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Abstracts from the 19th FGC

Abstract

Plenary and poster session abstracts from the 19th Fungal Genetics Conference

Plenary Session Abstracts at the 19th Fungal Genetics Conference at Asilomar

- Gene Regulation and Metabolism
- Cell Biology and Pathogenesis
- Evolution and Population Genetics
- Sexual/Asexual Reproduction

Plenary Session: Gene Regulation and Metabolism (Chair: Claudio Scazzocchio)

pH regulation in *Aspergillus nidulans* .

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The zinc-finger transcription factor PacC mediates pH regulation in *Aspergillus nidulans* and other ascomycetes. The 678-residue PacC primary translation product is inactive in structural gene regulation. Under ambient alkaline pH conditions, a signal provided by the six *pal* -gene pathway causes an unknown modification in the protein which makes it accessible to a proteolytic processing step. This limited proteolysis eliminates the ~60% residues of PacC at the carboxyl side. The processed protein (~residues 1 - 270) is fully active in structural gene regulation, activating alkaline-expressed genes (through 5'-GCCARG-3' sites) and repressing acid-expressed genes. Thus, the conformational change in PacC resulting from pH signal reception is the pH-sensitive step in the regulatory circuit. The C-terminal moiety of PacC mediates its negative action at least by masking PacC domains involved in transcriptional activation and presumably by preventing nuclear import of the full length protein, but does not appear to impair DNA binding. Point mutations and C-terminal truncations disrupt the interactions between the amino- and carboxyl-terminal moieties of the protein. *pacC* is itself an alkaline-expressed gene, but its mode of regulation is different from that of alkaline structural genes.

Developmental regulation of catalases in *Aspergillus nidulans.*

Rosa E. Navarro, Laura Kawasaki and Jesus Aguirre, Instituto de Fisiologia Celular, Univ Nacional Autonoma de Mexico.

A general hypothesis to explain microbial cell differentiation as a response to hyperoxidant states was derived from studies on sporulation in *Neurospora crassa.* Since catalases are ubiquitous enzymes that are central to cellular antioxidant responses, we have now approached this hypothesis by studying the function and regulation of catalases during *A. nidulans* asexual sporulation (conidiation). We have found two catalases in this fungus encoded by the *catA* and *catB* genes, whose predicted polypeptides are as similar between them, as they are to *E. coli* HPII catalase. The *catA* and *catB* expression is differentially regulated during growth and

development. The *catA* mRNA and protein appear during sporulation and are accumulated in both, sexual and asexual spores independently of the *brlA* regulatory gene, in a process that involves transcriptional and translational controls. In contrast, the *catB* mRNA and protein are very low in spores, accumulate in mature hyphae throughout conidiation and in response to H_20_2 both catalases can offer protection against H_20, at different stages of the life cycle. Although the increase in *CatB* activity and the appearance of *CatA* during sporulation is consistent with the occurrence of oxidative stress during development, our data indicate the operation of efficient alternative pathways for H_20_2 detoxification.

Genetic Regulation of *Aspergillus* Mycotoxin Biosynthesis.

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Aflatoxin (AF) and sterigmatocystin (ST) are polyketide mycotoxins derived from the same biochemical pathway found in several seed contaminating *Aspergillus* spp. The AF/ST biosynthesis genes are clustered in a ~60-75 kb DNA segment in all species examined to date. The magnitude of this cluster is well illustrated by the characterization of the *A. nidulans* ST gene cluster which contains 25 coordinately regulated transcripts most of which encode enzymes with functions required for AF and ST biosynthesis. In each cluster there is a positive-acting pathway specific regulatory gene, *aflR*, that encodes a sequence-specific DNA binding protein required for cluster gene expression. Regulation of this cluster is complex and involves nutritional factors, pH and life cycle controls. This complexity is illustrated by the fact that the ability of AflR to activate ST/AF gene expression is linked to regulation of asexual sporulation through a requirement for inactivation of an heterotrimeric G protein mediated signal transduction pathway.

White collar 1 and White collar 2 are partners in the blue light transduction pathway in *Neurospora crassa.*

Giuseppe Macino, Hartmut Linden, Paola Ballario. Universita "La Sapienza" Roma, Italy.

A saturating genetic dissection of "blind" mutants in *Neurospora crassa* has identified a total of two non-redundant loci *(wc1* and *wc2*) each of which is required for blue light perception/signal transduction. Previously, we demonstrated that *wc1* is a putative zinc-finger transcription factor able to bind specifically to a light regulated promoter. We have recently demonstrated using mutation analysis and *in vitro* DNA binding assays that *wc2* is the second partner of this two component light signal transduction system and encodes a functional zinc-finger DNA-binding

protein with a putative PAS dimerization domain and transcription activation domain. This molecular-genetic dissection of the two components of this light signal transduction system has elucidated a model whereby *wc1* and *wc2* interact via homologous PAS domains, bind to promoters of light-regulated genes and activate transcription. As such, this study provides the first insight into two interacting partners in blue light signal transduction in any organism and provides the molecular tools with which to dissect this engimatic process.

The circadian clock in Neurospora: light resetting of the oscillator and the control of gene expression.

Jennifer Loros, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755.

An important property of circadian oscillators is their ability to entrain to the daily light/dark cycle. The clock is a negative feedback loop wherein the *frq* gene, known component of the clock in *Neurospora crassa* encodes the FRQ protein which feeds back to turn off the gene, yielding the oscillation in *frq* transcript and FRQ protein that is the Neurospora clock. Light delivered at any point within the circadian cycle acts rapidly to increase the level of *frq* transcript. The magnitude of the light-induced increase in *frq* mRNA and the extent of clock resetting are correlated and the threshold, kinetics and magnitude of this response indicate elevation of the level of *frq* transcript in the cell is the initial clock-specific even involved in resetting of the clock by light. We are examining the roles of two photo blind strains, *band;white collar-1* (*bd;wc-1*) and *white collar-2 ;band;(wc-2 ;bd)* in light-resetting of the clock. By Northern analysis we find the transient, light induced accumulation of *frq* is blocked in *bd;wc-1* but not in *wc-2;bd* .

In constant light, *frq* mRNA remains at high levels in the *bd* strain, thereby suppressing the *frq* cycle. This sustained, light driven increase is blocked in both *bd;wc* strains. Both of the *bd;wc* strains failed to show a rhythmic phenotype when entrained by either light or temperature steps. In a separate effort aimed at understanding light regulation of rhythmic processes, circadianly expressed genes downstream of the clock, *eas (ccg-2*) and *ccg-1,* have been examined in a clock null strain and foun not to require a functional clock. Deletion analyses of the *eas (ccg-2*) promoter has localized cis-acting elements mediating clock, light, and developmental control. Primary sequence determinants of a positive activating clock element were found reside in a 45-base pair region close to the TATA box, and spatially distinct from sequence elements governing light regulation.

Plenary Session: Cell Biology and Pathogenesis (Chair: Hans VanEtten)

Animal pathogenesis.

David. W. Holden, Royal Postgraduate Medical School, London, UK.

Systemic fungal infections of humans caused by Candida spp., Aspergillus spp. and *Cryptococcus neoformans* are increasing in incidence, mainly in patients with impaired immune responses. Patient mortality is high because of difficulties in early diagnosis and the lack of effective and safe antifungal drugs. These fungi therefore pose a serious threat to human health, and although we have some understanding of the host defences which prevent infection in immunocompetent individuals, little is known of the molecular mechanisms of fungal pathogenesis. The antiphagocytic capsule of *C. neoformans* and melanin production by this fungus are important virulence determinants. A range of physiological attributes and specific gene products has also been proposed as virulence determinants of *Candida albicans*, but in most cases compelling evidence has been lacking because the molecular genetics of this diploid fungus have only been developed in recent years. The cell wall component chitin and a mannosyl transferase are required for the fungus to cause disease in experimental models of infection. We have identified a multigene family encoding six chitin synthases in *A. fumigatus*. Four of these genes have been disrupted and although two of the mutations lead to hyphal abnormalities, the mutant strains infect the lungs of immunosuppressed mice efficiently. We have developed a technique called signature-tagged mutagenesis (STM) which allows large numbers of uncharacterised insertional mutants to be tested simultaneously for loss of virulence. STM was originally applied to the bacterial pathogen *Salmonella typhimurium*, and has now been adapted for virulence gene identification in *A. fumigatus* and *Candida glabrata* as well as other bacterial pathogens.

The Fungal Cytoskeleton.

Berl R. Oakley, The Ohio State University, Columbus.

Microtubules and microfilaments comprise the two major cytoskeletal systems of filamentous fungi. Together they are essential for mitosis, meiosis, organellar movement, septation and polarized growth. For these two systems to function correctly, their assembly must be regulated spatially and temporally. The microtubule cytoskeleton is regulated in part by microtubule organizing centers such as the spindle pole body (SPB). SPBs nucleate microtubule assembly and are presumed to establish microtubule polarity. Their ability to nucleate microtubule assembly changes through the cell cycle. One of the major questions in microtubule research has been identity of the components of the SPB that nucleate microtubule assembly. We have discovered a novel protein, -tubulin that is ubiquitous in eukaryotes and is located most obviously at microtubule organizing centers such as the SPB. Disruption of the -tubulin gene of *Aspergillus nidulans* leads to blockage of nuclear division, failure of mitotic spindle formation and a transient blockage with condensed chromosomes. Accumulating data indicates that -tubulin rings at microtubule organizing centers nucleate microtubule assembly. In *A. nidulans,*

the majority of the -tubulin is associated with the nucleus (and thus, presumably, with the spindle pole body). Approximately 30% of the -tubulin, however, is present in the cytoplasm in complexes of a variety of sizes. We hypothesize that these complexes may be intermediates in the formation of -tubulin ring complexes. Supported by grant GM31837 from the NIH.

Motors and filamentous fungi.

Mike Plamann, In Hyung Lee, Peter Minke, and John Tinsley. Department of Biology, Texas A&M University, College Station, TX.

Mechanochemical enzymes ("motors") hydrolyze ATP and generate force relative to either actin filaments or microtubules. Actin-based movement is dependent on the myosin superfamily of motor proteins, while microtubule-based movement relies on the kinesin and dynein superfamilies of motors. Hyphae of filamentous fungi exhibit highly polarized growth which requires the directed movement of secretory vesicles to hyphal tips and the movement and positioning of organelles such as nuclei and mitochondria. Members of all three classes of motor proteins are currently being studied in filamentous fungi and have been shown to be required for a diverse array of functions including polarized growth and secretion, nuclear movement, and establishment of the mitotic spindle. We have been conducting a genetic analysis of cytoplasmic dynein, the most complex of the cytoplasmic motor proteins which is required for nuclear positioning in fungi. We have shown previously that the *Neurospora crassa ro-1, ro-3,* and *ro-4* genes encode subunits of either cytoplasmic dynein or the dynein-associated complex dynactin. We report here that five additional ropy genes encode novel proteins, one of which is required for the proper intracellular localization of cytoplasmic dynein and dynactin.

Cell wall biosynthesis in Filamentous fungi.

Oded Yarden, Adi Beth Din, Vered Ziv, Einat Yatzkan, Klaas Sjollema* and Hans Sietsma*, Dept. of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Rehovot, 76100, Israel and *Dept. of Plant Biology, University of Groningen, NI-9715 NN Haren, The Netherlands.

The biosynthesis of the fungal cell wall, the major determinant of the fungal cell shape, is spatially and temporally regulated. Components of the cell wall are synthesized in the cytoplasm, the plasma membrane and the wall itself. One of the components of the primary cell wall is chitin. The absence of chitin from plant and mammalian species makes the biosynthesis of this polymer an attractive target for developing strategies to interfere with fungal growth. In filamentous fungi the chitin synthase gene family is comprised of 5-7 genes, suggesting that chitin biosynthesis is complex. The regulation of chitin synthase activity involves both transcriptional and post-translational regulation. Compartmentalization may be one form of chitin synthase activity regulation, as at least some chitin syntheses are conveyed within vesicles in the fungal cell. Gene inactivation experiments indicate that not all chitin syntheses contribute equally to fungal growth or are essential for fungal development or pathogenesis. As filamentous proliferation is in most cases polarized, coordination of cell wall biosynthesis must be tightly

regulated and in concert with other cellular activities. Therefore, the study of genes and their products involved in the biosynthesis of cell wall components (e.g., chitin and glucan syntheses, hydrophobins) should be coupled with the analysis of other factors governing hyphal growth. Various elements potentially involved in transduction of signals governing cell wall biosynthesis and cell shape determination are being studied. Evidence concerning the involvement of ser/thr as well as other kinases and phosphatases in governing cell wall biosynthesis is accumulating, advancing our understanding of the linkage between cell wall biosynthesis and other cellular processes.

Plenary Session: Evolution and Population Genetics (Chair: John Taylor)

Fungal transposable elements and genome evolution.

M.J. Daboussi, Universite Paris-Sud, Orsay, France.

Many different classes of transposable elements which reflect the whole spectrum of eukaryotic transposons are now known in fungi. Most of our knowledge comes from species representing different ecological situations: plant pathogens, industrial and field strains, most of them lacking the sexual stage. A number of changes in gene structure and function have been shown to be TE-mediated: inactivation of gene expression upon insertion, DNA sequence variation through excision and probably by inducing extensive chromosomal rearrangements. Moreover, TEs may have other roles in evolution related to their ability to be transferred horizontally and to capture and transpose chromosomal host sequences thus providing a mechanism for dispersing sequences to new sites. However, the activity of transposable elements and consequently their proliferation within a host genome can be affected in some fungal species which undergo meiosis by silencing processes. It is now clear that transposable elements are ancient and ubiquitous components of the fungal genome with the potential to influence many aspects of fungal genome evolution. Our understanding of the biological effects of TEs on the fungal genome has increased dramatically in the past few years but much remains to be learned to understand the long term effects of TEs on genomes, populations and species.

Phylogenetics, RDNA polymorphisms and phylogeography of the phytopathogen *Fusarium.*

Kerry O'Donnell and Elizabeth Cigelnik, MPR, National Center for Agricultural Utilization Research, USDA/ARS, Peoria IL.

As part of a phylogenetically-based revision of *Fusarium*, we investigated the evolutionary history of the phytopathogenic *Gibberella fujikuroi* species complex by parsimony analysis of DNA sequences from multiple loci. Gene phylogenies inferred from 5 loci were generally

concordant, providing strong support for a fully resolved phylogeny of most species. The biogeographic hypothesis proposed from the phylogenetic evidence is based primarily on the formation of natural barriers associated with the fragmentation of the ancient super-continent Gondwanaland over the last 100 million years. Discordance of the nuclear RDNA internal transcribed spacer 2 (ITS2) gene tree with trees from the other loci is due to nonorthologous ITS2 sequences. The molecular evidence suggests that two highly divergent ITS2 types were combined by an ancient interspecies hybridization or gene duplication that occurred early in the evolutionary radiation of the *Gibberella* lineage of *Fusarium.* Only one of the two ITS2 types is discernible within each species when conserved ITS primers were used; however, the second ITS2 type was recovered from every strain tested with ITS2 type-specific PCR primers. Distribution of the major ITS2 type within the species lineages obscures true phylogenetic relationships because the major ITS2 type switched between a type I and type II sequence at least twice during the evolution of the *G.fujikuroi* complex. Remarkably, the two intragenomic ITS2 types have escaped concerted evolution within this lineage of *Fusarium.*

Clonality and Sex in Basidiomycete Populations.

Jim Anderson, University of Toronto.

The population structure of hymenomycete fungi is largely determined by two processes: vegetative growth and sexual reproduction. These fungi are territorial; each genetic individual arises in a unique mating event and then grows vegetatively to colonize a territory that can vary in size and shape. Diploid genetic individuals of *Armillaria gallica* thought to be at least one million mitotic cell generations old (ca. 1000 years) offer a rare opportunity to detect and estimate the rates of mutation, gene conversion, and mitotic crossing over in selected genomic regions under natural conditions. In effect, the history of genetic change within the individual should be evident in a spatial pattern reflecting growth from a point of origin. In contrast, most previous estimates of mutation rate in natural populations have been indirect; they are based on the frequencies of alleles whose precise origins in the population are unknown. In the sexual population of *A. gallica,* from which genetic individuals arise, genotype frequencies at nuclear loci do not deviate significantly from Hardy-Weinberg expectations and allele frequencies are not significantly different between localities in eastern North America. These observations are consistent with a high rate of gene flow among localities. Even mtDNA genotypes reflect the prevalence of genetic exchange and recombination in natural populations. Most pairs of non-contiguous mtDNA loci marked by RFLPs or nucleotide substitutions show no significant linkage disequilibrium.

Population genetics of *Cryphonectria parasitica:* Evolutionary inferences from studies of population structure.

Michael G. Milgroom, Cornell University, Ithaca, NY.

Analysis of multilocus genetic structure within populations can be used for making inferences about reproductive biology and the extent to which recombination shapes populations. The

chestnut blight fungus, *Cryphonectria parasitica,* has much variation in its multilocus genetic structure. A few populations are panmictic, with little evidence of nonrandom associations between loci (gametic disequilibrium). Other populations are nearly exclusively clonal, but the majority of populations are intermediate in structure. The structure of 11 populations of *C. parasitica* in Italy were analyzed based on frequencies of vegetative compatibility (vc) types. Genetic analyses were done to determine vegetative incompatibility (vic) genotypes for each vc type found in Italy. We found six independent, polymorphic vic loci, each with two alleles, however, only 20 of the 64 possible vc types were found in Italy. Most populations had significantly fewer vc types and less genotypic diversity than would be expected under random mating. Furthermore, 10 of the 11 populations had significant, but varying degrees of gametic disequilibrium. Very few recombinant genotypes were found in some populations, even with large samples, although sexual structures were commonly found in almost all populations. Approximately 25% of the perithecia of *C. parasitica* sampled in field populations appear to be self-fertilized, and even among outcrossed perithecia, there is evidence for inbreeding. The future challenge is to understand the genetic and environmental regulation of the breeding system in this fungus.

Pseudohomothallism and evolution of *Neurospora tetrasperma.*

Donald O. Natvig, David J. Jacobson*, Marian P. Skupski and Alena Gallegos. University of New Mexico and *Stanford University.

N. tetrasperma is self fertile as a consequence of each ascospore receiving one nucleus of each mating type. Spindle overlap at the second meiotic division and programmed nuclear movements lead to four heterokaryotic ascospores per ascus, instead of the eight homokaryotic ascospores typical of *N. crassa.* This reproductive system has been termed pseudohomothahism. Occasional single mating-type spores are self sterile and heterothalhc, suggesting the potential for outcrossing in nature. *N. tetrasperma* provides examples of both the power of evolutionary innovation and the constraints imposed by life-cycle biology. Recombination between *N. tetrasperma* mating-type chromosomes is suppressed relative to that observed for *N. crassa*. This suppressed recombination appears to reflect selection against homokaryotic (self-sterile) ascospores that would result from crossovers between mating-type genes and centromeres. Paradoxically, suppressed recombination, coupled with the unique ascospore development of *N. tetrasperma,* has produced wild-type strains with high levels of heteroallelism on the mating-type chromosome, but with virtually complete homoallelism for sequences on autosomes. Although *N. tetrasperma* appears to be well suited for facultative outcrossing in nature, laboratory outcrosses employing wild-type strains frequently result in high levels of sexual dysfunction, even when strains are closely related. We hypothesize that the observed high levels of homoallelism on autosomes reflects avoidance of heterokaryon incompatibility by *N. tetrasperma,* which is typically found in nature as a heterokaryon. Sexual dysfunction may reflect activation of heterokaryon incompatibility or other types of protoplasmic incompatibility. Efforts are underway to better define the genetic basis of sexual dysfunction and explore its role in the evolution of *N. tetrasperma.*

Plenary Session: Sexual/Asexual Reproduction (Chair:Mimi Zolan)

Meiotic initiation in Filamentous Ascomycetes.

Denise Zickler, Universite Paris-Sud, Orsay, France.

A cell lineage is part of the meiotic program of *Podospora anserina*, *Neurospora crassa*, *N. tetrasperma*, and *Ascobolus immersus.* After fertilization, only nuclei of opposite mating type form dikaryons which undergo karyogamy and meiosis, producing biparental progeny. Analyses of dikaryon formation showed that this process is a new example of asymmetric cell division linked to cell differentiation, including fixed patterns of nuclear division and migration with nuclear and cell death.

Functional mating type products are mainly required to distinguish self from non-self. When nuclei of *P. anserina* habor mutations in any one of the four genes that reside in the mating-type locus (one in *mat+* and three in *mat-*, in collaboration with M. Picard's lab), the mutant nuclei have lost the controls that prevent haploid and uniparental diploid meiosis in wild-type strains. These nuclei can enter meiosis (haploid meiosis) and spores, demonstrating that heterozygosity of the mat genes is not a prerequisite for meiosis and sporulation.

The three components of the cytoskeleton are interdependently involved in ascus morphogenesis. They are also implicated in the segregation of non-self nuclei: (1) Recognition of opposite mating type nuclei could be mediated by chromosomal DNA located at the nuclear face of interphase spindle pole bodies (2) Three developmentally regulated MTOCs are required for proper migration of nuclei (3) Nuclei of opposite mating type that will be contained in a single cell form interconnected astral arrays of microtubules prior to the formation of that cell.

Peroxisomes are required at two steps: at the stage of meiotic cell differentiation, when the haploid nuclei of opposite mating type undergo karyogamy, and for spore maturation. In homozygous *car1*$^{-}$rosses of *P. anserina*, the cells that would normally be committed to enter meiosis do not differentiate and instead resume dividing.

Recognizing homology before and during meiosis.

Patricia J. Pukkila, Lesley S. Benyon, Sylvia A. Frazier, and Janet L. Knight. University of North Carolina at Chapel Hill.

The source of specificity for meiotic chromosome pairing is not known. We have shown that prior to meiosis, a genome-wide homology search occurs in haploid nuclei of *Coprinus cinereus* which results in the methylation of duplicated sequences, a process termed MIP (methylation induced premeiotically). Genomic sequencing has confirmed that this methylation occurs predominantly at CpG dinucleotides. MIP can be monitored in a variety of cell types that never undergo meiosis including regenerated protoplasts recovered from dikaryotic mycelia, asexual

spores of the dikaryon (chlamydospores), and veil cells of fruit bodies. Thus, our system is ideal to explore the nature and extent of genetic overlap between premeiotic and meiotic homology recognition. We have analyzed two mutants that confer synaptic defects. The first *(rad3)* is MIP-competent, while the second *(bad27)* is MIP-deficient. If DNAs methylated by previous cycles of MIP are introduced into the *bad27* mutant background, the methylation is maintained. Our results suggest that either prior MIP is essential for synapsis, or that MIF and meiotic pairing are genetically related.

Genetics of Chromosome Structure and Segregation.

Gregory S. May and Paul Anaya, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

The condensation of chromatin into chromosomes prior to mitosis achieves several important goals. 1) It ensures that the DNA is not broken during segregation at mitosis. 2) It leads to the assembly of the kinetochore which interacts with the spindle microtubules and moves the sister chromatids to the poles. How higher order chromosome structure is achieved is poorly understood. Mitotic mutants and mutations that affect the fidelity of chromosome transmission are likely to alter chromosomal constituents that help assemble the chromosome. The *bimD6* mutation affects the ability of the chromosomes to interact with spindle microtubules. We have identified four extragenic suppressor genes to the *bimD6* mutation. One of these genes, *sudA,* is a member of the SMC family of proteins. SMC proteins are widely distributed and are known to function in chromosome condensation. A second gene, *sudD,* codes for another evolutionarily conserved protein. We have cloned the budding yeast and human homologues of *sudD* and begun working with these to determine this genes function. Curiously, the SUDD protein is cytoplasmic and not nuclear as would be predicted for a suppressor of a mitotic mutant. Data will be presented that suggests *sudD* and possibly *sudB* function not as chromosomal proteins but upstream in the chromosome condensation pathway.

Regulation of sexual development in basidiomycete fungi.

Regine Kahmann, Erika Regenfelder, Andreas Hartmann, Julia Kruger, Tilman Spelling and Michael Bolker. Institut fur Genetik und Mikrobiologie der Universitat Munchen, Maria-Ward-Str. 1a, D-80638 Munchen, Germany.

In a number of basidiomycete fungi the mating type loci encoding pheromones and receptors have been identified. While the complex structural organization of these loci is now established the question how the pheromone signals are used to control diverse intracellular processes are just beginning to be understood.

Our work concentrates on the phytopathogenic fungus *Ustilago maydis*. This fungus exhibits a dimorphic life style. Haploid sporidia grow yeast-like by budding and are non-pathogenic while the dikaryon grows filamentous and is able to induce tumors in maize plants. We will review the known contributions of the mating type loci to this morphological transition and then focus on an

analysis of the pheromone signalling cascade. In particular we will describe the characterisation of a G alpha subunit gene whose product plays an essential role in transmission of the pheromone signal. The G alpha subunit required for pheromone signaling appears to be essential for pathogenic development as well, althought this process does not require pheromone stimulation. Interestingly, when constitutively active, this G alpha protein affects tumor morphology. This may provide a handle to isolate molecules involved in the communication of *U. maydis* with its host plant.

Vegetative incompatibility: A question of identity.

N. Louise Glass, Biotechnology Laboratory and Botany Department, University of British Columbia, Vancouver, B.C. V6T IW3, Canada.

Vegetative incompatibility is responsible for intraspecific self/nonself recognition in filamentous fungi during the assimilative phase of growth. In *Neurospora crassa,* vegetative incompatibility can be caused by heteroallelism at any of at least 11 *het* loci, including the mating type locus. The mating-type locus encodes putative transcriptional regulators that affect expression of various target genes. A suppressor of mating-type associated incompatibility, *tol* has been cloned and encodes a putative 922 aa polypeptide; RIP mutants are fully compatible with both *A* and *a* strains, but have no other observable phenotype. We have also cloned and characterized two additional *het* loci, *het-c* and *het-6.* The *het-c*^{or} (for Oak Ridge) allele encodes a 966 aa coiled-coil, glycine-rich polypeptide that contains a signal sequence, suggesting that it is targeted to the fungal cell wall. RIP mutants in *het-c* are fully compatible, but have no other observable phenotype. Analysis of *N. crassa* strains indicates that *het-c* specificity is mediated by three alleles. Chimeric constructs localized the region determining *het-c* specificity to a highly variable domain of approximately 30 aa. Analysis of *N. crassa* strains, other Neurospora species and related genera show that the three allelic motifs that determine *het-c* specificity region are highly conserved.

Abstracts from the Thursday March 20 Poster session (Posters I)

Hyphal growth and polarity posters

Heather/Toyon/Acacia

Hyphal Growth and Polarity

1. The *CHK* Genes in *C. albicans*: OS1 Homologs important for Cell-Wall Assembly and Osmotic Tolerance.

J.Agnan, C. Korch, M. Schumacher, L. Alex[1], M. Simmons[1], C. Selitrennikoff. University of Colorado Health Sciences Center, Denver CO. [1]California Institute of Technology, Pasadena CA.

The aim of this research is to identify genes involved in cell-wall synthesis for the development of novel antifungal targets. We previously reported the cloning and characterization of the *osmotic-1 (os- 1)* gene, which is required for normal growth, cell wall synthesis, and osmotic tolerance in *Neurospora crassa.* Mutants of *os-1* have altered cell-wall compositions, are unable to grow on medium supplemented with 4% NaCl, and are resistant to dicarboximide fungicides, such as vinclozolin, iprodione and procymidone. *os-1* encodes an osmosensing histidine-kinase, believed to participate in a signal transduction cascade similar to the high-osmolarity glycerol (HOG) pathway of yeast.

We report the isolation, cloning, and sequencing of the *os-1* homologs from *C. albicans,* an opportunistic fungal pathogen responsible for a variety of diseases in humans. Three different genes were isolated and are believed to be members of a gene family. The *os-1* homologs in *C. albicans* have been designated *Candida histidine kinase (chk)* and the sequence obtained shows homology to the *N. crassa os-1* gene and to a sensor-regulator protein of *Saccharomyces cerevisiae, SLN* 1. These data indicate that the *chk* genes encode an osmosensing protein that is essential for normal growth and cellwall biosynthesis. Construction of *C. albicans chk* deletion mutants is currently underway.

2.The *Aspergillus nidulans sod^{VI}C* gene is involved in hyphal growth and encodes a subunit of the coatomer complex.

Susan J. Assinder, Susan L. Whittaker and John H. Doonan* School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, Wales, LTK; *John Innes Research Centre, Norwich, NR4 7LTH, UK.

The *A. nidulans mutation sod^{VI}C* (for stabilisation of disomy) causes a high frequency of aneuploid progeny (1). The mutation is lethal at 42 C but allows normal growth at 30 C. Upon upshift from 30 C to the sub-restrictive temperature of 37 C, disomic sectors are produced which carry an extra copy of chrVI. The disomic state is stable at 37 C (unlike the usual case for *A.*

nidulans aneuploids) but downshift to 30 C causes reversion to haploidy. Based on these properties, *sod*^{VI}*C* was originally presumed to be mutant in a function required for chromosome partitioning. However, cytological and molecular characterisation have shown that mutant strains are defective in hyphal extension. At 42 C, conidia arrest with 1-2 nuclei and fail to produce a germ tube. The chromosome mitotic index is similar to that of the wild-type, indicating that there is no cell cycle lesion.Temperature-shifts of pre-germinated conidia have shown the defect to be in hyphal growth rather than in a function specific to germination. The mutation has been complemented using a cosmid from a chromosome VI-specific library which has homology to a subunit of the human coatomer complex involved in the early secretary pathway.

(1)Upshall *et al.* (1979) In: Sebek (ed): Procs. 3rd Int. GIM Symp. pp 197-204.

3. Morphological changes during growth of *Ustilago maydis*.

Flora Banuett. Department of Biochemistry & Biophysics, University of California, San Francisco, CA 94143-0448.

Ustilago maydis is a Basidiomycete fungus that induces tumors in maize. It can grow as a unicellular haploid yeast-like form that is non-pathogenic and as a filamentous dikaryon that requires the plant for its growth. Growth of the hyphae in the plant leads to formation of tumors within which the fungus produces round diploid spores, the teliospores. In order to determine how the hyphae, which exhibit highly polarized growth, lead to formation of these round teliospores, a study of the infectious process was undertaken. The course of infection was followed from the time of inoculation of the fungus into the plant to the formation of mature telliospores. These studies and analysis of the *fuz1*$^{-}$ mutant led to the conclusion that a discrete developmental pathway is involved in teliospore formation (Banuett and Herskowitz, 1996 Development 1 22: 2965-2976). Progression of this pathway is arrested in the *fuz1*$^{-}$ mutant at a discrete step. This work also demonstrates that although different *a* alleles are necessary for filamentous growth in culture, they are not required for filamentous growth in the plant, suggesting that the *a* pathway may be activated by a plant signal.

In addition to this work, I will also describe the characterization of mutants with altered cell morphology as well as the changes in the actin cytoskeleton during the cell cycle and the life cycle.

4. *Neurospora crassa* chitin synthase 2 is expressed during aerial structure formation.

Adi Beth-Din, Vered Ziv and Oded Yarden, Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel.

In *Neurospora crassa* five chitin synthase (chs) genes has been identified so far. Gene inactivation experiments provided evidence for differential chs gene function. Null chs gene

phenotypes range from no observed morphological changes (when compared to wild type) to extreme abnormalities in hyphal growth. Under various laboratory growth conditions no change in phenotype was observed in strains in which *chs-2* had been inactivated. However, a substantial reduction in chs activity (as determined *in vitro)* could be attributed to the loss of *chs-2* expression. In order to advance our understanding of the possible role of *chs-2 in vivo,* we examined this gene and it's product during *N. crassa* growth and development. Aerial structures are richer in chitin than liquid-grown mycelium. In *in-vitro* assays, total chs activity was found to be much higher during aerial hypha formation than in mycelium growing in liquid culture. Polyclonal antibodies raised against an overexpressed fraoment of *chs-2,* identified a ~80Kd polypeptide in protein extracts of aerial hypha that

was absent from the mycelial extract. *acon-2, acon-3* and *fl,* mutants blocked at various staores of conidiation, were used to analyze *chs-2* transcription during aerial structure formation. *chs-2* transcript levels were found to be dramatically increased in stages of aerial hypha formation and major constriction development; We suggest that chitin synthase 2 expression is both temporally and spatially regulated.

5.Characterization of double disruptants of *chsA* and either *chsC* or *chsD* of *Aspergillus nidulans*.

Hiroyuki Horiuchi, Makoto Fujiwara and Masamichi Takagi. Department of Biotechnology, The University of Tokyo.

We have isolated four chitin synthase genes (*chsA, chsB, chsC,* and *chsD*) of *A. nidulans* and disrupted them. The disruptant of *chsB* showed severe defect of hyphal growth but the phenotypes of the other three disruptants were similar to that of the parental strain. We have constructed disruptants of two of three genes, *chsA, chsC* and *chsD*. Double disruptants of *chsC* and *chsD* (CD disruptants) showed no phenotypical change. While, efficiency of conidia formation of AD disruptants was about 10% of that of the parental strain and that of AC disruptants was less than 0.1% of that of the parental strain. AC disruptants showed pleiotropic defects, such as sensitiveness to high concentration of salts, SDS, Calcofluor white and Congo Red. Calcofluor white and Congo Red are known as chitin binding dyes. AC disruptants were also sensitive to a chitin synthase inhibitor, Nikkomycin Z. Moreover, abnormal structures of conidiophores of AC disruptants were observed by a scanning electron microscopy. These structures are similar to that of the medA mutant. These results suggested that *chsA* and *chsC* have important but redundant functions for tip growth of hyphae and formation of conidiophores.

6. Effect of water activity on the rate of hyphal growth and pectinase production by 2 deoxy-glucose resistant mutants of *Aspergillus niger*.

O. Loera-Corral, C.P. Larralde-Corona and G. Viniegra-González. Universidad Autonoma Metropolitana- Iztapalapa, México D.F. México.

Water activity (a_w) is a major environmental factor affecting the specific growth rate (m) and also the yields of enzyme production of molds such as *A. niger*, in terms of substrate or biomass changes ($Y_{p/s}$ or $Y_{p/x}$). Therefore, a_w depressants such as ethylene glycol (EG), can be used to select and screen for *A. niger* strains adapted to produce pectinases in solid substrates with low a_w values, i.e. coffee pulp. Antier *et al.* (1993) found that deoxy glucose resistant mutants from wild strain *A. niger* C28B25 were adapted to such purpose when selected at low a_w values (15% EG). Estimation of m can be done, according to Larralde (1996) by formula $m = u_r \, ln2 / [L_{av} \, ln(L_{av}/D_h)]$, where u_r=maximal rate of apical elongation, L_{av}= average length of distal branches, D_h= hyphal diameter. The patterns of specialized mutants AW96-4 and AW99-iii with respect to hyphal growth (m) and enzyme production ($Y_{p/s}$ or $Y_{p/x}$) using a_w =0.96 to 0.99 in a medium with pectin as sole carbon source, were compared to the patterns of wild and dikaryon strains, suggesting that growth and pectinase production are regulated by different sets of genes in a complex and non-coordinated fashion.

7. Evidence of *Cryphonectria hypovirus* replication in putative fungal transport vesicles.
Patricia M. McCabe and Neal K. Van Alfen. Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas.

The Chestnut blight fungus, *Cryphonectria parasitica*, contains a dsRNA virus, CHV1, which causes a reduced virulence of the fungus and changes in expression of specific fungal genes. Previously a host vesicle fraction was isolated and was shown to contain dsRNA. A similar fraction can also be isolated from non-viral infected strains, but in viral infected strains there is at least a 6 fold increase in the number of these vesicles. Viral replication has also been correlated with this vesicle fraction and the CHV I polymerase was also identified with these vesicles. Some of the genes which are down-regulated by the virus have been cloned and have been shown to be secreted proteins, some of which contain a signal peptide cleaved by a Kex2p-like serine protease. One of these proteins, cryparin, which is down-regulated in virus infected strains, has been found in this vesicle fraction. Treatment of the vesicles with proteinase K suggested that most of the cryparin is protected from the protease by the vesicles. When isolated with the vesicles, cryparin was not only present in its regular form, but a higher molecular weight glycosylated form was also isolated. Often on transport through a secretary pathway proteins are glycosylated, suggesting the vesicle fraction utilized by the virus for replication is a normal host secretary vesicle whose normal function is to transport, among others, cryparin.

8. Regulation of glucan synthase activity in *Aspergillus fumigatus.*

Pieternella C. Mol, Laboratoire des Aspergillus, Institut Pasteur, Paris, France.

The filamentous fungus *Aspergillus fumigatus* may cause pulmonary invasive aspergillosis which is a life-threatening infection in immunocompromised patients. To gain insights into the morphogenesis of this fungus and possible targets for chemotherapy, we study the structure and formation of the cell wall. Synthesis of (1,3)--linked glucan in the fungal cell wall is catalysed by the enzyme complex 1,3--D-glucan synthase (GS). Activity is stimulated by micromolar amounts of GTP, and as in *S. cerevisiae*, the enzyme complex contains a plasma membrane bound catalytic subunit that is activated by a GTP-binding protein. The latter can be substituted by the

homologous yeast protein, Rho1p, in an in vitro glucan synthase assay. Upon incubation of crude and detergent-solubilized membrane fractions with C3 exoenzyme from *C. botulinum* a single 21 kDa protein was ADP-ribosylated. A genomic library was screened using PCR fragments amplified from *A. fumigatus* DNA with oligonucleotide primers raised against the highly conserved GTP-binding and interaction regions of Rho-family genes. Two genes were cloned. Sequence analysis of RHO1 revealed a 950 nt region interrupted by 4 introns coding for a protein of 21.400 MW. RHO2 is 1022 nt long, interrupted by 6 introns, and encodes a peptide of 21.900 MW. Rho1p and Rho2p show highest identity with Rho1p of *S. pombe* and with Rho3p of *S. cerevisiae*, respectively. To identify all components of the GS enzyme-complex recombinant Rho1p has been isolated which will be used in the analysis of protein-protein interactions. Furthermore, we are studying the cellular localization of Rho1p and Rho2p.

9. Hyperbranching mutants of *Aspergillus nidulans.*

S. Pollerman and G. Turner, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, U.K.

Early studies on mycelial growth in *Aspergillus nidulans* led to a model for the duplication cycle of this organism which related hyphal elongation, mitosis, septation and branch development. More recently, commercial production of the edible mycoprotein Quorn™ *(Fusarium graminearum)* by continuous culture has led to the undesired selection of colonial mutants in fermenters, some of which exhibit an increased frequency of branching, and a number of genetic loci appear to be implicated. Hyperbranching mutants have also been isolated in *Neurospora crassa*, and the *cot-1* gene, encoding a protein resembling cAMP dependent protein kinase, has been cloned.

In order to understand better the control of branching frequency, and its relation to the duplication cycle in fungi, we have isolated and characterised a number of mutants exhibiting hyberbranching (Hbr) in *Aspergillus nidulans.* Most of these were identified by screening microscopically a collection of 1200 Ts mutants for the desired phenotype, and 25 have been selected for further study following backcrossing to the wild-type. The hyperbranching phenotypes have been confirmed by measurement of the hyphal growth unit G. Complementation tests and assignment of mutations to linkage groups should facilitate the isolation and molecular characterisation of some of these genes with the aid of the chromosome specific cosmid library.

10. Impairing calcineurin of *Neurospora crassa* reveal its essential role for hyphal Growth, morphology and maintenance of the apical Ca^{2+}-gradient.

Holger Prokisch[1], Oded Yarden[2], Margit Mischler[3], Maximilian Tropschug[3], Ilse Babette Barthelmess[1], [+]Institut fur Angewandte Genetik, Universitat Hannover, D-30419 Hannover, Germany; [2]Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Rehovot 76100, Israel; and [3]Institut fur Biochemie und Molekularbiologie, Universitat Freiburg, D-79104 Freiburg, Germany.

The function of *Neurospora crassa* calcineurin was investigated in *N. crassa* strains transformed with a construct for the inducible expression of antisense-RNA for the catalytic subunit of calcineurin (*cna-1*). Antisense-RNA expression, reduced levels of *cna1* mRNA and of immunodetectable CNA1 protein and decreased calcineurin specific enzyme activity, on induction medium only, were evidence that a conditional reduction of the target function had been achieved in antisense-transformants with multiple construct integrations. Induction conditions procured a growth arrest which indicated an essential function for the *cna- 1* gene of *N. crassa.* Growth arrest was preceded by increased hyphal branching, changes of hyphal morphology and concomitant loss of the distinctive tip-high Ca^{2+}-gradient typical for growing wild-type hyphae. This demonstrates, for the first time, a specific role of calcineurin in the precise regulation of apical growth, a common form of cellular proliferation. *In vitro* inhibiton of *N. crassa* calcineurin by the complex of cyclosporin A (CsA) and cyclophilin2O and increased sensitivity of the induced transformants to the calcineurin specific drugs, CsA and FK506, were evidence that the drugs act in *N. crassa,* as in T-cells and *Saccharomyces cerevisiae,* by inactivating calcineurin. Consistently, exposure of growing wild-type mycelium to the drugs led to a phenotype very similar to that of the *cna-1* antisense-mutants.

11. Directionality of growth in fungal hyphae.

M. Riquelme, C.G. Reynaga-Pena, G. Gierz and S. Bartnicki-García. University of California, Riverside.

Hyphal tips of higher fungi contain a characteristic phase-dark body: the Spitzenkörper (Spk). Former studies suggested a correlation between the position of the Spk and the direction of hyphal growth. We have studied Spk behavior in growing hyphae of *Neurospora crassa*. Hyphae of this fungus have a tendency to meander, yet they maintain an overall straight direction of growth. We used video-enhanced phase contrast microscopy to study in fine detail positional changes of the Spk in living hyphae of *N. crassa*. We found that Spk position and growth directionality were closely correlated. A change in growth direction, i.e. the establishment of a new axis, was correlated with a sustained shift of the Spk position away from the existing cell axis. By using a computer program based on the hyphoid model for fungal growth and morphogenesis, it was possible to duplicate the meandering behavior observed in living hyphal cells. This supports the idea that hyphal morphology is controlled by the Spk functioning as a vesicle supply center (VSC). Although external factors are known to affect directionality of hyphal growth (tropisms), we have no evidence to believe that they are the *primary* determinants of growth direction. Hyphal tips of *N. crassa* exposed to microtubule inhibitors became highly branched and lost their growth directionality, suggesting a role of the microtubules in maintaining growth direction. We propose that an intrinsic, cytoskeletal-based, mechanism keeps hyphae growing in a fixed direction while allowing minor reversible departures of the Spk away from its original trajectory.

12. Biochemical analysis and immunolocalization of ApsA, a protein involved in nuclear positioning in *Aspergillus nidulans.*

Nicole Sievers and Reinhard Fischer, Universitat Marburg and MPI fur terrestrische Mikrobiologie, Marburg, Germany

Nuclear migration is very important in many eucaryotic cells and absolutely crucial for apical extension of fungal hyphae. Microtubules and the microtubule dependent motor protein dynein are essential components for the translocation process. The regulation of the basic machinery and its coordination to other cellular functions like cell cycle, cell differentiation and morphogenesis are still unknown. In *A. nidulans* two genes were identified which seem to be involved in nuclear positioning, *apsA* and *apsB* (anucleate primary sterigmata). In the mutants nuclei are clustered in hyphae in contrast to evenly distributed nuclei in the wild type. In addition, the nuclear positioning defect leads to the formation of anucleate metulae and thus to a block of development.

The two genes *apsA* and *apsB* were cloned and sequenced. *apsA* encodes a 180 kD coiled coil protein with similiarity to the yeast nuclear migration protein NUM1. Amino acid sequence motifs suggest a role in signal transduction or an association to the cytoskeleton. The ApsA protein and a hemeagglutinine (HA) epitope tagged version of the protein were detected in Aspergillus protein extracts with polyclonal and monoclonal antibodies, respectively. The protein could further be detected in germlings, hyphae and conidiophores by immunofluorescence using HA-tagged ApsA and monoclonal anti-HA antibodies. ApsA was localized at the cell membrane with a spot like distribution. Similiar results could be obtained with ApsA tagged with the green fluorescent protein (GFP) of the marine yellyfish *Aquorea victoria.* Analysis of a cell cycle dependent regulation of ApsA are under way.

13. Investigating the nuclear cycle in *Ustilago maydis* vegetative cells and mating hyphae.

Karen M. Snetselaar, Michael P. McCann. St. Joseph's University, Philadelphia PA 19131.

Nuclear cycle events are traditionally studied using ^{3}H-thymidine, which cells take up from culture medium and incorporate into DNA, but fungi apparently do not incorporate exogenously supplied thymidine. Some methods used to study fungal nuclear cycles involve DNA extracted from synchronous cell cultures, but because we wish to correlate nuclear condition in *U. maydis* with nonsynchronous events such as bud formation, development of mating tubes and infection hyphae, we are using microdensitometry. Cells from liquid cultures were attached to microscope slides, stained with DAPI, and viewed using epifluorescence microscopy. Digital images of stained nuclei were captured, and densitometry software was used to quantitate their relative fluorescence. When nuclei were grouped by relative density, two peaks presumably corresponding to 1C and 2C amounts of DNA were observed. The methods used also allowed correlating measurements of nuclear density with cell morphology. *Ustilago maydis* sporidia completed DNA synthesis prior to visible evidence of bud formation, and cytokinesis was completed before DNA synthesis begins. Mid log-phase cultures had approximately equal numbers of cells with 1C and 2C nuclei, but as doubling times for cultures increased, the number

of cells in G1 increased dramatically, indicating that the length of G1 varies with growth conditions. Nuclei in cells induced by pheromone to form mating tubes are apparently arrested in G2 because they have the 2C amount of DNA.

14. Conventional kinesin from the plant pathogen *Ustilago maydis* is involved in vacuole formation and cytoplasmic migration.

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Using PCR a gene encoding a kinesin motor *(kin2)* was isolated from the dimorphic basidiomycete *Ustilago maydis.* The predicted amino acid sequence displayed a high similarity with the conventional kinesin from *Neurospora crassa (Nkin).* Purified Kin2 and Nkin proteins share some unique features as a high *in vitro* motility and the absence of light chains. Haploid strains carrying a deletion of the *kin2* gene were viable and showed no obvious defects. Upon fusion of compatible haploid wild type or kin2 mutant cells a filamentous dikaryon is produced but kinesin-deficient mutant hyphae were significantly shorter, curved, often branched and failed to create empty compartments that are normally left behind the growing tip cell. Moreover, kin2 mutant cells showed dramatically reduced pathogenity. A biologically functional kin2-GFP fusion protein displayed a diffuse distribution within the hyphal cells, suggesting that this kinesin transports small vesicles. Although kin2 sporidia grew apparently normal, these cells were defective in the accumulation of lucifer yellow in the vacuole. Moreover, kin2 mutant sporidia contained more but smaller and often misplaced vacuoles. In hyphae produced by kin2 mutants subapical vacuoles could not be detected. In wild type hyphae such vacuoles expand and approach a size similar to the empty compartments behind the tip cell. We suggest that Kin2 is involved in membrane traffic of vacuole precursors promoting the migration of cytoplasm during growth of the filamentous dikaryon.

15. Nuclear migration and positioning in *Aspergillus nidulans*.

Suelmann R.*; Galetzka D.*, Robertson L[1]. Timberlake W. E[1]. and Fischer R*. *Universitat Marburg

and MPI for Terrestrische Mikrobiologie, Marburg, Germany, [1]University of Georgia, Athens, GA.

Nuclear migration is important in many eucaryotic cells and crucial for apical growth of fungal hyphae. We have visualized this organelle movement by time-lapse video microscopy *in vivo* in *A. nidulans* using a GFP fusion protein localized in nuclei. First results will be presented.

Besides the microscopic characterization of the process the nuclear basis of the movement is of great importance. Essential components are e.g. microtubules and the microtule associated motor protein dynein. In addition two genes were isolated in *A. nidulans* which are involved in nuclear positioning and thus in regulation of this process. In contrast to wildtype hyphae where nuclei are evenly distributed, nuclei are clustered in mutant hyphae. Here, one of the genes *apsB* (anucleate primary sterigmata), was analyzed at a molecular level. The gene contains one 48 bp intron close to the 5' end of the transcript and encodes an open reading frame of 1052 amino acids. Secondary structure predictions suggest coiled-coil domain at the N-terminus of the hydrophilic protein. A HA:ApsB fusion protein was detected in crude cell extracts. *In vivo* localization experiment (HA:ApsB; GFP:ApsB) are under way. Potential interactions with the coiled-coil ApsA protein will be discussed.

16. Generation and Characterization of new mutations affecting tip growth and branching in *N. crassa* via insertional mutagenesis.

Michael Watters and Tony Griffiths, University of British Columbia, Vancouver B.C. CANADA.

Neurospora normally grows forming characteristically branched, spreading colonies. Many genes are known to affect this morphology altering either the branching pattern direction of growth or overall colony size. Reletively few of these genes however have been clones and their function in the cell determined. We have begun a study to induce new (or at least new alleles of) morphological mutations, using gene disruption by a plasmid transformed into cells. These mutants should have the advantage over existing alleles of being tagged with the inserted sequences. We plan to make use of these tags to aid in recovery of the affected genes. We have used this approach thus far to generate several mutants with a range of phenotypes and these have been partially characterized. The poster will report on a work in progress.

Gene Regulation posters

Gene Regulation

17. Hexokinase activity in *S. cerevisiae.* 1. The role of mitochondrial deficiency, glucose and ethanol concentrations.

Kamel A. Ahmed, Mohamed H. Hamoda, Ahmed N. Sharaf and Hassan A. Mohamed. Faculty Agriculture, University of Cairo, and National Res. Centre, Egypt.

The genetic regulation of hexokinase enzyme in *Saccharomyces cerevsiae was* investigated through the effect of the deficiency in mitochondrial function and regulatory effect of glucose and ethanol concentration as well as aeration. Petite mutants were induced by nicotine mutagen and identified in two genetically different haploid yeast strains. Petite mutants showed no chromosomal reverted genes, different patterns of sugar utilization, when tested on defferent carbon sources , and a variable increase in enzyme activity than that in its parental strains . Different types of diploids (normal mitochondrial function, partially or totally nonmitochondral function diploids) showed differences in their sporulation time where the petite diploid "non-functional mitochondrial diploid" never sporulated. Diploids exerted variable hybrid vigour in their enzyme production . Tetrad analysis of different diploids resulted in variable segregation patterns in the enzyme activity . Parental, petite mutants, diploids and tetrad segregants strains tolerated the increase in ethanol concentration up to 10% , glucose concentration up to 40% and aerobic or anaerobic conditions to different degrees which was reflected on their enzyme activity as a function of their chromosomal and cytoplasmic genes.

18. Characterization of the fluffy gene of *Neurospora crassa.*

Lori A. Bailey and Daniel J. Ebbole. Texas A&M University.

The fluffy (*fl*) mutant of *Neurospora crassa* blocks the development of macroconidia. Genetic mapping places *fl* between *mus-23* and *trp-3* on the right arm of chromosome III. A cosmid was identified that complemented both *mus-23* and *fl*. *Fl* was subcloned to a 4.5 kbp fragment and sequence analysis of this fragment revealed an open reading frame (ORF) containing a zinc finger DNA binding domain and two putative introns. The ORF has limited sequence similarity to *Nit-4* and *NirA*, regulators of nitrogen assimilation in *N. crassa* and *Aspergillus nidulans* respectively. We have sequenced four alleles of *fl*, and these data demonstrate that the predicted ORF is required for proper gene function. RIP inactivation of the 4.5 kbp fragment produced progeny with the fluffy phenotype. The sequence of the RIP mutant contained 74 mutations due to base pair changes including one changing the translational start codon and one destroying the zinc finger domain. Expression of *fl* appears to peak six hours after induction of development, and it is induced by nitrogen starvation.

19. A prion-like protein in the filamentous fungus *Podospora anserina*.

Joel Begueret, Carole Deleu, Virginie Coustou, Beatrice Turcq and Corinne Clave, Institut CNRS de Biochimie et Genetique Cellulaires, Bordeaux, France.

The *het-s* locus of the fungus *Podospora anserina* is involved in vegetative incompatibility. Coexpression of the two alternate alleles *het-s* and *het-S* is lethal leading to incompatibility between strains. Strains containing the *het-s* allele can exhibit two different phenotypes: either they are incompatible with strains containing the antagonistic *het-S* allele ([s] phenotype) or they are neutral in incompatibility ([s*] phenotype). This later phenotype can be propagated vegetatively and is maternally inherited through meiosis. However a [s*][s] switch can occur either spontaneously at a very low rate or at a high frequency after a contact with a strain which exhibits the [s] phenotype. Once induced, the [s*][s] transition spreads as an infectious process within the mycelium. We found that the HET-s protein is expressed at the same level in strains that display these alternate phenotypes leading to the conclusion that a post-translational modification of the protein is responsible for the difference between [s] and [s*] strains. These different results suggested that the HET-s protein should behave as the prion protein PrP: the protein present in [s*] and [s] strains should display different conformations and the [s*] molecule could be converted to the [s] form by a physical interaction between the molecules. Different results confirmed that the HET-s protein display properties common with the prion protein. i) the HET-s protein strongly aggregates in vitro and can form homodimers in vivo as demonstrated using the yeast two hybrid system. ii) the proteins present in [s*] and [s] strains display different sensitivity to proteolytic enzymes. iii) overexpression of the HET-s protein strongly enhances the frequency of the [s*][s] switch.

20. Cdc42-signaling during cytokinesis in *Ustilago maydis* .

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The phytopathogenic fungus *Ustilago maydis* exhibits a dimorphic life style. Haplold sporidia grow yeast-like by budding and are non-pathogenic. The dikaryon grows filamentous and is able to induce tumors in maize plants. To identify genes that are involved in this morphogenetic switch we have isolated a number of mutants with aberrant morphology. Among these we could identify two mutants that are affected in cytokinesis. Both the *donl* and *don3* mutants show normal nuclear division but the mutant cells fail to separate after bud formation. Cells remain connected through a septum that can be stained by calcofluor. The corresponding genes have been isolated by complementation. Molecular analysis of these genes revealed that the final step of cytokinesis seems to be regulated by a member of the Rho/Rac/Cdc42 family of small GTP binding proteins. The Donl protein shows high similarity to guanine exchange factors specific for these small Ras-like proteins, the *don3* gene codes for a kinase that is homologous to the yeast STE20 and the mammalian pak/p65 serine/threonine kinases. Using the yeast two-hybrid-system we could demonstrate that the *U.maydis* Cdc42 homologue interacts with both Donl and the

Don3. We propose that cell separation is triggered by a signal that is transmitted via Cdc42.

21. Mutations, pumps, antibodies, and polar growth.

Emma Jean Bowman, Forest J. O'Neill, and Barry J. Bowman, University of California, Santa Cruz, CA. 95064.

