range 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.
We are interested in the molecular mechanisms which guide the pathogen on this specific infection path. Two approaches will reveal new aspects for the understanding of directed growth mechanisms in this system.
In a forward genetics approach the creation of an insertional mutant library based on the Agrobacterium-mediated T-DNA-transfer is in progress. Mutants with impaired growth can be identified easily in an in vitro system for cultivation and infection of rye ovaries, which has been established by J. Scheffer (see also poster of Scheffer et al.). PCR techniques like TAIL-PCR and inverse PCR will be used to analyze new pathogenicity factors and directed growth related genes.
In a reverse genetics approach we are interested in the analysis of two signal chain components, homologous to the PAK kinases cla4 and ste20, which are influencing hyphal growth in several fungi. In yeast these kinases are activated by cdc42, a small GTPase. The homologous gene in Claviceps was isolated by J. Scheffer. A closer look to PAK homologous genes in C. purpurea, especially the cla4 homologue will contribute to the understanding of this unique system of directed growth during the infection process. A knock out approach is in progress. In addition yeast two hybrid interaction studies of cpcdc42 and cpcla4 will point out the function of these interesting signal chain components.

IVp-22

THE HISTIDINE-KINASE BCOS1 OF BOTRYTIS CINEREA IS INVOLVED IN FUNGICIDE RESISTANCE AND VIRULENCE

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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 Bcos1 gene where most of the ImiR mutations map. The Bcos1delta transformants are indeed resistant to dicarboximides, phenylpyrroles and are osmosensitive. Moreover they do not sporulate under all conditions tested suggesting a reduced fitness of Bcos1 disruptants. We investigated the pathogenicity profile of the same mutants and our tests show that they are severely impaired for infection, i.e. little or no penetration of intact plant cells, reduced lesion development on tomato and bean leaves. The BcOs1 histidine kinase therefore constitutes a major pathogenicity factor of B. cinerea.
Presumably it 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, BcOs1 does not have a detectable transmembrane domain. In order to investigate its subcellular localisation we used a BcOs1-GFP fusion. Our preliminary results show the fluorescence localised in the cytoplasm. 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.

UNUSUAL START CODON AND PROTEIN UNSTABILITY CAUSE LOW EXPRESSION OF THE PHOTO-CAROTENOGENIC REPRESSOR CrgA

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Mucor circinelloides responds to blue light by activating the biosynthesis of carotenoids. Gene *crgA* acts as a repressor of this light-regulated process, since its inactivation leads to over-accumulation of carotenoids both in the dark and the light. Site-directed mutagenesis identified two functional domains in the CrgA protein, a RING-finger domain and a glutamine-rich region. RING-finger motif defines a family of proteins that mediates ubiquitination, a process that, typically, targets proteins for proteasomal degradation. Thus, a role for the ubiquitin-proteasome pathway in the light regulation of carotenogenesis in M. circinelloides can be suggested.
The CrgA protein is present at extremely low levels in M. circinelloides cells. We have investigated the reasons of the low expression of CrgA. Site directed mutagenesis experiments indicate that translation of the crgA gene does not begin at the ATG codon previously proposed for the initiation of the CrgA protein, but it starts in an in frame GTG codon located 204 bp upstream of the ATG. The extension of the CrgA ORF to the GTG codon reveals the presence of an additional non-canonical RING-finger domain, which is conserved in the Blakeslea trispora crgA-homologous gene. GTG start codons, which have been described in a minority of eukaryotic genes, have been associated with a low efficiency of translation initiation. In addition, the CrgA protein seems to be very unstable. Expression of different constructs of the crgA gene in yeast cells indicates that wild-type CrgA is rapidly degraded. Deletion of the RING-finger domains dramatically increase the stability of the protein, suggesting that those domains are required for the rapid turnover of CrgA. Similar results were obtained when different mutant alleles of the crgA gene were expressed in M. circinelloides, in which a RING-finger mutant form, but not the wild-type CrgA, can be detected. Results indicate that the increased expression of the RING-finger-mutated CrgA protein results from post-transcriptional events and probably reflects an increase in its stability relative to the wild-type CrgA. Other RING-finger proteins have been demonstrated to mediate autoubiquitination in vitro and are themselves degraded via the proteasome pathway, being a functional RING-finger domain required for degradation. The role of the ubiquitin-proteasome pathway in the regulation of stability of the CrgA protein will be discussed.