Concanamycin A (CCA) is a potent specific inhibitor of vacuolar ATPases. Mutants were selected for growth on medium containing 1.0 uM CCA. Suprisingly, 65 of 66 mutations mapped in the region of the *pma1* locus, which encodes the plasma membrane H^+-ATPase. Plasma membrane H^+-ATPase activity in isolated plasma membranes from the mutants was 17-84% of the level seen in the wild type. The most interesting change in the plasma membrane H^+ATPase was in kinetic behavior. The wild-type enzyme showed sigmoid dependence on MgATP concentration with a Hill number of 2.0, while 7 of the 8 mutants tested exhibited hyperbolic kinetics with a Hill number of 1.0. One interpretation of these data was that the enzyme had changed from a functional dimer to a functional monomer.

Mutation of the plasma membrane H^+-ATPase did not confer resistance by preventing uptake of CCA. In the presence of CCA both wild type and mutant strains were unable to accumulate arginine, failed to concentrate chloroquine in acidic vesicles, and exhibited gross alterations in hyphal morphology, indicating that the CCA had entered the cells and inactivated the vacuolar ATPase. Instead, we hypothesize that the mutations conferred resistance because the altered plasma membrane H^+-ATPase could more efficiently rid the cell of toxic levels of Ca^{++} or protons or other ions accumulated in the cytoplasm following inactivation of the vacuolar ATPase by CCA.

22. Overproduction of aflatoxin precursors is associated with altered sclerotial development in *Aspergillus parasiticus.*

Perng-Kuang Chang[1], Peter J. Cotty[2], Deepak Bhatnagar[2], Joan W. Bennett[1] and Thomas E. Cleveland[1].

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A genetic relationship between aflatoxin biosynthesis and sclerotial development was tested in *A. parasiticus* SRRC 2043, an O-methylsterigmatocystin accumulating strain, by transforming it with the aflatoxin pathway genes, *aflR* and/or *aflJ*. Elevated production of aflatoxin precursors, norsolorinic acid, averantin, versicolorin A and O-methylsterigmatocystin, resulted from introduction of extra copies of either *aflR* or *aflR* plus *aflJ* in transformants, but not by introduction of *aflJ* alone. Similarly, sclerotial production on PDA increased only in the transformants receiving either *aflR* or *aflR* plus *aflJ* and not in the transformant which received *aflJ* alone. However, the sclerotial production of the *aflR* plus *aflJ* transformant was substantially decreased on CZ plates. Increased production of aflatoxin precursors was associated with

changes in sclerotial morphology; sclerotial shape became more elongated in transformants. Scanning electron micrographs showed that the sclerotia of the *aflR* plus *aflJ* transformant from PDA was not as compact as that observed for the wild-type strain. These results suggest a regulatory association between sclerotial morphogenesis and aflatoxin biosynthesis.

23. Regulation of the acetate utilization pathway in the basidiomycete *Coprinus cinereus.*

Pushpalata T. Chaure, Lorna A. Casselton and Ian F. Connerton, Univ of Oxford and Inst of Food Research, Reading, UK.

Growth on acetate as sole carbon source requires the induction of enzymes of the glyoxylate cycle and the acetate mobilising enzyme, acetyl CoA synthetase. Induction is dependent on the function of a regulatory gene, *acu-1*. *acu-1* was isolated by functional complementation in *C. cinereus.* DNA sequence analysis identifies a Zinc finger DNA-binding domain in its protein that has homology to the finger in corresponding acetate regulatory proteins of *Aspergillus nidulans* (facB) and *Saccharomyces cerevisiae* (cat8). Surprisingly, there is little other sequence conservation in the three proteins despite the obvious functional homology. We will present our sequence analysis of this gene and its predicted protein and describe experiments that confirm its regulatory role with respect to two of the key enzymes induced by acetate, acetyl CoA synthetase and isocitrate lyase. Elements within the promoters of the *acs-1* and *acu-7* genes have been identified as possible *acu-1* binding sites and these are also present within the promoter of *acu-1* itself indicating autogenous regulation. Other interesting aspects of *acu-1* regulatory function will be presented.

24. Protein-protein interactions between arginine biosynthetic enzymes of *Neurospora*.

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N-acetyl glutamate synthase (AGS) and *N*-acetyl glutamate kinase (AGK) are the first two enzymes in the arginine biosynthetic pathway in *Neurospora crassa*. AGS and AGK are encoded by two independent genes, *arg-14* and *arg-6*, respectively. Early biochemical and genetic evidence indicated that mutations in AGK affected both AGK and AGS activities, and that a wild-type AGK gene could restore AGS activity in *arg-6* mutants. In order to investigate the nature of this activation, we tested the possibility of protein-protein interaction between AGS and AGK proteins using the yeast Two-Hybrid System. We report the first molecular evidence for direct AGS and AGK interaction. AGS and AGK interaction is strong and visualization through a -galactosidase assay is rapid: blue colonies were observed in less than 30 minutes. The interaction domains of AGS and AGK have been identified using various deletion constructs. The interaction domain of AGS resides at the N-terminus and the interaction domain of AGK is at the C-terminus.

25. Arginine feedback resistant mutation in *Neurospora*.

Jessica Y. Chung, Suhn-Kee Chae* and Richard L. Weiss. Department of Chemistry and Biochemistry, University of California, Los Angeles, *present address, Pai Chai University, Korea

Mutations linked to the complex *arg-6* locus (*su(pro-3)*) are feedback resistant and suppress proline auxotrophic mutations *(pro-3). N*-acetyl glutamate kinase (AGK) is one of the proteins encoded by the *arg-6* gene and is inhibited by arginine. We postulated that the *su(pro-3)* mutation could be located at the arginine feedback site of AGK. In order to identify the feedback resistant site in AGK and the nature of *su(pro-3)* mutations, we cloned AGK from several *su(pro-3)* mutant stains. Genomic DNA from several *su(pro-3)* strains was isolated and PCR was performed. The PCR products were introduced into a *N. crassa* double mutant (*arg-6, pro-3*) and transformants were identified by arginine prototrophy. Transformants that contained the *su(pro-3)* mutant gene suppressed the proline requirement and were able to grow in the absence of proline. The *su(pro-3)* mutations were mapped to the N-terminus of AGK, and all *su(pro-3)* mutant genes have a single amino acid change from phenylalanine (F) to leucine (L) at amino acid 81. To confirm that the F to L mutation alone caused the *su(pro-3)* phenotype, wild-type AGK was mutated using overlap extension PCR mutagenesis. The mutated AGK was introduced into the *N. crassa* double mutant (*arg-6, pro-3*) and transformants were identified by arginine prototrophy. A single F to L change in the wild-type AGK gene resulted in the *su(pro-3)* phenotype: transformants grew in the absence of proline. Southern blot analysis verified the presence of the mutant AGK gene in transformants.

26. Transgene induced gene silencing "quelling" in *Neurospora crassa*.

Carlo Cogoni and Giuseppe Macino. Universita' "La Sapienza" Roma Italy.

Transgene-induced gene silencing of several genes used in transformation experiments in *Neurospora crassa* has been termed "quelling". In studies using the carotenoid biosynthetic gene albino- 1 as a visual reporter for quelling, silencing was found to be reversible, and reversion was accompanied by loss of exogenous copies. Gene silencing acts at a post-transcriptional level leading to a strong reduction of steady-state mRNA level of the duplicated gene. Silencing was shown to be a dominant trait, operative in heterokaryotic strains, indicating the involvement of diffusable, trans-acting molecules. The production of an unintended transgene derived sense RNA was found to correlate with the gene silencing.These findings are compatible with a model in which RNA-RNA interaction is involved in gene silencing.

The isolation of a transformant strain silenced for the albino-1 gene in which quelling is stable, allowed us to perform UV mutagenesis to isolate, by simple visual screening, mutant strains impaired in gene silencing. Among 120,000 survivors screened after exposure to UV, 25 strains showing the recovery of wild type (orange) phenotype were isolated. Genetic analysis indicates that all the strains, containing recessive mutations, fall into four different complementation groups. The isolation of the corresponding genes will be of fundamental importance in the elucidation of the molecular mechanism of gene silencing in *Neurospora* as well as in plants.

27. Clock Mutants and Light Entrainment in Neurospora.

Anne Cole, Susan Crosthwaite, Michael Collett, Jay Dunlap and Jennifer Loros. Dartmouth Medical School, Hanover, NH

A defining feature of circadian oscillators is their ability to be entrained by environmental stimuli. The most universal of these stimuli are light and temperature. In *Neurospora crassa,* the effects of light and temperature on the clock can be followed at the phenotypic level by monitoring the circadian conidiation rhythm, and at the molecular level by monitoring the expression of known clock components.

The clock in Neurospora is comprised of a negative feedback loop in which the *frequency (frq)* gene encodes the FRQ protein which, in turn, either directly or indirectly represses its own expression. With appropriate delays built in, this feedback loop yields the oscillation in the amount of *frq* transcript and FRQ protein which collectively comprises the Neurospora clock. Light delivered at any point within the circadian cycle acts rapidly to increase the level of *frq* transcript and thus resets the clock.

In two "photo-blind" strains, *white collar-1* (wc-1) and *white collar-2 (wc-2),* we have found that both light and temperature treatments that normally synchronize clock wild-type cultures do not result in a rhythmic phenotype. Furthermore, light induced accumulation of *frq* transcript is blocked in *wc-1*, and the sustained, light-driven increase in *frq* mRNA that is seen in wild type strains is blocked in *wc-2*. Both *frq* transcript and FRQ protein levels are low and arrhythmic in both of these mutants. These results together suggest that in addition to their role in light signal transduction, WC-1 and WC2 may be required for operation of a functional circadian clock.

In addition, we are currently using insertional mutagenesis to identify other genes involved in circadian rhythmicity. By screening for mutants with altered clock output phenotype, we hope to identify mutations which disrupt clock input, clock output and the central oscillator, as well as developmental mutants involved in conidiation. To date, several phenotypically arrhythmic strains have been isolated.

28. Studies on pectinolytic enzymes produced in submerged culture by mutant *exo-1* of *Neurospora crassa*.

Luciana B. Crotti, J.A. Jorge, H.F. Terenzi and Maria de Lourdes T.M. Polizeli. Depto. de Biologia - FFCLRP-USP; - Brasil.

The pectinolytic complex is composed by enzymes which degrade pectic substances and produce oligogalacturonates (hidrolases) or insatured products (lyases). These enzymes play an important role in processing and manufacturing industries as the agents of maceration, fruit juice

clarification, and others. The *exo-1* synthesized and secreted five to six times more pectic enzymes than the wild type. They are produced by many microorganisms, higher plants and some insects. the best condition to produce these enzymes was obtained in two-stage cultures: (1) pre-cultivation at 30 C for 24 h in Vogel's medium with 2% glucose; (2) transfer of the mycelial mass to fresh media with different carbon sources, and incubation for 72 h. The *exo-1* in this condition produced four extracellular pectinases (pectin-lyase, pectate-lyase and two polygalacturonase) those were purified in two peaks using DEAE- and CM-cellulose column and one intracellular polygalacturonase activity purified by DEAE-cellulose column. The optimum of temperature and pH for lyases activity were respectively 50 C and above 9.0, and for polygalacturonases activities, these values were about 40-45 C and 5.0-5.5, respectively. The amino acid composition of intracellular polygalacturonase was analysed and compared with a computer program (PROSEARCH) using as a data the molecular weight (31.565 Da) and pI (7.4) and this composition analysis resembled with a polygalacturonase of *Prunus persica* (peach).

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29. Characterization of the FluG protein required for asexual sporulation *in Aspergiflus nidulans.*

Cletus A. D'Souza, Bee Na Lee and Thomas H. Adams, Texas A & M University, College Station, TX.

We propose that the *A. nidulans* sporulation gene product FluG directs a low constitutive production of an extracellular diffusible factor with development initiating once a threshold level accumulates. Although FluG levels are relatively constant throughout the life cycle, development in submerged culture is limited by *fluG* expression levels because overexpression can obviate the need for air in sporulation. Deletion analysis has revealed that the C-terminal half bearing 28% identity with GSI-type prokaryotic glutamine synthetases is sufficient for development and its overexpression can cause spurious development in submerged culture. However, FluG appears to have a function distinct from glutamine biosynthesis because *flug* mutants are not glutamine auxotrophs. Moreover, the induced expression of the heterologous *Anabaena* glutamine synthetase in *A. nidulans* does not initiate sporulation. To identify other components acting downstream of FluG and presumably functioning in the response to the factor, we have isolated a dominant suppressor of a *fluG* mutation. Suppression results in precocious (conidiophores at the end of germ tubes) and prolific conidiation as well as inhibited hyphal growth on solid medium and in submerged culture. This indicates a bypass of FluG function as well as developmental signals acting upstream of FluG.

30. Processing and Function of a Polyprotein Precursor of Two Mitochondrial Enzymes in *Neurospora crassa*.

Lilian Parra-Gessert, Jay Fajardo, Kenneth Koo and Richard L. Weiss, Department of Chemistry & Biochemistry, University of California, Los Angeles.

In *Neurospora crassa*, the mitochondrial proteins *N*-acetylglutamate kinase (AGK) and *N*-acetyl--glutamyl-phosphate reductase (AGPR) are processed from a 96-kDa cytosolic precursor encoded by the ARG6 gene. The proximal kinase and distal reductase domains are separated by a short connector region. Processing was analyzed *in vitro* by import assays with isolated mitochondria and *in vivo* by immunoblot and N-terminal sequencing of processed products. Substitutions of arginines at positions -2 and -3 upstream of the N-terminus of the AGPR domain or replacement of threonine at position +3 in the mature AGPR domain revealed a second processing site. Substitution of arginine at position -22, in combination with changes at -2 and -3, prevented cleavage of the precursor. These results indicate a two-step proteolytic cleavage of the connector region at the sequences Arg-Gly Tyr-Leu-Thr at the N-terminus of the AGPR domain and at Arg-Gly-Tyr Ser-Thr located 20 residues upstream. Proteolytic cleavage at both sites was prevented by inhibitors of the mitochondrial processing peptidase. Enzyme assays and complementation studies have shown that misprocessed and unprocessed precursors yield catalytically active enzyme(s). The AGPR specific activity of strains expressing the unprocessed precursor is much higher than that of a wild type strain, suggesting that the function of the polyprotein precursor may be to protect the AGPR domain from proteolysis before reaching the mitochondrial matrix.

31. Coordinate induction of the genes encoding PKA subunits during sporulation in *B. emersonii.*

Suely L. Gomes Universidade de Sao Paulo, Sao Paulo, Brazil.

The aquatic fungus *B. emersonii* presents an interesting developmental cycle, which begins with the zoospore, a motile non growing cell, that is triggered to germinate by the presence of nutrients or the addition of cAMP, dfferentiating into the germling cell. This cell is capable of vegetative growth, which is characterized by intense nuclear proliferation, without cell division, leading to the formation of a multinucleated cell: the sporangium. At any time during growth, shortage of nutrients can induce the sporulation process, which culminates with the intrasporangial formation of the zoospores and their release from the mother cells. A single PKA is present in *B. emersonii*. Its activity is low in vegetative cells, rising sharply during sporulation, reaching maximum levels in the zoospores. After germination PKA activity decreases back to low basal levels. Work from our laboratory has shown that these variations in activity are due to a coordinate transcriptional control of the genes encoding the regulatory (R) and the catalytic (C) subunits. To investigate sequence elements common to both R and C gene promoters, which could be involved in the coordinate regulation of these genes, their 5' flanking regions were analysed by gel mobility shift and DNA-footprinting assays. It was determined that different DNA-protein complexes are generated when fragments of the R and C gene promoters are incubated with extracts from cells expressing or not expressing both subunits. Furthermore, DNaseI-footprinting experiments have indicated that a 7-nucleotide sequence element present in both promoters, was protected from DNase I digestion only by non-expressing cell extracts,

suggesting a negative control for the induction of R and C genes during sporulation.

32. Effects of removing the mitochondrial outer membrane protein TOM70 *Neurospora crassa.*

Leslie I. Grad, Roland Lill*, Walter Neupert* and Frank E. Nargang, University of Alberta, Edmonton, Alberta, Canada and *Institut fur Physiologische Chemie-Universitat Munchen, Munich, Germany.

Recognition, unfolding, insertion, and translocation of preproteins at the mitochondrial outer membrane is achieved by the TOM complex (**t**ranslocase of the **o**uter **m**embrane of mitochondria). The largest component of this complex, TOM70, is an integral outer membrane protein with a large cytosolic domain thought to serve as a preprotein receptor. To investigate the role of TOM70 in mitochondrial import, a *tom7O* null mutant was generated using the method of RIP (**r**epeat **i**nduced **p**oint mutations). In contrast to lethal phenotype of null mutations in TOM20 or TOM22, two other receptor components of the TOM complex, the effects of destroying TOM70 function are relatively mild. The TOM70-deficient mutant is still viable, has a growth rate about 60% that of wild-type, and produces few conidia. Unexpectedly TOM70-deficient mutant was also found to contain enlarged mitochondria with outer membranes more prone to breakage under stressful conditions than those of wild-type mitochondria. The null mutant has been successfully rescued by transformation with a wild-type copy of *tom7O* demonstrating that the effects of RIP are specific to only the *tom7O* gene. Preliminary assays of in vitro mitochondrial protein import suggest a significant decrease in the import efficiency of the ADP/ATP carrier protein (ACC).

33. *sreA*, a new GATA factor encoding gene of *Aspergillus nidulans*.

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GATA-binding proteins constitute a family of transcription factors that recognize the consensus motif GATA. This group includes a range of major regulatory proteins from organisms as different as fungi, mammals, birds, insects and plants. The different members of this protein family are related by a high degree of amino acid sequence identity within their DNA-binding domains. In *Aspergillus nidulans* at least three GATA binding activities other than the extensively characterized nitrogen regulatory protein AREA can be distinguished using gel mobility shift assays. As a prerequisite to defining the function and interaction of different GATA factors in *A. nidulans*, we employed different PCR approaches to isolate additional genes of this family. In the course of this study we identified a gene (*sreA*) encoding a new GATA factor of *A. nidulans*, containing two GATA type zinc fingers. The deduced amino acid sequence reveals 62% overall identity to SREP of *Penicillium chrysogenum*. Furthermore, the 200 amino acid sequence embracing the two zinc fingers and the intervening region displays 50% identity to

URBS1 from *Ustilago maydis*, a repressor of siderophore biosynthesis. Northern blot and cDNA analysis revealed two transcripts, 2.3 kb and 2.8 kb in length, due to two different major transcriptional start sites. If *A. nidulans* was cultured in low iron media, transcription of *sreA* was found to be repressed. In synopsis the data suggest a regulatory function of SREA in iron metabolism analogous to URBS1 in *U. maydis*.

34. Isolation of two new genes encoding subunits of the V-type ATPase of *Neurospora crassa.*

Ian Hunt and Barry Bowman. Department of Biology, University of California, Santa Cruz.

The vacuolar H^+-ATPase (or V-ATPase) is a large multimeric enzyme composed of several subunits that acidify intracellular compartments in eukaryotic cells and play an important role in a variety of cellular processes.

Here, we report the isolation of two new genes responsible for the expression of putative V-ATPase subunits in *Neurospora crassa.* The first gene (vma10) encodes a protein with a molecular weight of 13,693 Daltons. The protein (subunit G) contains 113 amino acids and is highly hydrophilic. With similar subunits reported in yeast, bovine and insect it appears this subunit is ubiquitous to all V-ATPases. The second gene (*vma9*) appears to encode a 19,125 Dalton protein that contains 177 amino acids and although observed in other organisms on SDSPAGE gels has yet to be isolated and sequenced. This report thus represents the first characterization of this subunit, tentatively called subunit H. The role these two subunits play with regard the structure-function of the V-ATPase is unsure. However, both show sequence homology to the b-subunit of the closely related F-ATPase that is thought to be involved in coupling proton conduction and ATP synthesis. We thus propose a similar mechanism.

Serendipitously, we have also isolated a gene that encodes an amino acid transport protein with a molecular weight of 65kDa. The protein shows no sequence identity with any of the known *Neurospora crassa* amino acid transporters but does have strong homology with several basic amino acid permeases in yeast. We are presently pursuing characterization studies of this protein.

*Both vma genes were originally identified from EST's sequenced by the Neurospora Genome Project, Department of Biology, University of New Mexico.

35. Molecular and functional analysis of the *hymA* gene in *Aspergillus nidulans.*

Marvin Karos and Reinhard Fischer, Universitat Marburg and MPI fur terrestrische Mikrobiologie, Marburg, Germany

The filamentous fungus *Aspergillus nidulans* is able to reproduce asexually with conidia and sexually with ascospores. In this study insertional mutagenesis was used to isolate mutants defective in asexual sporulation. One of these mutants was analyzed at a molecular level. In this strain hyphal growth rate was slightly decreased and branching frequency increased in

comparison to wildtype. The conidiophore development was specifically blocked at the metula stage. Metulae rather resembled hyphae, because they were elongate, multinucleate and septate. Thus, the gene was named *hymA* (hypha-like metula).

A 2.4 kb *hymA* complementing genomic DNA fragment was isolated which detected a 1.8 kb trancript in hyphae and in conidiophores. Sequence comparision of genomic DNA and corresponding cDNAs revealed 4 Introns between 58 and 65 bp in size. The 384 amino acid deduced HymA protein showed homology to proteins from *Saccharomyces cereviseae, Caenorhabditis elegans* and *Mus musculus.* The mouse protein is thought to be a Ca^{2+}-binding protein. The region harbouring the Ca^{2+}-binding site in the mouse protein was highly conserved in HymA. However, a functional role of HYMA in Ca^{2+}-dependent processes remains to be determined.

36. Catalase is specifically oxidized by singlet oxygen during conidiation.

Fernando Lledias, Shaday Michan and Wilhelm Hansberg, Instituto de Fisiologia Celular, Universidad Nacional Autonoma de Mexico.

We have proposed cell differentiation as an avoidance response to a hyperoxidant state. Total protein and specific enzymes were oxidized and degraded at the start of each of the three morphogenetic transitions of the conidiation process in *Neurospora crassa.* In this work, we asked if catalase was also oxidized at the hyperoxidant states of cell-differentiation.

Cat-l a and Cat-lc activity increased in hyphae at the prestationary growth phase and in the first two morphogenetic transitions of the conidiation process. With formation of conidia, in addition to a Cat-la and Cat-lc increase, Cat-le and Cat-2 appeared transiently. Conidia had over 60 times more catalase activity than exponentially growing hyphae. Increments were biphasic: after an initial rise, a decrease in specific activity coincident in time with the hyperoxidant state followed by catalase induction in the differentiated cell-structures. Accumulation of Cat-1 mRNA followed this biphasic activity pattern.

Purified catalase Cat-la gave rise to Cat-lc and Cat-le. Cat-la was shown to be oxidized through the sequential reaction of the four monomers with singlet oxygen, generating active catalase conformers with more acidic isoelectric points. Modification was brought about specifically by singlet oxygen, singlet oxygen scavengers prevented modification; superoxide and hydroxyl radical had no effect on electrophoretic mobility. Modified Cat-1 monomers had a similar molecular mass than the unmodified ones. Cat-2 from *N. crassa* and catalases from different organisms were also susceptible to modification by singlet oxygen.

37. Catalase is oxidized by singlet oxygen during germination.

Fernando Lledias, Pablo Rangel and Wilhelm Hansberg, Institute de Fisiologia Celular, Universidad Nacional Autonoma de Mexico.

Specific modification of Cat-1 by singlet oxygen occurred during the hyperoxidant states of the conidiation process. In this work we asked if a hyperoxidant state and Cat-1 oxidation by singlet oxygen also takes place during germination.

Neurospora crassa conidia did not germinate under an atmosphere of nitrogen or argon. However, they did germinate under N2 or Ar if a pulse of hydrogen peroxide or oxygen saturated water was given to generate a transient hyperoxidant state, measured by chemioluminescence.

Cat-l a stored in conidia was oxidized during germination giving rise to Cat-1c. This could be detected even when germinating in the dark. Under conditions of increasingly higher concentration of singlet oxygen, such as intense light, a source of singlet oxygen, or in carotenoid deficient mutant strains, a higher proportion of Cat-l a was oxidized to Cat-lc and Cat-le. Increased and precocious accumulation of Cat-1 mRNA was observed. Oxidized catalase conformers disappeared rapidly and new Cat-la appeared.

A hyperoxidant state is probably required for breakage of dormancy. Oxidation of Cat-la indicated intracellular generation of singlet oxygen during germination under all conditions tested. Modified Cat-1 conformers probably have a shorted half life than the unmodified enzyme. Carotenoids appeared to protect Cat-1 from in vivo modification by singlet oxygen.

38. Regulation of a conidiation gene, *con-10,* of Neurospora by starvation and stresses is unmasked by mutation of a developmental regulator, *rco-1*.

Kwangwon Lee and Daniel J. Ebbole, Texas A&M University.

The *Neurospora crassa con-10* gene encodes a small polypeptide that has homology with a general stress-induced gene, *gsiB,* of *Bacillus subtilis.* A regulatory gene, *rco-1,* controls *con-10* expression and sporulation in *N. crassa.* RCO1 appears to function as a repressor of *con-10* during mycelial growth. In wild-type strains, prolonged carbon starvation results in development and *con-10* expression, however, *con-10* is not highly induced by glucose starvation in the absence of development. In the *rco-1* mutant strain, *con-10* expression is rapidly and highly induced by carbon or nitrogen starvation and heat shock. Thus, in the wild-type, *rco-1* functions to repress *con-10* expression in mycelia and to block rapid induction of the gene in response to starvation and stresses.

39. Partial Purification and Biochemical Properties of Trehalose-6-Phosphate Synthase of *Neurospora crassa.*

Maria de Lourdes T.M. Polizeli, Luciana B. Crotti, J.A. Jorge and H.F. Terenzi. Dep. Biologia - FFCLRP-USP - Brasil.

Trehalose is synthesized in yeast from glucose-6-phosphate (G6P) and uridine-diphosphoglucose (UDPG) in a two step reaction catalyzed by the trehalose-6-phosphate synthase (Tre6P synthase)/trehalose-6-phosphate phosphatase (Tre6P phosphatase) complex. Previous report of our laboratory showed that the synthesis and breakdown of trehalose in *N. crassa* conidiospores submitted to temperature shifts. Tre6P synthase exhibited optimum of temperature and pH of 55 C and pH 7.0, respectively. During heatshock this activity increased about 100% simultaneously with accumulation of trehalose, independent of synthesis of protein. This metabolic event may be primarily due to temperature-dependent changes in the kinetic properties of Tre6P synthase. Different sensibility to inhibition by phosphate, F6P, ATP and T6P was verified when the enzyme was assayed either at 30, 40 or 50 C. The increase of temperature diminished inhibition by phosphate compounds. A similar difference in degree of inhibition was also observed between crude extracts of cells submitted or not to heat shock. Tre6P synthase was induced four-fold during germination in glucose, reaching in 6 h its maximum sp. act. In glycerol, the activity was 15-17 fold lower. The enzyme was partially purified by phosphocellulose chromatography and FPLC(BioGel SP5 plus). Tre6P exhibited Mwapp of about 430 kDa, suggesting its presence in a complex. Km and Vmax, were respectively 0.814 mM and 528 nmoles/min/mg prot for UDPG, and 9.5 mM and 77.7 nmoles/min/mg prot for G6P. Next we shall test whether the fundamental regulatory roles attributed to yeast Tre6P also apply to filamentous fungi. Support: FAPESP and CNPq

Oomycete posters

40. Genetic analysis and mapping of metalaxyl resistance loci in *Phytophthora infestans*.

Anna-Liisa Fabritius[1], Samuel Roberts[1], Richard Shattock[2] and Howard S. Judelson[1], [1]University of California, Riverside USA, [2]University of Wales, Bangor, UK.

Previous genetic studies have suggested that resistance by oomycetes to the phenylamide fungicide, metalaxyl, is controlled by single loci exhibiting incomplete dominance. To study if the same locus determines resistance in different strains of *Phytophthora infestans*, pedigrees in which that phenotype was segregating were derived from resistant isolates from Mexico, The Netherlands and the United Kingdom. The segregation of resistance in the progeny indicated that resistance was determined primarily by one locus in each isolate, and that some of the isolates were heterozygous and others homozygous for the resistant allele. DNA markers linked to resistance in the Mexican and Dutch isolates were obtained by bulked segregant analysis using random amplified polymorphic DNA (RAPD) markers, and mapped by RAPD or restriction fragment length polymorphism (RFLP) analysis in the three pedigrees. The results suggested that the same locus conferred resistance in the Mexican and Dutch isolates, based on the linkage of the markers and resistance in those isolates. However, in the British isolate those DNA markers were unlinked to the resistance gene. This implied that resistance can result from several different genes in *P. infestans*, or alternatively that the same gene is always involved but that chromosomal rearrangements exist within the species that altered its linkage relationship with the

DNA markers.

41. Genetic and physical variability at the mating type loci of Phytophthora.

Howard S. Judelson, Anna-Liisa Fabritius, and Thomas A. Randall, University of California, Riverside, California, USA.

A single but highly unusual locus determines mating type in heterothallic species of the oomyceteous genus, *Phytophthora.* DNA markers linked to the A1 and A2 mating types of *P. infestans* and *P. parasitica* were isolated and used to characterize the physical and genetic nature of mating type determination in these diploid species. Our data indicate that the A1 type is heterozygous for two mating type alleles (*M/m*) while the A2 type is homozygous (*m/m*). In *P. infestans*, the mating type locus segregates in a non-Mendelian manner consistent with its linkage to a system of balanced lethals. However, that locus displays normal segregation in *P. parasitica*, which raises the question of why and how such an unusual system evolved in *P. infestans*. Physical analyses of the chromosomal interval bearing the mating type locus of *P. infestans* indicated that the region was prone to duplication, transposition, deletion, and other rearrangements which could be related to the mechanism of evolution of the apparent balanced lethals; such aberrations were not observed for loci unlinked to mating type. In addition, genetic studies identified several strains in which the mating type locus had become duplicated or translocated to new regions of the genome. Chromosome walking studies are underway which should reveal the genetic and physical nature of both the mating type loci and the factors responsible for their unusual segregation in *P. infestans.*

42. Characterisation of genes encoding proteins present in large peripheral vesicles of *Phytophthora cinnamomi* zoospores.

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Zoospores of Phytophthora contain several characteristic types of peripheral vesicles. One of these, large peripheral vesicles (Lpv), has been proposed to act as a nutrient store and has been shown to contain three immunologically-related high molecular weight proteins. We have used antibodies directed against *P. cinnamomi* zoospores and cysts to isolate several cDNA clones encoding one of the proteins present in Lpv. Northern blot analysis demonstrated the presence of three large transcripts (9-14 kb) in mRNA isolated from hyphae which had been induced to form sporangia. Co-ordinate accumulation of the three transcripts occurred after induction of sporangial formation: no transcript was observed in uninduced hyphae and maximum transcript levels were seen 4-6 h after induction. Genomic Southern blots indicated that *P. cinnamomi* contains three *lpv* genes. Genomic clones representing two of the *lpv* genes were isolated and characterized by restriction mapping and partial DNA sequencing. The genes were >99% identical; the high degree of conservation extending at least 385 bp downstream of their polyadenylation sites. The *lpv* coding regions contained a variable number (approximately 9-15) of highly conserved 534 bp repeats, flanked by unique sequences. Variation in the number of

repeats in the *lpv* genes was responsible for the different sizes of the three transcripts and proteins. Database searches using the *lpv* nucleotide and deduced amino acid sequences failed to detect any similar sequences. We discuss the molecular events which may have been involved in the evolution of the *lpv* genes and the nature of the products of these genes.

43. Cloning of avirulence genes in *Phytophthora sojae.*

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There are 13 major resistance (Rps) genes in soybean against the oomycete pathogen *P. sojae,* and more than 37 races of the pathogen. We have demonstrated that avirulence against many of these Rps genes is controlled by single dominant genes, using crosses between three isolates of *P. sojae.* We are attempting to isolate the tightly linked Avr1b and Avr1k genes using a genetics-based positional cloning strategy. Towards this goal, bulked segregant analysis was used to find linked molecular markers. Two tightly linked Random Amplified Polymorphic DNA (RAPD) markers were used to start chromosomal walking. A BAC (Bacterial Artificial Chromosome) contig of 200 kb genomic region from Race 2 spanning 5 CentiMorgans was constructed. Restriction Fragment Length Polymorphism (RFLP) markers dispersed across this region were developed and analyzed on two F_2 populations derived from crosses between Race 2 and Race 7, and Race 2 and Race 19. These progeny segregate for Avr lb and Avrlk, respectively. A total of 110 F_2 progeny of cross Race 2 X Race 7, and 48 F_2 of cross Race 2 X Race 19 were characterized. Preliminary results suggest that both Avr gene loci are within a region of 150 kb with a possible location of the Avrlb locus within a single Notl fragment of 25 kb. Genetic analysis also indicates that mutation of Avrlb Race 2 does not involve insertion or deletion events. This enables isolation of functional Avr1b gene by cloning single Notl fragment from Race 7. Progress in isolating avirulence genes from *P. sojae* will be reported.

44. Molecular cloning and characterization of a cDNA that is differentially expressed in steroid hormone (antheridiol)-treated *A. ambisexualis* mycelia.

Julie C. Silver, Evangelia Tomais, Pavel Milman, Thilaka Ponnampalam and Shelley Brunt. University of Toronto, Scarborough.

In *Achlya ambisexualis* strain E87, sexual differentiation resulting in the formation of gametangia (antheridia), can be induced by adding the steroid hormone antheridiol to vegetatively growing mycelia in culture. Differential hybridization of cDNAs constructed from RNA populations isolated from hormone-treated and non-treated mycelia, yielded several clones. Among these was a cDNA clone representing an RNA present at very high levels in hormone-treated mycelia but at only basal levels in untreated (vegetative) mycelia. This cDNA clone was

used to isolate a genomic clone containing the 5' flanking region of the corresponding gene, in order to investigate the sequence motifs present. In the event that nuclear runon's confirm that the gene is regulated by antheridiol at the transcriptional level, the sequence information may help us identify an oomycete steroid-hormone response element. Both the cDNA and the corresponding gene are being sequenced. Preliminary sequence information (roughly 400bp/1.8kB) from the cDNA, was compared with sequences in Genbank. Identities over very small regions to three very different but nevertheless interesting proteins were found, including the Drosophila 7UP2 steroid hormone receptor, the *E.coli* (mitochondrial?) DNA polymerase III chi subunit and mouse and Anabaena P450 hydroxylases. Partial sequence information from the 5' flanking region of the gene revealed the presence of an oomycete transcription initiation sequence (INR) (as delineated by Pieterse, Govers, de Wit and colleagues). Similar INRs were found also in the 5' flanking regions respectively of *A. ambisexualis* genes encoding the chaperone and heat shock protein Hsp90, the mitochondrial chaperone and heat shock protein Hsp60 and the *A. ambisexualis* actin gene. Further sequencing of the above and the other differentially expressed cDNAs and genes isolated, should yield interesting information regarding the gene products involved in gametangial differentiation and the transcription factors which regulate their expression.

45. An AFLP linkage map of the oomycete *Phytophthora infestans.*

Theo Van der Lee and Francine Govers. Department of Phytopathology WAU and Graduate School Experimental Plant Sciences, Binnenhaven 9, 6709 PD Wageningen, The Netherlands.

We constructed a comprehensive molecular genetic linkage map of the heterothallic oomycetous plant pathogen *Phytophthora infestans.* The map is based on polymorphic DNA markers generated by the DNA f ingerprint technique AFLP (Nucl. Acids Res. 23: 4407-4414). AFLP fingerprints were made from single zoospore progeny and 73 Fl progeny from two field isolates of *P. infestans.* The parental isolates appeared to be homokaryotic and diploid, their AFLP fingerprints were mitotically stable and the segregation ratios of AFLP markers in the Fl progeny were largely Mendelian. Besides 185 AFLP markers, seven RFLP markers, four avirulence genes and the mating type locus were mapped. The markers are distributed over nine major and ten minor linkage groups and they cover in total a distance of 861 cM which is approximately 70% of the calculated genome size. Non-Mendelian segregation ratios were found for the mating type locus and thirteen AFLP markers all located on the same linkage group.

46. Antisense and sense mediated gene silencing as a tool to suppress production of INF1 elicitin of *Phytophthora infestans*.

Pieter van West, Sophien Kainoun, Koen de Groot and Francine Govers. Department of Phytopathology, Graduate School of Experimental Plant Sciences, Wageningen Agricultural University, The Netherlands.

Most *Phytophthora* and *Pythium* species produce 10 kDa extracellular protein elicitors, generally termed eliciting. Elicitins induce a hypersensitive response in a restricted number of plants, particularly in the genus *Nicotiana* within the Solanaceae family. Elicitins are thought to act as avirulence factors that restrict the host-range of the pathogen by triggering plant defense responses (Kamoun *et al.*, MPMI 6: 15-25; Yu, PNAS 92: 4088-4094).

Phytophthora infestans, the causal agent of the potato late blight disease, produces an elicitin named INF1. A cDNA clone encoding INF1 was isolated and characterized. Expression of the *inf1* gene was found in mycelium grown during various conditions, whereas *inf1* expression was not detected in sporangia, zoospores, cysts and germinating cysts (Kamoun *et al.*, MPMI 10: 13-20).

Since *P. infestans* is a diploid organism and homologous integration of introduced DNA has not yet been demonstrated, we adopted a gene silencing strategy to inhibit INF1 production. Therefore, *P. infestans* was transformed with constructs carrying strong oomycete promoters fused to the *inf1* coding sequence in both antisense and sense orientation. Expression of the integrated transgenes and the native *inf1* gene was studied and the production of extracellular INF1 protein was analyzed. With the phenotypic characterization of the transformants, the role of elicitin in host specificity should be determined unambiguously.

47. Genetic analysis of chemotactic response in *Phytophthora sojae.*

Wang, J., Wu, R., Cheung, W., Tran, P., Morris, P., Tyler,B. Department of Plant Pathology, University of California, Davis, CA 95616

Phytophthora sojae infects roots and stems of soybean plants, primarily by means of zoospores swimming in water in the soil or on the soil. *P. sojae* zoospores are attracted to the isoflavonoids daidzein and genistein which are present in soybean seeds and exuded by the roots. We have examined a wide variety of compounds having some structural similarity to genistein and daidzein including isoflavonoids, flavones, chalcones, stilbenes, benzoins, benzoates, benzophenones, acetophenones and coumarins, for ability to act as attractants or inhibitors for two isolates of *P. sojae*, race 2 and 6. Among the 59 tested compounds 43 compounds elicited some response from at least one race, and 30 compounds have been found that elicited different responses from race 2 and race 6. Genetic crosses between the two races has identified at least 3 genes responsible for the differences in specificity.

Posters from the 1st Aspergillus section

Do genetic variants of *Aspergillus fumigatus* found in Australia represent new species?

Margaret E. Katz, Martin McLoon, Stephen Burrows and Brian F. Cheetham, University of New England, N.S.W., Australia.

We have determined the DNA sequence of a 1.2 kb fragment from the alkaline protease (Alp) gene of two variant *A. fumigatus* strains, NSW3 and FRR1266. NSW3 was isolated from an

ostrich in a project aimed at developing a method to diagnose aspergillosis in farmed ostriches. NSW3 failed to yield a product in our initial PCR detection method. Southern blot analysis suggested that NSW3 differed from the majority of ostrich isolates of *A. fumigatus*. FRR1266 is an environmental isolate which, like NSW3, originated in New South Wales, Australia. In a survey of *A. fumigatus* strains from 6 continents, FRR1266 was reported to differ significantly from the other strains in isoenzyme, RAPD and RFLP pattern (Rinyu *et al*.(1995) *J. Clin. Microbiol.* 33:2567-2575). The DNA sequence of the NSW3 and FRR1266 Alp gene was compared with the sequences of a second ostrich isolate (QLD1) and the published Alp gene sequences of 3 human *A. fumigatus* isolates. The results showed that the 3 human isolates and QLD1 were virtually identical but NSW3 and FRR1266 differ from the others by >5% of the nucleotides that were analysed. The two variant strains differ but are more closely related to each other than to "standard" strains of *A. fumigatus*. Partial characterisation of the 18S rRNA genes of NSW3 and FR1266 revealed no differences between the variants and a standard strain. Differences in the restriction enzyme cutting sites within the 1.2 kb Alp gene fragment can be used in RFLP analysis of environmental and clinical isolates of *A. fumigatus* to determine the distribution of the genetic variants.

Regulation of extracellular protease production in *Aspergillus nidulans*

Margaret E. Katz, Patricia vanKuyk, Pam Flynn and Brian F. Cheetham, University of New England, N.S.W., Australia.

Work by ourselves and others suggests that the extracellular proteases of *Aspergillus* are regulated in response to five environmental signals: carbon, nitrogen and sulphur metabolite repression, pH control and induction by protein. We have studied the regulation of extracellular proteases in *A. nidulans* by identifying putative regulatory genes through genetic analysis and by characterisation of protease structural genes. Using several different strategies, we have isolated mutants with altered protease enzyme levels. Two of the 6 genes identified by genetic analysis have been cloned. Two classes of *xprG* mutants have been isolated-a mutant with increased levels of extracellular protease and mutants with a protease-deficient phenotype. The *xprG* gene is very tightly linked to (or is an allele of) the *sarB* gene. Mutations in *sarB* suppress a gain-of-function mutation in the *areA* nitrogen control gene. We have isolated and characterised two protease structural genes-*prtA*, a gene encoding a serine (alkaline) protease and *prtB*, a gene encoding an aspartic (acid) protease. An alkaline protease-deficient strain, constructed by gene replacement, has been used to show that the *prtA* gene product is the most abundant protease under a wide range of pH conditions. A 60 bp sequence in the 5' region of the *A. nidulans prtA* gene is highly conserved in *A. oryzae*, *A. flavus* and *A. fumigatus*. This high degree of sequence similarity suggests that this region is important in the regulation of the alkaline protease genes. Gel mobility shift assays have been used to identify proteins that bind to this conserved sequence. Experiments to isolate the genes that encode these DNA-binding proteins, using southwestern screening of cDNA expression libraries, are in progress.

48. Characterisation of the cAMP-dependent protein kinase catalytic subunit gene from the fungus *Aspergillus niger*.

Mojca Bencina[1,2], H. Panneman[2], G.J. Rujiter[2], M. Legisa[1], J. Visser[2]. [1]National Institute of Chemistry, Ljubljana, Slovenia; [2]Wageningen Agricultural University, Section Molecular Genetics of Industrial Microorganisms, Wageningen, The Netherlands.

The *pkaC* gene encoding the catalytic subunit of cAMPdependent protein kinase (PKA-C) was isolated from the filamentous fungus *Aspergillus niger.* An open reading frame of 1440 bp, interrupted by three short introns, encodes a polypeptide of 480 amino acids with calculated molecular mass of 53,813 Da.

The deduced catalytic core of PKA-C of *A. niger* shows extensive homology with the PKAC isolated from all other eukaryotes. The cAMP-dependent protein kinase catalytic subunit from *A. niger* has a 126 amino acids extension at the N-terminus compared to the PKA-C of higher eukaryotes that, except for the first 15 amino acids which are homologous to the *Magnaporthe grisea* PKA-C, shows no significant similarity to the N-terminus extension of PKA-C of other lower eukaryotes.

The cloned *pkaC* was used for transformiation of *A. niger* leading to increased levels of *pkaC* mRNA and PKA-C activity. Transformants overexpressing *pkaC* are phenotypically different with respect to gowth, showing a more compact colony morphology, accompanied by more dense sporulation. A number of transformants also showed a strongly reduced or complete absence of sporulation. The overexpression of *pkaC* in *A. niger* did not severely affect fungal viability but resulted in unstable transformants.

49. Fruitbody formation of *Aspergillus nidulans* tryptophan auxotrophic mutants.

Sabine E. Eckert, Christoph Wanke and Gerhard Braus, Georg-August-University Gottingen, Department of Microbiology Grisebachstrasse 8, D-37077 Gottingen, Germany

The filamentous fungus *Aspergillus nidulans* is a simple model organism for development and cellular differentiation. The molecular basis of its fruit body formation and the production of ascospores is largely unknown. We have started an analysis of the interplay between sexual development and metabolic pathways. We tested tryptophan requiring mutants of *Aspergillus nidulans* (Ref. 1, 2) for fruit body formation. The tryptophan mutants *trpA, trpB, trpC* and *trpD* are deficient in the structural genes for the biosynthetic enzymes of each step in the formation of tryptophan from chorismate. In addition, all mutant strains are unable to form fruit bodies on standard minimal and complete medium (Ref. 3).

Here we report that the strains carrying the mutations in the *trpA, trpC* and *trpD* locus can form cleistothecia on medium supplemented with indole, the strain *trpA* also on medium containing anthranilate, and all strains including the *trpB* strain on medium with 3-indolylacetic acid (heteroauxin). Therefore we conclude that the tryptophan biosynthetic pathway and the development of fruit bodies in *Aspergillus nidulans* are connected either by an intermediate or a derivative.

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         trpA, trpD             trpD, trpC,  trpC   trpB

Chorismate ------ > anthranilate --- > --- > ---> [indole] -- > tryptophan
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References:
1. Roberts, C. F. (1967): Genetics **55,** 233 - 239
2. Hutter, R. and demoss, J.A. (1967): Genetics **55,** 241- 247
3. Timberlake, W. E. (1990): Annual Review of Genetics **2,** 3 - 24

50. Suppressors of a temperature-sensitive allele of nimT^{cdc25} in *Aspergillus nidulans*.

Dorothy B. Engle, Xavier University

In order to identify new genes involved in cell cycle control, we are making extragenic suppressors that compensate for a mutation in a known cell cycle gene. The *nimT* gene encodes a homolog of fission yeast *cdc25* phosphatase. The phosphatase is required for entry into mitosis; it participates in the activation of the cyclin-dependent kinase (NIMX in Aspergillus) that triggers mitotic events. Extragenic, or second-site, suppressors of known mutations frequently encode proteins that interact with the original known protein.

The mutant allele *nimT23* causes a temperature-sensitive arrest in G2 phase. We mutagenized a *nimT23* strain, selected for survivors at restrictive Temperature (44 C), and distinguished revertants from extragenic suppressors by crossing to wild type. Chromosome mapping by diploid breakdown indicates that we have uncovered at least three different genes; they are located on chromosomes I, II and VI. The suppressor genes on chromosome VI are distinct from the *bimE* gene, another cell cycle gene located on VI. Further characterization of the suppressors, including dominance testing, is underway.

(Supported by NIH AREA grant GM49480-OlAl)

51. The construction of vectors and strains for efficient gene disruptions in *Aspergillus fumigatus.*

V. Gavrias, K. Thiede, N. Iartchouk, J.-A. Saxton, C. Hitchcock , W.E. Timberlake and Y. Koltin. ChemGenics Pharmaceuticals Inc., One Kendall Square, Cambridge , MA 01239. Pfizer Pharmaceuticals, Central Research Division, Pfizer Limited, Sandwich, Kent CT13 9NJ, UK.

The increasing medical significance of *Aspergillus fumigatus* as an opportunistic pathogen illustrates the need for the development of genetic tools applicable to this organism. To clone *A. fumigatus* genes coding for a variety of auxotrophic markers, we transformed *Saccharomyces cerevisiae* with an *A. fumigatus* cDNA library constructed in a yeast expression vector. By complementation of *S. cerevisiae* auxotrophies we isolated cDNA clones coding for HIS3, ADE2 and LEU2 *A. fumigatus* homologs. Sequence analysis of these clones indicates a high degree of similarity between the analogous proteins. Isolation of genomic clones by hybridization enables the construction of auxotrophic strains for efficient gene disruptions in *A. fumigatus* as well as making this organism more amenable to genetic manipulations.

52. Cloning of a gene that complements a camptothecin-sensitive mutant of *Aspergillus nidulans.*

Gustavo H. Goldman, G.C.M. Bruschi, C. C. de Souza & M.H.S. Goldman, FCFRP and *FORP, Universidade de Sao Paulo, Brasil.

Topoisomerases are enzymes that modify and regulate the topological state of DNA. Camptothecin is an anti-topoisomerase I drug. The filamentous fungus *A. nidulans* can grow on a high concentration of this drug but its topoisomerase-I is camptothecin-sensitive, Cellular sensitivity to the lethal action of this drug may be influenced by many factors that affect cleavage complex formation, its processing into permanent damage, or the cellular response to the permanent damage. We are interested in identifying additional factors of cellular sensitivity to camptothecin others than topoisomerase I. Towards this end, we decided to isolate camptothecin-sensitive mutants of the filamentous fungus *A. nidulans.* In one of these mutants, *sca299* (Sensitivity to camptothecin) hypersensitivity to camptothecin is cosegregating with sensitivity to EMS, MMS, actinomycin, and 4-NQO. Topoisomerase-I assays indicate that its topoisomerase-I activity is comparable to that of the wild type. We have cloned by DNA-mediated transformation the gene that complements the mutant *sca299.* Additional work will focus on further genetic characterization of this gene.

Financial support by FAPESP, CNPq-Brazfl and ICGEB-UNIDO

53. Genetic characterization of cycloheximide-sensitive mutants of *Aspergillus nidulans*.

Gustavo H. Goldman, C. C. de Souza, M. Hiraishi, C. H. Pellizzon & M. H. S. Goldman*, FCFRP and *FOR Preto, Universidade de Sao Paulo, Brasil.

During the last decades, the incidence of fungal infections has dramatically increased. *A. nidulans* is a non-pathogenic fungus with a powerful genetic system that provides an excellent model system for studying different aspects of drug resistance in filamentous fungi. As a preliminary step to characterize genes that confer pleiotropic drug resistance in *Aspergillus,* we decided to isolate cycloheximide-sensitive mutants of *A. nidulans.* The rationale for this approach is to identify genes whose products are important for drug resistance by analyzing mutations that alter the resistance/sensitivity status of the cell. Fifteen cycloheximide-sensitive *scy* mutants of *A. nidulans* were isolated and genetically characterized. Some of the *scy* mutants showed a complex phenotype of resistance or sensitivity to different drugs and/or stress conditions. Genetic analysis of six of them has defined six linkage groups designated *pdr* (pleiotropic drug resistance)*A-F*. One of these mutants, *pdrD,* shows cosegregation to cycloheximide, hygromycin, osmotic, low pH, and t-butyl hydrogen peroxidesensitivity. Fluorescent microscopy and transmission electron microscopy indicate that the vacuolar system of this mutant is fragmented. Additional work will focus on the cloning and characterization of the gene that complements this mutant.

Financial support by FAPESP, CNPq-Brazil and ICGEB-UNIDO

54. Fungal Gene Disruption by Transposon Mutagenesis in *E. coli*.

L. Hamer & J.E. Hamer, Dept. Biology, Purdue University, West Lafayette IN 47907.

Application of gene knock-out technology in filamentous fungi by homologous recombination generally requires initial construction of a disruption vector. This vector is composed of parts of the gene flanking an appropriate marker gene. We have constructed 2 tn5-containing plasmids, pLH1 and pLH3, specialized towards mutagenesis of fungal genes from the filamentous fungi *Aspergillus nidulans* and *Magnaporthe grisea.* pLHl contains the *argB* marker gene, and pLH3 contains the *Hyg* marker gene, functional in *A. nidulans* and *M. grisea*, respectively. We show that the *sepA* gene from *A. nidulans* cloned in *E. coli* can be readily mutagenized by pLH1. We have made multiple disruption derivatives of the *sepA* clone with the concomitant introduction of the *argB* gene. Using a 3.8 kb pBR322 derived backbone, hops into the ~ 6 kb gene were observed at a frequency of 0.25. The exact position of the disruption can easily be determined, due to the presence of SP6 and T7 priming sites at the ends of the intervening tn5/*argB* cassette. Because the disruption is confined by an insertion, the entire *sepA* gene provides the targetting sequence for later *in vivo* disruption of the *sepA* gene. In addition to speeding up the process of gene disruption, pLH1 and pLH3 are useful means of integrating marker genes in the plasmid backbone.

55. *sepA*, a New Member of an Conserved Family of Genes Involved in Cytokinesis.

L. Hamer, S Harris*, K Sharpless*, J.E. Hamer. Dept. Biology, Purdue U, W Lafayette IN 47907; *Dept. Microbiol, U Conn Health Ctr, Farmington CT 06030.

Cytokinesis in *Aspergillus nidulans* is an actin-dependent process coordinated with mitosis. Ts- mutations in *A. nidulans sepA* result in defects in both cytokinesis and polarized growth. At restrictive temperature, *sepA* Ts- mutants form multi-nucleate hyphae, which fail to undergo cell division and exhibit abnormal branching patterns. Temperature shift experiments suggest that the *sepA* gene product acts late during cell division. *sepA* has been cloned, and shown to encompass the previously described *figA* gene; thus *figA* is a truncated allele of *sepA*. Sequence analysis reveals that *sepA* is a member of a conserved family of genes, whose products appear to function in actin-dependent processes. In particular, *sepA* shares significant blocks of homology with genes involved in cytokinesis: *S. cerevisiae BNI1*, *S. pombe CDC12*, and *D. melanogaster diaphanous. sepA* deletion mutants displays depolarized growth and delays in septation. Our results suggest that *sepA* may play a role in mediating actin-polymerization, such as at the division site, and that *sepA* is required for maintenance of cellular polarity.

56. A role for the NIMA kinase in linking spore polarization to cell cycle progression in *Aspergillus nidulans*.

Steven Harris, Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06030-3205.

Aspergillus nidulans forms dormant conidia which contain a single nucleus arrested in G1 phase of the cell cycle. Nuclei re-enter the cell cycle during the process of condial germination. At the same time, the spore establishes a polarized axis of growth. Spore polarization was found to occur shortly after the completion of the first mitotic division in germinating conidia. Results described here suggest that two distinct mechanisms function to coordinate spore polarization with cell cycle progression.

Examination of the kinetics of polarization in a number of Ts mitotic mutants revealed that *bimE* mutants, which exhibit high NIMA kinase activity, fail to polarize. In addition, over-expression of the *nimA* gene also delays polarization in a dose-dependent manner. Finally, expression of a non-degradable version of the NIMA kinase completely inhibits polarization. These results have led to a model whereby NIMA kinase activity, which is required for entry into mitosis, also functions to inhibit spore polarization. Destruction of NIMA upon mitotic exit relieves the inhibition and allows polarization to proceed, thus linking it to the completion of the first nuclear division. Further experiments have shown that Ts mutants which fail to accumulate high levels of NIMA kinase must still progress through S phase before polarizing. Furthermore, Ts mutations which cause an irreversible arrest in S phase prevent polarization in a *nimA*-independent manner. These results suggest that an additional mechanism exists which prevents premature spore polarization during the first S phase in germinating conidia (when the NIMA kinase is normally inactive).

57. A novel zinc finger in the *Aspergillus nidulans nimO* gene is required for asexual development.

Steven W. James, Bryan A. Kraynack, Scott D. Wade, and Brett M. Forshey, Gettysburg College.