A NEW ROLE FOR HEXOKINASES IN FILAMENTOUS FUNGI

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Hexokinases catalyse the first step in glucose metabolism and have a regulatory role as the glucose sensors that trigger insulin secretion in mammals, and glucose repression of gene expression in many organisms. Sequence analysis of the *Aspergillus nidulans xprF* gene showed that the predicted gene product is a hexokinase-like protein, sharing highly conserved residues within the ATP- and sugar-binding domains of other, well-established hexokinases. In addition, the *xprF* gene product contains sequences not found in other hexokinases. Mutations in the *xprF* gene affect the regulation of extracellular proteases in response to carbon starvation and utilisation of certain nitrogen sources. The *xprF* gene does not appear to have a major role in glucose repression.

Genetic and biochemical evidence suggests that, unlike the other hexokinases that have been studied, XprF may have a purely regulatory role. We have shown, using gfp-tagged XprF, that the *xprF* gene product is found predominantly (or entirely) in the nucleus in all the growth conditions that were tested. No nuclear-specific hexokinases have been previously reported. Preliminary yeast two-hybrid experiments indicate that the XprF protein may interact with components of a signal transduction pathway.

Analysis of the *A. nidulans* genome sequence shows that a gene that we have designated *hxkC* encodes a second hexokinase-like protein containing a putative nuclear localisation sequence and atypical sugar-binding domain. We have generated *hxkC*$^-$ knockout strains and shown that the null mutant exhibits a phenotype, similar but not identical, to *xprF*$^-$ mutants. *Neurospora crassa, Magnaporthe grisea,* and *Fusarium graminearum* (but not *Saccharomyces cerevisiae*) possess homologues of both *xprF* and *hxkC.*

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IVp-25

THE ROLE OF OXYLIPINS AS DEVELOPMENTAL SIGNALS IN *ASPERGILLUS NIDULANS*

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The addition of molecular O_2 to polyunsaturated fatty acids (PUFAs), forming a class of molecules called oxylipins, is of particular interest in biological systems. Mammalian oxylipins, including prostaglandins and leukotrienes, mediate many immune and inflammation responses in animals. Plant oxylipins are products of pathogen or wound-mediated plant responses and regulate the expression of defense genes. Fungal oxylipins, including unique hydroxy-PUFAs as well as prostaglandins and leukotrienes, are abundant among fungi but their biological significance has not been well characterized. In the mycotoxigenic fungus *A. nidulans*, oleic acid and PUFAs are metabolized into a series of hydroxy-fatty acids called psi factors (precocious sexual inducer). Psi factors play a role as signal molecules that modulate the timing and balance of asexual and sexual spore development. The route of biosynthesis of oxylipins in *A. nidulans* is currently being elucidated. Three genes, *ppoA*, *ppoB*, and *ppoC*, encoding oxygenases similar in sequence to prostaglandin synthases, have been cloned and disrupted in *A. nidulans*. Deletion of both *ppoA* and *ppoC* increases ascospore production but decreases conidial production and mycotoxin synthesis whereas deletion of *ppoB* results in the opposite phenotype. Chemical analysis of the *Δppo* mutants revealed that are deficient in psi factor and prostaglandin production. Interestingly, *ppo* homologs are found in filamentous ascomycetes but not in yeast. Current efforts are focusing on the elucidation of the regulatory network that governs oxylipin production in order to understand how these molecules signal spore development and secondary metabolism in *A. nidulans, A. fumigatus* and *Fusarium spp.*

IVp-26

A SYSTEM FOR GENETIC DISSECTION OF THE PATHOGENICITY MAP KINASE CASCADE IN *FUSARIUM OXYSPORUM*

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MAPK cascades are eukaryotic signalling modules that mediate the transduction of signals from the cell surface to the nucleus, thereby controlling vital processes such as cell proliferation, differentiation, morphogenesis and death. The conserved Pmk1-type MAPK pathway is essential for plant infection in large number of biologically diverse fungal pathogens. The signals and upstream components that activate this pathway during infection, as well as the downstream targets that mediate pathogenicity are largely unknown. We previously found that the MAPK Fmk1 of the vascular wilt fungus *Fusarium oxysporum* is required for root penetration and invasive growth on plant tissue and regulates expression of genes encoding pectinolytic enzymes. Here we show that Δ*fmk1* strains have significantly reduced transcript levels of the *fpr1* gene, encoding a polypeptide that shares homology with the SCP extracellular protein family of plant PR-1 and human glioma pathogenesis-related proteins.
We are using the *fpr1* promoter (P*fpr1*) as a tool to establish a system for the genetic dissection of the Fmk1 MAPK cascade in *F. oxysporum*. To that end, we have fused P*fpr1* to the *nit1* gene encoding nitrate reductase and have introduced this construct into a *nit1* mutant, obtaining a reporter strain in which *nit1* expression will depend on the integrity of the Fmk1 cascade. This reporter system should allow the identification of genes encoding MAPK cascade components by selection of *nit1*-deficient mutants on chlorate and subsequent complementation with a genomic library and selection on nitrate as the sole nitrogen source. Our progress in validation of the system and in isolation of *F. oxysporum* mutants affected in the Fmk1 MAPK cascade will be detailed.

A NOVEL B-ZIP TRANSCRIPTION FACTOR FROM THE RICE BLAST FUNGUS, MAGNAPORTHE GRISEA, IS REQUIRED FOR PATHOGENICITY ON RICE

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Genes required for pathogenicity of M. grisea were identified using non-pathogenic mutants obtained by REMI-insertional mutagenesis. The number of lesions caused by mutant M763 on rice was dramatically reduced compared to wild type (-95%), and its colonisation of host tissues was also impaired (-80%). M763 was non-pathogenic on barley inducing a brownish host response on the leaf. The ORF inactivated in M763 (BIP1) encodes a transcription factor with a B-ZIP domain at its N-terminus. Orthologous genes were identified in N. crassa and F. graminearum genomes. These genes define a novel family of B-ZIP transcription factors that differ from known B-ZIP such as GCN4/CPC1 and YAP1. A $bip1\Delta$:hph deletion mutant was obtained by targeted gene replacement. This mutant is non-pathogenic on rice and barley, suggesting that the original insertion mutant M763 is not a null mutant. Cytological analysis of the penetration process showed that $bip1\Delta$:hph differentiated appressoria at normal rates that were unable to penetrate into host plant cells. This gene was shown to be only expressed in spores, appressoria and during plant infection. BIP1 is likely to control the expression of appressorial genes that are required for efficient penetration. Cell biology and expression studies are currently pursued in order to identify the cellular functions controlled by this gene. In particular, genes that are differentially expressed between wild type, bip1Δ:hph and M763 will be identified using a M. grisea genome-microarray.

ANALYSIS OF PROTEINS BINDING TO THE TTAGTAA ENHANCER SEQUENCE IN THE PENICILLIUM CHRYSOGENUM pcbAB PROMOTER BY SOUTHWESTERN

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In Penicillium chrysogenum the first two genes of the penicillin biosynthetic pathway, pcbAB and pcbC, are transcribed from a common intergenic region of 1013 bp. Analysis of the DNA sequence of this region reveals the presence of multiple consensus cis-acting regulatory sequences. We reported previously the presence of two regions (boxes A and B) which gave clear DNA-protein complexes with cell-free extracts. A DNA binding protein complex AG1 was shown to bind to box A containing a palindromic heptanucleotide TTAGTAA. Mutations of this sequences and in vivo studies supported its involment in the binding of a transcriptional activator named PTA1 (Kosalková, (2000) J. Biol. Chem., 275: 2423-2430)
Probe CAA3-4, consisting of a 35 bp fragment that includes TTAGTAA sequence we used for partial purification and identification of protein/s which form AG1 complex. EMSA assays performed with probe CAA3-4 and heparine-agarose purified protein extracts of cells grown on glucose for 24 h provided evidence of formation of two different DNA-binding complexes: AG1.1 and AG1.2. Competition assays and point mutation of probe CAA3-4 revealed that only AG1.2 is specific with this probe.
In order to identify proteins that bind to the heptamer enhancer sequence we used the Southwestern-blotting technique. Heparine-agarose purified protein extracts which form AG1.2 complex were subjected to SDS/PAGE, electroblotted on to a nitrocellulose sheet, renatured and probed with the ^{32}P-labelled CAA3-4 probe. This probe bound to three different proteins that appear to form a complex of enhancer-interacting proteins. The proteins are being identified by MS and de novo sequencing.