The *nimO* predicted protein of *Aspergillus* shares 29% identity with *DBF4,* a budding yeast G1/S regulator that controls DNA synthesis through its association with origins of replication and the G1/S-specific *CDC7* kinase. *nimO* also appears to control DNA replication, because ts-lethal *nimO*18 mutants are unable to synthesize DNA, and override normal checkpoint controls by progressing into mitosis with 1C DNA. *nimO* and *DBF4* are most conserved in a C-terminal region containing a single, novel Cys_2-His_2 zinc finger-like motif. In budding yeast this zinc finger is nonessential, as removal of the C-terminal -200 amino acids only mildly slows growth. Similarly, *alcA*-driven expression of a truncated *nimO* allele lacking the C-terminal 209 amino acids restored normal vegetative growth to *nimOI8* cells at restrictive temperature, with the striking exception that these strains were unable to form conidia. The zinc finger appears specifically necessary for asexual development, because the same aconidial phenotype was obtained by substituting one or both of the critical histidine residues of the zinc finger with glutamine. The zinc finger mutants could initiate the developmental pathway leading toward asexual reproduction, but aborted uniformly after producing conidiophores with several metullae. These results suggest a dual role for *nimO* in controlling DNA synthesis and asexual differentiation, and set the stage for biochemical and cell biological analyses of zinc finger function.

58. Keeping in shape: *Aspergillus* uses hypA-E to regulate morphology.

S. Kaminskyj and J Hamer. Dept Biol Sci, Purdue U, W Lafayette IN 47907-1392.

Fungal hyphae use polarized wall deposition for two morphological processes: tip growth and septation (cytokinesis) which defines subapical cells. Septation is dispensable for vegetative growth in A. *nidulans*, but essential for conidiation. In *A. nidulans* and other systems, cytokinesis is triggered temporally and spatially by mitotic nuclei. However, A. *nidulans* cells typically contain three or more nuclei, so there must be a mechanism determining which mitotic nuclei trigger cytokinesis.

Conditional ts- mutations defining genes named *hypA-E* grow slowly and have short cells and abnormal hyphal morphologies. *hypA-E* are dispensable for nuclear cycle progression and conidiation: all strains produce macroscopic colonies and viable spores after 3d incubation at 42 C. Thus, whilst *hypA-E* dramatically affect multiple facets of A. *nidulans* morphology regulation, they are not essential for viability or asexual reproduction.

In A. *nidulans*, subapical cells have a reversible nuclear cycle arrest. This is relieved by branching or by loss of *hypA-E* function due to temperature upshift. The latter induces apolar enlargement of subapical cells followed by mitosis and insertion of additional septa. Thus, *A. nidulans* seems to use *hypA-E* dependent cell volume control as a component of nuclear cycle regulation in subapical cells. However, double mutant analysis showed that *hypA* did not interact directly with *nimA, nimE, nimT, nimX, bimE.*

A cosmid complementing the *hypA* defect in co-transformations was identified after genetic mapping showed linkage to *sepA* and *lysF* on chromosome IR. The complemeting region was subcloned a 1.8 kb *Sac*I fragment. Sequence analysis suggests that *hypA* is a novel gene, and that A. *nidulans* uses previously unknown mechanisms to reaulate hyphal morphology and cytokinesis.

59. The gene product of *hapC* is a subunit of AnCP/AnCF, a CCAAT-binding protein in *Aspergillus nidulans*.

M. Kato, A. Aoyama, F. Naruse, Y. Tateyama, P. Papagiannopoulos[1], M. A. Davis[1], M. J. Hynes,[1] T. Kobayashi, N. Tsukagoshi. Nagoya University, Nagoya 464-01,Japan. [1] University of Melbourne, Parkville, Victoria 3052, Australia.

CCAAT sequences in promoter regions of many fungal genes such as the *taa* (Taka-amylase A) and *amdS* (acetamidase) genes play important roles in the determination of expression levels. We have shown that an *Aspergillus nidulans* CCAAT-binding protein (factor), AnCP/AnCF recognizes CCAAT sequences in several genes in *A. nidulans.* The *hapC* gene, an *A. nidulans* counterpart of the yeast HAP3 gene, was isolated and used to obtain *hapC* disruptants. No CCAAT binding activity was detected in nuclear extracts from a *hapC* disruptant. Taken together with the high similarity of *HapC* to the HAP3 protein this result suggests that the *hapC* gene product is a subunit of AnCP/AnCF.

To examine whether or not HapC is a component of the AnCP/AnCF complex, a recombinant MalE-HapC fusion protein was produced in *E. coli* and purified. AnCP was denatured in the presence of MalE-HapC, renatured, and used for gel shift assays. The shift band corresponding to a taa promoter-AnCP complex disappeared and a new band with lower mobility was observed. When anti-MalE antiserum was added to the binding reaction, the band was supershifted. These results indicate that the MalE-HapC fusion protein was functionally incorporated into the AnCP/AnCF complex bound to the CCAAT containing sequence. This clearly demonstrates that the *hapC* gene encodes a subunit of AnCP/AnCF.

Update on Pathogenicity Factors

61. Mitochondrial hypovirulence in KFC9, a *Cryphonectria parasitica* strain obtained from nature.

Dipnath Baidyaroy, David H. Huber, Dennis W. Fulbright and Helmut Bertrand, Michigan State University.

In C. *parasitica,* the chestnut blight fungus, a hypovirulence phenotype can be often transmitted from strain to strain by hyphal anastomoses (cytoplasmic transmission), and is caused by infection of the fungus by dsRNA viruses. However, hypovirulent strains have been isolated from nature which lack any dsRNA viruses but are capable of rendering virulent strains hypovirulent on hyphal anastomoses. These strains also show high levels of alternative oxidase activity, indicating that they must be deficient in cytochrome-mediated respiration and hence in mitochondrial functions. KFC9 is one such strain isolated from a healing canker on a tree in Michigan. Analysis of the mitochondrial DNA (mtDNA) of KFC9 and its derivatives (virulent strains rendered hypoviruient by contact with KFC9) revealed that a particular region of the mtDNA of KFC9 was responsible for the infectious hypoviruience phenotype. That region of the mtDNA was cloned and was found to be the small subunit rRNA gene with an inserted piece of DNA just 84 bp downstream of the beginning of the gene. This peculiar DNA is 973 bp long, has no sizable open reading frames and does not have homology to any existing sequence. The molecular basis of the infectious nature of this DNA is not clear at this time.

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62. Inhibition of pathogenicity of the rice blast fungus by *S. cerevisiae* -factor.

Janna L. Beckerman, Fred Naider*, and Daniel J. Ebbole. Texas A&M Universeity. *CUNY, Staten Island.

Magnaporthe grisea is a fungal pathogen of rice that forms a specialized cell necessary for pathogenesis, the appressorium. *Saccharomyces cerevisiae* -factor pheromone blocked

appressorium formation in *M. grisea* in a mating type-specific manner *in vitro* and protected plants from infection by a *MAT1 -2* strain.

Experiments with pheromone analogs suggest that the observed activity is due to the specific interaction of -factor with a *M. grisea* receptor. Culture filtrates of *MAT1-1* strains contained a putative pheromone activity that specifically inhibited appressorium formation of mating type *MAT1-2* strains. These findings provide evidence that a pheromone response pathway exists in *M. grisea* that can be exploited for plant protection.

63. Map-based cloning of pathogenicity determinants of *Nectria haematococca* MPI.

Joanna K. Bowen, Brian T. Hawthorne, Jonathan Rees-George, Rod D. Ball and Erik H.A. Rikkerink. The Horticultural and Food Research Institute of New Zealand Ltd., Mt. Albert Research Centre, Auckland, New Zealand.

Genetic analysis of pathogenicity of *Nectria haematococca* MPI (*Fusarium solani* f. sp. *cucurbitae*, race 1) on unwounded hypocotyls of *Cucurbita maxima* cv. Crown (buttercup squash) has demonstrated that it is controlled by a major effect virulence (MEV) gene superimposed on a quantitative background. A population of 800 random ascospore progeny was derived from a cross between a high pathogenicity and a low pathogenicity isolate. A colony pigmentation gene has been found to be closely linked to the MEV gene. Bulked DNA samples from progeny exhibiting the highest and lowest pathogenicity phenotypes are being screened to identify RAPD markers which are linked to the MEV locus. The high pathogenicity and low pathogenicity parents have different mini-chromosome profiles. However, the MEV gene does not appear to be located on a mini-chromosome. A partial BAC library with an average insert size of approximately 80 kb has been constructed from the high pathogenicity parent. A full library is under construction. The region containing the MEV gene will mapped and identified by chromosome walking and subsequent complementation of a low pathogenicity isolate.

64. Identification of virulence determinants of *A. fumigatus* using a REMI/STM approach.

J.S.Brown, A.Aufauvre-Brown, N.Tiffin, D.W.Holden, Dept. Infectous Diseases, RPMS, London UK.

Pulmonary infection due to *Aspergillus fumigatus* is a major cause of morbidity and mortality in immunocompromised patients. Research using *A.fumigatus* strains carrying disruptions in genes thought to have a role in virulence have generally failed to prove the importance of these genes in animal models infection. Recently our laboratory has developed a method, signature tagged mutagenesis (STM), which can identify avirulent mutants by screening pools of different mutants in a single animal. We have combined restriction enzyme mediated integration (REMI) with STM to screen mutant strains of *A.fumigatus* for loss of virulence. REMI was employed for single copy insertional mutagenesis of a clinical isolate of *A.fumigatus* using 96 plasmids, each carrying a different oligonucleofide tag and the *hph* gene conferring hygromycin resistance. Pools of 96 mutants each carrying a different tag were assembled by combining one transformant

from each different REMI transformation. Groups of 5 mice were inoculated by intranasal infiltration with spores representing all mutants from a particular pool. Fungal DNA recovered from infected lungs on days 3 and 4 post-inoculation was used as a target for PCR using primers to amplify the oligonucleofide tags. The PCR product was used to probe a dot blot membrane containing the original 96 tagged plasmids. Those tags present in the inoculum but not present in the recovered DNA will identify potentially avirulent mutants that can be further characterized. Using an avirulent *A.fumigatus* auxotroph we have shown the feasibility of STM/REMI screening to identify avirulent mutants from an inoculum pool of 90 mutants. By screening mutant pools we expect to identify new virulence genes and improve our understanding of the pathogenesis of pulmonary aspergillosis.

65. Development of a REMI-transformation procedure for the mutational tagging of genes in *Paecilomyces fumosoroseus*.

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We have developed a transformation system for an entomopathogenic fungus, *Paecilomyces fumosoroseus*, that relies on restriction enzyme-mediated integration, under the selection of the herbicide, bialaphos. This procedure is being used as a tool for the mutational tagging of genes and the creation of transformants deficient in some aspect of pathogenicity. Depending upon the enzyme and vector used, transformation frequencies were either unaffected or increased versus the non-REMI treatments. Restriction mapping and Southern hybridization analyses of transformant DNAs revealed that plasmids inserted randomly throughout the genome. However, results also revealed that integrations occurred other than by a simple ligation-repair model. From the pattern of REMI events revealed by Southern analysis we were unable to regenerate the original restriction sites of the plasmid with the enzyme initially used in the transformation procedure. Instead we observed that one or both sites were destroyed during the integration of the plasmid in the genome. We are now characterizing several pigmentation and pathogenicity mutants obtained with this transformation procedure.

66. The interaction between fungal polygalacturonases and plant PGIPs.

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Recognition of *endo*polygalacturonase (PG), a factor important for pathogenesis and therefore to be maintained by fungi during evolution for successful parasitism, may be exploited by plants as a clever strategy to establish incompatibility. Modular leucine-rich repeat proteins, structurally related to some of the resistance gene products recently characterized, recognize and inhibit fungal PGs. These proteins, named PGIPs have been found in the cell wall of many dicotyledonous plants and some monocotyledonous plants. We are studying at the genetic and biochemical level the interaction between PGs from different fungi and PGIPs of *Phaseolus vulgaris* L. We have introduced, by site-directed mutagenesis, different point mutations in the

PG from *Fusarium moniliforme* and produced mutated proteins with a range of activity between the 48% to 0% with respect to the wild type. The interaction between the variant enzymes and PGIP of *P. vulgaris* has been investigated using a biosensor based on surface plasmon resonance. All the variant enzymes were still able to interact and bind to PGIP with association constant comparable to that of the wild type enzyme. A family of *pgip* genes is present in the genome of *P. vulgaris* and the composition of the family is different among different cultivars. A major objective of our laboratory is to know how many PGIP proteins exist, under what circumstances they are expressed and their specificity. We have evidence indicating that the members of the gene family encode PGIP proteins with distinct specificities.

67. Differential Gene Expression During Infection by the Fruit-rot Fungus *Glomerella cingulata*.

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Glomerella cingulata (Colletotrichum gloeosporioides) is a fungal pathogen of pip and subtropical fruits. In New Zealand it is best known for causing bitter rot of apples. Penetration of the host surface involves spore germination followed by a morphological differentiation to form an appressorium. Appressorium formation is essential for infection, therefore genes expressed during appressoria formation are of interest as they may be potential targets for control of the fungus. To facilitate our studies of genes expressed during appressoria formation, apple wax is used to induce *G. cingulata* spores to form appressoria *in vitro.* In addition, *cap22* (Hwang & Kolattukudy, 1995) is being used as a control marker for appressoria formation.

Differential Display PCR has been investigated as a method of identifying genes uniquely expressed during appressoria formation. Currently, we have isolated two abundant surface proteins and we plan to study the expression of these proteins during appressoria formation.

Reference: Hwang, C.S. & Kolattukudy, P.A. (1995) *Mol Gen Genet* 247:282-294.

68. Gregatin-deficient mutants of *Phialophora gregata*.

Edmund H. Crane, and Charlotte R. Bronson, Iowa State University, Ames, IA 50011.

Phialophora gregata, a deuteromycete that causes Brown Stem Rot in soybean, produces a family of antimicrobial and phytotoxic furanones, collectively known as gregatins. These compounds have been proposed to cause the leaf necrosis and premature defoliation characteristic of Brown Stem Rot-infected soybeans. The role of gregatins in causing Brown Stem Rot symptoms is being tested using REMI mutagenesis. A transformation protocol for *P. gregata* was developed and used to insert pUCATPH containing hygB into wild-type *P. gregata*. Hygromycin-resistant transformants were screened for inability to produce gregatins as

demonstrated by loss of their ability to inhibit the growth of *Bacillus subtilis*; several putative gregatin-deficient mutants have been identified. Assays are in progress to verify the loss of gregatin production and to determine whether these mutants have reduced virulence on soybeans.

69. Disruption of a cutinase gene (*cutA*) in *Fusarium solani* f. sp. *cucurbitae* race 2 does not affect virulence and tissue specificity toward cucurbits.

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A 3.9-kb genomic DNA fragment from the cucurbit pathogen *Fusarium solani* f. sp. *cucurbitae* race 2 was cloned. Sequence analysis revealed an open reading frame of 690 nucleotides interrupted by a single 51-bp intron. The nucleotide and predicted amino acid sequences showed 92 and 98% identity respectively, to the *cutA* gene of the pea pathogen *F. solani* f. sp. *pisi.* A gene replacement vector was constructed and used to generate *cutA*$^-$ mutants which were detected using a PCR assay. Seventy-one *cutA*$^-$ mutants were identified among the 416 transformants screened. Vector integration was assessed by Southern analysis in 23 of these mutants. PCR and Southern analysis data show the level of homologous integration was 14%. Cutinase activity (as measured by non-specific esterase activity) was reduced in *cutA*$^-$ mutants to levels not significantly different from culture medium alone. Disruption of the *cutA* locus in mutants was confirmed by RNA gel blot hybridization. Neither virulence on *Cucurbita maxima* 'Delica' at any of six different inoculum concentrations, nor pathogenicity on intact fruit of four different species or cultivars of cucurbit or hypocotyl tissue of *C. maxima* `Crown' was found to be affected by disruption of the *cutA* gene.

70. Pathogenicity determinants of *Pyrenopeziza brassicae*.

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Pyrenopeziza brassicae is a hemibiotrophic ascomycete. It causes the disease light leaf spot of *Brassicas* and is thus economically important. Circumstantial evidence suggests a role for cutinase in penetration of the host cuticle. *P. brassicae* does not develop specialised infection structures indicating that breaching of the cuticle is mechanical, and SEM studies of infected leaves show that host entry is not via stomata. Liquid cultures induced with apple cutin show an increase in esterase activity which is absent in uninduced cultures. The esterase activity is inhibited by ebelactones A and B. Incubation of the culture filtrate with tritiated cutin caused the release of soluble radioactivity. However analysis of the products using TLC suggests that they do not correspond to those of cutin breakdown. Several approaches are being followed to try to detect a plant-inducible cutinase. Heterologous probing and a PCR directed approach have indicated that if there is a *P. brassicae* cutinase, then it lacks homology to those characterised to date. The current approach is the use of a subtractive cDNA cibrary. A subtracted library was

constructed to enrich for fungal and plant-inducible genes and which may include a cutinase gene. Differential screening has isolated several plant-induced fungal scenes and these are being further characterised.

71. Avirulence genes cloning in the rice blast fungus *Magnaporthe grisea*.

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Magnaporthe grisea is an Ascomycete responsible for the major fungal disease of rice. Three genetically independent avirulence genes, avrMedNoi-1, avrIrat7-1, avrKu86-1, were identified in a cross between isolates Guy11 and 2/0/3. Using 77 random progeny, we constructed a partial genetic map with 75 RFLP and 25 RAPD markers. Two avirulence genes mapped near chromosome tips (avrMedNoi-1 and avrKu86-1). The last avirulence gene, avrIrat7-1, mapped on chromosome one at 30 cM from avrCo39. Using bulk segregant analysis, we identified 16 RAPD markers linked to these avirulence genes (0-15 cM), some being completely linked to avrIrat7-1 or avrKu86-1. Most of these RAPD markers corresponded to junction fragments between *M. grisea* genome and known transposons. We screened a cosmid library of an avirulent progeny, either by hybridisation with single copy markers or by RAPD analysis of cosmid pools (repeated sequence markers). Contigs were started from markers linked to avrIrat7-1 and avrKu86-1. One of the cosmids from avrIrat7-1 locus confered avirulence to rice cultivar Irat7, when introduced by transformation in a virulent recipient strain.

72. Molecular genetics of turgor generation within the appressorium of *Magnaporthe grisea.* Katherine Dixon, Joke de Jong, B.McCormack, N.Smimoff, J.R.Xu* and N.J.Talbot. University of Exeter, Devon, UK. *Purdue University,USA

Magnaporthe grisea , in common with many plant pathogenic fungi, uses specialised cells known as appressoria to provide an entry route into its host plant. Appressoria are specialised, dome shaped cells which facilitate the infection of plants either by enzymatic means or via the generation of internal physical pressure, forcing a penetration peg through the rice leaf surface. We have shown that during the development of appressoria, the fungus accumulates a variety of solutes to extremely high concentrations. We suggest that accumulation of these solutes, contained within a semi-permeable melanised cell produces an osmotic gradient, drawing free water into the appressorium, inflating the cell and applying a physical pressure to the penetration peg. Biochemical analysis of the contents of appressoria have revealed the most abundant solute to be glycerol. In budding yeast, the HOG pathway (High Osmolarity Glycerol Pathway) is a signal transduction pathway which allows the cell to accumulate intra-cellular glycerol in response to external osmotic stress. We reasoned that *M.grisea* may use elements of the HOG pathway in order to regulate glycerol accumulation within appressoria during turgor generation. In order to test this hypothesis we have identified and characterised a homologue of the mitogen activated protein kinase (MAPK) encoding gene HOG1 in *M. grisea.* We have constructed a gene disruption vector and are currently working towards characterising a hog^- *M.grisea* strain.

73. Analysis of AVR1-CO39 avirulence gene locus from *Magnaporthe grisea.*

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A *Magnaporthe grisea* cultivar specificity gene toward rice cultivar C039 was previously mapped to one arm of chromosome I between RFLP markers CH5-120H and 5-10-F. These RFLP markers map 11.6 cM and 17.2 cM, respectively, on either side of *Avrl*-CO39. Using Achilles' cleavage methods, CH5-120H and 5-10-F were shown to be separated by 600 kb. A chromosome walk to *Avr1*-CO39 was initiated from these markers and over 500 kb have been covered in 20 walking steps. Cosmid clones cosegregating with *Avrl*-CO39 have been identified and selected clones obtained from the walk were shown to confer avirulence on rice variety C039 but not 51583 when introduced by transformation in the virulent *M. grisea* strain Guyl1. The DNA conferrring avirulence has been delimited to a 1.05 kb region. Four putative open reading frames were identified by DNA sequence analysis and the precise location of the *AVR1*-CO39 gene is being defined by transcript analysis, site-directed deletion of ATG codons and by introduction of frameshifts into each ORF. Hybridization studies of DNA of the virulent *M. grisea* strain Guy 11 indicates that approximately 20 kb of DNA at the *Avrl*-CO39 locus is deleted and that a repetitive DNA species is present at the deletion breakpoint. This deletion eliminated *AVR1*-CO39 from the Guy11 genome.

74. Catalase as a potential pathogenicity factor in *Clavicepes purpurea.*

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Claviceps purpurea colonizes young ovaries of grasses andcereals, finally establishing a stable, balanced interaction. In following up a combined biochemical, cytological and molecular genetic approach to indentify pathogenicity factors in the system C *purpurea*/rye, we found that *C. purpurea* - apart from cell wall degrading enzymes - secretes catalase activity in planta; this was shown by in situ activity staining, immunogold labelling, and biochemical analysis of infected tissue and honeydew (in comparison to axenic culture). We cloned a gene *(cat1)* coding for a catalase enzyme that most probably is secreted, since it contains a signal peptide and several glycosylation sites. We could show by RT-PCR that *cat1* is expressed in planta. A potential function of this enzyme during the parasitic cycle could be inactivation of active oxygen species generated by the host (peroxidase activity and lignification have been demonstrated as host defense reactions confirming the presence of $H_2 O_2$). Targeted gene disruption experiments combined with detailed cytological analyses are under way to clarify the role of the *cat1* product (or fungal catalases in general) during the interaction.

75. Isolation of genes involved in phytoalexin detoxification in *Leptosphaeria maculans* through differential display.

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Leptoshaeria maculans is a heterothallic ascomycete that causes blackleg or stem canker disease in oilseed rape and other brassica plants. Virulent strains of the fungus rapidly detoxify the phytoalexin brassinin, or its analog -chlorobenzyl dithiocarbamate. We have identified the major metabolic products of the pathway and deduced that at least three enzymes are involved. Differential display analysis was conducted on cDNA derived from mRNA isolated from 47 h fungal cultures, with and without exposure to 50 µg/mL -chlorobenzyl dithiocarbamate. Seventy-seven differentially amplified cDNA fragments were isolated using twelve anchored poly(T_{15}) primers and a mixture of twenty 9 base oligonucleotides (56 or 67% GC). Eleven of these cDNAs showed putative differential expression after rescreening by reverse-northern dot blot analysis. The cDNA fragments were purified from contaminants by PCR-SSCP (PCR-single-strand conformation polymorphism) and again subjected to reverse-northern dot blot analysis. Nine cDNA fragments with sizes ranging from 250-720 base pairs were subcloned and sequenced. The results of the characterization of the clones will be presented.

76. Isolation and characterization of 2 **xylanase genes from the fungal pathogen *Claviceps purpurea.***

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The phytopathogenic fungus *Claviceps purpurea* is a common parasite on grasses and cereals. It could be shown that while colonizing the *Secale cereals* ovary the fungus grows intercellularly as well as intracellularly. As the primary cell walls of monocotyledonous plants contain approximately 40% arabinoxylan, xylan-degrading enzymes probably play a role during infection of *Secale cereals.* 2 xylanase encoding genes (*xyl1* and *xyl2)* were isolated from a genomic library of *C. purpurea* using heterologous probes. *Xyll* exhibits great homology to family G xylanases whereas *xyl2* belongs to family F. Expression of both genes in planta were studied by RT-PCR showing that both *xyl1* and *xyl2* are expressed during all phases of infection of *Secale cereals.* Secretion of xylanases during the infection could also be demonstrated by tissue printing experiments using heterologous antibodies as probes (Lepping, unpubl.). A disrupted copy of *xyl1* was used to create a Xyl1 lacking mutant by transformation mediated gene replacement. Disruption of *xyl1* results in reduced total xylanase activity in axenic culture, Western analyses using monoclonal antibodies raised against *Trichoderma reesei* Xylanase Xynl as a probe show the disappearance of a crossreacting band with a MW of 30,000 strongly indicating that this is the *xyl1* gene product. In order to elucidate the role of xylanases for pathogenicity of *C. purpurea* the *xyl1* mutant is currently being examined in pathogenicity tests anct a *xyl2* as well as a *xyl1/xyl2* double mutant is created.

77. Isolation of genes from *Fusarium moniliforme* expressed during colonization of maize.

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Fusarium moniliforme is an economically important pathogen of maize and sorghum, causing stem, root and ear rot on its host plants. In order to understand the molecular processes conditioning pathogenicity of *F. moniliforme* fungal genes that show an altered expression pattern during colonization of maize stems were identified by differential display. Using 38 various primer combinations, five *F. moniliforme* cDNA fragments were isolated whose corresponding genes are exclusively expressed during fungal growth *in planta* as varified by Northern blot analysis. Sequence analysis of the cDNA fragments did not reveal any similarity to known genes, probably due to the shortness of the fragments (394 bp - 578 bp). Therefore, a cDNA libary is now screend to obtain longer clones.

78. Molecular studies on the cell wall degrading enzymes from *Botrytis cinerea* II: *In planta* analysis of the *Bcpel1* gene and the endopolygalacturonase gene family.

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B. cinerea causes grey mould, a plant disease resulting in serious damage on several economical important crops. During pathogenesis *B. cinerea* secretes a number of cell wall degrading enzymes. Despite much physiological and biochemical knowledge on these enzymes, there is no clear insight in the infection process. We started a molecular genetic approach resulting in the isolation of several genes (see abstract by Mulder *et al.).* Here we describe experiments performed on tomato leaves as well as the disruption of one of the isolated genes. Tomato leaves were inoculated with a conidial suspension (10^6/ml) . At several time points during the pathogenesis, samples were taken for RNA analysis. The results show that *Bcpga1* is expressed at the early stages of pathogenesis. *Bcpga1* is also expressed when grown on glucose medium. Therefore we decided to disrupt this latter gene. Two *Bcpga1* -null mutants were tested in pathogenicity assays. A lower amount of spreading lesions was found for the disruptants on detached tomato leaves (T = 20 C). In addition, assays perfomed at T = 4 C show that the outgrowth of lesions formed by the disruptant, was slower compared to the outgrowth of wild type lesions. Tests performed on ripe tomato fruits showed no observable difference in pathogenicity.

These data, combined with the *in vitro* expression data, indicate that BcPGl plays a role in the pathogenesis of *B. cinerea* on tomato leaves. Experiment aimed at getting further insight in the role of BcPGl in pathogenesis are in progress.

79. RAPD analysis of the New Zealand population of *Venturia inaequalis*.

Megan Hemming[1], Kim Plummer[2], Mike Pearson[2], Jo Bowen [1], Erik Rikkerink[1]. [1]HortResearch, Auckland; [2] University of Auckland, New Zealand.

Apple scab caused by *Venturia inaequalis* is the most commercially important fungal disease of apple. Currently none of the commercial apple cultivars used in New Zealand are resistant to the disease, and control in the orchard relies on a rigorous fungicide regime. Breeding resistance to *V. inaequalis* into commercial apple cultivars is a major focus of apple breeding programmes worldwide. Seven races of the fungus have been identified. Since the primary inoculum is derived from ascospores a high degree of variation in the fungus would be expected. We are currently investigating this variation at the molecular level. Monoconidial *V. inaequalis* isolates (39) were collected from infected leaves and fruit of 11 different apple cultivars from the major apple growing areas in New Zealand. RAPD analysis of these isolates revealed a high degree of variation. Only two isolates had identical banding patterns. Indeed, isolates from the same leaf produced different RAPD patterns. All isolates shared common major bands, some of which have also been reported by other researchers for *V. inaequalis* from a Swiss orchard. There appears to be no correlation between RAPD banding patterns and the origin of the isolates (apple cultivar or geographical location). We are investigating whether any markers can be used to identify particular sub-groups such as races.

80. Starvation and stress of *Cladosporium fulvum* - relations to pathogenesis.

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Cladosporium fulvum is a biotrophic pathogen of tomato *(Lycopersicon esculentum)* which lacks specialized infection structures and does not produce obvious extra-cellular cell wall degrading enzymes or host-specific toxins. It has been demonstrated in a number of plant-fungal interactions that genes expressed during starvation may also be those which are expressed during pathogenesis. On this basis, we have used starvation induced conditions to clone genes involved in pathogenesis.

Differential screening of cDNA libraries constructed from carbon and nitrogen starved mycelia has identified 34 starvation induced clones. To test the hypothesis that mRNAs induced during starvation are also induced during infection, Northern blots of starved, non-starved fungus, uninfected and infected plants taken at different stages of infection (7, 10, 12 and 14 days after inoculation) were made. 26 of the 34 clones were not onlv starvation induced but also plant induced. Sequencing has revealed homologies with an alcohol oxidase, aldehyde dehydrogenase, alcohol dehydrogenase, an ATP translocase and hydrophobins. Gene disruption experiments are currently in progress to establish the role of each gene in pathogenicity.

A high percentage of the carbon available to the fungus is converted to mannitol. The role of this and other polyols in pathogenic fungi remains unclear. We are investigating changes in polyol levels in *C. fulvum* under stress conditions to determine whether the high level of mannitol

produced by the fungus mediates some osmotic stress associated with growth *in planta,* or is involved in other aspects of pathogenesis.

81. Saponins and plant defence.

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Saponins occur in a great many plant species, and have been implicated as pre-formed determinants of resistance to fungal attack. The importance of the saponin-detoxifying enzyme avenacinase, produced by *Gaeumannomyces graminis,* in determining host range has been demonstrated. At least two other phytopathogenic fungi *(Septoria avenae* and *Septoria* lycopersici) produce saponin-detoxifying enzymes which are closely related to avenacinase, suggesting that common mechanisms for saponin detoxification may be widespread. The *S. avenae* and *S. 1ycopersici* enzymes detoxify the oat leaf avenacoside and the tomato -tomatine saponins respectively, and their roles in pathogenicity are currently being tested by the generation of fungal mutants. Expression of *S. lycopersici* tomatinase in the biotrophic pathogen of

tomato *Cladosporium fulvum,* which is not known to degrade -tomatine, gives increased pathogenicity to tomato. Interestingly, *C. fulvum* transformants expressing tomatinase are also able to cause significantly more infection than control strains in incompatible interactions, suggesting that saponins may also contribute to variety-specific resistance of tomato to *C. fulvum.*

82. Identification of pathogenicity related genes from entomopathogenic fungus, *Metarhizium anisopliae,* using differential display-RT-PCR and immunoscreening of an expression library.

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Although *Metarhizium anisopliae* is the best studied entomopathogenic fungus, there is limited information on specific gene expression during host-pathogen interactions. The objective of this study was to identify and isolate the full range of fungal genes specifically expressed during growth on insect host cuticle. Two populations of RNA, one from the fungus grown in the presence of the insect cuticle, the other from the fungus grown in the absence of the insect cuticle were compared and analyzed for differences in the expression of mRNA species. Both RNA populations were reverse transcribed and the cDNA were PCR amplified in the presence of arbitrary 5-end primers. The resultant PCR products were differentially displayed on polyacrylamide sequencing gels. Differentially expressed cDNAs were reamplified and sequenced. Along with differential display, immunoscreening of an expression library was performed with antibodies raised against the total spectrum of proteins secreted into cultures containing insect cuticle. We have identified and cloned a very broad spectrum of genes

including cuticle degrading enzymes, putative toxins and protein kinases by employing these two techniques. Analysis of these genes is facilitating a better understanding of the determinants of fungal pathogenicity to insects and is providing new tools for biotechnology, e.g.; the production of a transgenic baculovirus expressing a *Metarhizium anisopliae* toxin kills insects >50% faster than the wild type baculovirus.

83. Differential cloning and partial characterization of conidia germination and/or appressorium formation stage-specific genes of *Magnaporthe grisea*.

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During the infection process of *Magnaporthe grisea* on rice plants, the conidial germ tube

differentiates a specific infection structure, an appresorium, for penetration into the host. Formation of appressorium was observed not only on rice leaves but also on synthetic solid substrate like polycarbonate. Approaches toward isolation of genes specifically expressed during the differentiation stage of appressorium formation were made. A subtractive cDNA cloning strategy using combination of biotin labeled driver DNA method and adapter-primed PCR method was efficiently condensed the differential cDNAs those were presumably expressed during early stage of the development of conidial germ tube and/or appressorium formation. Part of the 700 colonies containing differential cDNA candidates were classified by cross colony hybridization and more than 50 independent clones' partial nucleotide sequences were determined. We have further analyzed some of those cDNA clones which showed differential expression by Northern hybridization and RT-PCR analysis. cDNA and genomic DNA structure and presumable character of those clones will be discussed.

84. From pisatin- and nystatin-resistance in Dictyostelium to the amino acid sequence similarity between Neurospora sterol C-14 reductase and human lamin B receptor.

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Dictyostelium discoideum, a cellular slime mold, can acquire resistance to toxic concentrations of pisatin (a pea phytoalexin) and nystatin (a polyene antibiotic) following pretreatment with sublethal concentrations of these compounds. Additionally, pisatin-pretreatment can induce nystatin-resistance but the converse is not true. We have isolated a mutant, *sup5*, that blocks inducible nystatin-resistance but does not affect inducible pisatin-resistance. We are using *sup5* to unravel an apparently unusual relationship between the pisatin- and nystatin-resistance mechanisms.

Mutations affecting the sterol biosynthetic enzyme, sterol C-14 reductase, block inducible pisatin-resistance in Dictyostelium. Remarkably, mutations in *erg-3*, the sterol C-14 reductase

gene of *Neurospora crassa* also confer a pisatin-sensitive phenotype. This suggests that inducible pisatin-resistance might be conserved between Dictyostelium and fungi. We cloned *erg-3* by complementation of its mutant phenotype and were surprised to find that the encoded protein shared ~40% amino acid sequence identity with the transmembrane (TM) domain of human lamin B receptor (LBR). To determine whether the human LBR TM domain is also a sterol C-14 reductase we have constructed recombinant genes that encode chimeric proteins comprising of Neurospora sterol C-14 reductase and human LBR TM sequences and are testing these genes for complementation of the Neurospora *erg-3* mutant phenotype.

85. Avirulence in *Mycosphaerella graminicola (*anamorph *Septoria tritici)* is controlled by a complex locus of tightly linked genes.

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Mycosphaerella graminicola is a plant pathogenic bipolar heterothallic ascomycete. We study the molecular genetic basis of specificity for wheat species and cultivars in this fungus, using induced necrosis and pycnidium formation as parameters. Isolate IP0323 is avirulent on cvs. Shafir, Veranopolis and Kavkaz whereas isolate IP094269 is virulent on these cultivars. Both isolates are virulent on cv. Taichung 29 and avirulent on cv. Kavkaz/K4500.

IP0323 and EP094269 were crossed and a random F_1 progeny was recovered. In addition, 32 BC_1 progenies were generated by back crossing F_1 individuals to either IP0323 or IP094269. Sixty F_1 and 60 BC_1 isolates, from three progenies, were assayed for avirulence on the aforementioned cultivars.

As expected, all progeny isolates were virulent on cv. Taichung. None of these isolates carried virulence for cv. Kavkaz/K4500, indicating that the parental isolates carry the same avirulence factor(s) for this cultivar. Avirulence for each of the differentiating cultivars inherited as a single gene. However, these avirulences cosegregated. Thus the entire F_1 andBC_1 progenies showed the parental types. This suggests that the avirulences for these cultivars are tightly linked. We hypothesize the presence of a complex locus of tightly linked avirulence genes since cvs. Shafir, Veranopolis and Kavkaz are considered to carry different resistance factors. Inheritance of other markers, such as the mating type genes and RAPDS, was independent of the avirulence loci.

Cell Biology and Pathogenesis Posters

86. Constructing a BAC contig of the *Phytophthora sojae* genome by repetitive sequence fingerprinting.

Felipe Arredondo, Alan Chan and Brett M. Tyler, Department of Plant Pathology, University of California, Davis, CA95616.

The more than 40 species of the oomycete *Phytophthora* cause serious diseases of a huge range of crop and ornamental plants. We are characterizing genes that control recognition, host specificity and pathogenicity in the soybean pathogen *Phytophthora sojae*. To facilitate isolation of such genes from *P. sojae* by map-based cloning, we are constructing a BAC contig of the entire genome of this organism. We have ordered a library of 7680 BACs of average size 55kb, spanning the 62 Mb genome 7 times. We are hybridizing the BACs with unique mixtures of random probes, most of them repetitive. We will use the subset of probes hybridizing to each BAC to identify overlapping BACS. Computer software has been developed to collect, simulate and analyze the data. We estimate that about 120 probe mixtures, each hybridizing to around 700 BACS, will be sufficient to enable us to quickly assemble the BACs into less than 100 contigs spanning >95% of the genome. More detailed restriction analysis will be used to consolidate the initial set of contigs. Correlation of the BAC contigs with the genetic map using PCR will enable rapid cloning of any genes placed on the genetic map.

87. Functional analysis of the *Magnaporthe grisea* avirulence gene AVR2-YAMO.

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The ascomycete *Magnaporthe grisea* causes disease on a wide variety of graminaceous hosts, although individual strains of the fungus are limited to infecting one or two grass species. Some examples of host-pathogen specificity in this system appear to fit the classical gene-for-gene hypothesis. The avirulence gene *AVR2-YAMO* confers avirulence towards the rice cultivar Yashiro-mochi, and the telomeric location of the *AVR2-YAMO* gene in some rice pathogens appears to account for frequent spontaneous mutation to virulence towards Yashiro-mochi. Transformation of virulent strains of *M. grisea* with a functional copy of the cloned *AVR2-YAMO* gene confers avirulence on Yashiro-mochi. Functional AVR2-YAMO genes have also been cloned from strains of *M. grisea* that infect *Digitaria* spp. or *Pennisetum* spp. and not rice. The cloned gene encodes a protein with a predicted molecular weight of 26 kDa that has some sequence similarities to neutral zinc metalloproteases. Mutation of specific amino acid residues in the putative zinc-binding region removes the ability to confer avirulence. Sequence comparisons with non-functional *avr2-yamo* homologs from rice pathogens indicate that maintenance of the putative zinc protease motif is essential. The temporal expression of the *AVR2-YAMO* gene is being studied by fusion of the AVR2-YAMO promoter to both the -glucuronidase gene and the green fluorescent protein gene. Initial experiments suggest *AVR2-YAMO* is expressed during infection suggesting a role in pathogenicity. Anti-AVR2-YAMO antisera has been raised in rabbits using a recombinant AVR2-YAMO protein antigen. It is unknown whether the *AVR2-YAMO* protein is secreted or remains within the fungus so both intracellular and secreted proteins are being analyzed. It is hoped to use this polyclonal anti-AVR2-YAMO antisera to localize expression.

88. Characterization of a new colonial mutant of *Neurospora crassa* .

Katrina D. Campsall, Paul Sallmen, and P. J. Vierula, Department of Biology, Carleton University, Ottawa, Ontario. KIS 586.

Sbr (small brown) is a new colonial mutant of *Neurospora crassa* which was recovered in a screen of transformants harbouring ectopic integrations of a hygromycin phosphotransferase (Hyg^r) gene construct. Insertional inactivation by a single copy of this drug resistance marker appears to be responsible for the mutant morphology. The *sbr* strain forms slow-growing compact colonies which become dark brown with age. Instead of elongate tubular hyphae, *sbr* grows by forming spherical buds. Each of these spherical compartments range from approximately 3 *u*m to 20 *u*m in diameter and are densely packed with nuclei. Immunofluorescence microscopy with an anti-tubulin monoclonal antibody revealed a scattered arrangement of microtubules. We have cloned *sbr* genomic DNA into a Lambda bacteriophage vector and have identified several putative clones from this genomic library that carry the Hyg^r gene construct. *Sbr* DNA flanking the marker has been further subcioned into a Bluescript plasmid. The DNA sequence is currently being analyzed.

89. Chitin biosynthesis in the phytopathogenes *Magnaporthe grisea* and *Botrytis cinerea.*

Florence Chappeland, Anne Vidal-Cros, Martine Boccara. Universite Pierre et Marie Curie, Paris, FRANCE

Two different approaches were used to study the relationshipbetween chitin synthase expression and virulence in the two filamentous phytopathogenes: *M. grisea* and *B. cinerea*. In the first approach, chitin synthase genes (*chs* or *csd*) fragments were amplified by PCR in M. grisea and *B. cinerea*. Amplified fragments corresponding to four different genes in *M. grisea* and three genes in *B.cinerea.* were characterized. The importance of each *chs* or *csd* gene during the vegetative cycle as well as during the infectious process is studied through transcription analysis and construction of disrupted mutants. In a second approach, a *B. cinerea* mutant affected in chitin biosynthesis was isolated by selection against Calcofluor. This mutant, which grows normally in culture, exhibits decreased virulence on grape and produces four times more elicitors of plant defence reactions than wild type strain. Preliminary biochemical characterization is in favor of a Csd3 phenotype(regulation of chitin synthase 3). The results of these two appoaches will be presented and discussed.

90. The *Ustilago maydis ubc1* gene is required for gall formation in maize.

Scott E. Gold[1], Steven Brogdon[1], James W. Kronstad[2], Franz Durrenberger[2] and Maria E. Mayorga[1]. [1]Department of Plant Pathology, University of Georgia, Athens, GA 30602-7274; [2]Biotechnology Laboratory, University of British Columbia, 6174 University Boulevard, Vancouver, B.C. V6T 1Z3, Canada.

Ustilago maydis, the causal agent of corn smut disease, is a semi-obligate plant pathogen. The haploid grows as saprophytic budding yeast cells on culture medium while the dikaryon formed upon mating of compatible haploid strains is filamentous and obligately pathogenic. We are interested in understanding the underlying genetic mechanisms involved in the dimorphic

transition and their impact on pathogenesis. Specifically, we have focused on genes, which when mutated, affect the ability of the fungus to express the filamentous phenotype. We employ, as our starting material, haploid strains disrupted in the adenylate cyclase gene (*uac1*) and therefore express a consitutively filamentous phenotype. Matings of two *uac1* mutant strains were previously shown to be unable to generate pathogenic dikaryons. Ultraviolet light induced and spontaneous second site suppressor mutants that have yeast colony morphology on solid media have been generated in a *uac1* disruption background. Earlier, we complemented one of these mutants and found that the corrective gene encoded the regulatory subunit of cAMP dependent protein kinase (*ubc1*). We have analyzed the dramatically reduced virulence of *ubc1* mutants. These mutants colonize the plant causing chlorotic symptoms but are unable to induce tumor formation in the host. The results taken together with past analysis of the *uac1* pathogenicity, suggest that the regulation of the cAMP pathway is critical for normal disease development. Our interpretation is that cyclic AMP dependent protein kinase activity is necessary during initial infection but should be reduced or eliminated for progression to gall formation.

91. Gene expression of the blackleg fungus *Leptosphaeria maculans* in the presence of alkenyl glucosinolates.

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Blackleg caused by the ascomycete *Leptosphaeria maculans* is the most economically important disease of oilseed Brassicas worldwide. Generally Indian mustard (*B. juncea*) is resistant to the blackleg fungus and consequently has been used as a source of resistance in canola (*B. napus*) breeding strategies. Indian mustard has high levels of alkenyl glucosinolates, sulphur-containing compounds that give mustard a pungent taste. Upon wounding of the plant, these compounds are cleaved by the plant enzyme myrosinase into glucose and gases such as isothiocyanates, which are toxic to many organisms including *L. maculans*.

Recently blackleg isolates that can attack some Indian mustard varieties have been found in Australia. These isolates may be able to tolerate or detoxify hydrolysis products of glucosinolates in the plant. We are using differential display to identify genes expressed by *L. maculans* isolates in the presence of glucosinolates. Such genes may be involved in detoxification of glucosinolates or evasion of their effects. A 1 kb mRNA is expressed only in the presence of 2-propenyl glucosinolate (5 and 10 microgram/ml) and myrosinase (5 microgram/ml). This message has a high degree of sequence similarity to a yeast 5S ribosomal DNA binding protein, which is transcribed at high levels as a response to growth in high concentrations of glucose. Other differentially expressed genes of *L. maculans* are being sought.

92. Genetic analysis of the dimorphic transition in *Ustilago maydis*.

Maria E. Mayorga and Scott E. Gold. Department of Plant Pathology, University of Georgia, Athens, GA 30602-7274

We are interested in understanding the genetic and biochemical mechanisms controlling fungal dimorphism. *Ustilago maydis*, the causal agent of corn smut disease, alternates between a unicellular, non-pathogenic yeast-like form and a dikaryotic, filamentous, pathogenic form. Because of well developed molecular genetic techniques, Ustilago can be used as a model fungal system to study the process of yeast-hypha transition and its importance in pathogenicity. Previously we obtained a mutant strain that exhibits constitutively filamentous growth in its haploid form. Complementation of this mutant led to the isolation of the gene encoding adenylate cyclase, *uac1*. Additional mutagenesis of this constitutive filamentous strain allowed the isolation of a large number of suppressor mutants lacking the filamentous phenotype, termed ubc, for Ustilago bypass of cyclase. Analysis of one of these supressor mutants led to the identification of *ubc1*, encoding the regulatory subunit of cAMP-dependent protein kinase. These genes are important not only for dimorphic switching but also in pathogenicity: mutations in *uac1* and *ubc1* appear to affect tumor formation in the plant. We are currently analyzing additional suppressor mutations in order to identify new genes in the cAMP signal transduction pathway involved in dimorphic growth in *U. maydis*. Microscopic analysis is being used to determine the effect of the mutations on cell growth and morphology. Characterization of some ubc class of suppressor mutants is described.

93. Insertional Mutagenesis, cloning and characterization of the *col 1* gene of the dimorphic fungal pathogen *Ophiostoma ulmi.*

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A colony mutant *col1*, of *Ophiostoma ulmi* was produced by insertional mutagenesis, which has a dramatically reduced mycelial growth rate and a near-normal budding cell growth rate. Genomic DNA flanking the inserted transforming DNA was recovered and used to isolate the uninterrupted DNA from a wild-type genomic library. The uninterrupted gene was isolated, and the sequence determined. The isolated *col 1* gene when transformed back into the yeast-like mutant restored the filamentous growth pattern. Analyses and comparison of the translated nucleotide sequence would suggest that the unique COL 1 protein shows some similarity to RNA and poly (A) binding proteins. The 600 amino acids at the carboxy terminus of the COL 1 protein align very well with the PRP24 (spliceosomal) protein of *Saccharomyces cerevisiae.* Attempts to complement the *col1* mutant with the *Prp24* gene, and to complement the yeast *Prp24* mutant with the *col 1* gene will be described. The open reading frame of the *col 1* gene was fused into the plasmid vector pJC40 for expression in *Escherichia coli.* A histidine-tagged protein was purified by affininty chromatography. This protein was used to prepare polyclonal antibodies against the COL 1 protein.

94. Cloning a gene which encodes a glycoprotein present at the fungal-plant interface formed in the *Colletotrichum*-bean interaction.

Sarah Perfect[1], J. Green[1], R.O'Connell[2]. [1]University of Birmingham, UK., [2]IACR-Long Ashton, UK.

Colletotrichum is a large genus of plant pathogenic fungi causing anthracnose on a wide range of crops. *C. lindemuthianum* is a hemibiotrophic species which causes anthracnose of bean, *Phaseolus vulgaris*. During the inital biotrophic stage of infection, the fungus differentiates infection vesicles and primary hyphae within host epidermal cells. These specialised intracellular hyphae invaginate the host plasma membrane, from which they are separated by a matrix layer. Monoclonal antibodies (MAbs) raised to isolated infection structures have been used to identify proteins present at the fungal-plant interface.

One of these MAbs, designated UB25, recognises a protein epitope in a 40kDa *N*-linked glycoprotein specific to intracellular hyphae. Indirect immunofluorescence and EM-immunogold labelling show that the glycoprotein is present in the infection peg, and the fungal walls and matrix surrounding the intracellular hyphae. However, it is not present in secondary necrotrophic hyphae, which suggests that it is specific to biotrophic infection structures. The glycoprotein may therefore be involved in the establishment and maintenance of biotrophy.

A cDNA library has been constructed from total RNA isolated from infected bean hypocotyl epidermis. The MAb UB25 has been used to immunoscreen the library and positive clones have been isolated and sequenced. Analysis of the deduced amino acid sequence revealed the presence of two distinct domains, one of which is proline rich and contains short repetitive motifs. The functional significance of this will be discussed. In addition, Southern analysis indicates that the glycoprotein recognised by UB25 is fungally encoded and is present in several *Colletotrichum* species.

95. Genetic and molecular evidence for regulation of genes involved in HC-toxin biosynthesis in *Cochliobolus carbonum*

race 1.

John W. Pitkin, Anastasia N. Nikolskaya, Joong-Hoon Ahn and Jonathan D. Walton. MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.

The Tox2 chromosome of *C. carbonum* race 1 ($Tox2^+$) contains race 1-unique genes required for HC-toxin biosynthesis and efflux. Crosses between different race 1 field isolates and between race 1 and race 2 ($Tox2^-$) isolates occasionally result in progeny with an altered virulence phenotype on maize. These strains have a "slow" virulence phenotype on maize due to a severely reduced ability to produce HC-toxin. Analyses of the progeny indicate that the new pathogenicity phenotype may be due to the loss of a gene responsible for the activation of at least two race 1-specific genes: *HTS1*, which encodes HTS, a cyclic peptide synthetase required for HC-toxin biosynthesis, and *TOXA*, which encodes a putative HC-toxin efflux pump. Comparisons of Tox2 chromosomal structure from various cross progeny has led to the preliminary identification of a 20-100 kb region as the site of the activator gene. We also have cloned a race 1-specific gene, *TOXE*, with sequence similarity to known fungal activator genes. Strains with *TOXE* gene

disruptions have a Tox2$^-$ virulence phenotype on maize. Unlike strains with the slow virulence phenotype, strains with *TOXE* disrupted have wild-type HTS activity yet make no detectable HC-toxin. The TOXE gene product may control race 1-specific genes other than *HTS1* and *TOXA*, such as those required for the synthesis of AEO, a novel amino acid in HC-toxin. The *TOXE* gene is present in the slow virulence progeny, thus there is evidence for at least two race 1-specific regulatory genes that affect HC-toxin biosynthesis.

96. Cytokines produced in mice infected by a virulent *Paracoccidioides brasiliensis.*

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Paracoccidioides brasiliensis is a pathogenic dimorphic fungus that reaches the lung of host through inhalation of conidia which can be destroyed by the host immune cells or transform in yeasts, that multiply and disseminate to other organs. The clinical symptoms of disease are characterized by a cronic granulomatous reaction, with empairment of the cell mediated immune response and exacerbate humoral immunity. The aim of this study was to asses the production of cytokines during the paracoccidioidomycosis evolution in several organs of *P. brasiliensis* infected mice by RNAm detection. In the first week of infection, we verified an accented increase in mRNA production for IL-2, IL4, IL-10 and IFN-, comparing with uninfected mice. We observed a decline of all cytokines at the fifth week, but this was always higher than control. In tenth week of infection we obtained an elevation in the levels of EL-10 mRNA, correlated with the number of fungi in organs. Through this study we suggest that occurs the activation of both T CD4 cells populations and possibly interleukin-10 is involved with cellular immunity reduced in paracoccidioidomycosis, by its immunosupressive properties, that favour the fungi multiplication. These results can contribute to a better understanding of immunopathogenesis of paracoccidioidomycosis.

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97. Biochemical and molecular characterization of non-pathogenicity in the *Glomerella magna*-cucurbit system.

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Biochemical and molecular analyses were performed on watermelon and cucumber plants subjected to four different conditions: 1- water controls, 2- inoculated with conidia from the non-pathogenic mutant (path-1), 3- inoculated with conidia from the wild-type (L2.5), and 4- inoculated under cross protection conditions. Cross protection involved exposure of plants to path-1 conidia for 48 hours followed by exposure to a lethal concentration of L2.5 conidia. The deposition of lignin, peroxidase activity, and specific plant defense related gene expression

indicated that the host defense system did not activate for 3 - 4 days after exposure to L2.5. In contrast, little to no defense system activation occurred in water control plants, plants colonized with path-1, or cross protected plants.

The genetic complexity of non-pathogenicity was assessed by the isolation of 20 non-pathogenic REMI mutants. Although the REMI mutants were capable of protecting plants against wild type disease, they varied in the efficiency of cross protection. Southern blot analysis of the REMI mutants indicated that both single and multiple integration events occurred, and that integrations occurred at several genomic locations. The phenotypic and genotypic variation between REMI mutants suggests that non-pathogenicity is genetically complex.

98. The production of auxin in transgenic fungi and its effect on fungal virulence and host specificity.

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As natural plant pathogens, fungi hold potential as biocontrol agents of weeds. Highly virulent fungal pathogens may be capable of eliminating large weed populations, while natural host specificity ensures that cultivar plants are left untouched. Most weed pathogens, however, are either not specific or not virulent enough to be effective as biocontrol agents. The insertion and high-level expression of virulence genes in fungi may be an effective means of elevating the virulence of weed-specific pathogens, rendering many weed pathogens better biocontrol agents.

In this study we tested the potential of the plant hormone indole-3-acetic acid (IAA) to enhance fungal virulence. The fungus *Cochliobolus heterostrophus* was transformed with the IAA biosynthesis genes *iaaM* and *iaaH* of the bacterium *Pseudomonas savastanoi*. Transgenic fungal isolates of opposite mating type containing either *iaaM* or *iaaH* were crossed and progeny containing both genes were obtained. Transformants that produce either indole-3-acetamide (IAM), IAA, or both were identified and their pathogenicity to host (corn) and non-host (pea) plants was tested. Isolates that contained both genes and produced IAA showed increased virulence on corn, while IAA had no effect on the pathogenicity of the fungus to pea plants. These results indicate that fungal virulence may be enhanced without affecting host specificity. This approach may open new ways for engineering superior biocontrol agents with the desired level of virulence and host range.

99. HCf-1 and HCf-2: two hydrophobins of *Cladosporium fulvum*.

Pietro D Spanu, Department of Plant Sciences, University of Oxford, UK

Fungal hydrophobins play a role in some plant-fungus interactions. They have been proposed to aid attachment to the host, to mediate specialised morphogenesis and have toxin activity. Their synthesis is induced during infection in ectomycorrhizal fungi. These proteins appear to be ubiquitous in higher fungi and may have diverse functions in different organisms. They are

characterised by eight cysteins at conserved positions and by having similar patterns of hydrophobicity; however sequence homology between different hydrophobins is very low, even from those of the same organism. *C. fulvum* is a hemibiotrophic pathogen of tomatoes and successful infection relies on the ability of the fungus to avoid perception by the host. Hydrophobins have been proposed to assist in this purpose. In this poster I am testing this hypothesis. Two hydrophobins (HCf-1 and HCf-2) are identified, partially purified, N-term sequenced and cloned. I shall present data on expression in vitro and in planta as well as preliminary data on the phenotype of the mutants which lack either of the two hydrophobins.