IVp-29

TISSUE ALKALINIZATION MODULATES SECRETION OF ENDO ?-1,4- GLUCANASE BY Aternaria alternata AS A MECHANISM FOR PATHOGENICITY IN FRUITS

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The phytopathogenic fungus Alternaria alternata produces one endo-?1,4 glucanase, encoded by AaK1, which is an important factor in disease development in persimmon fruits. During growth of A. alternata in media containing acidified yeast extract or cell walls from persimmon fruit, the fungus secreted ammonia and raised the medium pH. Increase in tissue pH was also observed when A. alternata infected in tomato, melon, lemon and pepper fruits. A rise in media pH from 3.8 to 6.0, in the presence of cell walls, induced the expression of AaK1, whereas a reduction of pH to 2.5 by the addition of glucose repressed transcription of AaK1 and its enzymatic production. When two isolates with differential pathogenicity on apples tissue were analyzed, they both had similar capability of ammonia secretion but differed in the overall level of expression of Aak1 at the different pH values. The highly pathogenic isolate had a higher level of AaK1 expression than the reduced pathogenicity strain. The results indicated that conditions affecting environmental pH modulate gene expression of AaK1 and virulence of A. alternata in fruit is affected by the enhanced expression of AaK1.

IVp-30

TISSUE ACIDIFICATION BY PENICILLIUM AS A MECHANISM TO ENHANCE PATHOGENICITY

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The phytopathogenic fungus Penicillium expansum acidifies the host tissue during the expression of pepg1, a gene encoding for polygalacturonase, affecting host maceration. The acidification is achieved by secretion of organic acid combined with the uptake of ammonia. Natural P. expansum isolates with increased pathogenicity accumulated higher amounts of gluconic acid and reduced the apple tissue pH to lower values than isolates with reduced pathogenicity. Glucose oxidase activity, involved in gluconic acid production, was detected in P. expansum decayed tissue but not in the healthy tissue of the same fruit. Reactive oxygen species resulting from glucose oxidase activity were easily detected in the decayed apple tissue. Growth of P. expansum in the presence of 6% sucrose showed high glucose oxidase activity, accumulation of gluconic acid and pH decline of the culture media. Fungal growth in the presence of reduce oxygen atmospheres (5% oxygen and 5% CO_2) inhibited glucose oxidase activity, gluconic acid accumulation and decay development. It is suggested that acidification of host tissue by gluconic acid enhance pepg1 expression, a gene encoding for polygalacturonase, involved in host maceration and fungal attack of P. expansum.

CELLULAR LOCATION OF THE palI AND palH, pH SIGNALLING GENES OF Aspergillus nidulans.

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Regulation of gene expression by ambient (extracellular) pH in Aspergillus nidulans is mediated by a signalling pathway composed of the products of the palA, B, C, F, H and I genes and the PacC zinc-finger transcription factor. This pH regulatory system ensures that extracellular enzymes(as well as permeases and exported metabolites) are produced under conditions of pH where they can function: acid phosphatase under acid conditions and alkaline phosphatase under alkaline conditions, for example. Both the predicted palI and palH proteins contain membrane spanning domains; four in PalI and seven in PalH. This suggests that one or both of these proteins may be present in the plasma membrane and may serve as the pH-sensing protein in the signalling pathway. To help determine the functions of these proteins, we have constructed strains of A. nidulans expressing palI and palH proteins fused to the Green Fluorescent Protein (GFP). Our results show that both the palI and palH GFP fusion proteins have a plasma membrane location. Interestingly, the palI GFP fusion protein appears in vacuoles when cells are transferred to alkaline conditions. It is under alkaline conditions that the pH signalling pathway is activated. This work was supported by NSF RUI grant 0108749, CICYT and Wellcome Trust grant 067878.

CHARACTERIZATION AND GENE DISRUPTION OF THE FST20 AND FST12 GENE FROM THE PHYTOPATHOGENIC FUNGUS FUSARIUM OXYSPORUM.

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Fusarium oxysporum is a ubiquitous phytopathogenic fungus able to infect an extremely wide range of crops. Fungi are capable of perceiving changes in the environment and adjusting their intracellular activities. In eukariotic cells, a family of serine/threonine protein kinases (MAPK) are involved in transducting a variety of extracellular signals and regulating growth and differentation processes. An important change is produced in fungal pathogens when the colonization of their hosts takes place, thus we are interested in the components of the environmental response pathway. A key link of the MAPK cascade with upstream elements seems to be the STE20 protein. STE20 is a serine/threonine protein kinase member of the p-21-activated kinase (PAK) family of protein kinases, shown to be activated in vitro by GTP-bound Cdc42. Previously, we reported the cloning of Ste20 and Ste12 homologs, named fst20 *and* fst12 respectively, from the plant pathogenic fungus *F. oxysporum*. In order to investigate the role of the fst20 and fst12 genes in the adaptative response of the fungus-plant interaction, gene disruption experiments have been developed using Agrobacterium tumefaciens-mediated transformation. The fst20 mutants show no obvious defect in pathogenicity and/or virulence. Furthermore, they do not show any indication of growth or sporulation impairment. We are currently screening the knockout mutants of fst12. Also, we want to analyse whether there is any differential expression of these genes under nutrient starvation conditions.

IVp-33

CELL WALL REMODELLING IN ASPERGILLUS NIGER IN RESPONSE TO CELL WALL STRESS

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The fungal cell wall is a highly dynamic structure: both its composition and architecture respond to internal and external stimuli to ensure the integrity of the cell wall. We are interested in the mechanism behind the response of fungi to cell wall disturbing agents. Therefore, the expression of several genes from Aspergillus niger involved in cell wall biosynthesis have been analysed. Some of these genes were heavily up-regulated after the cell wall integrity was compromised by the addition of CFW (Calcofluor white) to germlings. Northern analysis indicated that the mRNA levels of agsA, encoding an alpha-1,3-glucan synthase, and gfaA, encoding the glutamine:fructose-6-phosphate amino-transferase enzyme involved in de biosynthesis of UDP-N-acetyl-glucosamine, were induced 20-fold an 4-fold respectively. Both genes including at least 2 kb of their promoter sequences were cloned.
We have used the promoter sequence of the agsA gene to set up a genetic screen for the isolation of cell wall mutants. For that, the agsA promoter has been cloned in front of a selectable marker (amdS). The rationale of the screen is that a mutation that affects cell wall biosynthesis results in cell wall weakening and that this cell wall weakening triggers the expression of the amdS gene from the agsA promoter allowing the growth on acetamide as a sole N-source. 240 mutants were isolated that grew on acetamide and this collection of mutants were subjected to various secondary screens. We have focused our attention to a subset of 13 mutants which shown an osmotic remediable temperature sensitive growth defect at 37° C. Complementation of the mutants and the identification of the genes involved can potentially lead to the discovery of new cell wall biosynthesis related antifungal targets.

IVp-34

USE OF A GFP REPORTER SYSTEM TO STUDY THE pH REGULATION OF AN ACID EXPRESSED GENE FROM A NECROTROPHIC FUNGUS.

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The first barrier a phytopathogenic fungus encounters on its host is the plant cell wall composed of polysaccharides and several classes of structural proteins. During the interaction of the parasitic fungus with its host, the pathogen secretes a complet set of depolymerizing enzymes which not only contribute to produce an important assimilable nutrient source but also facilitate the colonization and the maceration of plant tissues.
Sclerotinia sclerotiorum a necrotrophic fungus secretes during pathogenesis several proteases including an acid protease ACP1 characterized by a very low optimal pH (pH 2.0) and an enzymatic activity not inhibited by pepstatin B a specific aspartyl protease inhibitor. Ambient pH plays a significant regulatory role in transcription of the gene acp1 encoding the protease ACP1 since no transcript is detected when the pH reaches values above 5. In order to investigate the molecular mechanisms which control the pH regulation of acp1, we have constructed a transforming vector in which the green fluorescent protein encoding gene was placed under the control of the promoter of acp1.This construction was used to transform Aspergillus nidulans wild type and two pH deregulated mutants characterized respectively by the mutation palA and pacC^{c}. The pH regulation of this reporter system was investigated under different conditions of ambient pH. The pattern of expression of the reporter in these different strains suggests that the pH regulation of the acidic gene acp1 is dependant on the pal transduction signaling pathway while the pH transcriptional transregulator PACC seems not to be involved in the pH regulation of the gene acp1. In order to confirm this result, the reporter gene gfp fused to a promoter region of acp1 characterized by the absence of the PACC binding site was introduced in an homologous context. The results will be presented.

IVp-35

CROSSTALK BETWEEN PKA AND MAPK SIGNALLING LEADS TO DIFFERENTIAL REGULATION OF PRF1 ACTIVITY

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The basidiomycete Ustilago maydis causes smut disease on corn. Prerequisite for infection are mating and fusion of two haploid cells resulting in a filamentously growing dikaryon. Mating is initiated by a pheromone signal which depends on crosstalk between protein kinase A (PKA) and mitogen-activated protein kinase (MAPK). During this process, pheromone response factor 1 (Prf1) activates transcription of different mating type genes by binding to pheromone response elements in their regulatory regions. Recently, we decribed a novel mechanism of crosstalk by demonstrating that the activity of this key transcription factor is regulated by PKA as well as MAPK phosphorylation. The differential phosphorylation by these kinases enables Prf1 to activate the transcription of distinct target genes. In addition we discovered that pheromone-induced gene expression occurs in strains with genetically ablated MAPK signalling. Currently, we are focusing on the signalling pathway that mediates this induced expression in the absence of MAPK signalling and the molecular mechanisms underlying PKA/MAPK crosstalk on the level of Prf1 phosphorylation.