100. Protease and carbohydrase strategies of *Aspergillus fumigatus,* phytopathogenic and entomopathogenic fungi.

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We compared saprophytes *(Neurospora crassa, Aspergillus nidulans*), an opportunistic human pathogen (*A. fumigatus),* an opportunistic insect pathogen (*A. flavus),* plant pathogens *(Verticillium albo-atrum, V. dahliae, Cochliobolus victoriae, Colletotrichum spp., Magnaporthe grisea, Nectria haematococca),* a mushroom pathogen *(V. fungicola)* and entomopathogens (*V. lecanii, Nomuraea rileyi, Beauveria bassiana, Metarhiziwn anisopliae)* in their abilities to degrade and utilize host-derived macromolecules (horse lung polymers, porcine mucin, hyaluronic acid, plant cell walls and insect cuticle).The major class of protease produced in most media by most plant pathogens and *N. rileyi* were broad spectrum basic trypsins; analogous peptidases produced by insect pathogens and *Aspergillus* spp. were specific for Phe-Val-Arg-X. Most plant pathogens produced additional very low levels of subtilisin-like proteases. Analogous enzymes comprised the major protease component secreted by *Aspergillus* spp., and entomopathogens. This provided them with much greater activity as *cf.* plant pathogens against elastin, mucin and insect cuticle. Plant pathogens and *Aspergillus* spp also produced high levels of several glycosidic enzymes on mucin and plant cell walls, which contain inductive carbohydrate substrates. Growth of *A. fumigatus* on mucin degraded mucin carbohydrates and mucin proteins by 40% and 75%, respectively. The residual mucin resisted further degradation because of the inability of the pathogens enzymes to remove bound sialic acid. Although they lack hyaluronidase, most of the fungi secreted a range of other enzymes on host-derived macromolecules e.g. phospholipases that are common components of bacteria as well as reptilian and invertebrate venoms. The wide distribution of these enzymes may help explain the pathogenicity of opportunists such as *A. fumigatus,* which are not subject to selection of specific virulence genes.

101. Differential gene expression in galled and asymptomatic tissues of loblolly pine infected with fusiform rust. Jaimie M. Warren, Alan L. Zwart, and Sarah F. Covert. The University of Georgia, Athens.

Cronartium quercuum f. sp. *fusiforme* is an obligate pathogen of oak *(Quercus* spp.) and pine *(Pinus spp.).* We hypothesize *C.q. fusiforme* causes gall on infected pine trees by altering the expression of pine genes that regulate cellular proliferation. The goal of this study is to identify

transcripts which are differentially expressed in asymptomatic and galled tissues of infected pine. We have approached this problem using RNA fingerprinting, a modification of the differential display technique originally described by Liang and Pardee in 1992. To date, preliminary screens have identified 30 differentially expressed cDNAs. Ongoing work focuses on screening for additional cDNAs, verifying differential expression, and determining genome of origin.

102. Transformation may enhance the virulence of *Nectria haematococca* on pea.

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Isolates of the fungus *Nectria haematococca* pathogenic on pea are able to detoxify the phytoalexin pisatin via a cytochrome P450-mediated demethylation. To examine the role of pisatin demethylating ability (Pda) in pathogenicity, mutants deficient in Pda were created by transformation-mediated gene disruption. All eleven of the Pda^- transformants were reduced in virulence on pea and a Pda^+ transformant used as a control had virulence equivalent to the Pda^+ recipients (*N. haematococca* isolates 77-13-4, 77-13-5, and 77-13-7). We now have begun to examine the virulence of 120 transformants in which integration of the transforming DNA did not disrupt the *PDA* gene locus. Many of these transformants produced lesions which were longer than those produced by the corresponding recipient isolates 77-13-5 and 77-13-7. In repeat assays of 49 of the putatively hypervirulent transformants 30% continued to produce lesions that were at least 30% longer than the wild type isolates. To test whether *N. haematococca* DNA sequences in the disruption vector pKOI had an impact on the transformants virulence, pCWHyg2, which lacks these sequences, was used to transform 77-13-7. In repeat assays some of these transformants also consistently produced lesions that were 30% longer than those made by 77-13-7. The enhanced virulence observed in these experiments may be isolate-dependent as the virulence of *N. haematococca* isolate 94-6-1 transformed with pCWHyg2 was the same as untransformed 94-6-1.

103. MAP Kinase Pathways in *Magnaporthe grisea.*

Jin-Rong Xu and John E. Hamer, Purdue University, West Lafayette, IN 47907.

Many plant pathogenic fungi, including *Magnaporthe grisea* -the causative agent of the rice blast disease, develop specialized structures to invade their hosts and undergo dramatical morphological changes to grow invasively in plants. Our research objective is to study genetic mechanisms regulating this plant infection-related morphogenesis. We have isolated a MAP kinase *PMK1* (Pathogenicity MAP Kinase 1) from *M. grisea* which is essential for appressorium formation and invasive growth in plants. Further characterization of the *PMK1* MAP kinase pathway is in progress and will be presented. In addition to *PMK1*, we isolated two other *M. grisea* MAP kinases. *PMK2* is 83% similar to *S. cerevisiae* HOGl gene, and 93% similar to *S. pombe styl* gene. PMK3 is 85% similar to *S. cerevisiae SLT2* MAP kinase gene. Five *PMK3* gene disruption mutants were isolated. *pmk3* mutants are nonpathogenic on rice plants, but make melanized appressoria on Teflon membranes or slideglass with the addition of 10 mM cAMP.

On onion epidermis, *pmk3* appressoria fail to penetrate, but elicit autofluorescence and papilla formation in onion epidermal cells. *pmk3* mutants are also dramatically reduced in conidiation, however, there is no obvious growth defect as measured by colony diameter on a variety of media. *pmk3* mutants are not temperature sensitive or infertile. It appears that *PMK3* regulates infection processes downstream of *PMK1,* and may play important roles in penetration, invasive hyphae differentiation and conidiation.

104. Comparative genetics of polyketide toxin production by *Mycosphaerella zeae-maydis* and *Cochliobolus heterostrophus.*

Sung-Hwan Yun, B.G. Turgeon, and O.C. Yoder. Department of Plant Pathology, Cornell University, Ithaca, NY.

M. zeae-maydis and *C. heterostrophus* race T are not closely related, however both appeared suddenly in the field in 1970, both are highly virulent to corn containing Texas male sterile cytoplasm (T) and both produce linear polyketides (PM- and T-toxin, respectively) which have similar structures and specifically affect T-corn. Molecular genetic analysis has shown that production of T-toxin requires a polyketide synthase (ChPKS1) and a decarboxylase (DECl). Genes encoding these enzymes are on different chromosomes and *ChPKS1* is flanked by A+T rich, repeated, noncoding DNA. To compare biosynthesis of PM-toxin with that of T-toxin, a PM-toxin-encoding gene *(MzmPKS1)* was cloned by PCR and compared to *ChPKS1*. The genes are similar in that six enzymatic domains and four introns are conserved. They differ in that they share only 62 % amino acid identity and there is no A+T rich DNA flanking *MzmPKS1*. Moreover, an additional ORF (RED l) located 5' of *MzmPKSl* is similar to reductases associated with bacterial and plant PKSs. These differences suggest that the two fungi did not appear suddenly as a result of acquiring the same gene(s) for polyketide production. Further, a gene cluster may be involved in PM-toxin biosynthesis. Targeted disruption of either *MzmPKS1 or RED1* causes loss of ability to produce PM-toxin.

Recombination

109. The *bad27* mutation in *Coprinus cinereus* is defective in both Methylation Induced Premeiotically (MIP) and meiotic chromosome synapsis.

Lesley S. Benyon and Patricia J. Pukkila, University of North Carolina at Chapel Hill, Chapel Hill.

In the basidiomycete *Coprinus cinereus* an epigenetic process known as methylation induced premeiotically (MIP) results in the preferential methylation of cytosines in the CpG dinucleotides of repetitive sequences. Here we document the first mutation in this epigenetic system. Strains homozygous for *bad27* (basidiospore development) develop normally but contain almost no *de*

novo methylation and their sexual spores arrest in pachytene of meiosis I with unsynapsed chromosomes. To monitor methylation patterns we chose to use the isoschizomers *Hpa*II and *Msp*I. Copies of the *trp1* sequence were inserted by homologous recombination and the levels of MIP evaluated. In two independent experiments, one involving a tandem triplication of the *trp1* sequence and the other a tandem duplication of *trp1,* highly significant differences in *de novo* methylation were detected between wildtype strains and strains homozygous for the *bad27-1* allele. We conclude that the morphological phenotype of our mutant results from the failure of a homology-based search as does the failure of the mutant to successfully carry out the *de novo* methylation process. Thus these two homology searches have a common genetic basis that includes the gene disabled by our mutation.

110. Molecular analysis of conversion events at the *am* locus of Neurospora.

Frederick J Bowring and David EA Catcheside, School of Biological Sciences, Flinders University, Adelaide, Australia.

In cross B163, heteroallelic am^1 am^6 and heterozygous for both conventional genetic flanking markers and closer molecular markers (Bowring and Catcheside 1996), we found that although 24 % of the conversion events that generated prototrophic recombinants were associated with an exchange of flanking genetic markers, the molecular markers were recombined in only 7% of convertants. We concluded that either conversion and crossing over result from different recombination pathways or that resolution of a common intermediate is biased in favour of preservation of the local parental marker association. We report here that natural polymorphisms distinguishing the parents of cross B163 also include sequences within and closely flanking *am*, supplying markers for determining the structure of conversion tracts in the progeny of cross B163. Conversion of am^6, the more distal allele, is more frequent than conversion of am^1 and is associated with a peak of conversion frequency ~ 100bp 5' of *am.* 37 of 61 continuous tracts converting am^1 extend less than the 741 bp maximum detectable with the available markers. Distal of *am* where additional markers are available, all nine crossovers that occurred in convertants were at least 115 kb away. We consider it implausible that exchanges at a distance two orders of magnitude further away than the modal length of conversion tracts are directly associated with the conversion event.

Bowring, FJ and DEA Catcheside. (1996) Gene conversion alone accounts for more than 90% of recombination events at the *am* locus of *Neurospora crassa.* Genetics 142: 129-136

111. Molecular analysis of *pan-2* prototrophs in *Neurospora crassa.*

Mary E. Case, University of Georgia, Athens.

Tetrad analyses of pantothenic acid-requiring mutants *pan-2* B3 and *pan-2* B5 indicated that 90 % of aberrant events arose by gene conversions and 10% by reciprocal recombination (Case and

Giles, CSHSQB 23:119-135, 1958). A molecular examination of prototrophs from crosses between *pan-2* mutants have confirmed these classical genetic studies. Since the original *pan-2* mutants were isolated in wild type 74 A, five new *pan-2* mutants were isolated in the wild type Mauriceville strain. These new *pan-2* mutants were crossed to a marked strain *ylo-1 pan-2* B3 *trp-2.* Prototrophs obtained from crosses with these mutants were phenotypically categorized as parental, *ylo*$^+$ *trp*$^+$ or *ylo trp.* or "recombinant" *ylo trp*$^+$or *ylo*$^+$ *trp* with respect to the flanking markers. DNA isolated from prototrophs from each of the these classes was digested with *Hind*III. Southern blots were done and the prototrophs were categorized as having either a 74A or Mauriceville restriction pattern. These molecular analyses indicate that over 90% of the events giving rise to prototrophs between the *pan-2* mutants arose by gene conversion not reciprocal recombination.

112. The use of the REMI technique to tag and mutagenize *rad12* and *rad12*-associated genes in *Coprinus cinereus*.

Martina Celerin, Teresa M. Niehoff, Miriam E. Zolan. Department of Biology, Indiana University, Bloomington, Indiana 47405

We are exploiting the synchronous meiosis of *Coprinus cinereus* to better understand the related processes of DNA repair and meiosis. Our laboratory has identified four genes, *rad3, rad9, rad11* and *rad12,* which are required both for survival of gamma irradiation and for meiosis in *C. cinereus.* Microscopic analysis of three *rad12* mutants showed that *rad12* chromosomes condense but do not synapse completely during prophase I of meiosis. In addition,*rad12* nuclei arrest at late prophase 1. We have initiated an approach to isolating the *rad12* gene and proteins which may be interacting with *rad12*. The strategy involves tagged mutagenesis using restriction-enzyme mediated integration (REMI) of transforming DNA followed by anchored PCR to retrieve the tagged gene. A self-compatible strain of *C. cinereus,* defective at both mating loci *(Amut Bmut),* and a plasmid harboring the hygromycin marker, linearized with the restriction enzyme *Kpn*I, were used for REMI transformations. The linearized plasmid and extra *Kpn*I were transformed into competent protoplasts. Hygromycin-resistant mycelial colonies, resulting from regenerated, transformed cells, were collected and induced to fruit using an established light and temperature regime. Of 2129 hygromycin-resistant transformants, 153 fruited as white mushrooms, an indication that these mutants are likely defective in meiosis and/or spore formation (W.J. Cummings, J. Crodian, L. Brunnick, M.E. Zolan, *in preparation).* To determine whether any of the 153 new, likely meiotic mutants are actually tagged alleles of *rad12,* we have mated each independently to two of the 15 known alleles of *rad12.* Of the 130 crosses that have fruited to date, 29 repeatedly produced white mushrooms, thereby demonstrating lack of complementation between the new mutant and the *rad12* mutation. We have so far identified 13 mutants which contain a single hygromycin insert that co-segregates with the sporeless, white fruiting body phenotype. Hence, the mutations in these strains are likely tagged. Currently, semi-random, two-step PCR (ST-PCR; Chun et al., 1996) is being employed to identify the tagged genes.

113. Involvement of homologous recombination in tandem repeat formation following integrative transformation in *Penicillium paxilli.*

Yasuo Itoh[1], and Barry D. Scott[2]. [1]Faculty of Science, Sinshu University, 390, Matsumoto, Japan. [2]Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand.

We have been attempting to clone genes involved in the biosynthesis of paxilline, a tremorogenic mycotoxin, by plasmid tagging. One mutant that did not produce the toxin was isolated by ectopic integration of plasmid pAN7-1. Analysis of the site of integration in this mutant revealed the presence of an extensive genomic deletion. This phenomenon was also observed in a set of pAN7-1 derived spore color mutants. We were therefore interested in furthering our understanding of the process of plasmid integration in this fungus. Transformations were carried out either with or without the addition of restriction enzyme, and 102 integration events analysed. In the absence of enzyme, 50% of the integrations were single copy events whereas addition of *Hind*III increased this ratio to 82 %. Of the 33 tandem repeat integrants obtained from transformations with and without enzyme, 88% were organised in a head to tail orientation with the remainder in inverted repeat configurations. Dephosphorylation of linearized pAN7-1 had no significant effect on either the transformation frequency or the integration profiles obtained, except that tandem repeats in the inverted repeat configurations were not obtained using dephosphorylated vector. These results suggest that the major mechanism for tandem repeat formation in *P. paxilli* is by homologous recombination of additional copies of the plasmid using the single integrated copy as substrate. Addition of restriction enzyme may provide a different pathway for plasmid integration.

114. Class I and Class II synaptic mutants in the basidiomycete *Coprinus cinereus.*

Janet Knight and Patricia J. Pukkila, University of North Carolina at Chapel Hill.

In the basidiomycete *Coprinus cinereus,* ultraviolet light can induce mutants that cannot successfully complete meiosis. These mutants are recognized phenotypically by a white cap which signifies a lack of spore production. Using electron microscopy and propidium iodide staining as characterization tools, these mutants can be divided into two distinct classes. Class I mutants are defined as those in which karyogamy and nucleolar fusion occur normally, and then subsequently arrest with no further progression through meiosis. These mutants exhibit very little structure in the form of synaptonemal complexes, and many times do not even initiate axial core assembly. Class 2 mutants, on the other hand, usually complete axial core formation and occasionally initiate small regions of synaptonemal complex. Of the ten *bad* (basidiospore development) mutants examined, four fell into Class I, five into Class II, and one exhibited wild type synapsis. None of these mutants is sensitive to ionizing radiation. Comparisons with previously published work indicate that mutants with defects in DNA repair have a different spectrum of synaptic defects than those observed here.

115. The hotspot paradox and the evolution of meiotic crossing-over.

R. Redfield, R. S. Myers and A. Boulton, Dept. of Zoology, University of British Columbia.

Studies of meiotic recombination have revealed an evolutionary paradox. Molecular and genetic analysis has shown that crossing over initiates at hotspots, by a recombinational-repair mechanism that replaces the initiating hotspot with a copy of its homolog. Computer simulations of large populations show that this mechanism causes active hotspots to be rapidly replaced by inactive alleles arising by rare mutation. Neither of the known benefits of crossovers (accurate segregation and genetic recombination) were sufficient to preserve active alleles in the face of this conversion. The paradox was partly resolved by introducing into the model an additional, non-meiotic function for hotspots, consistent with their observed association with functional sites in chromatin. Strong selection for this function could allow active hotspots to persist in spite of frequent conversion to inactive alleles. However, this explanation is unsatisfactory for two reasons. First, it is unlikely to apply to obligately-sexual species, because the viability selection needed to preserve many hotspots per genome (necessitated by observed crossover frequencies) would drive the species to extinction. Second, it fails to explain how natural selection could maintain such a genetically costly mechanism of recombination. Resolution of the paradox may require significant changes to the commonly-accepted models of meiotic recombination.

116. A meiosis-specific initiation site for recombination in the promoter region of the *niiA-niaD* gene cluster of *A. nidulans.*

Henk W.J. van den Broek, Hans Thijs and Theo Goosen. Dept. of Genetics, Agricultural University Wageningen, Dreyenlaan 2, 6703 HA Wageningen, The Netherlands.

Genetic markers typically show Mendelian segregation (2:2) in meiosis, but with a low frequency deviations are observed which are generally viewed as the consequence of gene conversion, in which one DNA duplex (the acceptor) is altered using a non-sister duplex (the donor) as a template. This interaction (which probably reflects the chromosomal search for homology during meiotic prophase 1) requires the formation of a heteroduplex tract between strands of non-sister chromatids that may include mismatches and the physical linkage of the homologues by Holliday junctions. Repair of these mismatches could result in aberrant segregation patterns.

Usually, polarity of gene conversion is observed (markers on one side of a locus are converted at higher frequencies than markers on the other side), which is interpreted as the result of fixed initiation sites for heteroduplex formation and distance dependent resolution. In the *ARG4* and HIS4 genes of yeast and the *niiA-niaD* gene cluster of *A. nidulans* these initiation sites are located in the promoter regions of these genes.

We have introduced molecular markers in the *niiA-niaD* gene cluster and used the resulting strains in two-point crosses. The results confirm the location of the initiation site in the promoter region and demonstrate that this site is meiosis specific. Furthermore they show that conversion tracts are small and that mutations included in a tract are always co-converted. Initiation of

mitotic recombination events does not take place at a specific site.

117. Molecular analysis of recombination events associated with the *cog* recombinator of Neurospora.

P Jane Yeadon and David EA Catcheside, School of Biological Sciences, Flinders University, Adelaide, Australia.

Multiple polymorphisms distinguish Emerson and Lindegren strains of *Neurospora crassa* within the *histidine-3* gene and in its distal flank. Restriction site and sequence length polymorphisms in an overlapping set of PCR products covering this region have been used to identify the parental origin of DNA in histidine-prototrophic recombinant progeny of crosses between the strains. 29% of conversion tracts are interrupted. Where the absence of *rec-2*$^{+}$ permits activity of the recombination hotspot *cog*, conversion appears to originate at *cog* and conversion tracts are up to 5.6 kb long. The chromosome bearing *cog*^{L} the dominant allele which confers a high frequency of recombination, is almost invariably the recipient of information. In progeny from *rec-2*$^{+}$crosses, conversion tracts are much shorter, most are not initiated at *cog* and either chromosome seems equally likely to be converted. Conversion is only infrequently associated with crossing over, suggesting that a conversion intermediate containing paired Holliday junctions may usually be resolved by a topoisomerase, or by scission of one junction and migration of the second to the resulting nicks. The low level of association between conversion and crossing over could be due to occasional scission of both junctions, resulting in a crossover in half of these events.

118. A Novel MCBL plates:co-induce nuclear membrane fusion and chromosome nondisjunctional recombination.

Yun-Can Ai, Fan-Mei Meng, Jin-Xian Luo,(Zhongshan University,Guangzhou,PR China), Christian P Kubicek, Robert Mach, Tanja Krupica,(TU Wien,Wlen,Austria)

MCBI, plates were constructed with Czapek's Minimal medium containing 0.1% (w/v) d-Camphor and 0.5-1.0 ppm Benomyl based on an assumption that d-Camphor could induce nuclear membrane fusion while Benomyl could induce chromosome nondisjunctional recombination.After the PEG-mediated fusion of UV-inactived prototrophic protoplasts between *Trichoderma reesei* and *Aspergillus niger*, the temporary heterokaryon could be induced on these plates at the same time. Thus serveral very stable fusants with dominance could be obtained from this fusion cross, for the first time. Three typical recombinants --after they had been undergone segregation over five years-- were confirmed under the approches of PFEE for chromosomes, RAPD-PCR for gene finger-prints especially Southern Blotting with cbhl, cbh2, egl, eg2, bgll probes for cellulose genes analysis.These results demonstrat a novel way for overcoming the several problems, such as incompatibility and no recombination, arose from the ordinary procedures It therefore would provide a novel approach for fungal genetics and biotechnology.

Neurospora posters

119. Phenotype of novel complex I mutants of *Neurospora.*

T. Almeida, M. Duarte, A.M.P. Melo and A. Videira. University of Porto, Portugal.

Respiratory chain complex I is an assembly of more than 30 polypeptide subunits located in the inner mitochondrial membrane. It couples electron transfer from NADH to ubiquinone to proton translocation across the membrane. The enzyme is constituted by two domains, the peripheral and membrane arms, that are assembled independently of each other. We are using the technique of repeat-induced point-mutations in order to inactivate specific complex I genes. We have isolated two mutant strains of *N. crassa* that lack two subunits bearing [Fe-S] clusters of the peripheral arm of complex I, a 24 kDa and a 21 kDa protein respectively. The former is apparently able to assemble an almost intact complex I while formation of the peripheral arm of the enzyme in the latter strain cannot be detected. In addition, lack of either protein seem to prevent the progression of *N. crassa* through the sexual phase of the life cycle. Further characterisation of the mutants will also be presented.

120. Neurospora NADP-glutamate dehydrogenase - mutational amino acid replacements and three-dimensional structure.

Ian F. Connerton and Angel Fuentes (Inst, of Food Res., Reading); John Fincham (Edinburgh Univ.); K. Yip, Tim Stillman and David W. Rice (Sheffield Univ.).

Numerous mis-sense *am* mutants have been characterised over the years in terms of their effects on GDH properties and their complementation relationships. Most of the corresponding amino acid replacements have been defined, initially by peptide sequencing but now mostly by DNA sequencing. Following the recent X-ray crystallographic analysis carried out in Sheffield on wild-type GDH, it becomes possible to position the mutant amino acid replacements accurately within the three dimensiona1 structure of the hexameric enzyme, and suggest reasons for their various effects on enzyme phenotype.

121. Signals for *de novo* cytosine methylation of DNA in *Neurospora crassa.*

Michael Freitag, Vivian Miao, Brian Margolin and Eric Selker, University of Oregon, Eugene.

Several Neurospora DNA sequences have been identified that act as portable signals for *de novo* cytosine methylation. "Signal DNA" typically contains C:G to T:A mutations introduced by repeat-induced point mutation. Consequently, such signal DNA has a higher number of T and A nucleotides and a higher density of TpA dinucleotides than unmutated Neurospora DNA. To identify critical characteristics of signal DNA we compared the level of *de novo* methylation induced by various mutated or unmutated Neurospora DNA, mammalian DNA or bacterial DNA that had been integrated into the Neurospora genome either at random or by targeting to the *his-3*

or *am* loci. To specifically test whether the number of T+A nucleotides or the density of TpA dinucleotides are important parameters for the induction of *de novo* methylation we further dissected one portable methylation signal, the - (zeta-eta) region, and assayed the ability of several short DNA fragments to induce *de novo* methylation.

122. Inositol 1-phosphate synthase of *Neurospora crassa.*

Barbara Hanson, Michelle Mathis, Barbara Stengel and Stephen J. Free, Canisius College and State University of New York,

Buffalo, NY.

Inositol 1-phosphate synthetase (IPS) is the enzyme that catalyzes the first step in the synthesis of inositol, a key growth factor and component of the phosphatidylinositol cycle in *Neurospora.* The DNA sequence of the inositol 1-phosphate synthase was determined from a clone that complemented a *Neurospora* inositol mutant (*inl* 89601). This clone was first isolated by Akins and Lambowitz (1985. Mol. Cell. Biol. 5:2272-2278). The DNA sequence containing the IPS gene in the clone was identified by its similarity to other IPSs in plants and other fungi. The IPS gene contained 1,614 base pairs that appeared to code for a protein containing 537 amino acids with a molecular weight of 58826.4. The amino acid composition of the protein indicated that 50.7% of the amino acids were hydrophobic. Comparison of the *Neurospora* IPS gene to other IPS sequences indicated that this sequence showed a higher homology to plants and algae rather than to other fungi such as *Saccharomyces* and *Candida.* Sequence data base searches of amino acid sequence databases available at the National Center for Biotechnology Information, NIH were done using programs based on the BLAST algorithm. The organisms with sequences that were most similar to *Neurospora* were *Brassica napus, M crystallinum,* and *Arabidopsis.* The sequences for IPS from *Candida* and *Saccharomyces,* two fungi, showed segment pairs that were about 25% less similar than the plants to *Neurospora.*

123. Mechanisms of polyamine regulation of ornithine decarboxylase synthesis in *Neurospora crassa.*

Martin A. Hoyt, Janet L. Ristow, and Rowland H. Davis, University of California, Irvine.

Ornithine decarboxylase (ODC), encoded by the *spe-1* gene of *Neurospora crassa* catalyzes the initial and rate-limiting step in the synthesis of the polyamines (putrescine, spermidine, and spermine). Ornithine deprivation, leading to polyamine starvation is imposed upon *aga* (arginaseless) mutants by growth on arginine. In response, ODC activity and spe-1 mRNA abundance increase about 12 fold.. The polyamine-mediated regulation of *spe-1* mRNA abundance relies on two mechanisms. First, spermidine, the major cellular polyamine, and sequences in the long *spe-1* 5'-untranslated region, act together to repress 6 fold the synthesis, processing, or nuclear export of *spe-1* mRNA. Deletion analysis shows that this sequence does

not affect the stability of the mRNA, which is intrinsically unstable. The second mechanism is a 2fold enhancement of this instability, imparted by putrescine addition, and probably targeted on the 3'-untranslated region of the mRNA.

124. Cloning and characterization of a *Neurospora crassa* MutS mismatch repair homolog.

D.H. Huber[1], G. Hausner[1], B. Seidel-Rogol[2], H. Bertrand[1]. [1] Michigan State University, East Lansing, and [2]State University of New York, Plattsburg.

Mismatch repair systems are involved in the faithful transmission of genetic information between generations by recognizing and correcting base pair mismatches in DNA. Genes encoding proteins homologous to the *E. coli* MutHLS repair system have been identified in several organisms. Mutations in mismatch repair genes produce mutator phenotypes. We have cloned and are characterizing a gene from *N. crassa* encoding a homolog of the *E. coli* MutS protein. The *N. crassa MSH* gene *(mut S* homolog) was identified using PCR with degenerate oligonucleotide primers based upon highly conserved regions found in MutS, the homologous yeast proteins, MSH1 and MSH2, and mammalian MSH protein Two cosmids were isolated from the Orbach/Sachs library using Southern hybridization with the cloned PCR product. Four subclones were then obtained from the cosmids that hybridized to the PCR probe. The deduced amino acid sequence from the region of MSH amplified by PCR is most similar to the corresponding sequence of the yeast MSH2 protein. The remainder of the gene is currently being sequenced. *MSH* maps to LG VII using the Metzenberg RF map.

125. Characterization of calcium and magnesium uptake in the vacuole of *Neurospora crassa.*

Kelly A. Keenan, Thomas Kirn, and Thomas Wisniewski, Richard Stockton College of New Jersey.

Calcium and magnesium uptake in the vacuole of *Neurospora crassa* was examined using cupric ion permeabilization system. The distribution of calcium and magnesium was examined and it was shown that 43.6 % of calcium and 79.5 % of magnesium was associated with the vacuole. A cellular fractionation experiment yielded the same distribution. When grown in minimal medium, the total pools were 0.17 *u*g/mg protein for calcium and 16.3 *u*g/mg protein for magnesium. Vacuolar uptake of calcium and magnesium was examined. Both metals were transported in a manner consistent with Michaelis-Menten kinetics and the Km values were 0.97 mM for Ca^{+2} and 0.45 mM for Mg^{+2}, Specificity studies indicate that there is a common permease for these two metals. The effect of pH on uptake was examined and was optimal at pH 6.0 for both metals. A series of mutants was isolated that had altered levels of calcium and magnesium pools. Vacuolar transport of both metals was examined and was shown to be reduced in two of the mutants.

126. Isolation and characterization of vacuolar basic amino acid permease mutants in *Neurospora crassa.*

Kelly A. Keenan, Jonathan Tayco and Zeljka Basic, Richard Stockton College of New Jersey.

Uptake of the basic amino acids into the vacuole of *Neurospora crassa* was examined using a cupric ion permeabilization system. The Km for lysine uptake was 1.4 mM and 11.1 mM for ornithine. Specificity studies suggest that there are distinct permeases for these two amino acids and that the lysine permease is inibited by arginine. The kinetic effect of arginine on lysine uptake was examined. A filtration enrichment procedure was used to isolate mutants in the basic amino acid vacuolar permeases. Vacuolar uptake was measured using a cupric ion permeabilization system. The mutants were grouped genetically according to complementation results and two groups were obtained. The first contains fourteen members and most of the mutants in this group failed to take up all three basic amino acids. Members of this group had a reduced level of vacuolar calcium transport. Two mutants are in complementation group 2 and these failed to take up ornithine but arginine and lysine uptake was normal. Vacuolar calcium uptake was normal. Mutants from both complementation groups have a visibly altered vacuolar morphology. Vacuolar ATPase activity was measured in members of both complementation groups.

127. Cloning and Characterization of the *aod-2* alternative oxidase regulatory gene of *Neurospora crassa* .

Tak Ko and Helmut Bertrand, Michigan State University.

Wild-type *Neurospora crassa* contains two respiratory pathways: the normal cytochrome pathway and the alternative pathway that is insensitive to cyanide and antimycin A, but is inhibited by hydroxamic acids. The alternative pathway diverges from the cytochrome pathway after the ubiquinone pool and is not linked to oxidative phosphorylation. The alternative pathway can be induced by treatment with chloramphenicol, oligomycin, or antimycin A. It is also induced in mutants which are deficient in *b*- and *a*-type cytochromes. The induction of alternative-oxidase activity requires two genes, *aod-1,* which encodes the alternative oxidase and *aod-2,* which is involved in the regulation of the *aod-1* gene on the transcriptional level. The *aod-1* gene is located on linkage group IV and has been recently cloned. The *aod-2* gene is located in linkage group II, 13.9 map units to the right of *thr-3* and 3 map units to the left of *arg-5.* We are attempting to clone the *aod-2*$^{+}$ DNA in two different ways: complementation of an *aod-2* mutant using *aod-1* promoter-driven expression of a reporter gene, in this case the bacterial hygromycin resistance gene as a method for selecting transformants and chromosome walking. Cloning and characterization of the *aod-2* gene which is known to affect the transcription of *aod-1,* will provide information about the possible mechanisms of communication between mitochondria and the nucleus.

128. Site-directed mutagenesis of the N-terminal domain (of F-Box)of the SCON-2 negative regulator leads to a novel regulatory phenotype.

Anuj Kumar and John V. Paletta, Dept. of Biochem. & Mol. Biol., Wright State University, Dayton, OH.

In the filamentous fungus *Neurospora crassa,* sulfur metabolism is controlled through an intricate multigene network which includes the sulfur regulatory genes *cys-3+* (which encodes a BZIP transcriptional activator) and the negative regulatory sulfur controller gene *scon-2+*. Sequence analysis of the SCON2 protein has revealed several striking protein motifs. Specifically, SCON2 contains an N-terminal domain subsequently identified within a number of eukaryotic regulatory proteins including the yeast cell-division-cycle protein Cdc4p and the Cdk regulatory subunit cyclin F. To assess the functional significance of this N-terminal domain (which has been recently renamed the F-box), we have undertaken an extensive mutational analysis. Site-directed mutagenesis of key residues within the F-box results in sulfur auxotrophy, with mutants unable to grow in the absence of a supplemental sulfur source such as methionine. In addition to this N-terminal domain (F-box), SCON2 also contains six repeated GP-homologous domains (or WD-40 repeats) spanning the C-terminal half of the protein. Previous studies have suggested a possible role for both the F-Box and P-transducin motifs in the mediation of protein-protein interactions. To address the possibility that SCON2 may function as part of a multiprotein complex, we have generated rabbit polyclonal anti-SCON2 antibody which is currently being used to characterize SCON2 binding properties in *N. crassa* cellular extracts.

129. Cloning of *Neurospora crassa* genes defective in DNA methylation.

Elena Kuzminova, Shan Hays and Eric Selker, University of Oregon.

Methylation of cytosines is the most common modification of DNA in eukaryotic organisms. The amount and distribution of methylated cytosines in the genome varies among eukaryotes. Most methylated sequences from *Neurospora* show RIP-like mutations. How the methylation patterns are established is not known. The biological role of methylation in *Neurospora crassa* is yet to be understood, since the *dim-2* methylation mutant, which completely lacks methylation in vegetative tissues, does not confer phenotypic changes in vegetative or sexual cycles. To understand the molecular basis for the lack of methylation in this mutant we are attempting to clone the *dim-2* gene. To this end we have mapped *dim-2* between *wc-1* and *un-10* on LGVII. Now we are trying to clone *dim-2* by chromosome walking from these two markers.

We have developed a mutagenesis protocol to identify more genes involved in cytosine methylation. We chose insertional mutagenesis because it yields mutants in which the mutated genes are marked, making them easier to clone. Since known *Neurospora* methylation mutants have no selectable or easy scorable phenotype *per se,* we are taking advantage of a *hph* (hygromycin B phosphotransferase) allele that is silenced by methylation in the *dim+* strain, leaving the strain sensitive to the drug hygromycin B. Subsequent to the mutagenesis, selection on hygromycin B allows only Hph+ (and thus Dim-) transformants to grow.

130. Temperature regulted alternative initiation of frequency protein mediates an aspect of temperature compensation in the *Neurospora* circadian clock.

Yi Liu, Norman Garceau, Jennifer Loros and Jay C. Dunlap. Dept. of Biochemistry, Dartmouth Medical School, Hanover, NH.

It has been shown that the *frequency (frq)* gene is a central component of the *Neurospora* circadian clock. The circadian oscillator in *Neurospora is* comprised of an auto regulatory feed back loop in which the amount of *frq* transcript is negatively regulated by its product, FRQ protein. Both *frq* message and FRQ protein oscillate with a period which is the same as that of the conidiation banding rhythm (Aronson et al., *Science* 263:1578-84; Dunlap, Annu. *Rev. Genetics* 30:579-601, 1996).

Based on DNA sequence information, *frq* transcripts were predicted to encode a single 989 aa protein. In this study, we found that there is an alternative initiation of FRQ translation: two FRQ forms, FRQ1-989 and FRQ100-989, are expressed as a result of alternative initiation from two of three in-frame initiation codons. Furthermore, we found that the ratio of two FRQ forms is modulated by temperature: high temperature favors the initiation of FRQ1-989, whereas at low temperature, FRQ100-989 is favored. In mutant strains lacking AUG#1, the circadian rhythm is lost at high temperature, whereas strains without AUG#3 became arrhythmic at low temperature. Our data also shows that this temperature-dependent synthesis of mutiple forms of FRQ reflects both quantitative and qualitative requirements for FRQ by the clock. Different amounts of FRQ are required at different temperatures and the two FRQ forms are also functionally distinct, so that an optimal clock requires both FRQ forms. Together, this represents a novel adaptive mechanism which allows *Neurospora* to keep its clock running over a wide range of temperatures.

131. Signal transduction proteins of *Neurospora crassa.*

Peter Margolis, Yanofsky Lab, Stanford University.

In response to environmental stress, vegetative cells of *N. crassa* can enter onto either of three developmental pathways. Recent work suggests that this process can be considered as a problem in signal transduction. I show here that *Neurospora* indeed possesses proteins homologous to those that control signaling in other systems. I have identified, using PCR with degenerate oligonucleotide primers, at least 13 putative *Neurospora* signal transduction genes, including some capable of encoding putative Ras proteins and members of MAP kinase cascades. The cloning of genomic DNA corresponding to these PCR-generated fragments will permit the mutagenesis (via RIP and marker replacement) and phenotypic characterization of these loci. Initial work has focused on a third *N. crassa ras* homolog *(ras-3)* that could encode a novel class of Ras protein [e.g. unusually large (34 kD), lacking a standard CAAX C-terminus, distinguishing sequence motifs].

132. Analysis of Neurospora ropy genes encoding novel proteins required for normal hyphal growth and nuclear distribution

Peter Minke, John Tinsley, In Hyung Lee, and Mike Plamann. Department of Biology, Texas A&M University, College Station, TX.

We have begun investigating the molecular mechanisms underlying hyphal growth and nuclear movement in filamentous fungi by studying a class of morphological mutants of *Neurospora crassa* called ropy. Ropy mutants exhibit a phenotype of curled, rope-like hyphal growth and abnormal nuclear distribution. Three *ro* genes have been found to encode subunits of either the microtubule-associated motor cytoplasmic dynein or the associated dynactin (dynein activator) complex. This report focuses on five additional genes which encode novel proteins. *ro-7* is predicted to encode a 70 kD protein distantly related to actin. In a *ro-7* mutant, cytoplasmic dynein heavy chain and p150Glued, the largest subunit of dynactin, accumulate at nuclear spindle pole bodies suggesting that RO7 is required for proper intracellular targeting of cytoplasmic dynein and dynactin. *ro-10* is predicted to encode a novel 24 kD non-essential protein and may be required for stability of dynactin as p150Glued is not detectable in a *ro-10* deletion strain. *ro-11* is predicted to encode a novel 75 kD non-essential protein that contains large regions of predicted coiled-coil structure with a highly basic C-terminal region. Intracellular location of dynein and dynactin is not disrupted in a *ro-11* deletion strain. *ro-2* and *ro-JT2* are predicted to encode novel proteins.

133. Genetic interactions among the *Neurospora crassa* circadian clock mutants.

Louis W. Morgan and Jerry F. Feldman, University of California, Santa Cruz 95064.

We have identified a series of epistatic and synergistic interactions among the circadian clock mutants of *Neurospora crassa* that indicate possible interactions among the various clock components encoded by these genes. *prd-6,* a new short period temperature-sensitive clock mutant, is epistatic to both *prd-2* and *prd-3* and the specific nature of the epistasis suggests a direct physical interaction between the *prd-6, prd-2,* and *prd-3* proteins. *prd-2* and *prd-3,* both long period mutants, show a synergistic interaction in that the period length of the double mutant strain is considerably longer than the expected value based on the period lengths of the single mutants. *prd-2, prd-3,* and *prd-6* also show significant synergism with frq^7, a long period allele of the *frq* locus, previously shown to play a central role in the *Neurospora* clock. In contrast, double mutants involving other clock mutations do not show either epistasis or synergism with each other or with the mutants listed above. These results suggest that the gene products of *prd-2, prd-3,* and *prd-6* play an important role in clock function and will shed light on the specific role of *frq* in clock organization.

134. An 88-kDa heat shock protein interacts with *Neurospora crassa* hsp30.

Nora Plesofsky-Vig and Robert Brambl, University of Minnesota, St. Paul.

We have identified proteins that interact specifically with hsp30 of *Neurospora crassa.* Affinity resins of recombinant hsp30, linked to either an Affi-Gel or a nickel resin, were incubated with soluble extracts from heat-shocked *N. crassa* cells. A 70-kDa and an 88-kDa protein were found to bind to the hsp30-affinity resin, but not to control resins. The 70-kDa protein was identified as hsp70 by N-terminal sequencing. For the 88-kDa protein, the N-termini of several tryptic peptides were sequenced. The encoding transcript, detected in Northern blots, is present at normal temperature, but is very strongly induced by heat shock. A 2.6 kbp cDNA was isolated from an *N. crassa* cDNA library and sequenced. The predicted amino acid sequence shows homology to hsp70 and especially to a recently identified class of proteins that includes mammalian hsp110, human hsp70RY, yeast SSE1/2, and Arabidopsis hsp91, which are distantly related to hsp70. The cDNA contains the entire coding region for hsp88 by structural criteria and the peptide's alignment with homologous proteins. This interaction of hsp70 and hsp88 with hsp30 may assist or regulate the activities of hsp30.

Agaricus and Basidiomycete posters

135. Genetics and breeding of *Agaricus.*

Leo Calvo-Bado, Simon Cutler, Mike Challen and Tim Elliott, Horticulture Research International, Wellesboume, Warwick, CV35 9EF, UK.

The genus *Agaricus* comprises c. 100 species and includes the cultivated mushroom *A. bisporus.* HRI has a genetics programme to develop technologies and approaches of value in strain improvement. Current aims are to develop a reproducible transformation system and to characterise and exploit the diversity present in the genus. Although transformation has been achieved in *A. bisporus* using the pAN7.1 vector, the system is not reproducible. We are developing transforming vectors, controlled by regulatory sequences from *Agaricus* genes, or based on *Agaricus* genes themselves. Putative regulatory sequences have been defined and vectors constructed in which promoter sequences have been fused to reporters. Protoplast-mediated transformation remains a problem with *Agaricus.* We have therefore developed ballistic delivery of DNA for use in mushrooms. Other *Agaricus* species provide unexploited genetic diversity; some may be directly cultivable whilst others may provide genes of agronomic value that can be utilised in *A. bisporus.* We are analysing *Agaricus* breeding systems using classical and molecular methodologies. These studies have revealed an unexpected complexity in some breeding systems where single spore isolates can fruit "normally" but heterokaryosis is still regulated by mating-type. Our poster illustrates the range and variety of studies being carried out at HRI.

136. The ATPase 6 gene in Agaricus, a comparison of a structural gene with the plasmid, pEM.

Becky Chiang, Mary Robison, and Paul A. Horgen. University of Toronto.

The ATPase 6 gene has been sequenced and analyzed from a representative sample of the genus, *Agaricus,* and from numerous isolates of *A.bisporus* (with different phenotypes, geographical

location, and reproductive strategies). We have integrated PCR with that of DNA cycle sequencing. DNA sequence comparison across the genus suggested a considerable amount of variability with transversions (pydmidine purine) being more common than transitions (purine purine or pyrimidine pyrimidine). We have compared the differences in primary nucleotide and primary amino acid sequence in the representative isolates across the genus. Within the 6 isolates of *A. bisporus* examined, one variable site was detected. Within this small sample, the difference was observed in the U3 cultivar, and a heterothallic isolate from the Sonoran Desert. From this small, but representative sample of the genus, we have constructed a phylogenetic tree comparing the sequences of the structural gene of ATPase 6 with the extra chromosomal plasmid, pEM. The results of this analysis suggests that the evolution of these two mitochondrial. genetic elements occurred quite separate from one another. The results from this analysis further supports the hypothesis that the integration of pEM homologous sequences into the mitochonddal genome was an ancient event probably occurring before the speciation of the genus. If pEM were of mitochondrial origin, one would predict that the evolutionary pattern should be similar to that of ATPase 6.

137. Cytological localization of an epitope-tagged Frtl fusion protein in the Basidiomycete *Schizophyllum commune.*

Gail E. Palmer, J. Stephen Horton, Dept. of Biological Sciences, Union College, Schenectady, NY, USA

Fruiting bodies (mushrooms) can be induced to form in normally nonfruiting homokaryotic strains of the Basidiomycete fungus *Schizophyllum commune* by the ectopic genomic integration of a cloned gene called *Frtl.* The *Frtl* gene encodes a predicted polypeptide of 192 amino acids, which has motifs which suggest that it is an ATP-binding protein. An important step in elucidating the mechanism of how *Frt1* influences mushroom development is to determine the cellular location of its protein product. A plasmid construct was made in which a triple hemagglutinin tag was fused inframe to the N-terminus of *Frt1*. *In vivo* function of the tagged transgene was confirmed by its ability to induce fruiting in homokaryotic transformation recipients. Hyphal cells from transformants were analyzed by immuno-EM and immunofluorescent microscopy, using a monoclonal antibody against the HA tag. The results of these experiments indicate that the Frtl protein is localized in the cellular membrane. The authors speculate that Frtl may be involved in a signal transduction pathway controlling the development of fruiting bodies in *S. commune.*

138. Parasitic interactions between thn and wild-type mycelia of *Schizophyllum commune*.

Frank H.J. Schuren, Swiss Federal Institute of Technology, Zurich, Switzerland.

Development of aerial structures in the basidiomycete *Schizophyllum commune* is regulated by the THN1 gene. A frequently occurring spontaneous mutation in this gene prevents formation of

aerial hyphae in a monokaryon and, if homozygous, the formation of aerial mycelium and fruit bodies in a dikaryon. Genes specifically expressed during formation of aerial structures (e.g. hydrophobin genes) are not expressed in mutant colonies. Other characteristics are the typical wavy (sometimes corkscrew-like) hyphae, faster radial growth rate than wild-type (although the biomass is only about half of that of wt colonies) and a typical pungent smell. Thn colonies were shown to produce excessive aerial mycelium when surrounded by an excess of wt colonies. When wt and thn colonies were grown together the growth rate of thn colonies was clearly increased, resulting in complete overgrowing of wt mycelium by thn hyphae when mixtures of both mycelia where grown from a single inoculation point. Since the growth stimulation (but not the excessive formation of aerial mycelium) could also be observed when thn and wt colonies were physically separated by a dialysis membrane (cut-off 6-8 kDa) it was concluded that low-molecular-weigth molecules were responsible for the observed effects.

139. Expression of heterologous genes in *Schizophyllum commune is* hampered by truncated transcripts.

Frank H.J. Schuren, Jeroen Bron and Joseph G.H. Wessels, University of Groningen, Groningen, The Netherlands.

GPD regulatory sequences were used to express a phleomycin resistance gene (Sh *ble)* in *Schizophyllum commune* resulting in high numbers of phleomycin-resistant transformants. Attempts to express heterologous genes coding for hygromycin B phosphotransferase (hph), geneticin, -glucuronidase (*uidA*) and B-galactosidase (*lacZ*) using the same regulatory sequences were not successful and no mRNA could be detected. GC-methylation could not be detected in any of the transformants. Cloning the *hph* and *uidA* genes in an internally deleted *GPD* gene resulted in truncated transcripts which ended within the 5'-parts of the heterologous genes. Cloning of the same genes as transcriptional fusions downstream of the Sh *ble* gene also resulted in truncated transcripts ending in the 5'-parts of these heterologous genes. Based on analysis of terminator regions of *S. commune* and the presented results, it is suggested that AT-rich sequences in heterologous genes are involved in generating these truncated transcripts thereby preventing expression of these genes in *S. commune.*

140. The molecular cloning and expression of laccases from the basidiomycete *Coprinus cinereus*.

Debbie S. Yaver, Sakari Kauppinen, Feng Xu, Kim Brown, Beth Nelson, Torben Halkier, Stephen Brown, Elizabeht Golightly, Sheryl Sandhal, and Palle Schneider, Novo Nordisk Biotech, Davis, CA and Novo Nordisk A/S, Denmark.

A laccase has been purified from the extracellular medium of a culture of the basidiomycete *Coprinus cinereus.* The protein is dimeric, comprised of two subunits of 63 kDa as determined by SDS-PAGE and ultra-filtration. On isoelectric focusing gels, two major bands with pIs of 3.7 and 4.0 are observed. The purified laccase has pH optima of 6 to 7 and 4 with syringaldazine and ABTS as substrates, respectively. Three laccase genes, *lcc1, lcc2* and *lcc3,* have been cloned

from *C. cinereus*. The nucleotide sequence of all 3 genes have been determined. Based on a comparison of the predicted proteins and the protein sequences determined from internal peptides of the purified enzyme, *lcc1* encodes for the purified enzyme. After the predicted signal sequence cleavage the mature proteins are 521, 499 and 501 for *lcc1, lcc2* and *lcc3*, respectively. Both partial cDNAs and full-length genomic clones have been isolated for all 3 genes, and based on the nucleotide sequences of the cDNA and genomic clones all 3 genes contain several introns. The *lcc1* gene contains 7 introns while both *lcc2* and *lcc3* contain 13 introns. The predicted mature proteins share identity to one another ranging from a low of 58% to a high of 80%. The predicted mature laccases also share identities with other fungal laccases. Interestingly, the predicted mature *lcc1* protein when compared to other fungal laccases contains a 23 amino acid C-terminal extension which is rich in arginine and lysine suggesting there maybe some C-terminal processing that occurs during its biosynthesis. The Lcc1 protein has been expressed in *Aspergillus oryzae*.

Abstracts from the Friday March 21 Poster session (Posters II)

Mating types

1. Mating system of *Glomerella cingulata.*

Cindy R. Cisar and David O. TeBeest, Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701.

Heterothallic filamentous ascomycetes typically have a one locus, two allele (idiomorph) mating system. In this study five heterothallic strains of *Glomerella cingulata (*anamorph: *Colletotrichum gloeosporioides)* from pecan were crossed in all possible combinations. Four of the isolates behaved as if they had a bipolar mating system while one isolate mated with all four of the other isolates suggesting that the mating system of *G. cingulata* is more complex. To further investigate the genetics of this mating system single ascospore progeny were isolated from each of the successful crosses and backcrossed with the original parental strains. In addition, a subset of Fl isolates was crossed with all five isolates from pecan and in all possible combinations with each other. The results indicate that progeny stably inherited the mating pattern of one of the original parents and that in each of the original crosses, a single locus which controls mating segregated 1:1. A model for the mating system of *G. cingulata* consistent with these data will be presented.

2. How nuclei with different mating types can recognize each other in a plurinucleate cell?

Sylvie Arnaise, Evelyne Coppin, Robert Debuchy, Denise Zickler and Marguerite Picard. Institut de Genetique et Microbiologie. URA CNRS 1354. Bdt. 400. F-91405 Orsay cedex, France.

In the heterothallic fungus *Podospora anserina*, fertilization leads to the formation of plurinucleate cells which contain a mixture of parental nuclei of opposite mating type termed *mat*+ and *mat*-. Then *mat*+ and *mat*- nuclei undergo a possible recognition which is followed by the development of specialized dikaryotic hyphae in which each cell contains a mat+ and a *mat*- nucleus. Caryogamy, meiosis and ascospore formation occur in these specialized hyphae.

Data indicates that there is indeed an intemuclear recognition process which is controled by the single *mat*+ gene and two of the three *mat*- genes. It also indicates that these mating-type genes, which encode transcription factors, have a nucleus restricted expression. We propose that this property allows the transcription factors to control the expression of specific markers on *mat*+ and *mat*- nuclei. These specific nuclear markers would be recognized by the cytoskeleton which would help to associate compatible nuclei during the recognition process, prior to their migration into the specialized hyphae.

We have demonstrated that one of the three *mat-* genes is not a *bonafide* mating-type gene, because it may be present in either or both parental nuclei without altering the progeny. Several lines of evidence suggest that this gene is required after the intemuclear recognition process, when the specialized dikaryotic hyphae start their development.

3. Expression of the mating-type genes in *Podospora anserina.*

Evelyne Coppin and Robert Debuchy. Institut de Genetique et Microbiologie. URA CNRS 1354. Bat. 400. F-91405 Orsay cedex, France.

The heterothallic fungus *Podospora anserina* has two mating-type alleles termed *mat+* and *mat-*. The *mat+* sequence contains one gene, *FPR1* while *mat-* contains three genes : *FMR1, SMR1* and *SMR2. FPR1* and *FMR1* are required for fertilization which is followed by mitotic divisions of the two parental nuclei inside the female organ. This leads to the formation of plurinucleate cells. *FPR1, FMR1* and *SMR2* control the recognition between nuclei of opposite mating type inside the syncytium. Then, pairs of compatible nuclei migrate into specialized hyphae, of which initial development requires *SMR1*.

Mature transcripts of the mating-type genes are detectable only in the fertilized female organ. We supposed that ectopic expression of *FMR1, SMR2* and *FPR1* would be deleterious to *P. anserina* in inducing untimely nuclear recognition. However, fusion of the mat gene coding sequences to the *gpd* promoter of *Aspergillus nidulans* does not impair their function nor have deleterious effect on *P. anserina.* Further characterization of vegetative expression of the *mat* genes by fusing their 5'untranslated region to a reporter gene shows that *FPR1 , FMR1* and *SMR2* are transcribed constitutively. The absence of their mature transcripts in the mycelium indicates that either they are degraded or the primary transcripts are not spliced at this stage. *SMR1* is the only mat gene specifically transcribed in the fruiting body.

The hypothesis of deleterious effect of ectopic expression of *FPR1, FMR1* and *SMR2* could not be tested with the help of a constitutive promoter because it does not overcome the post-transcriptional control of these genes.

4. Cloning of a putative mating-type gene from the Discomycete fungus *Tapesia yallundae*.

Paul S. Dyer[1], Paul Bowyer[2], John A. Lucas[2], John F. Peberdy[1]. 1University of Nottingham, [2]IACR Long Ashton, United Kingdom.

The Discomycete species *Tapesia yallundae,* causal agent of eyespot disease of cereals, exhibits a two-allele heterothallic mating system. Work is in progress to clone the mating-type genes (MAT1-1, MAT1-2) from *T. yallundae* for use in developing molecular markers to determine the mating-type of field isolates of the pathogen. A PCR-based strategy has been adopted using degenerate primers designed to amplify a partially conserved 270 - 280 bp region of the MAT1-2

locus from other Ascomycete species (*N. crassa*, *P. anserina*, *C. heterostrophus*, *S. pombe*). These primers were used successfully to amplify a 280bp fragment from one mating-type (MAT1-1) of *T. yallundae* which was cloned and sequenced. It contains a characteristic HMG-box motif and exhibits 35-40% amino acid homology to other Ascomycete MAT1-2 sequences in the partially conserved regions of the gene. The PCR fragment was used to probe Southern blots of genomic DNA from MAT1-1 isolates of *T. yallundae* and a single strongly hybridising band was detected. However, a similar signal was also detected in blots of MAT1-2 genomic DNA, indicating the presence of similar sequences in MAT1-2 isolates. Genomic libraries of *T. yallundae* MAT1-1 and MAT1-2 isolates have been constructed in lambda Zap and are now being screened with the PCR fragment to isolate the entire gene.

5. *Neurospora crassa* mating type: evidence for activation and repression by mating-type products.

Adlane V.B. Ferreira[1], Robert L. Metzenberg[2] and N. Louise Glass[1]. [1] Dept. of Botany, Biotechnology Lab, Univ. British Columbia, Vancouver, BC and [2]Dept. Biol. Sciences, Stanford University, Stanford, CA.

The *Neurospora crassa mt A* idiomorph contains three genes *mt A-1, mt A* 2 and *mt A-3*, that have amino acid sequence characteristics of transcriptional factors. *mt* A-1 controls mating identity and mating-type associated heterokaryon incompatibility. Mutations in *mt A-2 and mt A-3* genes were identified in a previously isolated A RIP mutant which is affected in post fertilisation functions. We have isolated specific mt A-2 and *mt A-3* mutants using Repeat Induced Point mutation (RIP). These mutants (A-2 ^{RIP} and A-3 ^{RIP}) are able to mate and produce abundant ascospores, in contrast to the A-2/A-3^{RIP} mutant. Heterokaryon tests showed that *mt* A-2 and *mt A-3* do not affect heterokaryon incompatibility. Northern analyses of genes expressed during sexual development (*sdv*) were performed using strains mutated in the *mt* A and *mt a* genes and a strain in which the mating-type sequences were deleted. Our results confirm that *mt A-1* acts as an activator and indicate that *mt A-2* acts as a repressor of most genes analysed. Some genes are expressed in the deletion strain suggesting that repression is necessary for regulation. The mating-type products may form transcriptional complexes to specifically regulate several genes. Identification of target genes of the mating-type products is the first step towards understanding gene interactions and how mating type controls sexual development in *N. crassa*.

6. Clues to the molecular basis of mutant B2-mating types of *Schizophyllum commune.*

Thomas J. Fowler,* Michael F. Mitton,* Natasha Motchoulskaia, * Lisa J. Vaillancourt[1] and Carlene A. Raper*. *University of Vermont and [1]University of Kentucky.

Previous molecular work on the *Schizophyllum commune* B mating-type locus shows that the locus is complex, encoding several pheromones and at least one pheromone-receptor. In the mating process, pheromones facilitate the donation of migrating nuclei from an individual to a compatible mate while the pheromone-receptor regulates the acceptance of migrating nuclei from a mate. Normally, each B specificity is self incompatible. Clones containing wild type B2 mating-type genes were isolated and subclones were derived that confer either B2 pheromone or

pheromone-receptor activity. Mutations within B2 yield a variety of phenotypes from constitutively self-activating to loss of some or all B2 activities. We are using functional assays and DNA sequencing to define the lesions. We hypothesize that B2(l), a mutant with constitutively self-activating B function, is the result of an altered gene that produces a pheromone able to stimulate the resident B2 pheromone-receptor. Mutation of the B2(l) strain resulted in a self-incompatible strain able to donate migrating nuclei to other specificities including its grand progenitor, B2, but unable to accept migrating nuclei from test mates. We suggest that this secondary mutant retains the primary gain of function mutation but has lost B2 pheromonereceptor function. We tested this hypothesis using the secondary mutant as a transformation recipient for a subclone from the B2 wild type locus that has pheromone-receptor activity. This resulted in constitutively self-activated transformants, confirming that pheromone-receptor activity was missing from the secondary mutant. Other mutants are being examined similarly.

7. Cloning and analysis of the mating-type genes from the homothallic ascomycete *Sordaria macrospora.*

Stefanie Pöggeler, Siegfried Risch, Heinz Dieter Osiewacz*, Ulrich Kuck. Lehrstuhl fur Allgemeine Botanik, RuhrUniversitat, 44780 Bochum, Germany; *AK Entwicklungsbiologie, J.-W.-GoetheUniversitat, Marie-Curie-Str. 9, 60439 Frankfurt, Germany

In the homothallic ascomycete *Sordaria macrospora* (Sordariaceae) a single ascospore gives rise to hyphae that are able to enter the sexual pathway and to produce fruiting bodies (perithecia) that enclose the meiotic ascospore progeny. To better understand the molecular basis of homothallism and to elucidate the role of mating-products during fruting body development, we cloned and sequenced the mating-type locus of *Sordaria macrospora.* In the *S. macrospora* mating-type region we were able to identify four open reading frames called Smta-1, SmtA-1, SmtA-2 and SmtA-3. Comparison of the *Sordaria* mating type locus to the functional regions of the mating-type idiomorphs from the heterothallic ascomycetes *Neurospora crassa* and *Podospora anserina* revealed that sequences from both idiomorphs (A/a and mat-/mat+, respectively) are closely linked in *S. macrospora.* For further investigation of the functional conservation of the *Sordaria* mating-type genes we transformed cosmid clones containing the mating-type locus of *S. macrospora* into mat- and mat+ strains of the closeley related heterothallic fungus *P. anserina.* The *Sordaria* mating-type genes were partially able to complement fruiting body formation in both mat- and mat+ strains of *P. anserina.*

8. Multiple *B* mating types generated by three sets of multiallelic genes.

John R. Halsall, Pushpalata T. Chaure, Ian F. Connerton and Loma A. Casselton, University of Oxford, Oxford, UK.

Pheromone signalling plays an essential role during and after mating in mushroom *Coprinus cinereus.* The genes that encode the pheromones and their cognate receptors are sequestered in the *B* mating type locus. Two asexual monokaryons must have different versions of the *B* locus to be compatible mating partners and thus generate the fertile dikaryon. Remarkably there are a

predicted 79 versions of the *B* locus. By cloning and sequencing two loci, *B6* and *B42*, w show that multiple *B* mating specificities are derived from three sets of paralogous genes, all of which are multiallelic. Each set of genes encodes a receptor and two pheromones and any one of these genes is effective in altering *B* mating specificity if introduced by transformation into a host monokaryon that has a different allele of the gene. Judged by transformation, *B6* and the uncloned *B3* locus have different alleles of all three sets of genes whereas *B42* shares one set with *B6* and one set with *B3.* We show that there is insufficient DNA homology between different allelic versions of each gene and immediate flanking sequences to permit cross-hybridization. This enabled us to use Southern analyses to demonstrate that five other uncloned *B* loci share at least one set of genes with *B42* or *B6.* We will compare sequences of allelic and non-allelic genes and describe experiments designed to determine how this large family of receptor proteins can be so specific in ligand recognition.

9. Targets for the bE and bW homeodomain proteins in *Ustilago maydis*.

Tina Romeis, Regine Kahmann and Jorg Kamper, Institut fur Genetik, Munchen, Germany.

In the phytopathogenic fungus *Ustilago maydis* the multiallelic *b* mating-type locus controls sexual and pathogenic development. The *b* locus encodes the two unrelated homeodomain proteins bE and bW. The bE and bW proteins form heterodimers via their highly polymorphic N-terminal regions (variable domains) but only when they originate from different alleles. The heterodimer is thought to act as a transcriptional regulator. We have constructed different single chain fusions in which the bE protein is linked via a flexible kink region to the bW protein (bE-k-bW). These single chain *b*-fusions are able to substitute for the active bE/bW heterodimer. Our data demonstrate that allele-specific dimerization via the variable domains is non longer prerequisite for activity if bE and bW proteins are fused; however, both homeodomains are needed for function. We were able to show specific binding of a truncated bE/bW fusion protein to the promoter regions of two genes in the a locus that had previously been shown genetically to be regulated by the bE/bW heterodimer. The binding site for the *b* fusion protein was determined using DNAse footprint and methylation interference techniques. Thus, the first targets for the complex of two homeodomain proteins in a Basidiomycete have been identified.

10. Molecular analysis of sexual morphogenesis in *Pyrenopeziza brassicae.*

Gurjeet Singh & Alison M. Ashby. Department of Plant Sciences, University of Cambridge, UK.

Pyrenopeziza brassicae is a hemibiotrophic fungal pathogen of oilseed rape and other brassicas. It is heterothallic, having two mating types designated MAT1-1 and MAT1-2. Sexual reproduction involves a complex yet co-ordinated pathway of development involving early interactions of the two mating types.

Physiological studies have established the involvement of a potential morphogen, termed sex factor (SF) in controlling development in *P. brassicae.* SF is a lipoidal component produced

during sexual development which has the capacity to promote fruiting body formation and inhibit asexual development.

Protein profiles of single mating type isolates induced with SF and individually picked fertile apothecia revealed the presence of a major protein termed sex factor induced (SFI 1) protein which was absent in asexually reproducing cultures. SFI 1 has been purified and internal amino acid sequence used to generate oligonucletide promers which generated a 700 bp PCR product against *P. brassicae* genomic DNA. When the 700 bp PCR product was used as a probe against genomic DNA of the two mating types of *P. brassicae* a different banding pattern was observed, indicating a physical linkage between the MAT loci and SFI 1 genes.

Cloning of the MAT loci from P. *brassicae* has involved heterologous probing using DNA probes from *Neurospora crassa* and *Tapesia yallundae*.

Current progress on cloning the MAT loci as well as cloning and further characterisation of the SFI 1 gene will be described.

11. Y and Z A mating-type proteins of *Schizophyllum commune* bind in all combinations *in vitro.*

Robert C. Ullrich, Yasuhiko Asada, Changli Yue, Jian Wu, Guang-Ping Shen and Charles P. Novotny, University of Vermont, Burlington.

The A locus regulates sexual development via two proteins, Y and Z. Each A mating type encodes a unique Y and a unique Z protein. We have expressed two isoforms of Y (Y4 and Y5) and two isoforms of Z (Z4 and Z5) as GST and 6HIS fusion proteins. These proteins have been used for affinity chromatography assays of protein binding. Each full-length Y or Z protein binds *in vitro* to itself and other Y or Z proteins regardless of the A mating type from which they are encoded (i.e., mating-type independent binding). Use of partial length polypeptides identified mating-type dependent binding. Mating-type dependent binding is observed only between Y and Z proteins encoded from different A mating types (e.g., Y4+Z5 or Y5+Z4). N-terminal regions of Y and Z proteins are responsible for mating-type dependent binding; the determinants of mating-type independent binding are encoded elsewhere within the proteins. Deletion analysis shows that the Y4 specificity domain (the region conferring recognition uniqueness to the Y4 isoform) is essential for mating-type dependent binding.

12. The specificity determinant of the Y mating-type proteins is also essential for Y-Z protein binding.

Changli Yue, Charles P. Novotny, and Robert C. Ullrich, University of Vermont.

In Schizophyllum, tightly linked Y and Z mating-type genes do not promote development in the combinations present in haploid strains (i.e., *self* combinations). When the *Y* and *Z* genes from two different mating types are brought together by the fusion of two haploid cells, the Y and Z proteins from different mating types recognize one another as *nonself,* form a complex and

activate development. We have made chimeric genes between *Y1, Y3, Y4* and *Y5* and examined their mating-type specificities by transformation and mating tests. These studies show that the specificity of Y protein recognized by Z protein is encoded within a short region of N-terminal amino acids. The critical region is not precisely the same in each Y protein and in each Y-Z protein interaction. For Y3 protein compared to Y4 protein the critical residues are in an N-terminal region of 56 amino acids (residues 17-72), with 40% identity and 65% similarity. Two-hybrid studies show that: (a) the first 144 amino acids of Y4 protein are sufficient to bind Z3 and Z5 proteins, but not Z4 protein, and (b) proteins deleted of the Y4 specificity region do not bind Z3, Z4 or Z5 protein. Thus the specificity determinant of the Y protein is essential for protein-protein recognition, Y-Z protein binding and mating activity.

13. Characterization of mating type genes in homothallic ascomycetes.

Sung Hwan Yuri, O.C. Yoder and B. Gillian Turgeon, Cornell Univ. Ithaca, NY.

Members of the genus *Cochliobolus* have different reproductive life styles, i.e., homothallic (self fertile) vs. heterothallic (self sterile) or sexual vs. asexual. We are exploring evolution of these strategies and have identified mating type genes *(MAT)* in heterothallic, homothallic, and asexual species. Here we describe *MAT* organization in a homothallic (C *homomorphus),* in a related heterothaflic (C *heterostrophus)* and in a distantly related homothallic *(Mycosphaerella zeae-maydis)* species. MAT genes are arranged in tandem in both homothallics, but differ strikingly in organization. In *C. homomorphus,* the counterparts of the *C. heterostrophus MAT* genes are fused into a single reading frame [5'-(*MAT-2/MAT-1*)- 3']. This finding is unprecedented and potentially useful since this novel gene organization may help unravel the genetic mechanism by which this homothallic species arose. Furthermore, to date, homothallic filamentous ascomycetes have been found to have either a single *MAT* gene, as in certain *Neurospora* spp, or opposite MAT genes as in *M. zeae-maydis* and most members of the Sordariaceae. The *C. homomorphus MAT* ORF encodes all of the *C. heterostrophus MAT-2* homolog except for 9 amino acids at the 3' end and 8 amino acids at the 5' end of the MAT-1 homolog. *M. zeae-maydis* has complete versions of the *C. heterostrophus* counterparts, however the *MAT* genes are separated by about 1 kb of unique, noncoding DNA; gene order is reversed compared to *C. homomorphus* [5'-(*MAT-1*)-(*MAT-2*)- 3']. A second ORF in the flanking DNA is conserved in both homothallic and heterothallic species. The remainder of the DNA flanking the two homothallic *MAT* genes is unique. These variable *MAT* flanking regions may be determinants of homothallism. Heterologous expression of homothallic *MAT* genes in a heterothallic genetic background and vice versa is underway.

14. Perturbation of expression of sexual pheromone precursor genes of the ascomycete *Cryphonectria parasitica* by a hypovirus.

Lei Zhang, Patricia McCabe, Pam Kazmierczak, and Neal K. Van Alfen. Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas.

There are a total of three pheromone precursor genes in both mating types of *C. parasitica.* In Mat I strains, only the *Mfl-1* gene is expressed while in Mat2 strains the *Mf2-1* and *Mf2-2* genes are expressed. The putative coding regions of *Mf2-1* and *Mf2-2* are typical of the prenylated pheromones that have been described from yeasts and other filamentous fungi. The gene *Mfl-1* has an ORF that encodes a protein of 530 amino acids. Within this ORF are seven repeats of the same decapeptide. Surrounding this decapeptide are the signals for post translational processing that have previously been described in the yeasts S. *cerevisiae* and *S. pombe.* Among these is the KR dipeptide sequence that directs cleavage by the Kex2p endoprotease. These three pheromone precursor genes are all down-regulated in strains infected with the hypovirus CHV I. The conserved sequences for Kex2p endoprotease maturation found in *Mfl-I* have also been identified in the leader peptide sequences of two other genes down-regulated by CHV1. One of these Kex2p-processed proteins that is down-regulated in virus infected strains has been found in host vesicles used by the virus for replication. We postulate that the virus perturbs expression of these genes by its replication on transport vesicles important for the processing of these proteins.

Fungal Genomes

15. Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins.

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There are at least ten plant diseases caused by *Alternaria* species in which host-specific toxins (HSTS) are responsible for fungal pathogenicity. Of these HST producers, seven are considered distinct pathotypes of the species called A. *alternata.* Inter- and intraspecific variation among *Alternaria* taxa, including HST producers, were determined by electrophoretic karyotype using pulsed-field gel electrophoresis. The seven pathotypes of A. *alternata* and nonpathogenic *A. alternata* had 9 to 11 chromosomal bands with estimated sizes ranging from 0.6 to 6.5 Mb. In contrast, *Altemaria* species that are morphologically distinct from *A. altemata* had 8 to 10 bands with sizes between 0.9 to 6.4 Mb; They could be differentiated from *A. alternate* on the basis of chromosome size polymorphisms. Comparison of karyotypes following Southern analysis using rDNA as a probe also revealed polymorphisms between *A. alternata* and other *Alternaria* species. The results were confirmed by DAPI staining and fluorescence in situ hybridization with the rDNA probe for chromosomes prepared by the germ tube burst method.

16. Replication mechanisms of circular fungal mitochondrial plasmids.

Dipnath Baidyaroy and Helmut Bertrand, Mchigan State University.

Fungal mitochondrial plasmids can be of three major types : (1) linear with a DNA polymerase and/or an RNA polymerase, (2) circular with a gene coding for a reverse transcriptase, and (3) circular with a novel DNA polymerase gene. These plasmids are generally present in high copy

numbers, have mitochondrial codon usage and are of unknown physiological significance. The mechanism of their replication remains to be understood. We are trying to characterize the replication mechanisms of the two groups of circular plasmids by analyzing replication intermediates using two-dimensional gel electrophoresis. So far, we have examined the Mauriceville plasmid (Gr. 2) from *Neurospora crassa* and the pCRYI (Gr. 3) from *Cryphonectria parasitica.* Contrary to previous studies (Maleszka, 1992, Biochem. Biophy. Res. Comm. 186:1669-1673), we found that the plasmids are present *in vivo* as multimeric circles instead of linear molecules of heterogenous sizes. In addition, we have obtained data indicating that the Mauriceville plasmid, despite containing a functional reverse transcriptase gene, replicates predominantly by a rolling circle mechanism which effectively generates numerous multimeric forms, some as big as octamers (~28.8 kb), of the unit length plasmid. On the other hand, the pCRYI plasmid, which is assumed to replicate by the action of its own DNA polymerase, seems to have a standard bidirectional replication with the predominant form of the plasmid being the circular monomer. Supported by USDA grant 95-37303-1785,

17. *Trichoderma reesei* sequences that enhance transformation frequency bind to the nuclear matrix.

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Australia, [4]VTT Biotechnology and Food Research, Espoo, Finland.

Three DNA fragments (*trs*l, 2 and 3) were isolated from the *T. reesei* genome by their ability to promote autonomous replication of plasmids in *S.cerevisiae.* The presence of the *trs* elements in transforming plasmids enhanced the frequency of transformation of *T. reesei* up to 5fold over plasmids without a *trs.* The *trs* elements did not promote autonomous replication of plasmids in *T. reesei.* The *trs* elements bound specifically to the isolated *T. reesei* nuclear matrix both *in vitro* and *in vivo* indicating that they are matrix attachment regions (MARs). The *trs* elements were sequenced and shown to contain 70%, 86% and 73% A+T over 2.9, 0.8 and 3.7kb respectively for *trs* 1, 2 and 3. *trs* 1 and *trs*3 were shown to be single copy in the *T. reesei* genome. A sequence previously isolated from *A.nidulans* that promotes autonomous replication of plasmids in *S.cerevisiae (ansl)* was also shown to be a MAR. Each *trs* contained sequence motifs commonly ascribed to MARs from other eukaryotes although we showed that the motifs were just as common in a series of randomly generated sequences of identical AT richness.

18. Genomic organization of genetically defined *Pneumocystis* populations.

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Pneumocystis organisms are the cause of a pneumonia in a wide variety of mammals that become immunocompromised. Gene sequence data show they are related to fungi but may represent an independent basal branch in the *Ascomycota.* Surveys of organisms obtained from the lungs of immunosuppressed rats in commercial colonies using CHEF-PFGE and gene sequencing techniques revealed the rats were infected with either of 2 apparent species of *Pneumocystis*, "prototype" or "variant" or a combination of both. Eight different forms of prototype (1 -8) and 2 of variant were defined by electrophoretic karyotyping. Prototype forms produced a total of 12-15 chromosome sized bands that separated between 300-700 kb in prototype forms 1-8 while 14-17 bands were observed in the 2 variant forms within the same size range. We are establishing chromosome numbers by hybridization of telomeric probes to Southern blotted restriction enzyme digested chromosome-sized bands excised from low melt gels (Mills et al. In Molec. Methods in Plant Pathol. Lewis Publishers, 1995) and linkage groups by hybridization of gene probes to Southern blotted karyotypes. At least 1 single copy gene has been localized to 8 of 14 bands in the karyotypes of prototype forms and to 4 bands of variant karyotypes. Gene probes hybridized to bands of similar sizes among the prototype karyotypes, but dramatic size differences were observed between prototype and variant populations, supporting the species distinctions detected by gene sequence comparisons.

19. Organization of the nitrate assimilation gene cluster of *Stagonospora (Septoria) nodorum*.

Simon B. Cutler, R. Neil Cooley and Christopher E. Caten, University of Birmingham, U.K.

The nitrate reductase gene (NIA1) of the phytopathogenic fungus *Stagonospora (Septoria) nodorum* has been cloned from a cosmid library by homologous hybridization with a PCR-generated probe. A 6.7kb fragment carrying the NIA1 gene was subcloned and partially characterized by restriction mapping. Sequencing of the gene indicated a high degree of homology, both at the nucleotide and amino acid levels, with nitrate reductase genes of other filamentous fungi. In addition, sequencing downstream of the NIA1 gene revealed another open reading frame which proved to be the nitrite reductase gene (NIA1) of the fungus. The nitrate and nitrite reductase-encoding genes of *S. nodorum* are contiguous and transcribed in the same direction. Furthermore, both genes contain consensus regulatory signals in the 5' flanking regions thought to be involved in the control of nitrogen metabolism.

The cloned NIA1 gene has been used to develop a gene transfer system based on nitrate assimilation. Stable mutants of *S. nodorum* defective in nitrate reductase activity were isolated by virtue of their resistance to chlorate. These were transformed back to nitrate utilization independently with the *Aspergillus niger niaD* gene and the *S. nodorum* NIA1 gene. Southern analyses revealed that transformation occurred as a result of integration of transforming DNA into the fungal genome. Moreover, it was shown that in many cases integration was targeted to the homologous sequence.

20. Preferentially expressed genes from the sexual stage of *Neurospora crassa*.

Patricia L. Dolan, Donald O. Natvig, Mary Anne Nelson, Department of Biology, University of New Mexico, Albuquerque, NM .

Neurospora crassa, a heterothallic filamentous fungus, undergoes a complex pattern of sexual development to form the perithecium (fruiting body) composed of several kinds of specialized tissue. In the Neurospora Genome Project (NGP) at the University of New Mexico, expressed sequence tags (ESTs) corresponding to three stages of the life cycle of *Neurospora crassa* (conidial, mycelial and perithecial) are being analyzed. Results of this pilot project identifying genes preferentially expressed during the sexual phase are presented. 557 partial complementary DNA (cDNA) sequences for 425 perithecial clones were determined using single-pass sequencing. For 29.5% of the sequences, highly or moderately significant matches to sequences in the NCBI database were detected. Approximately 59.2% of the ESTs correspond to previously unidentified genes. Genes involved in secondary metabolism were found only in mycelial and perithecial tissues. Genes encoding products required for transport were found primarily in mycelial and perithecial tissues. The majority of the genes from all three tissues were involved in metabolism or protein synthesis. In addition, the serial analysis of gene expression (SAGE) technique is being used to generate more extensive and precise information about mRNA abundance at different stages of the Neurospora life cycle.

21. Structural analysis of an unstable chromosome in *Nectria haematococca*.

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Certain isolates of *Nectria haematococca* mating population VI contain a dispensable 1.6 Mb chromosome that is unstable during sexual reproduction. Previous karyotype analysis of genetic crosses suggested that specific regions of this chromosome are particularly susceptible to chromosome breakage (Miao *et al.*, 1991 Science 254: 1773 - 1776). We are testing the hypothesis that repeated sequences contribute to the chromosome's instability. Using a chromosomespecific cosmid library, we created a contiguous cosmid map of the 1.6 Mb chromosome. The map consists of 8 contigs covering 1.3 Mb of the chromosome. We are using PCR and restriction enzyme mapping to order the contigs. Three repeated sequences that are each present in 5 to 7 copies on the chromosome were cloned during the mapping process. One of these repeats is homologous to *Fot1,* a transposable element in *Fusarium oxysporum.* To identify breakpoints and missing fragments, a variety of chromosomal derivatives are being used to probe the chromosome-specific cosmid library. Identified breakpoints will be mapped on a fine scale to determine if they are associated with repeated sequences.

22. Identifying sites of duplication-dependent DNA methylation in *Coprinus cinereus*.

Sylvia A. Frazier and Patricia J. Pukkila, University of North Carolina at Chapel Hill.

CpG methylation is known to be involved in gene inactivation and to be responsible for genomic imprinting for most eukaryotes. We wish to understand the consequences of cytosine

methylation in the fungal system *Coprinus cinereus.* It was shown previously that cytosine methylation can occur as a result of a duplication of the *trp1* gene in the *C. cinereus* genome. Using a genomic sequencing protocol, we have analyzed the *trp1* gene sequence of several clones representing one tetrad known to contain methylated cytosines. This tetrad which has both Trp^+ and Trp^- progeny was used to make a direct comparison of differences in methylation between silenced and unsilenced genes. The objective is to determine if specific methylated cytosines are necessary and sufficient to render the *trp1* gene inactive. Sequence analysis has confirmed that there is site specificity for cytosine methylation, and that this methylation occurs almost exclusively at CpG dinucleotides. Information on the location and extent of methylation in *C. cinereus* may give us clues about how gene silencing occurs in this species and in eukaryotes as a whole.

23. Generation and characterization of selenate-resistant mutants of *Magnaporthe grisea*.

Tyler L. Harp and James C. Correll, University of Arkansas, Fayetteville.

Numerous mutants have previously been recovered and genetically characterized in the fungus *Magnaporthe grisea.* In this study, selenate-resistant (*sel*) mutants of *M. grisea* were generated and characterized. Isolates representing several different MGR586 DNA fingerprint groups of *M. grisea* were grown on either rice bran agar (RBAS), potato dextrose agar, or a defined minimal medium, all amended with 0. 1% sodium selenate. Spontaneous fast-growing sectors were recovered from the restricted colonies after 7 - 28 days. All sectors were then transferred back to RPAS to verify that they were selenate-resistant (*sel*) mutants. All *sel* mutants were further characterized for their ability to utilize sulfur by growing them on NM amended with K_2S0_4 or a reduced sulfur (L-methionine) source. Mutants unable to utilize sulfate-sulfur were designated as sulfate non utilizing (*sul*) mutants. The majority of the sectors recovered were *sul* mutants. Broth growth-rate studies confirmed the inability of *sul* mutants to utilize sulfate sulfur; furthermore, growth of *sul* mutants were comparable to wild-type when given a reduced sulfur source. Generation frequency varied from 0.1 to 1.3 sectors per colony after 28 days on RBAS and mean sector frequency corresponded with MGR586 DNA fingerprint group. *Sul* mutants were stable and comparable in virulence to the corresponding wild type isolates on rice in greenhouse pathogenicity tests.

24. Analysis of expressed sequence tags (ESTS) corresponding to conidial, mycelial, and sexual stages of *Neurospora crassa*.

Seogchan Kang, Mary Anne Nelson, and Donald O. Natvig, Department of Biology, University of New Mexico, Albuquerque, NM.

Analysis of ESTs has been widely applied in many model organisms as a rapid method of gene discovery. We have undertaken an analysis of ESTs corresponding to three stages of the life cycle of *Neurospora crassa.* Three unidirectional cDNA libraries were constructed, using mRNA

isolated from germinating conidia, mycelia, and perithecia. Single-pass, partial sequencing of cDNA clones was used to rapidly identify duplicate clones present in the three libraries and to determine the nature of the encoded gene products. 1879 partial cDNA sequences for 1423 clones were determined. Contig analysis allowed the identification of 838 unique ESTs and 156 ESTs present in multiple cDNA clones. For about 33% of the sequences, highly or moderately significant matches to sequences in the NCBI database were detected. Approximately 57% of the ESTs show no similarity to previously identified genes, which was unexpected given the presence of the complete genome sequence of *Saccharomyces cerevisiae* in the database. Our result demonstrates the wealth of genes yet to be discovered, and supports the assertion that sequencing of genes from phylogenctically diverse organisms will yield novel insights into gene structure and function.

25. In vitro reconstruction of the *Neurospora crassa* genome.

H. Kelkar, M.E. Case, S. Covert, J. Griffith, C. Keith, M. Orbach, M. Sachs, and J. Arnold.

Physical maps of fungal genomes are opening up new research areas in genetics. We are building a physical map of the *Neurospora crassa* genome to study its distribution of repeated DNA and to contrast its distribution of repeats with that of the *Aspergillus nidulans* genome. We have expanded the Orbach-Sachs Library of 4800 clones in the Fungal Genetics Stock Center by 10,000 cosmid clones in the cosmid vector, pMOcosX. Average insert size is estimated to be 34 kb +/- 1 kb from EcoRI digestions. A second new cosmid vector has been created called LoristX6h. The cosmid vector Lorist6 was improved by inserting an adaptor (Orbach, 1994, Figure 1, Gene 150: 159-162) at the BamHI cloning site to allow half site fill ins (automatic insert size selection) as well as a hygromycin cassette (Carrol et al., 1994; Fungal Genetics Newsletter 41: 22) from plasmid pCB1004 for fungal transformation. A second cosmid library of 10,000 clones is being made in Lorist6Xh. The seven *N. crassa* chromosomes have been separated into 5 bands on CHEF gels, and the libraries are being probed with intact whole chromosomes corresponding to linkage groups I, V, IV, and III initially. A robot serving as an Intelligient System for Automated Assembly of Chromosomes (I.S.A.A.C.), for use by the fungal genetics community, is being constructed for ordering these libraries. (supported by NSF MCB-9630910 and BIR-9512887)

26. Proposal to sequence the genome of *Aspergillus nidulans*.

Doris M. Kupfer*, Sandra W. Clifton*, Rolf A. Prade, Bruce A. Roe*. *University of Oklahoma, Oklahoma State University.

A.nidulans is an ideal model organism for molecular and genetic studies of filamentous fungi. With the development of methods for rapid and accurate large scale DNA sequencing, the Aspergillus community has proposed undertaking sequencing the *A. nidulans* genome. We have begun three pilot projects as a prelude to the sequencing of the entire genome.

First, the sequence of the 38.8 Kbp cosmid, SW06EO8, from Chromosome VIIII was completed as a feasibility study. Thirteen ORFs were observed. Seven had homology with fungal and other higher eucaryotic genes. Six had no homologs in the public databases. Second, we are creating an Expressed Sequence Tag (EST) database by sequencing the vegetative and asexual *A. nidulans* cDNA library constructed by Rudolfo Aramayo. To date we have isolated over 5000 clones and sequenced 600. This data will be useful for analysis of the future genomic sequences and will be available on our www site, http://www.genome.ou.edu.

Third, sequencing of the 2.9Mbp Chromosome IV will commence upon completion of the EST database. We will shotgun shear and clone the entire chromosome via an approach similar to that used with great success in our laboratory to sequence the genomes of both *Neisseria gonorrhoeae and Streptococcus pyogenes.* Members from an ordered set of *A. nidulans* cosmids will be end sequenced to facilitate ordering of contiguous sequences generated from the random sequencing. At the culmination of the Chromosome IV project, a consortium of labs from the Aspergillus community will sequence the remaining seven chromosomes.

27. Optional introns in mitochondrial DNA of *Podospora anserina* are the primary source of observed polymorphisms.

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We have demonstrated that the significant differences in mitochondrial genome size among six races (M, T, U, B, W and Y) of *Podospora anserina* are primarily due to the presence and/or absence of introns, including four introns not previously known to be optional. Information from physical mapping of races M and T was used to identify regions likely to contain insertions or deletions, which were then characterized using PCR and sequence analysis. Newly confirmed optional introns are: the single intron in ATPase subunit 6 (ATPase6), the fifth intron of NADH dehydrogenase subunit 3 (ND3i5), the first intron of the large ribosomal RNA (r1i1), and the fifth intron of cytochrome oxidase subunit I (COIi5). In addition, an isolate resulting from a cross between races A and s (provided by L. Belcour), was shown to contain an novel intron pattern, resulting from a probable mobility event during mating (L. Belcour). We have also discovered that race M apparently exists in two forms.

These results bring to eight (including races A and s) the number of races characterized by mitochondrial intron content with a total of six known optional introns. Seven of the eight races contain a distinct set of introns, thus providing a reliable means for identification and comparison.

28. Multiple introns in the mitochondrial small subunit rDNA Gene of *Cryphonectria parasitica.*

Denise B. Searles, Claudia B. Monteiro-Vitorell and Helmut Bertrand, Michigan State University.

Ubiquity and conservation of the small subunit ribosomal DNA (SrDNA) have led to its extensive use in phylogeneties. However, this gene shows considerable variability in length and sequence composition, due in part to insertions. We are sequencing the mitochondrial SrDNA of *C. parasitica,* an ascomycetous fungus which causes chestnut blight in the American chestnut *(Castanea dentata).* Two introns have been found to date. The first is a 2.2 kb Group I intron, containing a 285 amino acid open reading frame (ORF). 1.8kb region of a second intron has also been sequenced and contains an ORF of 501 amino acids. Both ORFs contain a pair of LAGLI-DADG sequences, indicating that they encode maturases,

Insertions have been found in at least 17 positions in SrDNA sequences, and many fungi have multiple inserts in the nuclear SrDNA. However, ther have been few reported introns in mitochondrial SrDNA and this is the fir report of multiple introns in mitochondrial SrDNA. Further information regarding sequence analysis and secondary structure will be provided.

29. The *Candida albicans* genome project- First steps.

EJA Tait, MC Simon, S. King, AJP Brown, NAR Gow, DJ Shaw. University of Aberdeen.

A cosmid library of approximately 10-fold coverage has been constructed from *Candida albicans* strain 1161 and will serve as a starting point for physically mapping the entire *C. albicans* genome. The breadth and depth of genome coverage has been confirmed and investigations into the stability of the clones are described. DNA has been prepared from each clone and fingerprints generated busing a combination of restriction digestion and fluorescent labelling techniques. By alignment of these fingerprints sets of contibuous clones can be built up and then assigned to particular chromosomes. This will be folllowed by assignment of genes to individual clones thereby positioning these markers on the *C. albicans* genome. The computer software and data processing required to build the contigs are discussed.

30. Genome plasticity in *Mycosphaerella graminicola (*anamorph *Septoria tritici).*

Cees Waalwijk, Jos G.P. Koeken and Gert H.J. Kema, DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands.

Mycosphaerella graminicola is a bipolar heterothallic plant pathogenic ascomycete. A recently described crossing procedure enables genome analysis of this fungus showing specificity for Triticum species and cultivars. A molecular tetrad analysis enabled the identification of the pairs of ascospores within a single ascus. All isolates within this ascus contain a 800 bp RAPD marker produced with primer OPB-6. However, analysis of a progeny derived from a cross between isolates IP094265 and IP094266 revealed that the 800 hp RAPD markers inherited independently. Sequence analysis of both 800 hp fragments showed 91% identity. We hypothesize that both fragments inherited independently as a result of a translocation event, particularly since no segregation for the 800 bp fragment was observed in a cross between isolate IP094265 and isolate EP094268, also from the same ascus.

Pulsed field gel electrophoresis (PFGE) of the ascospore isolates derived from a single ascus, and RFLP analyses of these isolates and progenies of crosses between them were used to test this hypothesis and to study the inheritance of the supposed translocation. PFGE showed polymorphism among the monoascospore isolates, supporting the translocation hypothesis. The 800 hp fragment hybridized to multiple sequences on genomic blots. On PFGE blots these hybridization signals appeared to be evenly distributed among the chromosomes. Southern analysis with truncated sequences of the 800 bp fragment revealed less complex but still polymorphic multilocus haplotypes. The inheritance of these haplotypes and their relationship with the hypothesized translocation will be discussed.

31. Analysis of nuclear and mitochondrial DNA RFLPs of *Colletotrichum orbiculare* and allied species.

Lusike A. Wasilwa, James C. Correll, Doug D. Rhoads, and Teddy E. Morelock. Depts. of Plant Pathology, Biological Sciences and Horticulture. University of Arkansas, Fayetteville.

We have been using genetic and molecular markers to examine population diversity in several economically important *Colletotrichum* species. In this study, mtDNA and nuDNA RFLPs were analyzed from a worldwide collection of cucurbit isolates of *C. orbiculate* and allied *Colletotrichum* species. All isolates of *C. orbiculare* that were pathogenic on cucurbits belong to a single mtDNA haplotype. Nonpathogenic cocklebur isolates from Australia had a similar mtDNA haplotype, but could be distinguished by one additional restriction site. Several isolates of *C. lindemuthianum* and C *trifolii* had a similar mtDNA haplotype to pathogenic isolates of *C. orbiculate. C. acutatum, C. capsici, C. cingulata, C. gloesporiodes, C. graininicola,* and *C. truncatum,* each had distinct mtDNA haplotypes. Multiple mtDNA haplotypes were identified among isolates of *C. magna*, *Glomerella cingulata*, and *Colletotrichum* spp. recovered from cucurbit fruit, all of which were nonpathogenic on cucurbits. There was a correspondence between vegetative compatibility group and nuDNA RFLP haplotype among pathogenic races of *C. orbiculare.*

32. A novel approach to identify centromeric DNA of the filamentous fungal pathogen *Ashbya gossypii.*

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Ashbya gossypii has been isolated as a pathogen of cotton and citrus fruits. Additionally it has been shown to be a potent overproducer of riboflavin. Recent developments on its molecular genetics include a transformation system based on a dominant as well as on auxotrophic marker genes (1,2,3,4) and the demonstration of efficient homologous recombination in this fungus (5). In addition *S. cerevisiae* ARS-elements serving as origins of replication in yeast allowed the introduction of freely replicating plasmids in *A. gossypii* (1). These plasmids could be stably

maintained within a mycelium only upon selective pressure. *S. cerevisiae* centromeric (CEN) DNA positioned closely to the ARS-element rather decreased the stabilty of freely replicating plasmids as could be visualized under non-selective conditions. The identification of *A. gossypii* CEN DNA was fostered by the concurrent effort to gain insight into protein coding sequences of this fungus. This approach has been based on sequence analyses of a plasmid library containing genomic inserts of a size range from 3.5 kb to 5.5 kb. Up to now 650 plasmids were investigated by single-read sequencing 400-700 bases from both ends. Subsequent bioinformatics com-pared these single-read sequences (SRS) to the entries of available databases. At least 25 % of the open reading frames deduced from pairs of SRS derived from single plasmids displayed the same gene order as the homologous genes found in *S. cerevisiae* (synteny). Out of this data two pairs of SRS could be recognized that share significant homology and colinearity to genes located on both sides of cen-tromeric regions in *S. cerevisiae*. We have initiated a functional as well as structural analysis of these presumptive centromeric regions of *A. gossypii* .

(1) Wright and Philippsen, 1991 Gene 109: 99-105 (2) Steiner and Philippsen, 1994 MGG 242: 263-271

(3) Altmann-Jhl and Philippsen, 1996 MGG 250: 69-80 (4) Mohr, 1995 PhD-thesis, University of Basel, Switzerland

(5) Steiner *et al.*, 1995 Genetics 140: 973-987

33. Physical mapping of a developmental mutant deficient in appressorium formation in *Magnaporthe grisea* using BAC clones.

Heng Zhu and Ralph A. Dean, Clemson University, SC. 29634-0377.

Magnaporthe grisea (Hebert) Barr. causes rice blast, one of the most devastating diseases on rice *(Oryza sativa)* world wide. Previously, it was shown that an appressorium deficient mutant 243-7 was under single gene control, namely APPI. The gene was mapped to a central region of chromosome 2 flanked by several RFLP markers within 0.5 cM of the gene. To physically map this region, a BAC library of *M. grisea* was constructed containing 9216 clones with an average insert size of 120 kb, which represents greater than 25 genome equivalents. Four single-copy DNA probes were used to screen 4608 clones in the library and at least 6 overlapping BAC clones were found with each probe. These data indicate that the BAC library should be suitable for map-based cloning of *M. grisea* genes and physical mapping of the genome. A BAC contig covering the APP1 region has been constructed.

Fungal Transposons

34. Structure and distribution of Pcel elements in the white rot basidiomyce *Phanerochaete chrysosporium*.

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Phanerochaete chrysosporium strain BKM-F-1767 was previously shown to harbor a 1747 bp insertional mutation within lignin peroxidase allele *lipI2*. Designated Pcel, the element transcriptionally inactivated *lipI2*, but not *lipI1*. The mutant allele is inherited in a 1:1 ratio among haploid progeny. A second element derived from BKM-F-1767 has now been characterized. The nucleotide sequence of Pce2 is >99% identical to Pce 1. The elements are distantly linked showing approximately 85% cosegregation. Chef gel blots localized both elements to the same allelic homologue. Nucleotide sequence of regions adjacent to Pce2 showed no similarities with any known fungal genes. Ongoing studies with geographically diverse *P. chrysosporium* strains indicate wide distribution of elements nearly identical to Pce I /Pce2.

35. Evidence for horizontal transmission of the *Fot1* element between *Fusarium* species and discovery of a RIP-like process.

Marie-josee Daboussi and Thierry Langin, Universite Paris-Sud, Orsay, France.

To understand the evolutionary origin of the well-characterized *Fot1* mobile element identified in the *Fusarium oxysporum* species, we have undertaken an extensive survey of the *Fusarium* genus using Southern blot hybridization and Polymerase chain reaction (PCR) amplification to determine its distribution. The results showed that *Fotl*-homologous sequences widely distributed in the *F. oxysporum* species, are absent in the most closely related *F. moniliforme* species but present in some strains belonging to the distant species *F. solani* . The discontinuity in the distribution of the *Fot1* element together with the remarkably close similarity between *Fotl* sequences from distant species supports the conclusion that *Fot1* elements have been transferred horizontally among these species in the recent past. However, the fate of a *Fot1* copy in these heterologous species depends on the sexual state of the strain it entered. In the homothallic strain, *Neocosmospora* spp, analysis of the nucleotide divergence indicated that *Fot1* copies have probably been submitted to a RIP process. This result addresses the question of the occurrence of such phenomenon across the *Fusarium* genus.

36. *F. oxysporum* transposons as tools for the isolation of fungal genes.

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We have investigated the activity of the *impala* and *Fotl* transposons to develop a method for transposon mutagenesis in *Fusarium oxysporum.* A number of findings about their transposition are pertinent when designing gene tagging experiments. In general, excision is followed by integration at a new site offering the opportunity to screen thousands of strains for mutations in genes involved in many aspects of pathogenesis. Non-pathogenic mutants have already been

obtained and are under characterization. In order to use these elements for transposon tagging in different strains, experiments were designed to identify autonomous copies. Therefore, we employed a phenotypic assay for excision of the elements from the *nia* gene in different genetic backgrounds. Results obtained so far indicate that these copies transpose in different species thus providing a potential gene tagging system for filamentous fungi.

37. Distribution of the *restless* Transposon in Different *Beauveria nivea* strains.

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The *restless* element is an *Ac* like class 11 transposon of 4,097 bp, which was found in the filamentous fungus *Beauveria nivea* (syn. *Tolypocladium inflatum*). *This* strain (ATCC 34921) has been shown previously to carry about fifteen copies of the *restless* element (1). As a first step in the study of the evolution and distribution of this element, three other *B. nivea* strains from type culture collections were chosen for a molecular characterization. The *B. nivea* strains were investigated by Southern hybridization and PCR for the presence of copies of the *restless* element. The molecular analysis of the four strains with similar morphology includes differentiation by RAPD and a partial sequence comparision of the conserved tubulin gene. In contrast to the sequence analysis, which showed a high degree of similarity between all strains, the number of *restless* copies was divergent in all strains investigated. In addition to strain *B. nivea* ATCC 34921 with about fifteen *restless* copies we identified strains with only a single or a partial copy. These copies were further characterized by DNA and cDNA sequencing. The integration sites have been determined by inverse PCR. Finally, we identified a strain, which did not show any sequence homology to the *restless* element.

(1) Kempken F, Kuck U (1996) Mol Cell Biol 16: 6563-6572

38. A retroelement from *Ascobolus immersus* is not subject to "methylation induced premeiotically".

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The first evidence for the presence of transposable elements in mycelial fungi came from classical genetic analysis of two genetic instable stocks (28 and 50) of *Ascobolus immersus* [1]. The existence of transposable elements in mycelial fungi is well established know and a number of transposons have been characterized on the molecular level [e.g. 2, 3], which includes at least one retroelement from *A.immersus* stock 28 [cited in ref 4]. Differential hybridization was employed to identify repetitive DNA sequences from *Ascobolus immersus* stock 50, which has a different system of genetic instability than stock 28 [1]. One stock 50 specific repeated DNA sequence was further analyzed. Preliminary sequence analysis indicates the presence of long terminal repeats flanking the element, which presumably represents a retrotransposon. Most

interestingly, this element does not appear to be subject to "methylation induced premeiotically", a mechanism which is believed to inactivate repeated DNA sequences in *A. immersus [4]*.

References:[1] Nicolas A, Hamza H, Mekki-Berrada A, Kalogeropoulos A, Rossignol J-L (1987) Genetics 116:33-43- [2] Daboussi M-J, Langin T (1994) Genetica 93:49-59; [3] Kempken F, Kuck U (1996) Mol Cell Biol 16:6563-6572; [4] Rossignol JL, Faugeron G (1994) Experientia 50:307-317

39. Regulation of *Restless,* a fungal member of the *hAT*- transposon family.

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In the past years several fungal transposable elements have been identified [1]. We have isolated and characterized *Restless,* a new type of fungal class II transposons from *Tolypocladium inflatum* ATCC34921 (synonym: *Beauveria nivea)* which so far has not been found in any other fungus [2]. It carries short inverted repeats and eight basepair target site duplications, and encodes a large open reading frame which is interrupted by a single intron sequence showing a rare intron consensus sequence. The predicted amino acid sequence deduced from this frame shows significant homology to transposases of the *hAT* transposon family, e.g. the maize *Activator* element. Based on cDNA sequencing, alternate RNA splicing may lead to two different proteins, both encoded by the transposon. These proteins may be involved in regulation of transposition. To proof this hypothesis, proteins were overexpressed in *E. coli* and employed for gel shift and footprint analysis. From these data, a model will be presented for regulation of *restless* transposition.

References: [1] Daboussi M-J., Langin T. (1994) Genetica 93:49-59- [2] Kempken F., Kuck U. (1996) Mol Cell Biol 16:6563-6572

40. Promiscuous template switching by the Mauriceville plasmid reverse transcriptase and its role in senescence. John C. Kennell, Dept. of Biological Sciences, Southern Methodist University, Dallas, TX 75275

Variant forms of the Mauriceville mitochondrial (mt) plasmid of *Neurospora crassa* appear to cause senescence by integrating into mtDNA or by overaccumulating in the mitochondrion. The first step in senescence is the copying of mitochondrial transcripts by the plasmid-encoded reverse transcriptase (RT) and the incorporation of these cDNAs into the circular DNA plasmid. The RT can template switch directly from the 5' end to the 3' end of the plasmid transcript and, unlike strand switching associated with retroviral RTs, this reaction is not dependent on homology between templates. Without the specificity conferred by a template switching mechanism that requires sequence homology between templates, the plasmid RT can copy unrelated RNAs resulting in the production of suppressive forms of the plasmid and ultimately to

senescence. *In vitro* studies show that the plasmid RT can template switch at high frequencies and has less specificity for templates than is required for primer-independent cDNA initiation. Characterization of multimeric cDNA products showed that there are nontemplated nucleotides inserted at the junction sites, which may provide a clue concerning the mechanism of homology-independent template switching. Studies of senescent cultures show that formation of variant plasmids occurs at high frequencies and a new type of suppressive plasmid has been identified. The evolutionary significance of a homology-independent mechanism of template switching will also be discussed.

41. LINEs and SINES in *Magnaporthe grisea.*

Marc J. Orbach and M. Anthony Meyn. Department of Plant Pathology, University of Arizona, Tucson, AZ.

Several transposable elements have been identified in the rice blast pathogen, *Magnaporthe grisea,* in attempts to determine their role in the genetic instability observed in this fungus. We report the characterization of the first LINE retrotransposon found in *M. grisea*, that we have designated MGL, which is homologous to MGR583. This 6 kb element is highly conserved both in rice pathogens and pathogens of other grasses, although there is variability in copy number among the strains. Surprisingly, although full length transcripts have been observed, movement of MGL has not. MGL contains two open reading frames (ORFS) which overlap by a single base pair. The first ORF contains homology to retroviral *gag* proteins and the second to reverse transcriptase (RT) polypeptides. Most MGL copies are flanked by short, variablelength, direct repeats as is characteristic of this class of elements. The 3' end of MGL is characterized by variable numbers of a TAC repeat, a feature also observed in the SINE element, MG-SINE, of *M. grisea* (Kachroo *et al.* 1995). Sequence comparisons reveal that the last 240 nt of MG-SINE are 90% identical to MGL. This homology suggests a model for the origin of MG-SINE, and a mechanism for MG-SINE transposition that relies on the RT of MGL, and utilizes the end of MGL for initiation of reverse transcription. A revised distribution of these two elements will be presented.

Kachroo, P., Leong, S.A., Chattoo, B.B. (1995) Mg-SINE: a short interspersed nuclear element from the rice blast fungus, *Magnaporthe grisea.* Proc. Natl. Acad. Sci. USA 92:11125-11129

Sexual/Asexual Reproduction

42. *Aspergillus nidulans* maintains short telomeres throughout development.

Anamitra Bhattacharyya and Elizabeth H. Blackburn Department of Microbiology and ImmunologyUniversity of California, San Francisco, San Francisco, CA 94143-0414.

We have identified and cloned the telomeres of the filamentous fungus, *Aspergillus nidulans.* We have demonstrated that the telomeric repeat sequence is TTAGGG, identical to that found in vertebrates, including humans, and some lower eukaryotes. Three classes of cloned chromosomal ends were identified based on the telomere-associated sequences (TASs). One category of telomere clones was found to contain internal, variant TAAGGG repeats. The *A. nidulans* telomeric tract length is strikingly short, based on analysis of cloned ends (4 to 22 repeats) and from Bal3l digestion of native telomeres. We have also found that telomere length is remarkably stable in different cell-types and at altered growth temperatures, suggesting a highly regulated mechanism for length control.

43. Cloning, sequencing and structural analysis of nsdD gene that controls sexual development of *Aspergillus nidulans*.

Kap-Hoon Han, Kyu-Yong Han, Suhn-Kee Chae[1] and Dong Min Han. Department of Molecular Biology, Wonkwang University, Iksan, South Korea and [1]Department of Biochemistry, Paichai University, Tae-Jeon, South Korea.

Several mutants which never undergo sexual development (nsd) of *Aspergillus nidulans* have been isolated and characterized. Among them, a genomic DNA fragment (pNSD19) which complemented the nsdD19 mutation was isolated from nsdD19^{+} transformants by in vitro packaging after transforming with a genomic DNA library. When nsdD^{-} hosts were retransformed with pNSD19, four different kinds of phenotypes relating to the production of sexual organs were observed. Southern blot hybridization revealed that phenotypic variation was due to the differences in the copy number of the transformed nsdD gene. The nsdD gene has an ORF of 1,383bp, interrupted by three introns, encoding a protein of 461 amino acids. A putative 'zinc finger' DNA binding motif was observed in NsdD protein which was also found in GATA factor and AreA. The results suggested that the nsdD gene may play important roles in regulation of sexual development of *A. nidulans*.

44. Recurrent selection for fertility in *Nectria haematococca* MPI.

Brian T. Hawthorne, HortResearch, Auckland, New Zealand.

Selection for increased numbers of fertile perithecia in *Nectria haematococca* MPI was carried out for six generations of intercrossing among 18 to 24 parents (9-12 of each of the two mating types, MAT1-1 and MAT1-2) in each generation. Perithecia producing spore horns four weeks after spermatisation were scored as fertile. The parents were selected from progeny of the three most fertile crosses from the preceding generation. After six generations the mean numbers of fertile perithecia for the crosses within a generation had increased nearly threefold. Within a generation the difference between the least and most fertile crosses, as a fraction of the most fertile cross, reduced from 500% in generation 0 to 60% in generation 6. Variation in fertility of the reciprocal crosses, MAT1-1 x MAT1-2 and MAT1-2 x MAT1-1, which varied several-fold in early generations was reduced to virtually nil in generation six. The proportion of additive genetic variance decreased three- to four-fold between generation 0 and generation 2 and remained relatively constant in the subsequent generations. Confirmation of the increased

fertility was obtained by comparing the most fertile cross from each of the generations under common conditions.

The evidence from this study confirmed that there are genes additional to sex and mating type which influence both sexual compatibility and fertility in *Nectria haematococca* MPI.

45. Asexual sporulation in dikaryons and a dikaryon-like *AmutBmut* homokaryon of *Coprinus cinereus*.

Eline Polak[1], Ursula Kues[1], M. Hollenstein[1], Rene Hermann[1,2] and Markus Aebi[1]. Institute of Microbiology[1] and

Laboratory for Electron Microscopy I[2], Swiss Federal Institute of Technology, CH-8092 Zurich, Switzerland.

Monokaryons of *Coprinus cinereus* constitutively produce abundant vegetative spores (oidia) on specialised aerial structures, so called oidiophores. Although oidia formation in dikaryons is strongly repressed, a low amount of oidia is still produced, often upon light induction. Most oidia produced on dikaryons are haploid and uninucleate, and the ratio of the two possible oidia types is dependent upon the genotype of the dikaryon. Formation of the monokaryotic oidiophore can occur on a dikaryotic hypha suggesting a light-induced, regulated discrimination of the two nuclei in the initial steps of oidiophore development.

This light-induced oidiophore development was studied in the homokaryon AmutBmut. Unlike monokaryons and similar to dikaryons, oidia production in such *AmutBmut* homokaryons is repressed in the dark. However, upon light induction an *AmutBmut* homokaryon produces oidia in amounts comparable to monokaryons. Taking advantage of this phenomenon, oidia and oidiophore formation in an *AmutBmut* homokaryon is described morphologically and cytologically by light and electron microscopy. A model defining the different steps in this process will be presented.

46. Mating-type associated incompatibility in *Neurospora crassa*.

Patrick Ka Tai Shiu, University of British Columbia, Vancouver, BC, Canada.

In *Neurospora crassa*, mating is controlled by a single locus with two mating types, *A* and *a*. This mating type locus has an additional unique function during vegetative growth known as heterokaryon incompatibility: if hyphae of opposite mating types fuse during vegetative stage, the resulting heterokaryotic cells are inhibited in their growth. An unlinked mutation *tol* suppresses the heterokaryon incompatibility but does not affect sexual function.

Sequencing of the two mating type alleles, *A* and *a*, showed that the two alleles are totally dissimilar sequence of 5301 and 3235 base pairs respectively. The term "idiomorph" is coined to describe these dissimilar genes occupying homologous positions on a chromosome. A single

open reading frame (ORF) from each of mating-type idiomorph, *mt A-1* and *mt a-1*, confers both mating function and heterokaryon incompatibility. Both MT A-1 and MT a-1 contain a conserved DNA-binding motif, indicating that they could be transcriptional activators.

In this study, it was found that a putative LRR (leucine-rich repeat) in *mt A-1* is essential to the incompatibility function. *tol* was cloned by chromosome walking. It is the first suppressor of many incompatibility loci found in different fungi to be cloned. It encodes a 922-aa protein and contains a putative LRR and an amphipathic -helix. Further experiments will be done to characterize *tol* (including expression pattern, localization, and functional domains) and to study the mechanism of interactions among *tol* and the mating-type genes (using two hybrid system).