IVp-36

IDENTIFICATION OF PROTEINS THAT INTERACTS WITH THE PHOTOCAROTENOGENIC REPRESSOR CrgA OF Mucor circinelloides

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The crgA gene is involved in the regulation by light of the biosynthesis of carotenoids in the fungus Mucor circinelloides. Former characterization of its amino acid sequence identified several structural motifs found in regulatory proteins. The most relevant domain was a RING-finger domain at the amino terminus, which is essential for the crgA function in the carotene biosynthesis. RING-finger domains have been found in E3 ubiquitin ligases. E3 ubiquitin ligases interact with specific proteins targeting them for degradation in the 26S proteasome. A screening for CrgA-interacting proteins using the yeast two-hybrid system identified mainly proteins that either interact very weakly with CrgA or were not clearly related with the crgA function. Recent analyses of crgA indicate that crgA translation starts in an in frame GUG codon upstream of the first AUG codon, giving a protein with an additional putative RING-finger domain. Therefore, a new yeast two-hybrid screening was carried out using the protein with the two RING-finger domains as a bait. This screening identified three proteins that interact strongly with CrgA. Interestingly, these proteins interact weakly with the CrgA protein lacking the first RING-finger domain, which suggests that this RING-finger domain is important for the interaction. One of these proteins correspond to the peroxisomal targeting signal 1 receptor, which binds to proteins having this targeting signal and direct them to peroxisomal matrix. Since CrgA lacks this targeting signal, it can be speculated that CrgA controls the function of that receptor. The second protein is similar to the signal transducing adaptor molecule (STAM) from several organisms. These proteins bind to proteins with a molecule of ubiquitin, mainly activated receptors, directing them to degradation in lysosomes. The third protein does not show overall homology to any sequence protein, but it presents a putative CUE domain that binds monoubiquitylated proteins. Proteins having the CUE domain have been proposed to interact with ubiquitin-conjugating enzymes, collaborating in the process of ubiquination. Further analyses are in progress to characterise the role of the last two proteins in the crgA function.

IVp-37

MAPK SIGNALING IN THE ECTOMYCORRHIZAL SYSTEM TUBER BORCHII-TILIA AMERICANA L.

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Tuber borchii Vittad. is an ascomycetous fungus which forms ectomycorrhizae on the roots of angiosperms and gymnosperms. Ectomycorrhizae formation is a highly regulated process that is accompanied by molecular reorganization of both partners during symbiosis. In the pre-symbiotic phase, T. borchii-Tilia americana L. are able to ''interact'' before any physical contact. For this reason we focused our attention on the possible signaling processes taking place between the two symbionts.
From a cDNA library screening we isolated a 1713 bp cDNA clone containing an ORF of 1056 bp encoding a protein of 351 amino acid with an estimated mass of 40661 Da, that is 84% identical and 87% similar to Gibberella fujikuroi MAPK.
The T. borchii gene, designed TBMK1, is homologous to Fusarium oxysporum FMK1 and Pyrenophora teres PTK1, both involved in the infection procedure of their host plants. TBMK1 gene contains three introns of 53 bp, 54 bp and 55 bp, respectively.
The aminoacidic TEY motif of TBMK1 is preferentially phosphorylated when T. borchii interacts with its symbiotic plant, or when radical exudates are added in the mycelial growth medium.
The immunoblot analyses, using an anti-ERK antibody recognising the TEY aminoacidic motif, evidenced the presence of two ERK forms. This result led us to suppose that at least another ERK protein family carrying the amino acidic TEY motif should be expressed in T. borchii, since TBMK1 is present in genome as a single gene.
The data obtained for the ectomycorrhizal fungus T. borchii are similar to those reported for other pathogenic fungi suggesting a common and ancestral infection process, subsequently evolved in different mechanisms of interaction: the symbiotic and the pathogenic.
Further studies will be carried out in order to define the MAPK scaffold activation during the pre-infection stage of the T. borchii life cycle.