47. Analysis of *A. nidulans* genes involved in meiosis: *uvsC*, a homologue of yeast *RAD51*.

Diana van Heemst, Klaas Swart, Christa Heyting, Henk W.J. van den Broek and Theo Goosen

Dept. of Genetics, Agricultural University Wageningen, Dreyenlaan 2, 6703 HA Wageningen, The Netherlands.

We cloned the *uvsC* gene of *Aspergillus nidulans* by complementation of the *A. nidulans uvsC114* mutant. The predicted UVSC protein shows 67.4% sequence identity to *S. cerevisiae* Rad51 and 27.4% sequence identity to *E. coli* RecA proteins. Transcription of *uvsC* is induced by MMS, like transcription of *RAD51* of yeast. Similar levels of *uvsC* transcription were observed after MMS induction in a *uvsC*$^{+}$ strain and the *uvsC114* mutant. The coding sequences of the *uvsC114* allele have a deletion of six base pairs, which results in a deletion of two amino acids and a replacement of one amino acid in the translation product. In order to gain more insight in the biological function of the *uvsC* gene, a *uvsC* null mutant was constructed by replacing the complete *uvsC* coding sequences for a selectable marker gene. The *uvsC* null mutant was more sensitive to both UV and MMS than the *uvsC114* mutant. Meiotically, the *uvsC114* mutant arrested in prophase-I. The *uvsC* null mutant arrested at an earlier stage, before the onset of meiosis. One possible interpretation of these meiotic phenotypes is that the *A. nidulans* protein homologous to Rad51 of yeast has a role both in the specialised processes preceding meiosis and in meiotic prophase-I.

In order to isolate more genes involved in meiosis by complementation, we started a mutant screen, which so far has resulted in a few dozen specific meiosis mutants. Part of those have now been characterised by complementation and cytology. Of one mutant, blocked in karyogamy (*karA*), the corresponding gene has been isolated.

Neurospora/Aspergillus Model Systems

48. Links between cell biology and fungal development.

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Fungi have been used as model organisms to study cell biology and differentiation of eukaryotic cells for many years. E.g. molecular anlyses of mitosis, meiosis or organelle movement revealed generally valuable insights into these evolutionarily conserved processes. Fungal development has also been studied in several fungi on a molecular level. Besides "true" developmental genes which are specifically involved in the regulation of morphogenesis, the number of characterized genes necessary for basic cellular processes and for fungal development is increasing. The aim of the talk will be to emphasize recent advances in understanding some links between cell biology and differentiation processes in fungi.

49. Characterization of the *car1* gene of *Neurospora crassa*; A putative peroxisome assembly factor gene.

Kelly A. Howe and Mary Anne Nelson, University of New Mexico.

Peroxisomes are the least characterized organelles of the cell; they received little attention until the discovery of their role in a specific group of human genetic disorders. The most severe of these disorders Zellweger Syndrome which is lethal shortly after birth. The disease is characterized by a lack of peroxisomes in cells and a subsequent loss of vital peroxisomal functions. It is now known that peroxisomes contain enzymes involved in many metabolic pathways of eukaryotic cells, including -oxidation of fatty acids. Peroxisome biogenesis and the role of the organelle in development are still poorly understood, but microorganisms have provided good model systems for the study of peroxisomes. We have cloned a peroxisome assembly factor gene, *car1,* from the filamentous fungus *Neurospora crassa.* The *car1* gene is a putative peroxisome assembly factor with significant homology to a conserved family of proteins related to the human PAF1 (Peroxisome Assembly Factor 1) protein. The PAF1 protein is known to be involved in peroxisome biogenesis and the PAF1 gene is one of the genes known to be responsible for human Zellweger Syndrome.

The *car1* gene has been RIP-disrupted and partially characterized in *N. crassa.* It's amino acid sequence encodes two membrane spanning domains and a zinc finger region that are all conserved among the PAF1-related proteins. A *car1* mutant strain has been isolated exhibiting a mild form of RIP-disruption of the *car1* gene and potential partial peroxisome function based on selection procedures. Here we report the sequence analysis and partial characterization of *car1* function in *N. crassa.*

50. Involvement of the *Aspergillus nidulans* anaphase promoting complex/cyclosome in a surveillance pathway blocking entry into mitosis in the absence of NIMA function.

C. Mark Lies, J. Cheng, S. Venkatram, and P. M. Mirabito, School of Biological Sciences, University of Kentucky, Lexington, KY.

In *Aspergillus nidulans,* initiation of mitosis normally requires the function of the cell cycle regulated NIMA kinase. The requirement for NIMA function can be relieved by mutational inactivation of the *bimA* or *bimE* genes, suggesting that BIMA and BIME are components of a

G2 checkpoint that prevents entry into mitosis in the absence of NIMA function (the NIMA checkpoint). Genetic and biochemical data indicate that BIMA and BIME are components of the Anaphase Promoting Complex/Cyclosome (APC/C), leading us to propose that the APC/C itself regulates the NIMA checkpoint. If so, then all loss of function APC/C mutants should be defective in this checkpoint. We have tested this hypothesis by using reverse genetics. We have cloned an *Aspergillus* gene corresponding to the human APC/C gene, HCDC16, which we propose to call *bimH.* Strains containing an *alcA::bimH* fusion as their only intact *bimH* gene die on glucose medium with a complex mitotic phenotype, consistent with BIMH being an essential component of the APC/C. We find that *nimA5, bimH*$^{-}$ double mutants arrest in mitosis. These results are consistent with a model in which the APC/C is part of a surveillance system that prevents entry into mitosis until NIMA function is complete.

51. Swollen cell (swo) mutants of *Aspergillus nidulans.*

Michelle Momany, Gretel Abramowsky, Jered Brown, and Patrick Westfall, University of Georgia, Athens, GA.

Fifty-two mutants showing limited growth and a swollen cell phenotype at restrictive temperature (*swo* mutants) have been identified from a collection of temperature-sensitive *A. nidulans* mutants based on stereoscope examination. Phenotypes of putative *swo* mutants at restrictive temperature range from conidia which swell but do not send out germ tubes to hyphae with irregular swellings. Such phenotypes may be associated with defects in cell wall synthesis. Analys's of progeny from *swo* x wild-type crosses showed that 17 of the putative *swo* mutants resulted from single gene mutations. The *swo* mutants are currently being analyzed for response to osmoticum and known inhibitors of cell wall synthesis.

52. Dynamics of actin and chitin ring formation during cytokinesis in *Aspergillus* nidulans.

Michelle Momany[l] and John E. Hamer[2], [1]University of Georgia, Athens, GA and [2] Purdue University, West Lafayette, IN

Previous work has shown that septation in the filamentous fungus *Aspergillus nidulans* superficially resembles cytokinesis in animal cells in that both are dependent on mitosis and require actin. We show that a ring of actin is the precursor to the chitin ring seen in mature septa and that actin and chitin co-localize during an intermediate phase of septum formation. We present evidence supporting a contractile actin ring in septation and suggest a structural role for the chitin ring. By employing the microtubule depolymerizing drug benomyl we show that microtubules are required for both actin ring formation and the progression from the early actin ring to the chitin ring. In contrast to studies in yeast cells, our results suggest that an intact mitotic spindle is required for actin ring formation in *A. nidulans* and that a mitotic checkpoint may prevent further progression of cytokinesis in the absence of an intact mitotic spindle.

53. Overlapping binding sites for the CreA repressor and AmdX and AmdA activator proteins in the 5' region of the *amds* gene of *Aspergillus nidulans.*

Rachael L. Murphy, Alex Andrianopoulos, Janynke L. Brons, Meryl A. Davis and Michael J. Hynes, Department of Genetics, University of Melbourne, Parkville 3052, Victoria, AUSTRALIA.

The acetamidase gene *(amdS)* of *Aspergillus nidulans* is subject to complex transcriptional control. One important regulator is the *creA* gene product which mediates carbon catabolite repression of *amdS. The amdA* and *amdX* positively acting genes were identified as regulators of *amdS* following the isolation of gain-of-function alleles of these genes.

The CreA, AmdA and AmdX proteins each contain two C2H2 zinc finger DNA binding motifs. Alignment of these domains reveals extensive similarity particularly in residues known to be involved in contacting DNA. Consequently, the three proteins are predicted to bind to very similar target sites which are compatible with the published CreA binding site consensus sequence.

We present results showing that *in vitro* DNA binding of the CreA, AmdA and AmdX zinc fingers to the *amdS* 5' region can be localised to two sites containing predicted consensus binding sequences. The *in vitro* data is consistent with results of *in vivo* experiments suggesting that CreA, AmdA and AmdX act through these sites. Competition for binding site occupancy by multiple regulators may therefore play a role in the regulation of *amdS* and possibly other CreA-regulated genes.

54. *Grisea,* a nuclear gene of *Podospora anserina* involved in the control of differentiation and senescence, encodes a putative transcription activator.

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In *Podospora anserina* all wild-strains are characterized by a limited lifespan which is controlled by both, mitochondrial genetic traits as well as by a number of nuclear genes. Recently, we reported the cloning of the nuclear gene *grisea*. Mutation of this gene leads to a significant increase in lifespan and affects morphogenesis (spore color, sexual reproduction). A molecular characterization of *grisea* suggested that it codes for a copper-activated transcription factor. This factor appears to be important for a tight regulation of the cellular copper level and is thus linked to metabolic pathways leading to the generation of reactive oxygen species. Four point mutations were identified in the sequence of the cloned *grisea* region of the mutant. Three of these changes are located in the sequence upstream of the start codon and one mutation at the 5'-boundary between the first exon and the single intron of this gene. The latter mutation was shown to result in an RNA splicing defect. The characteristics of long-lived mutant *grisea* and those of the putative GRISEA protein suggest a number of candidate target genes, the expression of which may be controlled by GRISEA. Data of approaches to identify and to characterize individual genes of this type and to unravel unknown parts of the molecular network involved in the genetic control of differentiation and senescence in *P. anserina* will be presented.

55. Mutations in the *Aspergillus nidulans bncA* gene uncouple the nuclear division and cell division cycles.

Renata Castiglioni Pascon*, Aline A. Pizzirani-Kleiner*, Bruce L. Miller**. *Universidade de Sao Paulo, S.P., Brazil;

** University of Idaho, Moscow, ID.

The *Aspergillus nidulans bncAl* (binucleate conidia) mutant was first described as a single mutation located on chromosome IV that caused the formation of approximately 25% binucleated and 1% trinucleated conidia. Further analysis has shown that this mutation also affects conidiophore morphology. Metulae and phialides are elongated and have incorrect number of nuclei. Philalides also show internal septation. Diploid construction and cytological analysis of the conidia indicate that *bncAl* is a recessive mutation. Heterokaryon analysis using a binucleated strain and a uninucleated strain with contrasting auxotrophic markers produced prototrophic conidia at a high frequency compared to control heterokaryons, suggesting that more than one nucleus of each genotype migrated from the phialide to the conidium. The vegetative growth rate of the *bncAl* mutant was the same the wild-type. DAPI/Calcofluor double staining showed the nuclei of *bncAl* to be fairly normal in number, distribution and shape in the hyphal tip, but in the older elements nuclei appear to undergo continued, asynchronous divisions or, are mostly misshapen and fragmented. In general the mutant strain has a greater number of nuclei per hyphal element. The cell division cycle was followed during conidial Germination. We observed a high frequency of young trinucleated germlings and older germlings with odd numbers of nuclei. Results suggest that unlike the wild type, early nuclear divisions are not synchronous in the same germline. The information available suggests that the gene product of *bncAl* is required during the early staizes of germination and throughout development, affecting both vegetative tissue and the asexual reproductive apparatus. Moreover, *bncAl* may have a role in controlling nuclear division and septation. These questions will be addressed.

56. The Inquiry Track approach to learning principles of genetics.

Patricia J. Pukkila and Marshall Hall Edgell, University of North Carolina at Chapel Hill.

Advanced genetics courses for both undergraduate and graduate students usually provide active educational experiences that capture a sense of how professionals pursue the discipline. The challenge is to bring these useful approaches into large "lecture" courses at the beginning level. Our goals are for these students to feel entitled to seek information, to analyze the methods used to derive the "facts", to assess the validity of conflicting viewpoints, and to communicate their conclusions effectively. Accordingly, we have asked students to use reading guides to synthesize information from the textbook prior to class, to compare their answers to these assignments with those of neighboring students in the class, to participate in class discussions that develop a consensus, to revise their assignments to abandon erroneous views and/or incorporate new

information, to explore cause and effect using computer simulations, to prepare written analyses of the conceptual difficulty of past exam questions, to research and then discuss conflicting points of view concerning issues in the field that are currently unsolved, and to take the intellectual risks that are necessary to learn to think effectively in this discipline. Our methods have dramatically increased student-student and student-faculty interactions in classes of 150 students, and we conclude that the Inquiry Track approach could be effective in classes of any size.

57. Genetics of two incompatibility genes at *het-6* in *Neurospora crassa*.

Myron L. Smith, Nadereh Mir-Rashed, Cristina O. Micali, Sheryl P. Godkin, David J. Jacobson* and N. Louise Glass[#]. Carleton University, Ottawa, Canada. *Stanford University, Stanford, CA. [#]U. of British Columbia, Vancouver, Canada

The heterokaryon incompatibility system of *Neurospora crassa* provides an excellent model for the molecular, genetic and biochemical study of non-self recognition. *N. crassa* has at least 11 heterokaryon incompatibility (*het*) loci (including *mt*). Cell fusion between two individuals that differ at any one of these loci triggers an incompatibility response that results in little, or no growth of heterokaryons. This response reduces cytoplasmic contact and may, therefore, prevent the spread of disease elements among individuals. We have cloned and sequenced two genes, *het-6V* and *het-6J*, at the *het-6* locus on LGII that appear to be involved in heterokaryon incompatibility function. A difference in alleles at *het-6V* results in no appreciable growth of heterokaryons. Heterokaryons with different alleles at *het-6J* can grow for a few days before a rapid and complete growth cessation occurs. From DNA sequence data and transcript analysis, *het-6V* contains no introns and putatively encodes a 400 amino acid polypeptide. A 300+ open reading frame has been identified at *het-6J* . Neither *het-6V* nor *het-6J* ORFs have striking sequence identity to data-bank entries. Subclones that confer the *het-6J* incompatibility function (based on transformation assays) also complement strains carrying the temperature sensitive mutation, *un-24*. Sequence analysis of the mutant *un-24* allele is nearly complete. Southern blot analyses of *N. crassa* populations reveal multiple RFLPs in the regions of *het-6V* and *het-6J*. Both genes have homologous counterparts in other Neurospora species. We will present a preliminary analysis of the distribution of alleles at both genes in a world-wide population sample of *N. crassa*.

58. Tyrl5 phosphorylation of p34^{cdc}2 regulates septation and development in *Aspergillus*.

Xiang S. Ye, Sarah Lee McGuire[1], Tom Wolkow[2], John E. Hamer[2] and Stephen A. Osmani, Geisinger Clinic. [1]Millsaps College; [2] Purdue University.

Conidia of *A. nidulans* undergo polarized growth, producing multinucleate filamentous hypha which are then compartmentalized by septation. Septation is normally dependent on mitosis as germinating spores unable to complete mitosis fail to septate. We demonstrate that the dependency of septation on mitosis is established by Tyrl5 phosphorylation of p34^{cdc2}. p34^{cdc2} is inhibited after Tyrl5 phosphorylation by ANKA^{wee1} and activated by Tyrl5 dephosphorylation by

NIMT^{cdc25}. At 42 C *nimT23* cells arrest at G2 with Tyrl5 phosphorylated p34^{cdc2} and no septation occurs. At 37.5 C, *nimT23* cells undergo mitosis and grow at near wt rate but are unable to form septa. Addition of 6 mM HU, which increases Tyrl5 phosphorylated p34^{cdc2} levels but does not arrest mitosis, also suppresses septation. Conversely, deletion of ANKA^{wee1} or a Tyrl5 mutant p34^{cdc2} fully complements the *nimT23* septation defect and, furthermore, cells unable to tyrosine phosphorylate p34^{cdc2} undergo septation without mitosis. Such strains form multiple septa in the conidiophore stalk and vesicle, which normally never septate. Tyrl5 mutant p34^{cdc2} strains also form highly abnormal conidiophores and conidiate poorly. Thus, septation and cellular differentiation are regulated through Tyr 1 5 phosphorylation of p34^{cdc2} in *A. nidulans.*

59. Analysis of loci on linkage group VIL of *Neurospora crassa*.

Thomas J. Schmidhauser and Dan Chen. The University of Southwestern Louisiana.

Genetic Mapping places the *lys-5* and *un-4* loci on LGVIL of *Neurospora crassa* with *un-4* mapping 2 mu to the right of *lys-5*. We have cloned both genes and present cloning and RFLP analysis data. DNA sequence analysis of cDNA isolates is also presented.

Biotechnology

61. Tapping fungal diversity for drug discovery

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Many of today's drugs are fungal natural products and most of these drugs have been isolated from species which can be easily cultured. However, culturable fungi constitute only a fraction of the fungal taxa. Due to technical limitations, unculturable and slow growing microorganisms have received little attention for drug discovery. While there is little doubt that many new agents will continue to be discovered from culturable fungi, there is a clear need to expand our potential drug source. Recent advances in our understanding of fungal genetics has opened a new avenue, known as combinatorial biology, to allow the expansion of the fungal drug source. Combinatorial biology comprises at least two approaches. The first captures genetic diversity from unculturable fungi through genetic engineering. The second approach directly manipulates gene(s) in specific classes of secondary metabolites, such as polyketides and non-ribosomal peptides. At ChemGenics Pharmaceuticals Inc., we are employing these approaches in our drug discovery efforts.

62. Identification of genes differentially expressed in biobleaching cultures of *Trametes versicolor*.

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Trametes versicolor has the ability to selectively bleach and delignify both hard and soft wood kraft pulps without significantly decreasing the quality or yield of the resulting fiber. Traditional biochemical techniques have identified one enzyme essential for this process(manganese peroxidase) and one probably essential (laccase). In order to identify other enzymes involved in the biobleaching and delignification of hard wood kraft pulp (HWKP) we performed a differential display polymerase chain reaction (DDPCR) screen for genes elevated in expression in the presence of HWKP. The primary screen of RNA isolated from cultures grown in the presence and absence of HWKP identified 74 messenger RNAs produced in increased abundance in the presence of HWKP. The differential expression of 12 of these messages has been confirmed by a secondary DDPCR screen of RNA isolated independently under identical conditions. We have cloned 6 of the cDNA fragments identified in the secondary screen for sequence and northern blot analysis to identify the gene products and confirm their elevated expression.

63. *Trichoderma reesei* EGIII expression and modification.

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Trichoderma reesei is an important producer of cellulases for industrial uses. It has been reported to produce two cellobiohydrolases, CBHI, CBHII and four endoglucanases, EGI, EGII, EGIII and EGV. EGIII is a small 23 kDa enzyme that differs from the other Trichoderma cellulases by having a comparatively high pI and lacking a separate cellulose binding domain. We have overexpressed EGIII by placing the gene under control of the strong *cbhI* promoter. While there exist two potential glycosylation sites in the egIII gene, the protein has been thought to be unglycosylated. When EGIII is expressed under CBHI control, it is initially produced as a glycosylated protein of 28 kDa. It appears to be deglycosylated by an EndoH like activity post secretion. The glycosylated enzyme can be purified using ConA Sepharose. The glycosylation can be removed with *Bacillus* EndoH. Both enzymatic activity and heat stability appear to be unaffected by glycosylation.

64. Isolation and expression of the APS kinase (sD) gene of *Aspergillus nidulans*.

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As part of a study on supply of cysteine as a precursor for penicillin biosynthesis, we are investigating the sulphate assimilation pathway of *Aspergillus nidulans*, for which a number of structural and regulatory genes have now been isolated, Mutations in the *sD* (APS kinase) gene, which lead to a requirement for methionine, map approximately 3cM from *benA.* Cosmids known to contain *benA* were probed with the APS kinase gene of *Saccharomyces cerevisiae,* and a hybridizing cosmd selected. The *sD* gene was identified by transformation of an *sD50* mutant and subsequently sequenced. Sequence comparison was made between a number of APS kinase genes, and the C-terminal region of the *A. nidulans sC* (ATP sulphurylase) gene, which resembles APS kinase, and may be involved in allosteric regulation of ATP sulphurylase by PAPS. Although the sulphate assimilation pathway is repressed by methionine, transcription of the *sD* gene is not noticeably affected. The *sD* gene has been expressed in *A. nidulans* under the control of the stronger alcA promoter, and the effect on pathway flux and penicillin titre will be examined.

65. Protein glycosylation in *Aspergillus nidulans*.

C. Joshua Eades and William E. Hintz, University of Victoria.

Glycosylation can have major effects on the structure and function of proteins, influencing the stability, antigenicity, and biological activity of the protein. We are currently cloning and characterizing the various enzymes of the N-glycosylation pathway of *Aspergillus nidulans* to allow production of heterologous glycoproteins having uniform carbohydrate moieties. The processing of mannose residues occurs early in the glycosylation pathway and can play a major role in the final carbohydrate structure of N-glycans. Historically, -mannosidase enzymes have been classified into two groups based on functional and sequence similarities. Certain -mannosidases, including the rat ER/cytosolic and yeast vacuolar enzymes, have been more difficult to classify and may consitute a third group. These cytosolic -mannosidases may play a catabolic role in the ER 'recycling' system and may facilitate glycoprotein breakdown in the ER by removing mannose residues from aberrant proteins. We have recently cloned a gene for an -mannosidase from the filamentous fungus *Aspergillus nidulans* which appears to belong to this third group of cytosolic -mannosidases. This gene has a similar length and shows considerable homology with both the rat and yeast 'cytosolic' -mannosidases. A phylogenetic comparison of all the cloned -mannosidases revealed three distinct groups. We suggest that cytosolic -mannosidases represent an evolutionarily independent group.

66. Overexpression of heme biosynthesis pathway genes results in increased heterologous hemoprotein production in *Aspergillus oryzae.*

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Genes encoding the first two enzymes in the heme biosynthesis pathway were cloned from *Aspergillus oryzae* in order to investigate 1) the effects of their overexpression on heterologous hemoprotein production and 2) the regulation of heme biosynthesis in filamentous fungi. The first enzyme, 5-aminolevulinate synthase (ALAS), is a nuclear-encoded mitochondrial enzyme which catalyzes the condensation of glycine and succinyl-CoA to form 5-aminolevulinate. The second enzyme in the pathway, porphobilinogen synthase (PBGS), is a cytoplasmic enzyme which catalyzes the formation the monopyrrole, porphobilinogen, from two molecules of 5-aminolevulinate. ALAS is the rate-limiting enzyme in mammals while PBGS is thought to be the rate-limiting step in yeast. The A. *oryzae* genes encoding both enzymes, *hemA* (ALAS) and *hemB* (PBGS), were cloned and exhibit significant identity to their respective homologues from other organisms. Integration of multiple copies of the *hemA* gene, under transcriptional control of the TAKA promoter, resulted in a 2-fold increase in heterologous peroxidase production, while integration of a similar *hemB* construct had little effect. These data indicate that genetic manipulation of heme biosynthesis results in increased heterologous hemoprotein production.

67. Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains .

Robin J. Gouka, Peter J. Punt, Johanna (H.) G. M. Hessing and Cees A.M.J.J. van den Hondel. TNO Nutrition and Food Research Institute, Department of Molecular Genetics and Gene Technology, PO Box 5815, 2280 H*V* Rijswijk-, the Netherlands

Filamentous fungi, especially members of the genus *Aspergillus,* are able to secrete large amounts of homologous proteins into the medium which makes them attractive as a host for protein production. In contrast, heterologous proteins are very poorly produced and secreted.

The obective of our research is to obtain more insight in the parameters that influence heterologous protein production in *Aspergillus awamori.* To investigate this, a systematic analysis was carried out in which the expression levels of a number of different fungal and non-fungal genes were analyzed. This method is based on the single copy integration of different expression cassettes at the *pyrG* locus of *A. awamori*

Differences in expression mainly occured at the steady state mRNA level, varying from high mRNA levels for genes of fungal origin to low levels for genes of non-fungal origin. With one gene, encoding plant *Cymamopsis tetragonoloba* -galactosidase, no full length mRNA could be detected. With RT-PCR and nuclear run-on transcription assays it could be demonstrated that incorrect processing of full length mRNA was probably occurring, resulting in the lack of about 900 nt in the mRNA. By changing the DNA sequence of the gene improved levels of full length mRNA could be obtained.

In most cases the protein levels corresponded to the amount expected on basis of the mRNA levels. Only in the case of human interleukin-6, relatively high mRNA levels were obtained, whereas, only very low amounts of protein could be detected.

To further investigate the problems observed for plant -galactosidase and human IL6, gene fusions with the *A. niger* glucoamylase gene *(glaA)* were constructed. Data on improved mRNA and protein levels will be presented.

68. Studies on the production of single-chain antibody fragments by *Aspergillus* species.

Hanny (J.)G.M Hessing[1], Leon G.J Frenken[2], Marian van Miuijlwijk-Harteveld[1], Wouter Musters[2] and Cees A.M.J.J. van den Hondel[1]. [1]Department of Molecular Genetics and Gene Technology, TNO Nutrition and Food research, PO box 5815, 2280 H*V* Rijswijk, The Netherlands. [2]Unilever Research Laboratorium, Vlaardingen, The Netherlands.

Filamentous fungi, such as *Aspergillus niger* and, *Aspergillus awamori,* are able to produce extracellularly significant amounts of homologous proteins. Furthermore, they have the capacity to secrete heterologous proteins although to a much lesser extent. We investigated their capacity for production of single-chain antibody (scFv-) fragments. These fragments comprise the variable fragment of an antibody heavy chain connected via a linker peptide to a variable fragment of a light chain. We studied a.o. the production of scFv-anti Hen egg white lysozyme [scFv-LYS; (1)] as a model. For expression of the scFv encoding sequence expression cassettes were made in which the gene to be expressed was flanked by efficient expression signals such as the *A. niger* glucoamylase promoter (2) or signals derived from the *A. awamori* 1,4--endoxylanase A gene (3). Transformants comprising multiple copies of the expression cassette produced up to 10 mg/l scFv-LYS in the presence of a signal sequence. However, when scFv-LYS was expressed as a fusion protein with glucoamylase - a protein which is well secreted by A. *niger*- production levels increased at least five-fold. To obtain mature scFv-LYS, a KEX2 cleavage site was inserted between the glucoamylase and the scFv-LYS sequences. More data on the production of scFv-LYS and on the production of other scfv-antibody fragments will be presented.

1. Ward, S. et al.,- Nature 341 (1989) 544-546; 2.Verdoes, J C et al.,- J of Biotechnology 36 (1994) 165-175; - 3. J. G.M. Hessing et al.,- Curr. Genet. 26 (1994) 228-232

69. Gene disruption as a method of fungicide target validation.

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With increasing knowledge of the physiology and biochemistry of phytopathogenic fungi an expanding number of potential targets for biochemical fungicide design can be identified. To prevent wasted resources it is important to test at an early stage whether each putative target is in fact essential for infection and disease development, i.e. to validate the target. We are using *Stagonospora* (*Septoria*) *nodorum* to explore the use of gene disruption as a general method of fungicide target validation.

In initial studies, nitrate reductase was chosen as a hypothetical target because the gene (*NIA1*) has been cloned from *S. nodorum* and disruptants should have a readily detectable phenotype (chlorate resistance and nitrate non-utilising). Three approaches to targeted disruption of the *NIA1* gene have been tried: (1) cotransformation, (2) integrative gene disruption, (3) one step gene replacement; with each approach hygromycin resistance was used as the selectable marker. Around 2% of transformants from the cotransformation approach became chlorate resistant and Southern analysis confirmed disruption of the resident *NIA1* gene. These disruptants retained full pathogenicity thereby invalidating nitrate reductase as a fungicide target. Transformants from the other two approaches are presently being screened for disruptants. To test this approach on a realistic target, we are cloning chitin synthase genes from *S. nodorum*. These clones will be used to disrupt resident chitin synthase genes and the disruptants assayed for pathogenicity. Reduction in pathogenicity would identify a major chitin synthase in this species that might be constitute a valid fungicide target.

70. Isolation of hazardous chemicals-induced mRNA in white-rot fungus *Coriolus versicolor* using differential display

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White-rot fungi such as *Pheanerochaete chrysosporium* and *Coriolus versicolor,* a ligniolytic basidiomycete, has been extensively studied for its ability to degrade hazardous chemicals. Hazardous chemicals degradation by the fungi involves complex families of secreted phenoloxidases. The transcriptional regulation of the genes in hazardous chemicals stress has been studied. In this study, we searched for hazardous chemicals specific genes using the mRNA differential display technique on *C. versicolor* exposed to pentachlorophenol (PCP). Eleven partial cDNA fragments were cloned and DNA sequences of the five fragments were further analyzed. Four of the clones represent novel genes that has not been identified previously. One of them show strong sequence homology to enolase and is up-regulated in PCP-treated *C. versicolor*.

71. Expression cloning and characterization of full-length *cel45* cDNAs from *Myceliophthora thermophila, Thielavia terrestris* and *Acremonium* sp. reveals several conserved regions common to fungal family 45 cellulases.

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The glycosyl hydrolase family 45 is defined as a group of cellulases containing a highly conserved amino acid sequence at the NH2-terminus. To date, only one bacterial and four fungal endoglucanases belonging to this family have been reported. We have constructed directional cDNA libraries from cellulase induced mycelia of *Myceliophthora thermophila, Thielavia terrestris and Acremonium* sp. in the yeast expression vector pYES 2.0, and isolated 12 full-length family 45 cellulase (CEL45) cDNAs by functional expression in the yeast *Saccharomyces*

cerevisiae. The positive clones were identified using 0. 1 % AZCLHE-cellulose as substrate in agar plates. Yeast colonies producing cellulose activity were surrounded by a blue halo. Nucleotide sequence analysis of the cDNAs classified the clones into four groups representing transcripts of four genes; *cel45* of *M. thermophila, cel45* of *T. terrestris,* and *cel45A* and *cel45B* of *Acremonium* sp. The *T. terrestris* and *Acremonium* endoglucanases are composed of a catalytic core domain (211, 212 and 213 residues, respectively), a linker and a cellulose binding domain (CBD). In contrast, the *M. thermophila* CEL45 endoglucanase contains a 209-residue catalytic core, but neither a linker nor a CBD. Comparison of the deduced amino acid sequences with the endoglucanase V from *Humicola insolens* and *Kfaml* - encoded endoglucanase V from *Fusarium oxysporum* revealed a sequence similarity of 68-81 %. Besides the consensus sequence, several additional, highly conserved regions can be found in the core region, which together with the conserved cysteines and glycines reflect the structural relatedness of the family 45 endoglucanases. By comparison, the Ser and Thr rich linker regions are less similar varying both in length and composition, while the CBDs are both similar to one another and to other fungal CBDS.

72. Purification and molecular cloning of acid stable xylanase from *Penicillium sp.40*.

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Faculty of Bioresources, Mie University, Tsu, Mie, 514 Japan.

We have isolated *Penicillium sp.40* which can grow in acidic medium at pH2.0. This *Penicillium sp. 40* produces a xylanase in the xylan medium as a carbon source.We purified this xylanase (XynA) by DEAE-Toyopearl column and Superdex200pg column. The optimum pH of XynA is 2.0 and XynA is stable at pH2-5, indicating that XynA is acid stable enzyme. The molecular mass of purified XynA was 25 kDa on SDS-PAGE gel. The expression of XynA is induced by xylan and b-methyl-D-xyloside and repressed by glucose. Also, the expression of XynA is dependent on pH of the culture medium, suggesting that expression of XynA is controlled by multiple factors. To know the expression mechanisms of XynA, we isolated the gene encoding XynA (xynA). A 4 kb of XbaI fragment was isolated and the nucleotide sequence of this fragment was determined. The xynA appeared to be 721bp long and was interrupted by a single intron of 58bp. The xynG1 encodes a polypeptide composed of 221 amino acids which contained a putative signal sequence composed of 32 amino acids in the N-terminal region. N-terminal amino acid sequence determined by purified XynA showed perfect match to the deduced amino acid sequence from nucleotide sequence. XynA showed 73% amino acid identity with the XynC of acidophilic *Aspergillus kawachii* and showed strong similarity to the other fungal xylanases classifed as family G.

73. Heterologous expression of human tumor necrosis factor in *Aspergillus niger*.

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The human tumor necrosis factor (hTNF) monomer is a 17 kDa non-glycosylated protein. The active form of this important cytokine is a trimer with a wide range of biological activities. We

decided to express hTNF in the GRAS filamentous fungus *A. niger*, because *Aspergillus* expression-secretion system has proved to be able to produce therapeutically important proteins as cheaply as industrial enzymes, even though it cost more time and effort than initially hoped. On the other hand also has to be taken in account, it has not yet been generalized to a wide range of heterologous protein products. For heterologous expression of hTNF in *A. niger* the same strategies were used, which were proved to be the most successful for several other non-fungal proteins, including (i) the use of protease deficient host strain AB1.13, (ii) the use of strong fungal transcription control regions and efficient secretion signals of the *A. niger* glucoamylase gene, (iii) the use of gene fusion with the *A. niger* glucoamylase GII form as a carrier-gene and (iv) the introduction of large number of gene copies due to the *amdS* selection marker.

74. Fungi from geothermal soils in Yellowstone national park.

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Eighteen species of thermotolerant and thermophilic fungi representing nine genera were isolated from geothermal soil depths of 5 to 20 cm. The soils ranged in temperature from 16 to 91 C and pH from 2.58 to 5.79. Temperature and pH optima *in vitro* culture of these fungi ranged from 25-45 C and 3.0-6.0 respectively. Temperature growth ranges were 5-37 C for thermophilic fungi. Transect studies indicated that a higher number of fungi were obtained from soil core samples in and below the root zone of the tropical plant *Dicanthelium lanuginosum.* Extracellular enzyme analysis revealed that 8 of these fungal species were capable of secreting proteases and/or cellulases at 35 C. Chemical analysis of soil samples indicated that high levels of phosphorus, lead, iron and sulfur were present. Isolates representing 13 species were able to grow on media containing 1 mg/ml of iron and 200 *u*g/ml of lead.

75. Cloning of glycolytic genes and their 5' flanking region from *Aspergillus oryzae*.

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Aspergillus oryzae is considered a favorable host for heterologous protein production (Barbesgaard et al. 1992) owing to its ability to secrete large amounts of proteins (Christensen et al. 1988) and wide range of application in food industries. We have already cloned the genomic enolase gene (*enoA*) and corresponding cDNA from *A. oryzae* (Machida et al. 1996). The *enoA*

gene was highly expressed (approximately 3% of total mRNA) and the 5'- flanking region of the *enoA* gene may be useful for foreign gene expression in *A. oryzae*.

In *Saccharomyces cerevisiae*, most of the glycolytic genes are regulated by the general transcription factor, *Rap1p* (Chambers et al. 1995). We examined the DNA-binding activity specific to *Rap1p*-binding sequence in *A. oryzae* crude extract using 36 bp DNA fragment containing *S. cerevisiae* ENO1- UAS. Several strong binding activities were detected, however, none of them was sequence specific. This suggests that the recognition sequence of the factor in *A. oryzae* glycolytic genes may be different from that of *S. cerevisiae* or that the general transcription factor, such as *Rap1p*, may not be present in *A. oryzae*.

We cloned several glycolytic genes from *A. oryzae* by the random sequence analysis of *A. oryzae* cDNA or by PCR using mixed primers which have the sequence homologous to the corresponding genes from several other organisms. We cloned the 5' flanking region of the genes from *A. oryzae* genomic DNA by PCR-based DNA walking using primers designed from their cDNA sequences. Sequencing is under progress.

76. The characterization of the second laccase gene (CVLG1) from white-rot basidiomycete *Coriolus versicolor*.

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A white-rot basidiomycete *Coriolus versicolor* secretes laccase during wood decay. Many studies show that laccase plays an essential role in fungal brakedown of lignin. The extracellular laccases from *C. versicolor* were fractionated as laccase I, II and III by using anion exchange chromatography. Laccase III which is the most abundant isozyme among them has been characterized biochemically and genetically. In this study, second gene (CVLG1) coding for laccase isozyme has been isolated from *C. versicolor*. CVLG1 is interrupted by eleven putative introns and exhibits high similarity (70%) with laccase III gene (CVL3). From the deduced amino acid sequence, CVLG1 product is predicted to be a preprotein of 526 residues with twelve N-glycosylation sites. Genomic southern analysis showed that CVLG1 and CVL3 are not allelic. From the CHEF analysis, CVLG1 and CVL3 were mapped on distinct chromosomes. It has been cleared that laccases of *C. versicolor* were encoded by a gene family.

77. An expression system based on the improved promoter containing multiple copies of the conserved sequence in the amylase genes of *Aspergillus oryzae*.

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Aspergillus oryzae produces a copious amount of enzymes that are important in Japanese fermented food production such as *sake, shoyu* and *miso* manufacturing. Among them, enzymes involving in the degradation of starch are most important. These include -amylase, glucoamylase, and -glucosidase, which are known to be produced in the presence of starch or malto-oligosaccharides such as maltose, maltotriose, and isomaltose, but not of glucose. We found that there exist three highly conserved sequences, designated Region I, II, and III, in the promoter regions of the genes encoding -amylase (*amyB*), glucoamylase (*glaA*), and glucosidase *(agdA)* of *A. oryzae.* Deletion and insertion analyses of the promoter regions were carried out and indicated that Region III is mainly involved in high-level expression and maltose induction of the amylase genes (Minetoki *et al., Curr. Genet.,* **30,** 432-438, 1996). To enhance the promoter activity, multiple (6-12) copies of the fragment comprising Region III were inserted into the *agdA or glaA* promoter. Promoter activities were assayed by GUS expressed under control of the improved promoters and were shown to be elevated by 3-5 fold higher than those of the intrinsic promoters. Using the improved *agdA* promoter, we could achieve a remarkably high yield of native proteins, of which secreted level is very low by nature in wild type of *A. oryzae*, and observed concomitantly a significant decrease in -amylase and glucoamylase yield in the transformants. Northern blot analysis showed that transcriptional levels of *amyB* and *glaA* in the transformants were extremely reduced, suggesting that titration of a common regulatory protein(s) involved in high-level expression and maltose induction of the amylase genes may occur. In addition, advantages of conventional solid-state culture system for protein production will be presented.

78. An *Aspergillus fumigatus* extracellular phytase with high activity.

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In 1968, an isolate of *Aspergillus niger* (*ficuum*), NRRL 3135 was identified as the producer of the most active extracellular phytase. It produces 12.5 nkat/ml of enzyme in the crude culture filtrate. Since then, the gene for this enzyme has been cloned in NRRL 3135, over expressed, and marketed commercially. In this period of almost 30 years, no other extracellular phytase has been reported to equal the yield of phytase produced by this isolate. The reason for its unique high activity level remains unknown. Recently, in a survey of other Aspergillus species, an isolate of *A. fumigatus* was identified as another high producer of phytase. When this isolate was grown under the identical conditions as NRRL 3135, it produced 11.0 nkat/ml of phytase in the crude culture filtrate. By employing PCR technology we are cloning the phytase gene from this fungal isolate and examining it for domains it shares with NRRL 3135 that may be associated with high levels of enzyme activity.

79. Characterization of a heat tolerant *Aspergillus terreus* phytase.

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The animal feed industry is seeking a heat tolerant phytase for use as a feed additive. Phytic acid, the major storage form for phosphorus in soybean seeds, is undigestible to monogastric animals. Phytase makes the phytin phosphorus in soybean meal available and thus reduces phosphorus contamination of the environment from animal waste. A heat tolerant phytase would survive the brief period of elevated temperature necessary for animal feed production, such a phytase would be more cost effective to use with current animal feed production methods. We have identified several isolates of *Aspergillus terreus* that secrete phytase with a higher thermal stability than found in the previously reported *A. niger* (*ficuum*) NRRL 3135 phytase. The heat tolerant phytase from one of these isolates is being characterized and its gene cloned. We are determining the molecular basis of this heat stability, i.e., primary structure, disulfide bonds, etc., in order to engineer an enzyme with both high phytase activity and heat tolerance.

80. Expression of -lactam biosynthesis genes of *Acremonium chrysogenum.*

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The filamentous fungus *Acremonium chrysogenum* is the most important producer of the -lactam antibiotic cephalosporin C. Many attempts have therefore been made to achieve a detailed understanding of the biosynthesis of this metabolite. For several -lactam genes a clustered organization has been shown. The *pcbAB* and *pcbC* genes as well as the *cefEF* and *cefG* genes are transcribed from a bidirectional promoter region (for review, see [1]; [21). We have constructed chimeric reporter genes to investigate the effect of potential controlling sequences of the promotor regions. For this purpose non translated regions of both pairs of genes have transcriptionally been fused to the *lacZ* reporter gene derived from plasmid pSI8.8 [3]. Further on we show that regulation of *cefEF/cefG* gene expression is different to the expression of genes, which are specific for penicillin biosynthesis in other filamentous fungi.

[1] Martin JF et al. (1 994) Antonie van Leeuwenhoek **65:** 227-243

[2] Brakhage AA, Turner G (1995) In: Kuck (ed) The MycotaII. Springer Verlag, Berlin pp 263-285

[3] Menne S, Walz M & Kuck U (1994) Appl Microbiol Biotechnol **42:** 57-66

81. Isolation of a -tubulin gene conferring resistance to carbendazim in the phytopathogen *Septoria tritici*.

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Infection of wheat by the fungal pathogen *S. tritici* has not been well studied at the molecular level. A transformation system is an essential tool in the molecular analysis of pathogenesis, especially in systems recalcitrant to genetic analysis. In our laboratory and elsewhere (Pnini-

Coben *et al* 1996) *S. tritici has* been transformed to hygromycin B resistance. Transformation based on the homologous MBC-resistant allele of the -tubulin gene has been established in a number of fungal systems. This selectable marker has the advantage of being of potential use as a reporter gene *in planta.*

The -tubulin gene of a carbendazim resistant (MIC > 10 ug ml^{-1}) isolate of *S. tritici* (ST16) was isolated by screening an EMBL3 genomic DNA library with a homologous PCRgenerated probe. An 8.1 kb Sall fragment common to a number of positive clones, and which hybridised to the PCR probe, was subcloned into pUC18 (pST-BT.1). Sequence analysis of pSTBT. 1 indicated the presence of the whole of the -tubulin gene with at least 400 bp of sequence upstream of the start codon. pST-BT.1 was transformed into the carbendazim sensitive (MIC = 0. 1 ug ml^{-1}) *S. tritici* strain 12-3B.8 by PEG treatment of protoplasts. Transformants were directly selected on 1 ug ml^{-1} carbendazim and arose at a frequency of between 0.6 and 2.2 per *ug* DNA. Results of studies of carbendazim sensitivity of the transformants, and the nature of the integration events, are presented.

Pnini-Cohen, S. *et al (1* 996). Phytopathology. **86:** S40.

82. Characterization of superoxide dismutases from *Trichoderma harzianum.*

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UT Superoxide dismutases, which transform the toxic superoxide radical to hydrogen peroxide and divalent oxygen, produced by two biocontrol strains of *Trichoderma harzianum* were characterized. The metal prosthetic groups of the three common types of superoxide dismutases (SODs); copper-zinc (CuZnSOD), iron (FESOD) and manganese (MnSOD) were differentiated by selective chemical inactivation. CuZnSODs were inactivated by treatment with cyanide, hydrogen peroxide or diethyldithiocarbamate. MnSODs were inhibited by azide and FeSODs, if present were sensitive to azide and hydrogen peroxide treatment. The strains studied were *T. harzianum* from soil suppresive to *Rhizoctonia solani* plant disease and its'rhizosphere competent mutant generated by nitrosoguanadine (T95). Both strains produced CuZnSOD and MnSOD isozymes. Strain T95 produced an additional CuZnSOD isozyme. Exposure of mycelia in liquid cultures to paraquat, increasing intracellular superoxide concentrations, resulted in increased SOD activity. The intensity of the MnSOD isozyme increased with paraquat exposure. PCR of genomic DNA with primers specific for conserved sites in MnSOD amplified two bands, 294 bp and 378 bp in size, that appear to be different gene products. The PCR products had homology to MnSOD genes from yeast, corn, tobacco and a thermophilic bacterium.

83. Analysis of the -amylase production in a transformed *Aspergillus oryzae* strain.

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For industrial application of recombinant strains genetic stability is a very important aspect especially related to continuous cultivations. In this study we investigated the production of -amylase in chemostat cultures of a transformant of a wild-type strain of *A. oryzae* containing additional copies of the -amylase gene. To quantify strain instability a parameter has been introduced which is characteristic of the rate of instability and has different values depending on the nitrogen source during the cultivations. From the cultivations several mutants have been isolated and further characterised with regards to their -amylase production, to their resistance to the marker used for transformation, and to the number of copies of the -amylase gene.

84. Expression of *Aspergillus niger* Glucoamylase in *Fusarium graminearum*.

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The filamentous fungus, *Fusarium graminearum,* has been shown to be an excellent host for the production of heterologous proteins. Transcriptional control elements derived from a *Fusarium oxysporum* trypsin gene have been utilized to achieve the expression of many different proteins. Transcription systems that can be regulated by simple media changes are often useful in a number of protein expression regimes. Therefore, the regulation of the trypsin promoter in F. graminearum was investigated. The *A. niger* glucoamylase (GLA) gene was utilized as a reporter gene in transformants of *F. graminearum.* Functional glucoamylase protein was produced in these transformants. Northern analysis of RNA extracted from cultures grown under different conditions indicated that considerable levels of steady state RNA were observed when nitrate was used as the nitrogen source and no, or only a low level of RNA, was observed when ammonium salts were used. GLA RNA levels from cultures growth in glucose/nitrate medium were similar to those from succinate/nitrate medium suggesting no strong repression of the promoter by glucose. The GLA RNA present in wild type *A. niger* is found in two different forms as a result of differential splicing of intron E. The GLA RNA produced in *F. graminearum* was also observed to be present in two forms consistent with this differential splicing.

85. Extrachromosomal Replication of Transforming DNA by a Filamentous Fungus.

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Effective molecular genetic engineering of filamentous fungi to increase their utility and to further our understanding of their natural roles has been, as compared to bacterial and yeast studies, difficult. Among the factors causing this are: 1) many filamentous fungi can not be transformed in an efficient manner, and 2) there are almost no broad host range plasmids with the properties required by molecular genetic methods. Any significant improvements in these, as applied to molecular engineering of filamentous fungi, would be expected to open vast opportunities in terms of our understanding and exploitation of these organisms. We have

discovered a filamentous fungus, *Pestalotiopsis microspora*, that can be transformed with an unusually high efficiency using methods which give much lower efficiencies, or sometimes fail completely, with other filamentous fungi. A combination of Southern blotting and phenotypic stability experiments has established that *P. microspora* transformants replicate transforming DNA in an extrachromosomal mode, rather than by the integration route used by nearly all other filamentous fungi. To accomplish this, *P. microspora* recombines transforming DNA, which does not contain a replication signal recognizable by filamentous fungi, with its own to yield autonomously replicating plasmids. These plasmids display remarkable structural stability and the ability to replicate over extended periods of growth. Thus, *P. microspora* is an unusual filamentous fungus in that it readily accepts exogenous DNA and, in effect, synthesizes from this, its own autonomously replicating plasmids. These are precisely the type of DNAs that are, arguably, required to advance fungal molecular genetics.

86. Expression and secretion of a functional, humanized, IgG monoclonal antibody from a *N. crassa* heterokaryon culture. W. Dorsey Stuart, Douglas C. Vann , Elie Kato, Charles A. Long, Glenn M. Magyar, Doreen Morris, Faye Nagano, Stephen Buczynski, Gordon Edlin and Helen Tu. Neugenesis Corporation, Honolulu, Hawaii.

Genes coding for the kappa and gamma chains of a humanized monoclonal antibody (type IgG_1) were each inserted separately into an expression vector and cotransformed into *Neurospora* host cells with mutations at the *his-3* and *trp-1* loci. The cotransformations were designed such that kappa chain transformants would grow on tryptophan only and the gamma chain transformants would grow on histidine only. Putative kappa chain transformants were further screened by ELISA assay of media for production and secretion of the humanized kappa chain protein. A stable producer of kappa chain was then fused in Vogel's minimal medium in a microtiterplate format with putative gamma chain transformants. The cells readily formed heterokaryons and their media was screened by ELISA for production of IgG. One strain which secreted both kappa and gamma chains was chosen for further study. Media from this strain produced intact, assembled IgG_1 as demonstrated by Western blot analysis of reduced and native media proteins. The IgG_1 was bound by protein A and showed functionality by antigen specific binding. Additional characterization of the IgG_1 molecule is underway.

87. Development of a novel fungal expression system for combinatorial biology.

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ChromaXome Corporation (CXC) is conducting pioneering research into combinatorial biology, which combines the power of molecular genetics and biodiversity to produce novel chemistries for drug discovery. Our collaboration with Bristol-Myers Squibb which employs terrestrial actinomycetes and CXC's combinatorial biology technology has proven to be extremely successful. As a result, we have embarked on a program to extend this technology to fungi.

Fungi are widely recognized as valuable sources of chemically diverse secondary metabolites. Furthermore, heterologous gene expression among the major fungal groups, particularly those of the Ascomycetes and Deuteromycetes, is well documented, and a wide variety of genes have been cloned via heterologous complementation. Therefore, heterologous expression of secondary metabolite genes with the appropriate fungal host should be possible, and thus provide access to novel natural products for pharmaceutical development. We report here on a fungal expression system designed to exploit these aspects of fungal biology. This system utilizes a cosmid-based expression vector, PombeCOS-U, and the fission yeast, *Schizosaccharomyces pombe* as a host. To demonstrate the potential utility of this system, we have examined the expression of the polyketide 6-methylsalacylic acid derived from *Pencillium patulum* source DNA.

88. Expression of the GFP protein in *Aspergillus oryzae*.

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The Green fluorescent protein (GFP) isolated from the marine cnidarian *Aequorea victoria*, was subcloned into a fungal expression vector placing it under the control of the TAKA amylase promoter and the AMG terminator. This vector when used to transform *Aspergillus oryzae* failed to direct expression of the GFP protein. Northern blots revealed that GFP specific mRNA was present but that a substantial portion of the mRNA population was not of the expected size. 3-Prime RACE analysis was performed on mRNA from an *A. oryzae* transformant which contained the GFP expression vector. DNA sequencing of the resulting RACE clones revealed deletions within the coding sequence. The deleted regions contained fungal consensus intron sequences at their 5-prime and 3-prime ends. In order to correct this aberrant splicing, a new GFP allele was constructed in which the consensus intron sequences were removed and the GC content of the coding sequence increased. Transformants which contained this new expression vector produced functional GFP, as determined by fluorescence spectra of intracellular protein extracts.

89. Expression of cellulases genes of *Phanerochaete chrysosporium in* Aspen wood chips.

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The white-rot basidiomycete *Phanerochaete chrysosporium has* been intensively studied for its ability to degrade lignocellulose. Genes encoding cellulolytic enzymes characterized to date include six closely related cellobiohydrolase I clones *(cbh1s),* a cellobiohydrolase 11 *(cbh2),* and a cellobiose dehydrogenase (*cdh*). Owing to the inherent complexities of 'natural' substrates (e.g. woody tissue), studies of transcriptional regulation of these genes have been limited to submerged cultures with synthetic media. We describe a generally applicable methodology for

the quantitative assessment of fungal mRNAs in wood. In brief, the approach involves magnetic capture of PolyA RNA followed by competitive RTPCR. The techniques were applied to analyses of *cbh* and *cdh* transcripts in *P. chrysosporium* colonized Aspen wood chips. Transcript patterns in wood were substantially different from those previously observed in submerged cultures. Results provide a foundation for directed strain improvement.

90. The xylanolytic transcriptional activator *xlnR* of *Aspergillus niger.*

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Little is known about pathway-specific induction of extracellular enzyme systems due to the inability to select for, and complement in, mutants with a negative phenotype. A system has been developed that allows both the isolation of regulatory mutants, as well as the cloning of these regulatory functions by mutant complementation. A xylan induction-responsive element of the endo-xylanase gene *xlnA* gene of *A. tubingensis* has been used to isolate *A. niger* mutants lacking xylanolytic expression. The cloning of the xylanolytic transcriptional activator *xlnR* was done by complementation and subsequent recovery of the introduced wild-type copy of the mutant regulation allele. *A. niger* XYLR will be discussed on the basis of mutant characterisation and the primary structure. A model for the regulation of the *A. niger* xylanolytic sytem will be presented.

91. -Xylosidase activity, encoded by *xlnD,* is essential for complete hydrolysis of xylan by *Aspergillus niger,* but not for induction of the xylanolytic enzyme spectrum.

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Two proteins exhibiting -D-xylosidase activity were identified upon fractionation and purification of a culture filtrate of an arabinoxylan-grown *Aspergillus niger*. A single band of 110 kDa by SDS-PAGE was obtained in both cases and these were active on xylo-oligosaccharides but not on xylan. Partial *xlnD* cDNA clones were immunochemically identified and isolated from a cDNA expression library. Sequence analysis showed that all cDNA clones correspond to a single gene. A genomic clone was isolated and overexpressed in *A. niger* and *A. nidulans*. The *xlnD* gene has an ORF of 2412 nucleotides, encodes a protein of 804 amino acids and contains a potential signal peptide of 26 amino acids. This results in a mature protein of 778 amino acids with a predicted molecular mass of 85 kDa and an isoelectric point of 4.5. The protein is N-glycosylated and contains 15 potential N-glycosylation sites. Sequence similarity is found with -D-glucosidases both of bacterial and fungal origin. Both -xylosidase proteins purified have high activity on theartificial substrate para-nitro-phenyl- -D-xylopyranoside (XylNp) and a side activity on para-nitrophenyl--L-arabinofuranoside (AraNp) and para-nitro-

phenyl- -D-glucopyranoside (GluNp). *A. niger* strains, in which the *xlnD* gene was disrupted, accumulate mainly xylobiose and xylotriose when grown on xylan and have no significant -xylosidase activity in the culture medium, indicating that this gene encodes the major extracellular -xylosidase.

92. Coordinate regulation of expression of a cytochrome P450 enzyme system in *Aspergillus niger*.

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Cytochrome P450 enzyme systems comprise two elements; cytochrome P450 reductase (CPR), a generally acting electron donor and the reaction specific cytochrome P450 enzyme. In previous work we have identified the *A.niger* cytochrome P450 gene encoding benzoate para-hydroxylase (*bpha*) and the gene encoding cytochrome P450 reductase (*cprA*). Expression of both genes was shown to be regulated at the transcriptional level by benzoate. However, some indications were obtained that regulation also might occur at post-transcriptional level. To study the exact mechanism underlying the regulation of gene expression of both genes, the gene control region of both genes were fused to a reporter gene followed by generation of progressive deletions. Using this strategy we were able to identify regions (Benzoate Responsive Region = BRR) in both gene control regions involved in benzoate dependent induction of gene. Cloning of this DNA region upstream from a minimal promoter conferred benzoate inducibility on this promoter. Another mechanism involved in regulation of the BPH enzyme system is the use of different promoters[1]). Clear differences in mRNA size was observed between *cprA* and *bpha* mRNA obtained from induced and from non-induced mycelium. Using 5'-RACE we were able to determine the different transcription start points.

[1]) Promoter is defined as the part of the gene expression control region where the general transcription factors and the RNA polymerase assemble.

93. Heterologous protein expression system using a lipase gene, *mdlA*, from *Penicillium camembertii.*

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The mono- and diacylglycerol lipase (MDGL)-encoding gene, *mdlA*, from *Penicillium camembertii* (Pc) has advantageous characteristics for utilizing to heterologous protein expression. First, *mdlA* promoter functons efficiently in *Aspergillus oryzae* (Ao) as well as Pc, and is also functional in *Saccharomyces cerevisiae* (Sc). Secondly, the MDGL Pro-form is processed at a Lys-Arg site in the C-terminal region in both Pc and Ao. These findings led us to develop a novel heterologous protein expression system using the *mdlA* gene. In this system, a target protein can be expressed in three microorganisms, Pc, Ao and Sc by the introduction of a

same expression cassette. The host organism is selected according to the purpose and is introduced with the cassette by an appropriate vector. The *S. cerevisiae* host is suitable for the plate colony-screening of large numbers of randomly mutagenezed target proteins. As a model protein, we have examined the expression of two filamentous fungal proteins, *Myrothecium verrucaria* bilirubin oxidase (BO) and *Eupenicillium brefeldianum* ascorbate oxidase (ASO) and an anti-bacterial protein, sapecin, from insect, *Sarcophaa peregrina.* The BO cDNA was efficiently expressed in Pc (1.0 g/L) and Ao (0.3 g/L), and Sc transformant could be conducted to plate-colony assay. The ASO gene was also expressed efficiently in this system. The expression and secretion of sapecin was examined by the use of a fusion gene, in which mature sapecin cDNA was connected at the Lys-Arg site of *mdlA.* The Pc transformant having the fusion gene secreted correctly processed recombinant sapecin with a yield of 7 mg/L and a normal biological activity.

94. A palindromic CCAAT-motif is involved in the regulation of the *Trichoderma reesei cbh2* (cellobiohydrolaseII-encoding) gene.

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The cellulase system of the filamentous fungus *Trichoderma reesei* consists of several cellobiohydrolases, endoglucanases and B-glucosidases, encoded by separate genes, which are coordinately expressed in the presence of cellulose or the disaccharide sophorose. In order to identify nucleotide-motifs involved in the induction of cellulases we prepared protein extracts from induced and non induced mycelia of *Trichoderma reesei* QM 9414, and used them for electrophoretic mobility shift assays (EMSA) with a 90 bp fragment of the *cbh2* promoter. This assay detected different, specific protein-DNA complexes present when using cell-free extracts from glucose, lactose or sophorose grown cultures. Using various overlapping fragments and competitive oligonucleotides, the DNA target motif for these protein complexes was shown to be a palindromic CCAAT-box. A similiar motif is also present in the 5' noncoding region of another sophorose inducible gene (*xyn2*). To study the DNA-protein complex formation more in detail, the binding motif was elucidated by EMSA using native and mutated oligonucleotides and by *in vitro* DNA-footprinting with crude extracts.

Aspergillus

95. The gene product of *hapC* is a subunit of AnCP/AnCF, a CCAAT-binding protein in *Aspergillus nidulans*.

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CCAAT sequences in promoter regions of many fungal genes such as the *taa* (Taka-amylase A) and *amdS* (acetamidase) genes play important roles in the determination of expression levels. We have shown that an *Aspergillus nidulans* CCAAT-binding protein (factor), AnCP/AnCF recognizes CCAAT sequences in several genes in *A. nidulans.* The *hapC* gene, an *A. nidulans* counterpart of the yeast HAP3 gene, was isolated and used to obtain *hapC* disruptants. No CCAAT binding activity was detected in nuclear extracts from a *hapC* disruptant. Taken together with the high similarity of *HapC* to the HAP3 protein this result suggests that the *hapC* gene product is a subunit of AnCP/AnCF.

To examine whether or not HapC is a component of the AnCP/AnCF complex, a recombinant MalE-HapC fusion protein was produced in *E. coli* and purified. AnCP was denatured in the presence of MalE-HapC, renatured, and used for gel shift assays. The shift band corresponding to a taa promoter-AnCP complex disappeared and a new band with lower mobility was observed. When anti-MalE antiserum was added to the binding reaction, the band was supershifted. These results indicate that the MalE-HapC fusion protein was functionally incorporated into the AnCP/AnCF complex bound to the CCAAT containing sequence. This clearly demonstrates that the hapC gene encodes a subunit of AnCP/AnCF.

96. Regulation of Septum Formation in *Aspergillus nidulans* by a DNA-damage Checkpoint Pathway.

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Cytokinesis (septation) in *Aspergillus nidulans* germlings is delayed until three rounds of nuclear division have occurred. Conditional mutations in the *sepB* gene block septation but allow germinating conidia to complete the first three rounds of nuclear division. This phenotype can be mimicked by germinating wild-type conidia in the presence of sub-lethal concentrations of the DNA damage-inducing agent diepoxyoctane (DEO) and the replication inhibitor hydroxyurea (HU). The effect of the *sepB* mutation and the response to DEO are both suppressed under conditions where inhibitory phosphorylation of $p34^{nimX}$ kinase does not occur, indicating that this kinase is a target of the machinery regulating cytokinesis. The *sepB* phenotype and the DEO response can also be suppressed by loss-of-function mutations in the DNA damage checkpoint genes *uvsB* and *uvsD*, suggesting that the pathway which determines the appropriate timing of cytokinesis is the same pathway which prevents mitosis in the presence of DNA damage. A model which accounts for the regulation of septum formation by $p34^{nimX}$ will be presented.

We have initiated two screens to identify additional components of the regulatory network which controls septation. For one screen, a collection of mutants which are sensitive to low levels of MMS and HU has been generated. This collection has yielded several mutants which form septa inappropriately (i.e. in the presence of DNA damage). In a second screen, HU-sensitive pseudorevertants of the Ts *nimT23* mutation were collected and are being analayzed for the precocious formation of septa. Details of these screens will be presented.

97. Identification and characterization of *Aspergillus nidulans* genes required for establishment and/or maintenance of Cell polarity.

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Establishment and maintenance of cell polarity are essential for the growth and development of the fungus *Aspergillus nidulans.* We have identified four complementation groups of temperature sensitive polarity defective (*pod*) mutants that are unable to establish and maintain cell polarity based on the following criteria: (i) failure of germinating spores to produce a germ tube, (ii) enlargement of spores with continued growth in a depolarized manner, and (iii) accumulation of multiple nuclei since pod mutations should not affect nuclear division. We have characterized the phenotypes of these mutants in detail and are currently initiating attempts to clone the *pod* genes.

As an alternative approach, we are cloning genes known to play an essential role in cell polarity in other organisms. One such gene is CDC42, originally identified in the budding yeast *Saccharomyces cerevisiae.* Cdc42p is a member of the ras superfamily of low-molecular-weight GTP-binding proteins. Yeast *cdc42* mutants display an inability to establish cell polarity during budding. Functional homologues of Cdc42p have also been identified in such varied species as *S. pombe, C. elegans, Drosophila,* and human cells suggesting that it may be a fundamental component of the mechanism controlling cell polarity in all eukaryotes. It is reasonable to expect that the homologue of the CDC42 gene in *A. nidulans* is required for polarized vegetative growth and/or conidiophore development. We have cloned the *A. nidulans* homologue of CDC42 and have begun characterization of the null phenotype and the constitutively activating and inactivating mutations of Cdc42p.

98. The sequence of *pal*F, an environmental pH response gene in *Aspergillus nidulans.*

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Aspergillus nidulans, and probably all other living organisms, alter gene expression as an adaptive response to environmental pH changes. The *pal* genes probably constitute part of a signaling pathway that responds to changes in environmental pH. To molecularly characterize the influence of external pH in the secretion of enzymes by filamentous fungi, we have cloned and sequenced the *pal*F gene of *Aspergillus nidulans*. cDNA clones were obtained from a lgt10 cDNA library and sequenced, showing a nucleotide sequence of 3,320 bp. A major 2,325 bp long ORF was identified and its translation resulted in a putative protein of 775 aa, with a predicted molecular mass of 84,086 Da. A large (828 bp) 5'-UTR was observed and a putative polyadenylation signal was identified in the 3'-UTR. This large 5'-UTR is very unusual for fungal transcripts and is the largest thus far noted. The *pal*F nucleotide sequence did not show any clear homology with any known nucleotide sequence and the putative PALF protein shared some sequence similarity with the putative products of *Saccharomyces* chromosome VII ORFs

YGL045w and YGL046w. The similarities seen between the predicted PALF protein and these ORFs are restricted to regions of 18 to 57 aa in length and had similarities of 43 to 72%.

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99. Characterization of *sepH,* a gene encoding a protein kinase required for septation in *Aspergillus nidulans.*

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We are investigating the process of septum formation in the filamentous fungus, *Aspergillus nidulans*. The hyphae of *A. nidulans* are partitioned into multinucleate compartments by the formation of septa. The earliest visible sign of septation is the assembly of an actin ring which anticipates the site of septum formation. Following the completion of nuclear division, the actin ring decreases in diameter and cell wall material is deposited to form the septum (M. Momany and J.E. Hamer, submitted). We have isolated a collection of ts mutants defective in septum formation (Harris *et al.*, 1994. *Genetics* 136:517532). One of these mutants, *sepH*, appears to be required late in the process of septum formation. This mutant is unable to form septa but displays no defects in growth or nuclear division, and thus produces long, multinucleate hyphae at the restrictive temperature. The *sepH* gene has been cloned and encodes a protein kinase with similarity to the protein kinase encoded by *cdc7* in *S. pombe*, which is also required for septum formation. Phenotypic analysis of the *sepH* null mutant indicates the presence of faint actin rings. Based on these results, a model for SepH function will be proposed.

100. The regulator of nitrate assimilation in Aspergillus nidulans binds a completely asymmetrical sequence as a dimer.

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NirA protein mediates nitrate induction of the *niaD, niiA* and *crnA* genes of *Aspergillus nidulans,* encoding respectively nitrate reductase, nitrite reductase and a nitrate permease. We have studied in detail the mode of binding of NirA to its cognate DNA target sequences. The sequence bound by NirA is the asymmetrical sequence 5'CTCCGHGG3'. Four such sequences are present in the intergenic region which lies between the divergently transcribed *niiA* and *niaD* genes. We have studied the binding of NirA to the region by DNaseI and methylation protection, and by methylation, depurination and depyrimidation interference, the seven bases of the consensus sequence interfere strongly. Methylation interference reveals that the phosphate contacts are clearly asymmetrical. We have also shown that the NirA protein binds as a dimer. This was shown by the expression of two NirA partial peptides of different lengths in an in vitro transcription/translation system. The formation of the heterodimer is evident in gel shifts assays.

We have shown that the putative coiled-coil domain, carboxy terminal to the DNA binding domain can replace the cI dimerisation element in an in vivo lambda immunity assay. We have also carried out a mutagenesis analysis of the NirA dimerisation domain. These data show an unprecedented mode of binding of the NirA protein to its cognate DNA.

101. Improved enzyme expression in *Aspergillus oryzae* due to vector construction and chromosomal *amdS* deletion.

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A. oryzae has been used industrially to produce heterologous proteins of commercial interest. We have investigated the effects on heterologous expression of different selectable markers and the topology of the transforming DNA on the expression of a heterologous lipase in this fungus. Different vectors were used to transform A. *oryzae* hosts and approximately 100 transformants of each were tested in shake flask cultures for their ability to produce extracellular lipase. We report here the distribution of lipase yields observed from the transformants. The following observations were made: i. lipase yields of single vector transformants are on average higher than those produced by cotransformants; ii. lipase yields of transformants generated using a linearized expression cassette which does not contain *E. coli* vector sequence are on average higher than those produced by transformants generated with circular plasmid; iii. linearization of the expression plasmid leads to an increase in transformation frequency but does not have an effect on lipase yields; iv. the production of lipase is increased on average using *amdS* versus *pyrG* as the selectable marker; v. the presence of a truncated *amdS* promoter leads to an increase in lipase production on average. We have also examined the effect of disrupting the chromosomal *amdS* gene because background growth is observed with *A. oryzae* on plates with acetamide as the sole nitrogen source.

The presence of a *amdS* locus in the strain to be transformed shifts the distribution of lipase production towards the higher end and reduces background growth.

102. Identification of Protein kinase C in *Aspergillus nidulans*.

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A 600 base pair (bp) PCR product was amplified from *Aspergillus nidulans* genomic DNA using degenerate oligonucleotide primers designed to eukaryotic Protein kinase C (PKC) gene sequences. This product was cloned and sequenced and its predicted amino acid sequence has 90% identity with the pedicted amino acid sequence (aa 837-942) from an *Aspergillus niger* PKC gene (Morawetz et al., 1996. Mol. Gen. Genet. 250: 17-28). The PCR product was labelled and used to probe a Southern blot of restricted *A. nidulans* genomic DNA revealing a single BamH1 digestion product of 6kb. Immunoblotting using anti-PKC antisera raised to a conserved PKC peptide reveals protein bands at 140, 50 and 40kDa in *A. nidulans* membrane protein fraction and 140, 90 and 40kDa in *A. nidulans* cytosolic protein fraction. The effect of two PKC inhibitors

(chelerythrine chloride and bisindolylmaleimede) and a PKC activator (a phorbol ester) on the growth and morphology of A.nidulans were assessed. No significant effects on specific growth rate in *A. nidulans* were observed. However, germlings grown on the two inhibitors were consistently smaller than controls and germlings grown on the activator were consistently larger than controls. This could indicate a role for PKC in spore germination.

103. Analysis of the protein secretion pathway of *Aspergillus niger.*

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Filamentous fungi, Such as *A.niger,* secrete large amounts of proteins into the culture medium. However, little information is available on the molecular mechanisms of protein secretion in these organisms. We have started research to analyse the mechanism of protein targeting/secretion and to identify and resolve potential bottlenecks for efficient protein overproduction [(1,2)].

I To identify the different compartments of the secretion pathway and to study the routing of proteins to these compartments, several genes encoding compartment specefic proteins have been isolated. The cloning and characterisation of the *A.niger bipA* gene, encoding the major ER-chaperone, will be presented together with initial results aimed at the overexpression of the ER-chaperone to improve the yields of secreted heterologous protein.

II Based on research carried out in *S. cerevisae,* defined mutations in the secretion pathway will provide important information about the organisation of the pathway. Therefore, the cloning of several various genes encoding small GTP-binding proteins involved in the vesicle transport between the different compartments of the secretion pathway, was started using heterologous hybridisation and PCR-based approaches. The cloning of several of these small GTPase encoding genes will be presented together with initial results on *A. niger* strains carrying mutant alleles of one of these genes *(sarA)*

(1) Punt, P.J et al (1994) Anthony van Leeuwenhoek 65.- 211-216. (2) Gouka et al. (1997) Appl Microbiol Biotechnol, in press

104. *sepA* is Necessary for Normal Cytokinesis During Vegetative Growth and Conidiation in *Aspergillus nidulans*. Kathryn E. Sharpless and Steven D. Harris, Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06030-3205.

Ts mutations in the *Aspergillus nidulans sepA* gene affect a variety of morphogenetic processes in hyphal cells. Specifically, these mutations prevent septation and lead to an inability to maintain hyphal polarity. *A. nidulans* is dimorphic, in the sense that the pattern of cell division is altered during conidiophore formation to a mode which is more similar to that of budding in yeast. We have used two approaches to analyze the effect of *sepA* mutations on cell division during conidiation.

Strains in which the regulation of the important developmental genes, *brlA* and *abaA*, is altered undergo inappropriate development. The induction of *alcA::brlA* leads to the formation of single spores at hyphal tips. Furthermore, these spores are subtended by "collar-like" septa, which appear similar to those formed in normal conidiophores. Induction of *alcA::abaA* leads to the formation of unusually thick septa throughout the hyphae. We have found that the formation of septa in *alcA::brlA* and *alcA::abaA* is dependent upon the presence of a functional *sepA* gene product.

A deletion mutant of *sepA* grows slowly and is capable of making conidia, although fewer than normal. Analysis of conidiophores in the *sepA* deletion mutant revealed that many undergo uncharacteristic branching. In addition, many spores fail to bud properly resulting in the production of multiple connected spores. These results suggest that the *sepA* gene product is required for cytokinesis in both hyphal cells and in developing conidiophores. Furthermore, they also indicate that *sepA* is necessary for maintenance of polarity during conidiophore development.

105. Meiotic Mutants of *Aspergillus nidulans*: Phenotypes, Gene Localization and Gene Cloning.

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More then twenty meiotic mutants of *Aspergillus nidulans* have been isolated (identified by the lack of ascospores in well developed cleistothecia) and tested for complementation. Eighteen different complementation groups were identified. Five mutant genes were assigned to linkage groups. Three mutants were characterized cytologically: one appeared to be blocked at karyogamy (karA1), another at prophase I (meiC8) and a third one at metaphase I (meiB7). The karA1 mutant has been transformed with a linkage group VI specific cosmid library and the complementing wild type gene has been isolated. Further characterization of this gene is in progress.

106. Isolation and characterization of *Aspergillus nidulans* delayed conidiation mutants.

J. Wieser and T. Adams. Texas A&M University College Station, TX 77843.

The formation of conidiophores in A. *nidulans* is a precisely regulated event that occurs within the context of a radially expanding colony. Conidiophores typically differentiate 1-2 mm behind the leading edge of vegetatively growing hyphae. Mutations in any of four *A. nidulans* genes *flbB,flbC,flbD,* and *flbE* result in fluffy colonies that are delayed at least 24 hours in their ability to form conidiophores. This delay in conidiophore initiation gives rise to colonies in which conidiophore development occurs 12-15 mm behind the leading edge of the radially growing hyphae. Each of the four genes is required for normal timing of *brlA* expression indicating a potential role for each in activation of this important developmental regulatory gene. Wild-type copies of all four genes have been isolated and transcripts of all the genes were shown to be

present throughout the *A. nidulans* life cycle. The deduced *flbC* protein sequence predicts a polypeptide with significant similarity to C2H2 zinc finger proteins, while the *flbD* protein sequence predicts a polypeptide with significant identity at its N-terminus to the DNA binding domain of the *myb* family of transcription factors. The sequence analysis of *flbB* and *flbE* is in progress. The genetic interactions among *flbB, flbC, flbD,* and *flbE* and the other genes required for development is discussed.

107. Effects of two mutant actin alleles on growth and morphology in *Aspergillus nidulans.*

Tom D. Wolkow and John E. Hamer, Purdue University, West Lafayette, IN 47907

The mycelium of *Aspergillus nidulans* is composed of multinucleate cellular compartments delimited by crosswalls called septa. Initiation of septum formation occurs in germlings which have achieved a minimal cell size and undergone at least one nuclear division. Evidence suggests that the future site of septum formation in *A. nidulans* is marked by an actin ring. The actin ring forms over a mitotic nucleus and persists until cytokinesis and septation begin, at which time the actin ring invaginates, leaving behind a ring of chitin and other septal material. The placement of septa in mycelium, and presumably the actin ring, can be manipulated by altering the distribution of nuclei. Two mutant alleles of actin were constructed (based on previous experiments in *Saccharomyces cerevisiae)* to test the role of actin in determining the site of cytokinesis and septation in *A. nidulans.* Although each allele was designed to be temperature-sensitive, they arrested growth shortly after germination at the permissive temperature. Germlings displayed morphologic abnormalities but did not appear compromised for septum formation or positioning.

108. Characterization of the *Aspergillus oryzae brlA* gene by its overexpression and disruption

Osamu Yamada, Byung Rho Lee, Katsuya Gomi and Yuzuru Iimura National Research Institute of Brewing

Aspergillus oryzae conidiospores (called tane-koji in Japan) have been used for making koji as a supplier of hydrolytic enzymes and nutrients in the Japanese fermentation industry, such as sake, soy sauce and miso. It is therefore, of great importance in tane-koji manufacturing that industrial strains of A. *oryzae* can produce conidiospores efficiently. To understand the mechanism and control of *A. oryzae* conidiation, at first we cloned *brlA* gene from A. *oryzae* RIB40. The *brlA* gene of *A. oryzae* contained an open reading frame coding for 421 amino acid residues and the deduced amino acids sequence revealed 70% homology with *A. nidulans* BRLA. To investigate the function of the *A. oryzae brlA* gene, we constructed (-amylase promoter-*brlA* fusion gene (P*amyB-brlA)* and introduced into *A. oryzae* niaD300. Interestingly, these transformants of the P*amyB-brlA* fusion gene could form conidiophore even in the submerged culture supplemented with maltose under control of the -amylase promoter. Furthermore, the *brlA* gene disruptant of *A. oryzae* could not develop any conidiophore, indicating that the *A. oryzae brlA* gene is also main regulator of conidiation.

Evolution and Population Genetics

110. Clonal origin and phylogeny in *Sclerotinia sclerotiorum*.

Ignazio Carbone and Linda M. Kohn, Dept. of Botany, Univ. of Toronto, Erindale College, Mississauga, Ontario, Canada, L5L 1C6.

Sclerotinia sclerotiorum is a cosmopolitan ascomycetous plant pathogen with a wide host range. This fungus reproduces asexually by sclerotia and sexually by self-fertilization. Both mycelial compatibility testing and DNA fingerprinting with a dispersed nuclear repetitive element have shown that *S. sclerotiorum* has a predominantly clonal population structure with many unique genotypes. We are using a phylogenetic approach to study whether mutation or recombination is the dominant evolutionary force that is giving rise to new genotypes. If mutation is the diversifying force, then clones should be related to each other in clonal lineages and phylogenies will be well-supported. If recombination is giving rise to new genotypes, then clonal lineages should fall apart and phylogenies will be poorly resolved. So far we have identified three phylogenetically informative regions in *S. sclerotiorum*, the intergenic spacer (IGS) of the nuclear ribosomal DNA repeat, a portion of the gene encoding the translation elongation factor 1 alpha, and one anonymous nuclear region. A parsimony analysis of nucleotide variation at all three loci for a sample of 42 isolates of *S. sclerotiorum* from a Canadian canola field showed one most parsimonious tree with a consistency index of 0.857. To further test our hypothesis of clonality, we will compare the length of our tree with tree lengths obtained from randomized data sets and also perform a likelihood analysis for each locus separately and for all three loci together. We are also extending this study to other populations of *S. sclerotiorum* from both wild plants and other agricultural hosts.

111. ITS sequences from the arbuscular mycorrhizal fungus, *Gigaspora margarita*.

Luisa Lanfranco, Silvia Perotto and Paola Bonfante. Dipartimento di Biologia Vegetale dell'Università and Centro Micologia del Terreno -C.N.R.- Viale Mattioli 25, 10125 Torino, Italy.

Arbuscular mycorrhizal fungi are obligate endosymbionts, colonizing the roots of almost 80% of land plants. They belong to the order Glomales, Zygomycetes, with at least 150 species. Each asexual spore contains thousands of nuclei, with a relatively large amount of DNA (about 10^9 bp/nucleus) when compared to other fungi. Several PCR based-techniques have been recently applied to explore the genetic diversity of these fungi. The ITS (Internal Transcribed Spacers) of the nuclear ribosomal unit have proved to be very informative to reveal species-specific DNA polymorphisms. It has been proved that a single spore of several *Glomus* species presents slighly different ITS sequences. In order to study genetic variability and to devise molecular tools to study species diversity in the field, we have investigated polymorphism of the ITS region of different isolates of Gigasporaceae. Multispore DNA preparations were amplified with ITS1/ITS4 universal primers. Single bands were obtained, about 550-600 bp in size. The fragment of *Gigaspora margarita* was cloned and after sequencing of different clones, at least three different sequences were obtained. By comparison with sequences in data banks, two primers were designed and tested for their capability to detect specifically *G. margarita* during the symbiotic phase. A similar approach has been used to investigate the species *Glomus*

versiforme.

112. Molecular phylogeny of the Agaricales based on 25S rDNA sequences.

Moncalvo, J. M., F. Lutzoni, S. Rehner, J. Johnson, and R. Vilgalys, Department of Botany, Duke University.

The identity of natural groups within the order Agaricales were addressed using ribosomal DNA sequences. Approximately 900 bases of the 5' end of the nuclear-encoded large subunit RNA gene (25S rDNA) were sequenced for over 300 taxa, selected broadly across all major families within the Agaricales. A phylogenetic tree was estimated from 154 of the most diverse taxa using maximum parsimony. Many groups were supported by moderate to high bootstrap levels, or else are consistent with morphologically-based classification schemes. Some well recognized groups were the families Amanitaceae, Coprinaceae (excluding *C. comatus* and subfamily Panaelideae), and Agaricaceae (excluding the Cystodermateae), and the genera *Tricholoma, Termitomyces* and its ally *Podabrella*, *Pleurotus* and *Hohenbuehelia*, etc. Nonmonophyletic groups revealed were the families Tricholomataceae and Hygrophoraceae, and the genera *Clitocybe* and *Marasmius*. This first-order estimate of phylogenetic relationships among major lineages will serve as a starting point for further comparative studies on mushroom biology and evolution.

113. Hypermutability as a source of genomic variation in the homothallic fungus Basidiobolus.

Rex Nelson and Bruce Cochrane. Department of Biology, University of South Florida, Tampa, FL 33620.

Members of the genus *Basidiobolus* (Ediam, 1886) are filamentous saprophytic fungi. This genus has a sexual phase but is considered homothallic and as such, its reproduction is essentially clonal. Genetic studies of isolates recovered from the environment demonstrate that there exists a great amount of variation within and between populations of this fungus. RAPD analysis of isolates recovered from the University of South Florida's Ecological Study Area indicate that virtually each isolate is genetically unique. Sequencing of a fragment of the chitin synthase gene revealed an extraordinarily high number of silent site differences among isolates. In order to determine whether sequence variation is generated during sexual reproduction sequences were obtained from single zygospore cultures derived from a single saprobic isolate. The sequence obtained from one of the progeny isolates differs by 10% from the parental sequence. All differences were silent with respect to amino acid sequence. Sequencing of a fragment of an unrelated gene similar to the RNA binding domain of the RNP1 gene of *Saccharomyces cerevisiae* reveal no sequence differeces between the parent and progeny. The spontaneous mutation rate to resistance to miconazole was also determined from assay of conidia germination on selectective media. This rate approached 2×10^{-5}. Implications of these observations on the

genetic structure of *Basidiobolus* will be discussed.

114. Fungal pathovars from north American host species are distinct from those isolated from hosts not native to this continent.

Michael H. Perlin. University of Louisville, Louisville, KY 40292.

Molecular markers were used to compare haploid strains of *Microbotryum violaceum (Ustilago violacea)* derived from different host species. This fungus is an heterobasidiomycete which forms its spores in the flowers of over 200 species of Caryophylaceae (Pinks). On the other hand, each strain of the fungus has a host range limited to one or a few plant species. Although different *M. violaceum* strains, even from different hosts, are morphologically similar and can apparently initiate mating, inter-pathovar matings seldom result in "normal" progeny; often passage through host plants, when successful, leads to aneuploid or polyploid sporidia . In order to establish relationships between and among different pathovars, haplotype analysis was carried out on sporidia derived from eight different host species. Comparisons were made using electrophoretic karyotypes and by phylogenetic analyses of two introns and an intervening exon of their respective -tubulin genes. Strains from the same or similar host species had similar chromosome profiles and similar, if not identical, DNA sequences in the region examined. Most striking, were differences for both electrophoretic karyotype and DNA sequence polymorphisms between the strains derived from North American host species and those from hosts native to Europe. Thus, reproductive isolation has apparently resulted in major polymorphisms between the North American *M. violaceum* strains and their European cousins.

115. Fungal Class C Cellulases.

Alan Radford, Biology Department, University of Leeds and Maristella Azevedo, Cell Biology Department, University of Brasilia.

We have now sequenced two CBH1 isozymes from *Humicola grisea* and one from *Neurospora crassa* and have a second equivalent *N. crassa* clone. Seventeen more fungal examples are sequenced, including both EG1 and CBH1 from *Trichoderma reesei* and other *Trichoderma* species, an EG1 from *Humicola grisea*, EG1 and CBH1 from *Fusarium oxysporium*, CBH1 from *Penicillium janthinellum*, *Cochliobolus carbonum* and *Cryphonectria parasitica*, and a series of CBH1 isozymes from *Phanerochaete chrysosporium*. Sequence alignment and phylogeny using CLUSTAL suggests that the ancestral class C cellulase was a CBH1 (exocellulase or cellobiohydrolase) which is found in both ascomycetes and basidiomycetes. By interstitial deletion, an EG1 (endocellulase) clade has arisen in ascomycetes and their now imperfect relatives, in *T. reesei*, *T. longibrachiatum, H. grisea* and *F. oxysporium*. The seven CBH1 isozymes of the basidiomycete *P. chrysosporium* form a single clade, and appear to have amplified within this line. The two CBH1 isozymes of *H. grisea* are closely related to each other, and to the single known CBH1 sequences from *N. crassa* and *F. oxysporium*. The single CBH1 of the two *Trichoderma* species, *C. parasitica, C. carbonum* and *P. janthinellum* forms a distinct

branch. Most class C cellulases have a hinge region and a C-terminal cellulose-binding domain (CBD). However, these have been lost independently within several clades.

Population Genetics

116. Development of a genus-specific genetic marker for identification of a phytopathogenic fungus.

Elisa M. Becker and William E. Hintz, University of Victoria.

The basidiomycete fungus *Chondrostereum purpureum* is being developed as a biocontrol for forest weed species. To fulfill registration requirements we are conducting provincial and national population surveys, environmental fate studies and efficacy trials. During these studies *C. purpureum* as well as many other fungi are routinely isolated from wood. The mycelia of many fungi are morphologically similar on agar media and traditional biochemical tests are not specific enough to resolve fine differences, hence a PCR-generated marker that could be used to distinguish *C. purpureum* from other fungi was desired. Our recent work has revealed relatively little variation in the intergenic spacer region (IGS) of the ribosomal DNA (rDNA) of isolates of *C. purpureum* collected from several regions of the world. We hypothesized that a small portion of this region would be a good target for the design of diagnostic primers specific for *C. purpureum.* A 500 base-pair fragment of the rDNA IGS was subcloned and the terminal sequences used to design sequence-characterized oligonucleotide primers for amplification of specific regions (SCAR). This SCAR primer pair has been used to successfully amplify a 500 base-pair fragment from every *C. purpureum* isolate screened to date. Furthermore, this fragment was not amplified from any other fungal species tested including those which are closely related to *C. purpureum* and/or found in a similar ecological niche. These diagnostic primers are now being used to routinely screen DNA from all putative *C. purpureum* cultures collected.

117. Molecular population genetics of the pathogenic fungus, *Coccidioides immitis:* recombination, distinct populations, and cryptic speciation.

A. Burt, V. Koufopanou, G.L. Koenig, B. M. Dechario, T.J. White and J.W. Taylor. University of California, Berkeley; Imperial College, Silwood Park; Roche Molecular Systems, Alameda, CA.

Understanding the reproductive mode (clonal or recombining), and the population structure of fungi is important to studies of their identification, molecular development, epidemiology, and control. With the ascomycete pathogen, *Coccidioides immitis*, we used phylogenetic and population genetic analysis of 14 loci (defined as base subsituations or small length mutations in usually arbitrary DNA), to show that Arizona *C. immitis* recombine in nature. Using 11 of these loci and population genetic analysis (Wright's Fst), we now show that gene flow among *C. immitis* from Arizona, California and Texas is significantly reduced, particularly to and from

California. Subsequent analysis of ca. 2400 nucleotides from parts of five genes in 17 widespread *C. immitis* confirms recombination among *C. immitis* individuals in-California and out-of-California, and demonstrates genetic isolation of the two groups over an estimated 8 myr. Eight fixed nucleotide differences between the groups facilitate group-specific identification and make it desirable to include representatives of both groups (species?) in efforts to control this fungus by drugs or vaccines.

118. Multiallelic molecular markers for strain typing and epidemiology studies of fungal populations.

Dee A. Carter and Nai Tran Dinh. Microbiology Department, University of Sydney, NSW, Australia.

Multiallelic molecular markers such as microsatellites and short repetitive sequences are being widely applied to population and genetic studies of many eukaryote species, but their application to the study of fungi has been relatively limited. As these markers are analysed by directed PCR amplification they can be detected in minute amounts of even extensively degraded DNA, making them ideal for the study of hazardous pathogens or organisms that are difficult to culture. In addition, these markers may be amplified from impure DNA samples so they can be applied to obligate pathogens and commensals without the need to eliminate host tissue. Here we report the development of multiallelic markers from the genomes of *Histoplasma capsulatum* and *Aspergillus parasiticus*, and show how these markers can be used to distinguish isolates of these fungi at the individual or clonal level.

119. Mitochondrial DNA haplotypes in clonal and sexual populations of *Phytophthora infestans.*

Pia D. Gavino and William E. Fry, Dept. of Plant Pathology, Cornell University, Ithaca NY.

The diversity in nuclear DNA (nDNA) and mitochondrial (mtDNA) was assessed within clonal and sexual populations of the late blight fungus, *Phytophthora infestans.* Every isolate tested (n=60) from central Mexico, the center of origin of *P. infestans,* had a different nDNA fingerprint. Surprisingly, all these isolates had the same mtDNA haplotype (Type A). The high nDNA diversity and low mtDNA diversity was consistent with the absence of recombination in mtDNA and the uniparental inheritance of mtDNA in sexual populations of *P. infestans.* In northern Mexico, however, two mtDNA haplotypes (Types A and B) were identified within clonal and sexual populations (n=30). Type B appears to have evolved from Type A through an insertion of about 2 kb and rearrangement in the mitochondrial genome. Types A and B were also found in other clonal and sexual populations of *P. infestans* worldwide. Type A was found in most of the old clonal lineage (US- I genotype) and in recombinant isolates recently found in British Columbia (n= 18). Both haplotypes were likewise present in recent clonal lineages in Latin America (n=33) and in sexual populations in Europe (n=14) and in the Pacific northwest of the United States (n=26). These findings support the hypothesis that the mtDNA haplotype B of *P. infestans* may have originated from northern Mexico.

120. Wright's fixation index analysis reveals the probable mating system for 16 species of *Phytophthora.*

Stephen B. Goodwin, USDA-ARS/Purdue University, West Lafayette, Indiana.

Approximately half of the 67 species of *Phytophthora* are heterothallic; the rest are homothallic. If hetero- or homothallism dictates the mating system, there should be almost no heterozygosity in populations of homothallic *Phytophthora* species due to self fertilization. In contrast, heterothallic species should contain high levels of heterozygosity. However, levels of heterozygosity within species of *Phytophthora* so far have not been analyzed. To test whether there are differences in mating system, Wright's fixation index [$F = 1 - (H_{Obs} / H_{Exp})$, where H_{Obs} is the observed heterozygosity and H_{Exp} the expected heterozygosity assuming random mating] was calculated for 16 species of *Phytophthora* by reanalysis of previously published data. As expected, fixation indices were near 1.0 for four of the six homothallic species. Fixation indices for four of the ten heterothallic species were between zero and 0.3, as expected for random mating populations. The remaining species could be divided into three groups. One group, consisting of three hetero- and one homothallic species, probably had a mixed mating system with intermediate fixation index values near 0.5. A second group of heterothallic species had high fixation index values similar to those for homothallic species, probably due to asexual reproduction or inbreeding. A third group contained two heterothallic species with negative fixation index values. Deviations from expectation probably were due to asexual reproduction, incorrect scoring of some isozyme data, or possibly a Wahlund effect. Many *Phytophthora* species probably have a mixed mating system in nature that cannot be predicted on the basis of hetero- or homothallism. However, this conclusion is preliminary and must be confirmed by analyses of larger samples from carefully defined populations.

121. Mathmatical and computer modeling of the spread hypovirulence in fungal populations.

Susan Kelling, Keith Klein and Marty Wolf, Mankato State University, Mankato MN, 56002.

We employed a multiple population growth model to simulate the effect of the release of hypovirulence factors into a population of a plant pathogen. The model followed logistic growth kinetics with discrete generations for ease of analysis. The following variables were tested for their effect on the predicted spread of hypovirulence: reproductive rate of infected and uninfected individuals (rate for infected individuals < rate for uninfected individuals),number of genotypes (vegetative compatibility groups, VCG), transmission rate of hypovirulence within VCG and between VCG's (rate within>rate between), migration rate of individuals between populations, and recovery rate from hypovirulence infection. The model was realized on a

massively parallel processor and run as a simulation for many generations. The simulations predict that recovery rate is the single best predictor of the success of hypovirulence as a control method.

122. Classical and molecular genetic analyses of *Colletotrichum* spp.

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The genus *Colletotrichum* contains plant pathogens which cause disease on a number of economically important crops worldwide. *C. acutatum* f sp *pineum is* capable of killing *Pinus radiata* seedlings. This pathogen was first noted in New Zealand (NZ) in the 1960's. In the mid 1980's, a *Colletotrichum* species caused widespread dieback of tree lupin *(Lupinus arboreus)* in NZ. Tree lupins are cocultivated with *P. radiata*, providing nitrogen for young plantations. Tree lupin dieback had not been previously recorded in NZ, however the causal fungus had similar biology to the pine pathogen. It was proposed that the lupin pathogen may have arisen from the NZ pine pathogen population. Sequence analysis of the D2 region of rDNA of various NZ *Colletotrichum* taxa grouped the pine and lupin pathogens with three fruit rotting taxa. We have used morphological characters, as well as vegetative compatibility (VC) groups, RAPD analysis, and pathogenicity on lupin and pine, to investigate taxonomic relationships between the five taxa. All five taxa could be differentiated on the basis of colony morphology and RAPD banding patterns. All isolates from lupin had identical RAPD patterns. Isolates from pine and the three fruit rotting taxa were able to complement each other, however the isolates ex-lupin were all in one, separate VC group. Pathogenicity tests showed host specificity of the isolates from lupin and pine.

123. Differentiation between physiological races of *Fusarium oxysporum* f.sp. *dianthi* by pathogenicity assay, random amplification of polymorphic DNA (RAPD) and distribution of the *Fot1* transposable element.

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RAPD fingerprinting was used in combination with pathogenicity assay on differential cultivars and analysis of the distribution of the *Fot1* transposon to characterize a representative collection of Fusarium spp. isolates from diseased carnation. In *F. oxysporum* f.sp. *dianthi*, isolates were clustered in three RAPD groups: group 1 included isolates of race 1 and 8; group 2 was formed by isolates of race 2 and single representatives of race 5 and 6; group 4 included isolates of race 4 only. No correlation was found between RAPD data and geographic origin of the tested *Fod* isolates, as representatives of pathotype 2 isolated in Italy, Israel and Japan presented the same

amplification profile. Four isolates showing a low level of pathogenicity on all tested carnation cultivars shared an identical amplification pattern and are probably saprophytic *F. oxysporum.* Finally, two *F. redolens* isolates from Japan and seven non-pathogenic isolates of *F. proliferatum* collected from diseased carnation in Italy, Israel, Spain and The Netherlands were clearly recognized according to their RAPD fingerprint. The distribution of the transposable element *Fot1* was determined by Southern hybridization on DNA restricted with *Xho*I, which has no sites in the *Fot1* sequence. *Fot1* copy number varied between 1 and 6, with insertion sizes comprised between 2 and 12 kb. The *Fot1* hybridization patterns led to the same clustering based on RAPD fingerprinting. Inverse PCR experiments are being carried out to clone flanking genomic DNA from different races and design race-specific primers.

124. Genetic Variation in *Rhizoctonia solani* AG1 using DNA-Based Genetic Markers.

Rodney E. Pettway, U. Liane Rosewich, H. Corby Kistler, University of Florida, Gainesville, and Bruce A. McDonald, Texas A&M University, College Station.

To study the population genetic structure and genome organization of *Rhizoctoni solani* AG1, a *Pst*I library was constructed from the DNA of an isolate that originated from Texas. Using these clones as probes we have screened the DNA from two sets of isolates. The first set consisted of 14 isolates from five fields each located in a different county in Texas. *Eco*RI-digested DNA was hybridized to 27 probes, whereby 88% of the probes were polymorphic and 40% were highly repetitive (>50 bands). Only two isolates, collected from the same field had the same multilocus haplotype. The clonal nature was confirmed by using one of the repetitive clones as a DNA fingerprinting probe. The second set of isolates consisted of seven isolates from different states (Arkansas, Mississippi, Alabama, Texas and Louisiana). These were tested with a subset of nine probes. In addition to *Eco*RI, *Pst*I, *Hind*III and *Xho*I were used to determine optimal probe-enzyme combinations. Among the 27 probe-enzyme combinations tested,19 detected RFLPs among the seven isolates, whereby *Hind*III and *Xho*I seemed to detect RFLPs most efficiently. Even though alleles were shared between isolates originating from different states, a sharing of haplotypes was not detected. Probes developed in this study will be used to test for heterozygosity and random mating.

125. Genetic analysis of *Cantharellus cibarius* populations in rain forests of the pacific northwest.

Rusty Rodriguez, Regina Redman, Judy Ranson, and Roger Hoffman, NBSC, Biological Resources Division, United States Geological Survey, Seattle, WA 98115

Five clusters of *Cantharellus cibarius*, that occurred within a 30 meter diameter, were analyzed by PCR amplification to determine the genetic structure of this population. The number of fruiting bodies varied from 15 to 54 per cluster and the location of each fruiting body relative to a benchmark placed in each cluster was recorded for Geographic Information System (GIS) mapping. A small non-lethal sample was collected from each fruiting body for DNA extraction. Arbitrarily primed PCR (apPCR) was used to identify 15 products that were polymorphic in a

subset of these samples. The apPCR products were cloned and sequenced in order to design marker-specific PCR primer sets for dual primer PCR (dpPCR) of all individuals in the five clusters. These analyses indicated that each cluster of fruiting bodies was genetically distinct and representing five different populations. In addition, each population expressed varying levels of genetic diversity indicating that, although each population represented highly related individuals, the individuals were not clonal.

126. Morphological and molecular characterization of isolates of *Fusarium oxysporum* f.sp. *radicis-lycopersici* from Florida.

U. Liane Rosewich, Rodney E. Pettway, H. Corby Kistler, Univ of Florida, and Talma Katan, Volcani Center, Bet Dagan, Israel.

Work is in progress to elucidate the population genetic structure of the tomato pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici.* Isolates have been collected from all major tomato growing counties in Florida. Characterization of vegetative compatibility groupings (VCG) of 102 isolates revealed, that most isolates (81.4%) fell into VCG 0094 which previously has been only reported for Belgian isolates. A previously undescribed VCG, tentatively assigned 0098, is also widespread in Florida. In addition, molecular markers are being developed and tested to characterize the collection. Single-copy probes developed from a genomic library have yielded to date only a limited amount of polymorphic markers. Only 17 out of 42 probe-enzyme combinations were polymorphic, whereby most polymorphisms were caused by a single isolate. Clonal relationships were better visualized by using a moderately repetitive fingerprinting probe (pEY10). Seven different haplotypes could be differentiated among 19 isolates, selected from VCG 0094. Electrophoretic karyotyping also revealed polymorphisms. Visualizing chromosomes < 1.5 Mb, four karyotypes were found among the eight strains examined, all belonging to VCG 0094.

127. Influence of geographic distance and mating strategy on the genetic population structure of wood decay basidiomycetes.

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The extent to which populations of wood inhabiting basidiomycetes differentiate gentetically on various geographical scales is not fully known. In *Heterobasidion annosum*, a widespread pathogenic species, and *Fomitopsis pinicola*, a common saprotrophic decay fungus, evidences from spore dispersal gradients and DNA fingerprinting indicate that distances above 500 km may be needed in order to find strong differentiation among populations. In *H. annosum* we have indications of relative frequent rematings to occur within single substrate units. Expansion of the geographical range of the fungus following the last ice age as well as host species preferences are

likely to contribute to present day population structure. In the largely non-outcrossing *Stereum sanguinolentum,* near-clonal vc-group lines can be isolated from widely seperated locations in Northern Europe. Data from DNA markers are compatible with strong inbreeding within the vc-groups of *S. sanguinolentum.*

128. Sexual reproduction of *Botrytis cinerea* and population structure in Champagne: two sibling species?

Giraud Tatiana, Fortini Dominique, Leroux Pieffe and Brygoo Yves. INRA.

B.cinerea is a haploid, filamentous, heterothallic ascomycete. It attacks a wide range of plants in temperate regions and causes grey mould on many economically important crops, such as grapes. Previous studies have shown that this species has a great genetic diversity and morphological variability, for which the usual explanations are heterokaryosis and aneuploidy. Sexual reproduction is not commonly invoked as a cause for genetic diversity because, although apothecia are produced in laboratory for any strain, its sexual organs have rarely been observed in field. We used 15 markers (RFLP markers, sensitivity to fungicides and presence of two transposable elements, *Boty* and *Flipper),* to analyze 356 field isolates, from four different places in Champagne, collected in 1994 and 1995, from three different types of wineyard. The markers revealed that *B. cinerea* contained a large amount of intra-population genetic variation. The two transposable elements were found to be associated : one group of isolates had both transposable elements, the other had none. These groups were subsequently shown to be different for all the other markers using tests of difference of allelic frequencies. Morphological characters (e.g., spore length) corroborated this structure of the population of Champagne. RFLP markers showed genetic recombination in both groups of isolates, using the number of haplotypes, and tests of gametic desequilibrium. We conclude that there are two sympatric populations of *B. fuckeliana* in Champagne. One species seems to be local and well-adapted, while the other one is likely to be a migrant population, less homogeneous and less fit on grapes in Champagne.

129. Spore-killing in *Podospora anserina:* an overview.

Marijn van der Gaag, Fons Debets, Jessica Oosterhof and Rolf Hoekstra. Dept. of Genetics, Wageningen Agricultural University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands.

Spore-killers are segregation distorters found in several ascomycete fungi. In *Podospora anserina* spore-killing can be identified by the abortion of two of the four dikaryotic spores within the ascus. In the post-meiotic phase the spore-killer allele prevents the formation of spores not containing the spore-killer allele. In case of a cross-over event, ascospores end up with a 'killer' and a 'sensitive' allele. The ascus contains then the normal amount of four spores, the sensitive allele is 'saved'. The further the spore-killer allele is localised from the centromere on a chromosome, the higher percentage of four-spored asci within a fruiting body, and the less efficient the killer-gene. Backcrosses of the heterokaryotic spores from four-spores asci with the parental strains reveal that the sensitive alleles remain undamaged by the spore-killing action. At the moment seven different types of spore-killer genes can be identified in *P.anserina,* of which

6 recently isolated from nature. The newly isolated Psk7 killer is apparently identical to a French killer strain originating from 1969. All spore-killer types can be distinguished from each other by killing percentage, localisation on linkage groups, and killing interaction among each other. Some sort of linear killing order or dominance seems to exists, when different killer types are crossed with each other. Still most of the natural population consists of strains sensitive to spore-killing, coexisting with the spore-killer strains. No resistant strains have yet been found nor fixed killer genes. Meiotic drive elements only work within an outcrossing population, however *P. anserina* is a secondary homothallic fungus, and mainly reproduces by selfing.

130. The dynamics of linear plasmids in *Podospora anserina.*

Marijn van der Gaag and Rolf Hoekstra. Dept. of Genetics, Wageningen Agricultural University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands.

A natural population of recently isolated strains of the ascomycete *Podospora anserina* were screened for homologues of the linear longevity inducing plasmid pAL2-1. Of the 78 wild-type isolates 14 hybridised with a pAL2-1 specific probe, however only one of the plasmid inhabiting strains showed the long-lived phenotype. Also some strains were found which contained a related plasmid family, instead of a single plasmid. The inheritance of plasmid ladder containing strains was also investigated. The plasmid was inefficiently sexually transferred to the next generation. Not only a loss of the plasmid family members could be observed, also about 20% plasmid-free ascospores were produced. No significant difference in amount of plasmid-free spores between normal binucleate and uninucleate ascospores was found. The linear plasmids are transmitted maternally. Furthermore, horizontal transfer experiments showed that the linear plasmid could easily infect plasmid-free strains, in both vegetative compatible and incompatible situations. The ascospores produced by the 'plasmid-free' strains in the horizontal transfer experiments, also showed presence of the linear plasmid, indicating that infected maternal tissue directly leads to the production of plasmid containing spores.

131. Poulation structure of the black *Aspergilli*.

Anne D. van Diepeningen, Alfons J.M. Debets, Klaas Swart and Rolf F. Hoekstra. Department of Genetics, Agricultural University Wageningen, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands.

A survey was made of natural populations of the soilborne fungus *Aspergillus niger*. Representatives of the whole range of black *Aspergilli* were found to be able to utilise media containing 20% tannin, showing the utility of these media for exclusive selection of black *Aspergilli*. We made use of this unique characteristic to selectively isolate *A. niger* from nature. 642 Strains were isolated from soil samples of different countries throughout the world and some sites were sampled over a range of years. All isolates were classified according to their mitochondrial restriction fragment length polymorphisms. A majority of mitochondrial types occurred all over the world among populations and similar frequencies of mitochondrial patterns were observed. More endemic -possibly recent characters can also be found. Infections with mycoviruses occur in 10% of all isolated black *Aspergilli*, irrespective of sampling site,

mitochondrial background or year of sampling.

132. Heterokaryon incompatibility blocks virus transfer among natural isolates of black *Aspergilli*.

Anne D. van Diepeningen, Alfons J.M.Debets and Rolf F. Hoekstra. Department of Genetics, Agricultural University Wageningen, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands.

Somatic (also heterokaryon or vegetative) incompatibility in black Aspergillus strains was examined using nitrate-nonutilizing mutants selected on chlorate medium. Pairings of complementary mutants showed that in natural populations of the asexual black *Aspergilli*, somatic incompatibility between different strains is the rule. Among strains that appear related on basis of mitochondrial RFLP classification and (sometimes) mycovirus patterns, even isolated from the same site, rarely somatic compatibity was observed. Moreover, even heterokaryon self-incompatibility occurs. Mycoviruses are present in a considerable fraction of the sampled natural population, but surprisingly horizontal transfer of mycoviruses is strongly limited - at least under laboratory conditions - to the rare compatible combinations of strains. Thus, unlike in other fungal species, somatic incompatibility in black *Aspergilli* efficiently blocks virus transfer. Viruses present in black *Aspergillus* isolates are stably transmitted to asexual progeny.

133. Trans-species genetic polymorphism at the vegetative incompatibility locus(*het-c*) *in Neurospora*.

Jennifer Wu, Sven Saupe and N. Louise Glass, University of British Columbia, Canada

In *Neurospora crassa*, genetic control of vegetative incompatibility results from genetic differences at one or more *het* loci. Eleven *het* loci have been identified so far in *Neurospora crassa*. The *het-c* locus has been molecularly characterized and encodes a 966 aa glycine-rich polypeptide. Specificity at *het-c* is mediated by at least three different allelic types *(het-*C^{OR} - type, *het-*c^{PA} - type and *het-*C^{EM} - type). A variable domain of 27 - 34 aa in HET-c, HET-C^{PA} and HET-C^{EM} was sufficient to confer allelic specificity. Sequences of the specificity region from isolates of eight species of *Neurospora* and four strains of *Gelasinospora* have been determined and analyzed. Pairwise and maximum-likelihood comparison of these alleles reveals two interesting features: (I)These alleles are highly conserved over a range of divergent taxa. Ancient allelic polymorphisms have been maintained among contemporary species. (2) Interspecific similarities at *het-c* are greater than intraspecific similarities. The data suggest that in *Neurospora*, the existence of genetic polymorphisms associated with the specificity region of *het-c* predates speciation and also the origin of genus.

Mating Type Evolution

134. The Mixed Mating System of *Cryphonectria parasitica.*

R. E. Marra and M. G. Milgroom. Department of Plant Pathology, Comell University, Ithaca NY.

Cryphonectria parasitica, the ascomycete that causes chestnut blight, has been shown in laboratory crosses to conform to a heterothallic mating system. When perithecia were sampled from North American populations, a significant portion (~25%) appeared to be the result of self-fertilization, suggesting a mixed mating system. Ascospores from a single perithecium were considered the result of self-fertilization if there was no segregation at 5 to 7 vegetative compatibility (vc) loci, 6 unlinked RFLP loci, and 8-15 (or more) unlinked fingerprinting loci. However, progeny arrays from most putatively selfed perethecia segregate approx. 1:1 for mating type, even though the maternl parents of these perithecia usually express only one or the other mating type in the lab. Results from lab matings correlate 100% with southern hybridizations using a *C parasitica Mat-2*-specific probe, a 280-bp conserved HMG domain. Although selfing in the lab is rare, 5 individuals have selfed, and the progeny of lab selfs segregate for mating type, based on both mating phenotype and HMG hybridizations. Most of our laboratory experiments begin with single conidia, which are single-celled. We tested the hypothesis that the conidia of individuals that can self are multinucleate by studying the conidia from three selfing strains. Two thousand conidia from each of these three strains were examined using epifluorescent microscopy, and we could not reject a null hypothesis that conidia are uninucleate. Additionally, no segregation was observed for DNA fingerprints or the HMG domain in ten conidia from each of these three strains, suggesting that mating type segregation does not occur somatically.

135. Isolation of compatible strains of *Podospora anserina* with the same mitochondrial rearrangement: analysis of the effects of sexual reproduction on mitochondrial inheritance and life span.

Margaret E. Silliker and Eric M. Nelson, DePaul University, Chicago, IL.

This lab has previously described a stop/start longevity mutant, Mn19, of *Podospora anserina.* It is unusual in that it has a stable mitochondrial genome which is a rearrangement of the wild type, juvenile, race A mitochondrial genome. The Mn19 genome consists of two non-overlapping circular molecules which most likely arose by recombination between short sequence repeats. Attempts to transmit the Mn19 rearrangement to progeny for genetic analysis have resulted in preferential inheritance of wild type genomes, even when wild type genomes are rare in the mate (detected only at the level of PCR). In order test whether the process of sexual reproduction was contributing to the restoration of the Mn19 mitochondrial genome we wanted to cross the Mn19 mutant with itself. However, Mn19 was isolated as a monokaryotic strain of the (+) mating type. Here we describe a confrontation cross where perithecia were produced exclusively on the Mn19 side of the cross. Spores were isolated and the progeny were screened by PCR in order to detect isolates with only the Mn19 rearrangement and the (-) mating type. One such strain was identified and crossed with the original Mn19 strain. Wild type (un-rearranged) sequences have been amplified from the majority of the progeny, though the rearrangement was transmitted to some strains. We are currently measuring the life span phenotypes of these progeny.

136. Phylogeneties and evolution of mating type genes.

B. Gillian Turgeon, Cornell Univ. Ithaca, NY. and Mary Berbee, Univ. of British Columbia, Vancouver, BC.

We are exploring the evolution of reproductive strategies in sexual and asexual ascomycetes by comparing mating type *(MAT)* gene sequences. Although *MAT* sequences are powerful tools for molecular evolution studies, their usefulness has been limited by difficulties in cloning them from a wide array of fungi. We have developed an efficient PCR-based procedure for cloning MAT genes across genus lines from both sexual and asexual species. With *MAT*, ITS, and *GPD* sequence data we are inferring phylogenetic histories. Preliminary comparisons of distances among species pairs suggest that phylogenies from the three regions will be congruent. Trees from ITS sequences divide *Cochliobolus* species into two groups; one contains serious cereal pathogens like *C. heterostrophus* and C. *carbonum* and tends to have *Bipolaris* asexual states. In the second group, serious pathogens are lacking and *Curvularia* asexual states are common. Using the phylogeny, we will ask whether *MAT* genes have co-evolved with genes for primary metabolism. The fungi chosen for this study (Pleosporaceae) have different reproductive strategies, ie., sexual vs. asexual or homothallic (self fertile) vs. heterothallic (self sterile). As sequence data are accumulated we will ask if asexual species arise from sexual, if homothallics arise from heterothallics, and if *MAT* genes in sexual and asexual fungi are evolving at the same rate. Finally, because *MAT-1 and MAT-2* genes never recombine (they are dissimilar sequences occupying the same genetic locus), each gene has a completely clonal phylogenetic history. Conflicting phylogenetic histories may suggest interspecific mating.

Horizontal Gene Transfer

137. Natural variants of the kalilo senescence plasmid.

Cynthia He, Natasja de Groot and Tony Griffiths. Botany, UBC, Vancouver, Canada.

The prototypic death plasmid 'kalilo' was originally found in Hawaiian strains of *Neurospora intermedia.* Each isolate bearing the plasmid shows a strain-specific lifespan. Death is by integration of the plasmid into mtDNA. Although kalilo-homologous plasmids are not common, several examples have been reported (Arganoza et al. 1994). We have characterized several of these, and they fall into four types. 1) the protoypic kalilo; this has now been found in *N. intermedia* (Hawaii), and *N. tetrasperma* (Moorea-Tahiti). 2) LA-kalilo, a form with a modified 60 bp region in the TIRs; this has been found in *N. tetrasperma* (Louisiana) and *N. crassa* (Haiti). 2) Short-kalilo, a form with a 700 bp deletion in the TIRs; found in *N. discreta* (Thailand and Ivory Coast). 4) Gel-kalilo, a form with complex differences in the TIRs; found in Gelasinospora only (Louisiana). The coding regions of all types contain two open reading frames showing little variation. The poster will describe the detailed structure of these plasmids and discuss the significance of their curious distribution.

Abstracts from the Saturday March 22 Poster session (Posters III)

Signal Transduction

A. nidulans calmodulin-dependent protein kinase (ACMPK), exhibits tyrosine kinase activity.

B.M. Manolas, Rose Antilus and D.C. Bartelt. Department of Biological Sciences, St. John's University, Jamaica, NY.

Previous studies have shown that ACMPK has substrate specificity similar to calmodulin (CaM)-dependent protein kinase II (CaMKII). Like other CaM-dependent protein kinases, the optimal substrate consensus sequence for ACMPK is R/K-X-X-S/T, a sequence present in autocamtide 2 (K-K-A-L-R-R-Q-E-T-V-D-A-L). Other protein S/T kinases including cAMP- dependent protein kinase and phosphorylase kinase have recently been shown to phosphorylate tyrosine (Y) residues in peptide substrates such as angiotensin II (N-R-V-Y-V-H-P-F). Protein tyrosine kinase activity is Mn^{2+} dependent. We have examined the ability of ACMPK to phosphorylate tyrosine-containing peptides and studied the effects of divalent cations on kinase activity. ACMPK phosphorylates both angiotensin 11 and alpha-neo endorphin (Y-G-G-F-L-R-K-Y-P-K) but not a peptide containing the phosphorylation site of $pp6O^{src}$ (R-R-L-I-E-D-A-E-Y-A-A-R-G). Mn^{2+} enhances both the threonine and tyrosine kinase activity of ACMPK. Phosphorylation of Threonine/serine and tyrsolne by ACMPK is Ca^{2+}/CaM- dependent.

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1. Protein kinase C, a novel component of blue light transduction pathway in *Neurospora crassa.*

G. Arpaia, C. Catalanotto, F. Cerri and G. Macino.

In the ascomyces *Neurospora crassa* blue light influences developmental processes, production of carotenoids and the entrainment of the circadian rhythm. Genetic aproach for the identification of components of the blue light transduction pathway, never gave clear indications on the characteristics of new molecules other than *wc-1* and *wc-2*. In the present study we used a pharmacological approach to screen a wide range of second messengers and chemical compounds capable to interfere with the activity of well known signal transducers in vivo. By this aproach we identify the Protein Kinase C as an additional component of the light transduction cellular machinery. We tested specific inhibitors (Calphostin C and Chelerythrine chloride) or activators of PKC (1,2-Dihexanoyl-sn-Glycerol) and monitored the effect on the blue light-stimulated transcription of the gene albino-3.

PKC role is carried out both during the vegetative growth and conidiation. In the first case PKC is responsible for the desensitization to light, an adaptative response: inhibitors of the enzyme inhibit the decay-phase in the kinetic of light induction of the *al-3*(m) transcript. During conidiation, instead, PKC activity is necessary for the light induction of the *al-3*(c) conidiation-specific transcript. In this phase, PKC inhibitors totally abolish light-induced *al-3*(c) expression, but not conidiation-driven transcription of the gene.

We cloned the gene coding for the *Neurospora crassa* PKC and its characterization is actually under way.

2. Blue light signal transduction: The white collar-1/white collar-2 dimerization system.

P.Ballario, H.Linden*, D.Gallie G. Macino* Universita' "La Sapienza" Roma Italy.

Both White collar-1 and White collar-2 genes products are necessary for light perception in *Neurospora crassa* since mutations in either one of the two genes cause the total blindness of the fungus. As the *wc* mutants are defective only in blue light induced processes, their products are considered to be specific elements of the blue light transduction pathway. Extensive screenings for other mutants with a "full blind" phenotype have been unsuccessful. The WC-1 and WC-2 genes have been recentely isolated (1, 2) and although no overall homology between them was identified they share some common domains. WC proteins are putative transcriptional factors characterized by amino terminal activation regions and a carboxy terminal Zn finger DNA binding domain. Both Zn finger binding domains expressed in *E.coli,* are able to bind in vitro to the promoter of the light inducible *al-3* gene. In addition both WC proteins show homology to the dimerization domain termed PAS (Per,Arnt,Sim) (3). PAS domains are present in several proteins, mostly transcriptional factors, able to form homo and heterodimers. The human AHR (aryl hydrocarbon receptor) containing a PAS domain shows the highest degree of amino acid conservation with WC-1 PAS or WC-2 PAS. This receptor is able upon the binding with xenobiotic metabolites (i.e. dioxin) to dimerize with ARNT (its nuclear translocator protein).The heterodimeric protein once translocated in the nucleus, recognized the XRE (xenbiotic recognition element) of certain promoters through the bHLH binding domains present in both proteins. AHR and ARNT are both able to homo and heterodimerize. We have in vitro evidences that WC-1 and WC-2 are able to form homo and heterodimers, and also to be engaged in heterodimeization with AHR in vitro. The implications of this finding for the the construction of a model of blue light transduction in Neurospora will be discussed.

1)P.Ballario, G.Macino (1996) EMBO J , 15,1650-1657

2)Linden H. and Macino G.(1997)EMBO J, 16, 98-109.

3)Huang Z.J., Edery I., Rosbash M.(1993)Nature, 364, 259-262

3. Characterization of *Neurospora crassa* transport mutant *nap*.

Tatiana A. Belozerskaya, Tatiana V. Potapova, Natalla N. Levina, Natalia E. Petrova and Yuri V. Ershov. A.N. Bach Inst Biochem., RAS, *A.N. Beloversky Inst Phys.-Chem Biol., MSU Moscow, Russia.

To elucidate the role of the plasma membrane transport systems in the blue light signal transduction chain leading to biosynthesis of carotenoid pigments, electrophysiological characteristics, intracellular ATP content and ability to synthesize carotenoids have been studied

in the *N. crassa* transport mutant *nap*. Resting membrane potential value of the mutant *nap* measured with the aid of intracellular microelectrodes turned out to be about 1.5 times lower than the wild-type. In spite of this fact the plasma membranes of the mutant *nap* responded to the blue light treatment by hyperpolarozation. Its value was the same as in the wild type and appeared to be energy dependent and thus connected with the functioning of H^+ ATPase. Intracellular ATP content of the mutant strain was about 1.5 times higher than in the wild type. Proportional about two times increase of all the carotenoid fractions was observed in the mutant *nap*. Thus the intensity of transport processes throught the plasma membrane does not seem to be limiting for the light induced carotenoid acccumulation in *N. crassa.* The critical factor for the process of carotenogenesis appear to be the photoinduced changes in the electrical properties of the plasma membrane.

4. Cloning and sequencing of the gene encoding the catalytic subunit of a cAMP-dependent protein kinase in *Candida albicans*.

Monikca Cloutier*, Beatrice B. Magee and Luc *. Laval University*, Quebec, Canada; University of Minnesota, MN, USA.

The cloning and sequence analysis of a gene that encodes the catalytic subunit of a cAMP-dependent protein kinase (PKA-C) in the human pathogen *Candida albicans* is reported. Two highly conserved amino acid segments (IYRDLKP and GTHEYLAPE; respectively subdomains VI and VIII of the conserved regions from catalytic domains through the kinase family) served to design two oligonucleotides that were used to PCR amplify a 150 pb fragment. Sequence analysis of this fragment showed it to be 83% identical to that of the *Saccharomyces cerevisiae* TPK2 gene. This amplicon was used to screen a *C. albicans* genomic fosmid library. One of the three positive cosmids obtained was mapped using several restrictions endonucleases. The PKA-C gene was localized by Southern analysis. Two overlapping DNA fragments (a 1.7 kpb *Pst*I and a 3.0 kpb *Eco*RI/*Kpn*I) were subcloned and used to determined the sequence of the gene. An ORF of about 1,2 kb was detected by sequence analysis. Comparison of the predicted amino acid sequence showed it to be 80% identical to that of TPK2 from *S. cerevisiae*. Construction of homozygous null mutant for PKA-C is underway using the "URA-blaster" technique. This should allow us to verify the implication of PKA-C in cellular morphogenesis and virulence in *C. albicans*. (Supported by MRC grant #MT-12892).

5. Spore germination and trehalose metabolism in *Aspergillus nidulans.*

Christophe d' Enfert and Thierry Fontaine. Labratoire des Aspergillus, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, Cedex 15.

Althought conidial germination is a key developmental stage in the life cycle of Aspergillus species, it remains poorly understood at the molecular level. Trehalose is a non-reducing disaccharide found at high concentrations in Aspergillus conidia and rapidly degraded upon

induction of conidial germination. Trehalose-degradation is concomitant with the accumulation of a glycerol pool in the conidia. Our aim is to understand the role of the trehalose and glycerol pools wth respect to conidial germination and to identily the signal transduction pathways that are responsible for activating trehalose breakdown and that might control other events required for the achievement of conidial germination. Two *A. nidulans* genes encoding trehalases have been cloned and sequenced and *A. nidulans* strains with a null mutation in either gene have been obtained. *treA* encodes an acid trehalsae that is localized in the conidial wall and that is similar to *S. cereviasiae* vacuole trehalase. Disruption of *treA* results in *A. nidulans* strains that are unable to grow on trehalose as a carbon source and that are not affected in the mobilasation of intracellular trehalose during conidial germination. *treB* encodes a cytoplasmic neutral trehalase highly homologous to the two *S. cerevisiae* neutral trehalases that are activated by the cAMP-dependent pootein kinase (PKA) and catalyze intracellular trehalose breakdown in budding yeasts. Disruption of *treB* results in *A. nidulans* strains that are unable to degrade intracellular trehalose at the onset of conidial germination and that do not show the transient accumulation of a glycerol pool, suggesting that this pool results from the entry into glycolysis of the glucose formed from trehalose. The involvement of a neutral trehalase is consistent with a role for the PKA pathway during the early events of conidial germination.

6. Four protein kinsase homologs in *Ustilago maydis.*

Ge Yang, Franz Durenberger, Ann B. Orth* and James W. Kronstad. Biotechnology Laboratory, University of British Columbia, Vancouver, BC V6T 1Z3 Canada and *DowElanco discovery Research, Indianapolis, IN 46268-1054 USA

Recently, the cyclic AMP pathway and a protein kinase (*adr-1*) in *U. maydis* have been implicated in dimorphism and fungicide resistance. We are interested in additional homologs of protein kinase A (PKA) and protein kinase C (PKC) for their potential roles in morphogenesis and as fungicide tergets. A 150 bp PCR fragment, which contains a PKC homolog, was used as a probe to isolate four cosmid clones carrying additional protein kinase genes. The four cosmids have been subcloned and partially sequenced. The sequence information indicates that one of the genes may encode another catalytic subunit of PKA. The other three genes appera to encode PKC homologs, with strongest homology to *ypk1*, *ypk2* and *sch9* genes in *Saccharomyces cerevisiae*. The disruption constructs have been made to knock out the four genes in diploids and/or haploids. The results of the gene disruption experiments will be reported.

7. G protein a subunit genes of *Trichoderma harzianum* and *Cochlioholus heterostrophus.*

Benjamin A. Horwitz, Department of Biology, Technion, Haifa, Israel.

G genes with features of the Gi class have been isolated from T. *harzianum*[1] and *C. heterostrophus*[2] . Both have high homology to *gna1* of *Neurospora crassa;* intron positions are conserved between the two species, and there is no evidence for more than one gene in either. *Trichoderma species* are soil saprophytes or mycoparasites.A brief pulse of blue light (200 *u*mol m^{-2}, delivered over ns to min) induces synchronous conidiation of *T harzianum* even under

otherwise favorable conditions. Loops, coils and branches are formed in response to the proximity of host hyphae. Experiments with activators of animal cell G proteins suggest that G- is involved in mycoparasitism rather than in the blue light response. In *C. heterostrophus,* a foliar pathogen of corn, disruption of the G subunit gene by homologous recombination results in the loss of the ability to form appressoria on a glass surface. Furthermore, crosses in which even one member of the pair lacks G produce infertile pseudothecia. The pleiotropic nature of the G null mutants suggests that the unique consequence of each signal is determined not by G, but by proteins with which it interacts.

1. V, Rocha, J. Inbar, I. Chet, A. Herrera-Estrella and B.A. Horwitz (in preparation)

2. B.A. Horwitz., A. Sharon, S. Lu, O. C. Yoder and B.G. Turgeon (in preparation)

8. Biochemical Analysis of Heterotrimeric G Protein Regulated Events in the Filamentous Fungus *Neurospora crassa.*

F. Douglas Ivey and Katherine A. Borkovich. University of Texas Medical School- Houston.

Heterotrimeric guanine nucleotide binding proteins (G proteins), consisting of of , and polypeptides, regulate a vast range of processes from muscle contraction and vision in mammals to mating in yeast. Through coupling to seven helix transmembrane receptors, G proteins relay ligand/receptor binding events to downstream proteins or enzymes known collectively as effectors. *Neurospora crassa* possesses at least three known G subunits, Gna-1, Gna-2, and Gna3. Gna- 1 was the 1st reported microbial subunit to be a member of any mammalian G family. The G_i subfamily members are thought to participate in diverse functions including controlling ion channels and regulating phospholipase activity. It was previously shown that deletion of *gna-1* in *N. crassa* results in vegetative growth defects that include sensitivity to hyperosmotic media and abnormal formation of aerial hyphae. More importantly, deletion of *gna-1* in *N. crassa* results in female sterility. Recently, the levels of second messenger molecules have been investigated using several approaches. The deletion of *gna-1* appears to lead to observable changes in hyphal-tip calcium levels as observed using chlor-tetracycline fluorescence. Because the levels of inositol 3-phoshate affect Ca^{2+} levels in many eukaryotic cell types the levels Of EP3 in both *gna-1* mutants and in control strains were measured. In addition, pharmacologic agents that act on cellular Ca2+ levels appear to differentially affect the growth of *gna-1* and control strains. Currently, biochemical methods are being applied to determine if any of the observed *gna-1* mutant phenotypes are related to changes in intracellular or extracellular cAMP levels. The goal of this research is to correlate changes in second messenger molecules with G proteinmediated signalling events.

9. Withdrawn

10. Fil1, a G-protein subunit that mediates dimorphic switching of *Ustilago hordei.*

Amnon Lichter and Dallice Mills. Department of Botany & Plant Pathology, Oregon State University, 2082 Cordley Hall Corvallis, Oregon, 97331-2902,

A constitutive mutation, *fil1*, causing filamentous growth in the haplophase of the dimorphic smut fungus *Ustilago hordei*, was previously shown to be genetically associated with a 50 kb deletion in a 940 kb chromosome. The effect of this mutation could be transiently relieved by adding cAMP, or adenylyl cyclase stimulators, or a cAMP phosphodiesterase inhibitor to the growth media, suggesting that a gene that functioned upstream in the cAMP cascade was deleted. Representational difference analysis (RDA) was modified to construct a chromosome subtraction library enriched for deletion-specific sequences that were used to identify homologous genomic DNA in a cosmid library. The cosmid clone, pOSU2100 and a derivative plasmid with a 2.1 kb insert, converted the transformants from the filamentous to the sporidial cell type. The 2.1 kb insert contained a single open reading frame of 354 amino acids that encodes a putative subunit of the heterotrimeric G-proteins. FIL1 displayed high amino acid sequence identity to Gpa1 of the basidiomycete *Cryptococcus neoformans*, CPG-2 of the ascomycete *Cryphonectria parasitica* and intermediate homology to Gpa2 of *Saccharomyces cerevisiae*. *FIL1* was verified to be deleted in the mutant and to be present as a single-copy gene in the wild-type. Wild-type strains transformed with *FIL1* were suppressed for filament production which can normally be induced in minimal medium with limiting levels of nitrogen. These strains, including the complemented mutant were mating-competent with a wild-type partner. The effect of FIL1 on the pigmentation pattern of *U. hordei* is demonstrated.

11. Fruit body development of *Sordaria macrospora:* Isolation and molecular characterization of a regulatory gene.

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We have chosen the homothallic ascomycete *Sordaria macrospora* as a model organism for investigating fruit body development. Here we present the molecular characterization of the sterile mutant *pro1*, which formes only protoperithecia. Complementation transformation of this mutant was performed with an indexed genomic cosmid-library [1, 2], leading to the isolation of two overlapping cosmid clones. The minimal complementing region was defined on a 2.3 kb DNA fragment, which subsequently was sequenced. This analysis was completed by the generation and molecular characterization of partial cDNA clones. Restriction and hybridization analysis of mutant *pro1* indicates a deletion of more than 11 kb, including the complementing region. Database search of the deduced amino acid sequence of the complementing DNA fragment reveals a region of significant homology to a DNA-binding motif found in the GAL4 transcription factor from yeast [3]. Functional studies are underway to identify domains in the polypeptide that are involved in fruit body formation of this ascomycete.

[1] Walz M, Kuck U (1995) Curr Genet 29:88-95

[2] Poggeler S, Nowrousian M, Jacobsen S, Kuck U, submitted

[3] Pan T, Coleman JE (1990) Proc Natl Acad Sci USA 87:2077-2081

12. A *ras* homolog of *Neurospora crassa* regulates apical growth.

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We cloned and characterized a *ras* homologue gene, termed NC-*ras2*. The predicted protein product of this gene is composed of 229 amino acid residues and contains all the consensus sequences shared by the *ras* protein family. An NC-*ras2* disruptant showed morphological characteristics very similar to that of the *smco7* mutant. Nucleotide sequence analysis revealed that the *smco7* mutant harbored a single base deletion in the NC-*ras*2 gene which is predicted to result in the truncation of the protein product. Introduction into the *smco7* mutant of an NC-*ras*2 clone yielded stable transformants with the wild type phenotype. The *smco7* mutant exhibited very slow hyphal growth. The *smco7* mutation also caused both the changes in the pattern of hyphal growth and the defects in cell wall synthesis. Both the diameter and the length of the apical compartment were shorten in the hyphae of the *smco7* mutant.

These results suggest that NC-*ras*2 is identical to *smco7*, and that the signal transduction pathway mediated by the NC-*ras*2 protein regulates the apical growth of hypha by regulating the transport of apical vesicles containing cell wall material in *N. crassa.*

13. Cloning and sequencing of the gene encoding the regulatory subunit of a cAMP-dependent protein kinase in *Candida albicans*.

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We have isolated and sequenced the gene encoding a cAMP-dependent protein kinase regulatory subunit (PKA-R) from the opportunistic human pathogen *Candida albicans*. This gene is suspected to be involved in the control of cellular dimorphism, a putative virulence trait. A genomic cosmid library was screened with a probe (generated by PCR) derived from two almost perfectly conserved amino acid segments of the cAMP binding sites from *Saccharomyces cerevisiae*, *Blastocladiella emersonii* and mouse PKA-R genes. Five positive clones were recovered. The PKA-R gene was localized by Southern hybridization. A 5.5 kb *Pst*I fragment was isolated from one of the positive fosmids, subcloned in pUC19 and mapped using several restriction enzymes. Two *Eco*RI subfragments (0.75 and 1.85 kb), each one containing part of the PKA-R gene, were subcloned in pCRScript and sequenced. The PKA-R gene is 1380 nucleotides long with a single copy per haploid genome. Nucleotide sequence analysis has shown that the predicted protein comprises 459 amino acids, giving an average molecular mass of 55 kDa with an overall sequence identity of 62% with its homologue in *S. cerevisiae*. The promoter region contains the canonical CAAAT and TATA boxes. The presence of a serine

residue in the phosphorylation site suggests that this *Candida* gene encodes a type II regulatory subunit. We will also report on the work in progress to inactivate the PKA-R gene in *C. albicans*. (Supported by MRC grant #MT-12892).

14. Characterization of the role of the cAMP pathway in vinclozolin resistance *of Ustilago maydis.*

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Ustilago maydis, the basidiomycete smut pathogen of maize, grows as a yeast while haploid and filamentously when dikaryotic. Recent work from this laboratory has shown that the cAMP pathway plays a role in the dimorphic switch. Mutants defective in adenylate cyclase and protein kinase A (PKA) have altered cell morphology. In addition, it has been reported that a catalytic subunit of PKA, encoded by *adr1*, may also play a role in fungicide resistance. To determine whether other components of the cAMP pathway could play a role in vinclozolin resistance, mutants in adenylate cyclase and PKA, which were previously generated by our laboratory, were grown in media containing various concentrations of vinclozolin. A disruption mutant of the *ubc1* gene, which encodes the regulatory subunit of PKA, showed increased resistance tovinclozolin compared to wild type cells. To explore the role of the cAMP pathway in vinclozolin resistance, the *ubc1* disruption mutant strain has been mutagenized and several mutants have been isolated which show decreased resistance to vinclozolin. Further analysis of these mutants will provide a better understanding to the role of the cAMP pathway in fungicide resistance.

15. Signal transduction in *Trichoderma harzianum*: Role of a G protein.

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Trichoderma harzianum a biocontrol agent of phytopathogenic fungi which is attractive as a model for the study of diferentiation processes, such as sporulation and apresorium formation. Biochemical evidences suggests that a trimeric G protein regulates apressoria formation and induction of conidiation by light.

As a first step towards the elucidation of the possible signal transduction pathway in *Trichoderma* we have cloned the gene encoding the Ga subunit, PCR technology was applied to generate a small fragment of the gene using oligonucleotides designed based on two of the highly conserved regions of Ga proteins. This product was used as a probe to obtain a *T. harzianum* genomic clone and its corresponding cDNA. The expression pattern of the gene under several conditions including those used for the induction of sporulation and appresorium formation was analyzed. Additionally, transgenic *Trichoderma* strains were obtained using a

series of plasmids which will allow us to determine the role of the G protein in light induced conidiation and apresorium formation.

16. Dissecting the signaling pathways between oxalic acid production and sclerotia development in *Sclerotinia sclerotiorum*.

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The production of oxalic acid has been demonstrated previously to be an essential pathogenicity determinant in disease interactions between *Sclerotinia sclerotiorum* and its hosts *Phaseolus vulgaris* and *Arabidopsis thaliana.* UV induced mutants of *S. sclerotiorum* selected for their inability to produce oxalic acid, are pleiotropically deficient in sclerotia development. We have initiated studies to determine the molecular basis for these observations and to dissect the molecular relationships between these two phenotypes. To determine if oxalic acid plays a direct role in sclerotia development, wild type *S. sclerotiorum* has been transformed to express a bacterial oxalyl decarboxylase gene to lower or eliminate oxalic acid accumulation. Several sclerotia minus transformants have been obtained by this approach and are currently being evaluated to determine if they express the transgene and whether they have altered levels of oxalic acid accumulation. Additional work has focused on endogenous signal transduction as related to development. In these studies, several classes of signal transduction effectors, including drugs which affect cAMP levels, protein kinase activities, and calcium homeostasis, were examined for their effects on oxalic acid production and sclerotia morphogenesis. Addition of exogenous 8-Br-cAMP, or treatment with caffeine, 3-isobutyl-l-methylxanthine, or sodium fluoride, which raise endogenous levels of cAMP, resulted in the reduction or complete inhibition of sclerotia development without affecting the production of oxalic acid. These results suggest that cAMP mediated signaling may also play a role in the switch from mycelial growth to sclerotial development.

17. Lovastatin may interfere with the functions of M*Ras*l and 3 and triggers a cell death process resembling apoptotis in *Mucor racemosus.*

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The filamentous fungus *Mucor racemosus* possesses three Ras genes, M*Ras*l, 2 and 3, which have striking similarity in nucteotide sequence to each other and to mammalian *Ras* genes. The *Ras* gene family is implicated in the pathophysiology of cell transformation and the pathogenesis of human cancer. Differences between M*Ras*l and 3 have been observed in the pattern and level of transcript and protein accumulation, post-translational modification as well as the protein complexes formed in vivo, suggesting that the each M*Ras* gene may have a different function. Lovastatin, an indirect inhibitor of protein farnesylation, affected the processing of as M*Ras*1, blocked the expression of M*Ras*3 and caused the complex M*Ras*l/ p2O to disappear. Concurrently it blocked sporangiospore germination and decreased the growth rate of *Mucor racemosus.* Lovastatin at high concentration (250 uM) caused the loss of cell viability accompanied with cell shrinkage, increased cell density and cytoplasm condensation and

triggered intensive DNA fragmentation to nucleosomes and nucleosome multimers with profound laddering at late stages. The specific morphological and biochemical events seen in *Mucor* cell death, particularly DNA fragmentation, resemble the best known characteristics of classical apoptosis in mammalian cells and to our knowledge represents the first observation of programmed cell death in filamentous fungi. These findings provide insights into the origin of programmed cell death and support the idea that controlled cell suicide machinery originated in unicellular organisms and was selected through evolution for the optimal adaptation to the environment.

18. Isolation, functional analysis and overexpression in mammalian cells of a *ras* gene from *Colletotrichum trifolii*.

Gina Truesdell, Clinton Jones, Todd Holt and Marty Dickman, University of Nebraska-Lincoln.

Ras proteins are ubiquitous molecular regulators of signal transduction pathways in eukaryotic cells. Ras proteins transduce external signals across the plasma membrane by cycling between inactive GDP-bound and active GTPbound states. The importance of Ras in regulating growth in mammalian cells is demonstrated by the finding that point mutations which cause constitutive activation of Ras lead to a transformed phenotype. To study the function of Ras in the life cycle of the alfalfa pathogen *Colletotrichum trifolii,* a cDNA encoding a Ras homolog, designated *CT-ras,* was cloned. *CT-ras* is a constitutively expressed single-copy gene and its deduced protein sequence is similar to Ras proteins from other organisms. To determine if *CT-ras* is functional, its activity was tested in a mammalian system. Activating mutations equivalent to those identified in animal tumors were introduced into the coding region by site-directed mutagenesis and the modified sequences were expressed in NIH 3T3 cells. Transfected cell lines expressing the modified fungal genes were identified. *In vitro* assays show these cells display characteristics of transformed cells, indicating activated *CT-ras* likely functions as an oncogene. The ability of these cells to form tumors *in vivo* is being tested. In addition, the consequence of constitutive Ras activation in *C. trifolii* is being investigated by expressing the modified genes in the fungus.

19. MAP Kinase Pathways in ***Magnaporthe grisea.***

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Many plant pathogenic fungi, including *Magnaporthe grisea*- the causative agent of the rice blast disease, develop specialized structures to invade their hosts and undergo dramatical morphological changes to grow invasively in plants. Our research objective is to study genetic mechanisms regulating this plant infection-related morphogenesis. We have isolated a MAP kinase PMKL (Pathogenicity MAP Kinase 1) from *M. grisea* which is essential for appressorium formation and invasive growth in plants. Further characterization of the *PMKL* MAP kinase pathway is in progress and will be presented. In addition to *PMK1,* we isolated two other *M. grisea* MAP kinases. *PMK2* is 83% similar to *S. cerevisiae HOGl* gene, and 93% similar to

S.pombe styl gene. *PMK3 is 85%* similar to *S. cerevisiae SL T2* MAP kinase gene. Five *PMK3* gene disruption mutants were isolated. *pmk3* mutants are nonpathogenic on rice plants, but make melanized appressoria on Teflon membranes or slideglass with the addition of 10 mM cAMP. On onion epidermis, *pmk3* appressoria fail to penetrate, but elicit autofluorescence and papilla formation in onion epidermal cells. *pmk3* mutants are also dramatically reduced in conidiation, however, there is no obvious growth defect as measured by colony diameter on a variety of media. *pmk3* mutants are not temperature sensitive or infertile. It appears that PMK3 regulates infection processes downstream of *PMK1,* and may play important roles in penetration, invasive hyphae differentiation and conidiation.

20. Analysis of the function of Gna-1, a G protein a subunit from *Neurospora crassa*.

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Heterotrimeric GTP binding proteins, which consist of , and subunits, are crucial intermediates in signal transduction pathways. Upon ligand stimulation, exchange of GDP for GTP on the a subunit causes activation of the subunit and heterodimer. Hydrolysis of GTP by the subunit returns the subunit to its inactive conformation, causing it to reassociate with complex. An subunit with a mutation which causes defective GTPase activity is constitutively active. *Neurospora crassa* Gna-1 is a member of mammalian G 1 superfamily, which consists of G_i, G_o, G_t, and G_z subclasses. *gna-1* mutants have a slower apical extension rate on solid medium and produce aberrant, infertile perithecia during the sexual cycle. We have introduced two kinds of GTPasedeficient mutations into a *gna-1* mutant by targeted integration at the *his-3* locus. In comparison to isogenic wild type controls, both of the activated alleles cause a dramatic increase in the amount and length of aerial hyphae. However, these strains are otherwise indistinguishable from wild type. The above results imply that Gna-1 has functions independent of subunits, since the *gna-1* activated allele strains have different phenotypes from the *gna-1* mutant strain. To investigate the evolutionary relationship between Gna-1 and mammalian G_i proteins, we transformed mammalian G_i, G_o, G_t, and G_z genes into the *gna-1* strain. Preliminary data shows that some of the mammalian G subunits are able to partially complement the *gna-1* defects in either the vegetative or sexual cycle.

21. Molecular cloning and characterization of cAMP-dependent protein kinase (PKA) genes from *Colletotrichum trifolii*.

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Colletotrichum trifolii is the causal agent of alfalfa anthracnose. Pharmacological data have strongly suggested that cAMP-dependent protein kinase (PKA) is involved in *C.trifolii* morphogenesis, including conidia germination and appressorial development. In order to dissect the function of PKA at molecular level, we have isolated and sequenced the genes for both PKA catalytic subunit and regulatory subunit, which are encoded by separate genes. Sequence analysis indicated high homology with PKA genes from other fungi. RNA blots using total RNA from different developmental fungal stages showed that the expression level is highest in conidia,

relatively lower in germinated conidia and appressoria, and lowest level of expression was found in vegetatively grown mycelia. Antibodies were generated against the catalytic subunit, and similar expression patterns were also observed in a Western blot. These results indicated that PKA is likely to be important in *C.trifolii* morphogenesis and the expression of PKA genes are regulated at transcriptional level. A heterologous complementation experiment showed that *C.trifolii* PKA catalytic subunit could complement *S.pombe* PKA mutation. Further functional characterization is underway for antisense analysis of catalytic subunit gene and gene-replacement analysis of regulatory subunit gene.

22. *Neurospora crassa* type 2A protein phosphatase is involved in hyphal growth .

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The type 2A serine/threonine protein phosphatase (PP2A) holoenzyme consists of a core complex comprised of a 36 kDa catalytic subunit (C) tightly associated with a 65 kDa regulatory subunit (A). This dimeric core can be complexed with a third, variable, subunit (B), which in higher eukaryotes has been shown to control enzyme activity and specificity. In the presence of the PPI (type 1 phosphatase) and PP2A inhibitors cantharidin (100 mM) or calyculin A (250 nM), abundant cell leakage and abnormal swelling events were observed, respectively. Both inhibitors induced multiple branching of hyphae. We have isolated and analyzed two of the PP2A holoenzyme components. A PCR approach, employing the use of degenerate oligonucleotide mixtures, was used to isolate the genes encoding for the PP2A catalytic and variable regulatory subunits from *N. crassa*. In contrast to other organisms, *N. crassa* apparently has only one gene (designated *pph-1*) encoding for the C subunit. *pph-1* was mapped to LG-IVR. Nonetheless, a cross-hybridizing, structurally related, gene encoding a novel PPT-like serine/threonine protein phosphatase was also identified (*ppt-1*, mapped to LG-VR). Transformants in which *pph-1* had been disrupted could be maintained only as hetrokaryons which contained additional, intact, copies of *pph-1*. RNase protection and phosphatase activity assays showed differential *pph-1* transcript and PP2A activity levels during conidial germination. Ectopic integration of *pph-1* in a *pph-1*-disrupted strain brought about altered growth and sensitivity to cantharidin. *rgb-1* (mapped to LG-IL), a gene encoding for the B regulatory subunit, is highlly similar to those found in other organisms. Attempts to obtain strains in which *rgb-1* had been disrupted were unsuccessful, suggesting that this gene is essential.

23. Isolation and characterization of *flbA* suppressor mutants.

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Initiation of A. *nidulans* asexual reproductive development requires the control of proliferative growth and production of an extracellular signal that activates development. We have shown that two genes, *flbA* and *fadA*, have a major role in determining the balance between growth and sporulation. *fadA* encodes the - subunit for a heterotrimeric G protein and continuous activation

of FadA blocks sporulation while stimulating proliferation. *flbA* encodes an A. *nidulans* RGS (regulator of G protein signaling) domain protein that antagonizes FadA-mediated signaling to reduce proliferation and allow development. Interestingly, this role for FlbA in negatively affecting FadA signaling is necessary for biosynthesis of the mycotoxin sterigmatocystin (ST) as well as sporulation. To better understand FlbA function and other aspects of FadA- mediated growth control, we have isolated and characterized mutations in five distinct loci (designated *sfaA - sfaE)* that suppress a *flbA* loss-of-function mutation (*flbA*98). These suppressors overcome the requirement for *flbA* in both sporulation and ST biosynthesis. *sfaB8, sfaC67, sfaD82,* and *sfaE83* mutations are dominant to wild-type whereas *sfaAl* is semidominant. *sfaA1* also differs from other suppressors in that it cannot suppress the *flbA* deletion (and is therefore allele specific) whereas all the dominant suppressors can. Only *sfaE83* can suppress the dominant activating mutation in *fadA* indicating that *sfaE* may play a unique role in *fadA-flbA* interactions that control growth and sporulation. Finally, asexual sporulation directed by all suppressor mutations requiresflug, indicating that no suppressor can bypass the requirement for development-specific activation.

Photobiology

Cloning analysis of light signal transduction through NDP kinase in *Neurospora crassa*.

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We have established that 15 kDa proteins (psl5) showed increase in the phosphorylation in response to blue light (420 nm)(1). The psl5 protein was partially purified from the crude extract of mycelia to 200-fold. The partially purified fraction contained nucleoside diphosphate kinase (NDP kinase) activity forming CTP, GTP and UTP from CDP, GDP and UDP in the presence of [gamma- ^{32}P-]ATP. The partially purified fraction was further separated by a Tricine SDS-PAGE and the psl5 showed autophosphorylation activity. From the protein band ps 1 5 was extracted and hydrolyzed by chymotripsin. The polypeptides were further fractionated by a reverse phase HPLC. Amino acid sequence of one of the polypeptide was completely coinsided with that deduced from the cDNA for NDP kinase; NCNDKI (No.D88148) isolated by our group. The null type mutant of the phosphorylation of NDP kinase, designated to be delta-ps15-1 was isolated. From the mutant mRNA was isolated and by use of RT- PCR, the cDNA was cloned. The delta-ps15-1 included aminoacid replacement from Pro-72 to His-72. The amino terminal part of Pro-72 formed Beta-sheet, and carboxy terminal part formed alpha-helix. The amino acid replacement from Pro to His may cause aberrant folding of the protein leading complete loss of the phosphorylation activity of it. The results of RT-PCR identified two species of mRNA, both of which was cloned and resulted to detect alternative splicing in the mRNA. Southern blot analysis showed single copy of NDP kinase in Neurospora crassa genome, and the genomic DNA was isolated and sequenced. Two introns and one alternative splicing between the two introns could be detected. The consensus sequence for ATP-binding and the mutation site of Pro-72 were spliced out.

(1) Oda, K. and Hasunuma, K. (1994) FEBS Lett. 3459 162-166.

Molecular analysis of light signal transduction regulating NTP- binding, ADP-ribosylation and phosphorylation of proteins in *Neurospora crassa.*

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We have established an *in vitro* system to analyze molecular mechanism of light signal transduction by use of the crude membrane and the soluble fractions of mycelia of Neurospora crassa. The irradiation of the crude membrane fraction containing 10^{-7}M[alpha-^{32}P]ATP or [alpha-^{32}P]GTP by UV-A (370 nm) stimulated the increase in the binding of ATP and /or GTP to proteins with molecular masses of 53 kDa, 77 kDa, 83 kDa and 129 kDa(l). Blue light (420 nm) irradiation of the soluble fractions in the presence of 10^{-7}M[^{32}P]NAD stimulated the ADP-ribosylation of 38 kDa and 56 kDa proteins. A blind mutant, *bd lis-3* showed reduced light response in the ADP-ribosylation of the 56 kDa protein(2). Blue light (420 nm) irradiation of the membrane fraction in the presence of 10^{-8}M[gamma-^{32}P]ATP stimulated the increase in the phosphorylation of 15 kDa proteins (psl5)(3). We have isolated a null type mutant with no phosphorylation of psl5, designated to be delta-ps15-1, which was mapped on LGVR, 22% distal to *al-3* and 24 % proximal to *his-6.* The delta-ps15-1 showed abnormal light response to the morphogenesis of perithecia. In darkness wild type formed perithecial beak at random places on the perithecia. However, under the light illumination parallel to the solid medium wild type formed the beak at the top of the perithecia. Such a light induced ordering of the positioning of perithecial beak was designated as "polarity of perithecia". The delta-ps15-1 completely lacked such a light induced polarity of perithecia (submitted).

(1) Oda, K. and Hasunuma, K. (1 993) Cytologia 5 8, 231-240.

(2) Oda, K. and Hasunuma, K. (1994) FEBS Lett. 345, 162-166.

(3) Mizoguchi, K. et al. (1 993) J. Photochem. Photobiol., in press.

24. Is there a link between photolyases and blue light reception in fungi?

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Trichoderma harzianum is a common filamentous fungus which lives in the soil as a mycoparasite or saprophyte. As a mycoparasite, *Trichoderma* has potential to control soil-borne fungal diseases. Furthermore, a brief pulse of light induces sporulation, making the organism a convenient model for the study of morphogenesis. There is evidence that some blue light receptors in higher plants share homology with DNA photolyases, an enzyme that uses visible light to repair one of the major forms of UV-damage to DNA. We used action spectroscopy to study whether photoreactivation of UV-damaged spores, and the induction of sporulation, could share the same photoreceptor. The action spectra of sporulation and photoreactivation turned out to be very different and provided evidence that the *Trichoderma* enzyme belongs to class I photolyases. In parallel with the photobiological studies, we isolated and characterized the

complete DNA photolyase gene of *T. harzianum* . The sequence of the gene indicates that it belongs to class I photolyases, confirming the photobiological approach. The expression of the corresponding mRNA during light induced sporulation and germination was analyzed The photolyase mRNA is detectable in inmature spores and it is abundant in mature spores. The message is also detectable in germinating spores until germ-tube emergence. These data suggest a role of the enzyme in repairing DNA-UV damage in spores and germlings.

25. Circadian clock mutants of *Neurospora crassa.*

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The *frequency* (*frq*) gene has been shown to be a central component of the Neurospora circadian clock. frq mRNA and *FRQ* protein are components of a negative feedback loop which comprises the circadian clock in Neurospora (Loros, Seminars in Neuroscience 7: 3-13, 1995). *frq* expression cycles with a period identical to that of the Neurospora circadian cycle. In addition *frq* expression is rapidly induced by light. Induction of *frq* by light resets the clock in a time of day dependent manner, either advancing or delaying the clock towards circadian dawn or dusk (Crosthwaite et al., Cell 81: 1003-12, 1995). Continuous light suppresses clock function, due to induction of *frq* and FRQ to high levels suppressing *frq* cycling.

The *lis* (light insensitive) mutants express the circadian rhythm under continuous dim light, a condition when the function of the clock is normally repressed in Neurospora. *frq* mRNA in the *lis* mutants is indeed induced to high levels by light, as in wild type, but still cycles with the appropriate period under these conditions. Thus it may be that the circadian oscillator is altered in some manner in these mutants such that high levels of *frq* no longer suppresses clock function, although the clock is still reset when first exposed to light, alternatively, perception of light levels may be altered.

In addition we are using insertional mutagenesis to identify other genes involved in circadian rhythmicity. By screening for mutants with an altered conidiation rhvthm or altered responses to environmental stimuli we hope to identify mutations which alter clock input, output, or the central oscillator itself, and thus further our molecular dissection of the circadian clock in Neurospora.

26. Photomorphogenesis mutants of *Phycomyces*.

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The sporangiophores of the zygomycete *Phycomyces blakesleeanus* grow out into the air lifting a sporangium filled with spores. *Phycomyces* develops two types of sporangiophores:

Macrophores are giant sporangiophores that grow several cm long guided by many stimuli. The microphores are dwarf sporangiophores, about 1 mm long. At a certain stage in its development, the mycelium becomes photosensitive: blue light governs the production of the yellow pigment, -carotene (photocarotenogenesis), and the development of the sporangiophores (photomorphogenesis). Illumination increases the production of macrophores and decreases the production of microphores.

The *mad* mutants were isolated because of the defective phototropism of their macrophores. There are ten unlinked *mad* genes. Mutations in genes *madA* and *madB* are defective not only in phototropism, but in the mycelial responses, photocarotenogenesis and photomorphogenesis.

The *car* mutants suffer various structural and regulatory alterations in the biosynthesis of -carotene. The mutants have revealed that -carotene is not required for photocarotenogenesis or phototropism, but is essential for photomorphogenesis.

We have designed an experimental procedure to isolate mutants affected in photomorphogenesis. We have isolated three such mutants and characterized their photomorphogenesis, photocarotenogenesis, and phototropism. The threshold for photomorphogenesis is 10 to 1000 times higher in the mutants, but their phototropic response is unaffected. In addition, one of the mutants is also affected in photocarotenogenesis. Thus, the genes that we have identified by mutation are specific for mycelial photoresponses and presumably encode new proteins involved in the phototransduction chain. None of the mutants resemble the phenotype of previously isolated *mad* mutants which suggests that our search is far from complete.

27. Mutants in light regulation of fruitbody development in *C. cinereus*.

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During the life cycle of *Coprinus cinereus*, two compatible monokaryons with different mating types fuse to form a dikaryon in order to accomplish fruitbody development and sexual reproduction. Compatibility is controlled by the two mating type loci, *A* and *B*. Specific mutations in *A* and *B* can transform a monokaryon into a so called *AmutBmut* homokaryon that produces fruitbodies like dikaryons. Fruitbody development in *C. cinereus* is controlled by temperature, nutrition and, at two different stages (initiation and fruitbody maturation), by light signals.

In our current project, we use an *AmutBmut* homokaryon to study regulation of fruitbody development in a homogenetic background. We submitted the strain to a REMI and UV mutagenesis. Fruiting of isolated mutants was tested on yeast extract-malt-extract-glucose (YMG) agar supplemented with tryptophan. Mutants were grouped according to their defects in fruitbody development (primordia initiation, primordia maturation, fruitbody maturation, spore formation). 35 out of 179 REMI mutants and 20 out of 114 UV mutants unable to initiate fruitbody development on YMG-trp do also not fruit on horse dung, the natural substrate of *C. cinereus.* Among these, we hope to find mutants with defects in the light pathway. One special class of mutants with an unusual light response form in the light so called etiolated stipes, extra

long stipes with undeveloped caps. This phenotype normally occurs if a wild type strain is left in the dark after primordia induction. We describe one REMI mutant of this class in more detail. Interestingly, the defect in light response in this mutant can be reversed by changing the polarity of gravity (turning fully developed cultures by 180 degrees).

28. Blue light overrides mating type repression of asexual sporulation in *Coprinus cinereus*.

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The basidiomycete *Coprinus cinereus* undergoes a life cycle with transitions between two types of mycelia. The monokaryotic mycelium constitutively produces abundant vegetative spores (oidia) on aerial hyphae. A dikaryotic mycelium arises after fusion of two compatible monokaryons. Oidiation is repressed in dikaryons. Upon light induction, fruitbodies are formed in which karyogamy and meiosis occur. In addition, light can induce the formation of haploid, monokaryotic spores on a dikaryon.

Developmental processes in *C. cinereus* are controlled by two distinct mating type loci, *A* and *B*. To form a dikaryon two nuclei with different *A* and *B* specificities have to be present in the same cell. Specific activating mutations in both mating type loci lead from a monokaryon to a homokaryon that forms fruitbodies like a dikaryon but also produce oidia in amounts comparable to monokaryons. However, in contrast to monokaryons, oidiation in *AmutBmut* is repressed in the dark and is induced by light. Extensive studies on *Amut* homokaryons, *Bmut* homokaryons, "*A* on" transformants of monokaryons and dikaryons show that the *A* mating type loci is responsible for repression of oidiation in the dark. Light overrides this *A* mediated repression and the *B* mating-type locus seems to modulate this light effect. As in fruiting, it is blue light that induces oidiation.

29. Light/dark cycles and the control of meiosis in *Coprinus cinereus*.

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In the synchronous meiotic system of *Coprinus cinereus*, meiosis is controlled at meiotic S and at metaphase I, and light intensity and light/dark cycles play an important role. The time of exposure to light requried to enter meiotic S is inversely proportional to the exponent of light intensities. The shift-up experiments showed that the critical light dependent period is 10h before the initiation of meiotic S-phase.

The control of entry to meiotic metaphase is also light intensity and strain dependent. The London strain is light/dark blind while the Java and Japanese strains are dark dependent under moderate to high light intensities; a 3 h dark period is essential for proper progression to metaphase I and its effect is very stage specific. It is quite possible that the dark period signals

the cellular processes leading to metaphase events. Cytological studies showed that chromosomes are normal in meiotic prophase but are unable to undergo condensation under arrest conditions. Genetic crosses and backcrosses showed that light blindness is dominant and appeared to be a single gene inheritance.

30. Light-dependent conidiation in *Aspergillus nidulans* is influenced by *fluG* and *veA* gene expression.

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Conidiation in *Aspergillus nidulans* is induced by exposure to red light. Using a strain that contains a blue light gain-of-function mutation, we have isolated a mutant, designated *fluG*701, that fails to conidiate in red light. The *fluG* gene has been shown by Lee and Adams (1994, Genes Dev. 8, 641-651) to be necessary for the synthesis of a small diffusible factor that is required for the initiation of asexual development. Although conidiation in a null *fluG* strain can be rescued by growth next to a wild-type colony, conidiation in the *fluG*701 strain cannot be rescued by the extracellular factor. Moreover, the loss of transcriptional negative autoregulation observed with other *fluG* mutant alleles is not observed in a *fluG*701 strain. The *fluG*701 mutation has been shown to be a G to A transition that replaces glutamic acid at amino acid position 467 with lysine.

The *veA1* mutation allows conidiation to occur in the absence of light. The *veA* gene has been cloned, but the predicted ORF shows no homology to other known sequences. We have isolated *veA1* suppressors, one class of which are alleles of *fluG*. These suppressors show varying degrees of rescue by the extracellular factor and do not show transcriptional negative autoregulation. The nucleotide identity of these suppressors has been determined and suggests, first that the amount of available *fluG* product influences light-dependent conidiation and second, that light-dependent functions are located in the C terminal portion of the fluG protein. These data indicate that *fluG* and *veA* have a genetic interaction. Although no evidence for a physical interaction has been demonstrated, one potential model is that veA and fluG operate together to interpret the light signal required for the production of the extracellular factor and initiation of conidiation.

Gene Regulation and Metabolism

31. The carbon catabolite repressor *Cre1* in *Trichoderma reesei* requires phosphorylation for its binding to the corresponding consensus sequence.

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In the industrial important fungus *Trichoderma reesei* glucose repression is mediated by the C2H2 Zn-finger protein *Cre1*, which binds to sites in the promoter of genes sensitive to glucose. In yeast, the homologous MIG1 protein is thought to recruit SSN6 and TUP1 to form the

repressor complex. Derepression is mediated via the SNF1 gene encoding a protein kinase. In contrary, we could prove that phosphorylation of the *Trichoderma reesei* Cre1 protein leads to strong binding to the corresponding consensus sequence 5' SYGGRG 3'. A GST::Cre1 fusion protein, derived from a 700 bp DNA fragment encoding the Zn-finger region and the RGR1-like region of Cre1, was phosphorylated using protein kinase A catalytic subunit from porcine heart and dephosphorylated alkaline phosphatase from bovine intestinal mucosa. Only phosphorylated fusion protein showed binding to the corresponding consensus sequence. Similiar results were achieved by employing a thrombine-cleaved GST::Cre1 fusion protein.

32. Effects of amino acid substitutions in l tubulin on sensitivities to the antimicrotubule drugs benomyl and griseofulvin in *Coprinus cinereus.*

Tadashi Matsuo, Akira Kiba, Yuki Yamamoto, Hajime Muraguchi and Takashi Kamada, Okayama University, Okayama 700, Japan.

We first analyzed the wild-type 1 tubulin gene of *Coprinus cinereus* and then examined 26 1 tubulin mutants obtained as benomyl-resistant strains, griseofulvin-resistant strains, or extragenic suppressors of a mutation in a gene whose product appears to interact with 1 tubulin. Most of the mutations altered sensitivities to both benomyl and griseofulvin, regardless of how they had been identified. The 26 mutations all resulted in amino acid substitutions in 1 tubulin. The mutations were found in three clusters within the protein (445 amino acids): near the N-terminus (residues 3-50), near the middle (198-222) or at residue 350. These three regions overlap or lie close to the three colchicine-binding regions. We also found that a missense mutation at residue 350, which confers benomyl-resistance and heatsensitivity, could be suppressed by amino acid substitutions at several additional sites in 1 tubulin. This finding suggests that missense mutations in both residues that interact with the drug and also in residues distant from the binding sites can affect sensitivity to the drug.

33. Regulation of the *Neurospora crassa* Alternative Oxidase gene *aod1*.

Lesley LT. McLean and Frank E. Nargang, University of Alberta, Edmonton, Alberta, Canada

Alternative oxidase is encoded by a nuclear gene, *aod1,* and functions as a second terminal oxidase, transferring four elections directly from reduced ubiquinone to oxygen. This bypasses two sites of ATP production as well as the sites of action of respiration inhibitors Antimycin A and cyanide. In *Neurospora,* alternative oxidase is not seen under normal physiological conditions, but is induced if respiration through the standard mitochondrial electron transport chain is compromised. The signal(s) sent from respiration deficient mitochondria to the nucleus are unknown at this time. A cyclic-AMP response element (CRE) was found 750 bp upstream from the *aod1* transcription start site and was shown to bind specifically with proteins from *Neurospora* cell extracts. However, transformation of *aod1*$^{-}$ cells with constructs lacking the CRE upstream of *aod1* gave isolates with alternative oxidase activity that is regulated in the wild-type fashion under conditions examined thus far. Similar studies using progressively shorter upstream regions in *aod1*$^{+}$ constructs have shown that 250 bp are capable of providing control in the wild-type fashion. Work is currently under way to study induction quantitatively using the

reporter gene lacZ. Identification of the factors involved in the regulation of *aod1*, and the method of regulation of those factors, may allow the dissection of signaling from the mitochondria to the nucleus in times of respiratory distress.

34. Nitrate utilisation in *Aspergillus nidulans:* Binding of the pathway specific activator *NirA* to its target sequence in vivo is dependent on induction and *AreA*.

Frank M. Narendja[1], Markus F. Wolschek[1], Jan Karlseder[2], Christian P.Kubicek[1], Claudio Scazzocchio[3] and Joseph Strauss[1].

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We have adapted the method of in vivo DMS footprinting to be suited for filamentous fungi using LMPCR, initially developed for mammalian cells. In order to obtain equally in vivo methylated DNA, we used germinating conidia with germ tubes of 4-6 cells for methylation instead of mature mycelium. We applied this technique to study the regulation of the nitrate utilisation cluster in *A. nidulans* as it represents a well characterised model system. Preliminary experiments determining the optimal time points for methylation following induction and/or repression showed that the response of *A. nidulans* cells to changes in nutritional composition is in the range of minutes only.

In vivo footprinting analysis of the bidirectionally functional NirA site 2 revealed that: (1) the consensus target sequence determined with the truncated protein in vitro (5`CTCCGHGG3`) is recognised by the native NirA protein in vivo, (2) binding of NirA in vivo is strictly dependent on the presence of inducer, (3) addition of ammonia to induced germlings results in dissociation of NirA, and that (4) under no condition NirA associated with its DNA target in a mutant carrying a non functional areA allele.

35. Role of negative charges in the *N. crassa* mitochondrial protein import receptor TOM22.

Frank E. Nargang, R. Gary Ritzel, Doron Rapaport, Walter Neupert, and Roland Lill. University of Alberta, Edmonton, Alberta and Institut fur Physiologische Chemie, Munich, Germany.

TOM22 is a mitochondrial outer membrane protein whose amino terminal domain extends into the cytosol and acts, in conjunction with TOM20, as a receptor for precursor proteins destined to be imported into mitochondria. The first 47 amino acids of the protein contain 18 negatively- and zero positively-charged residues. It has been suggested that this region of TOM22 interacts with mitochondrial targeting signals, which are thought to form positively charged amphipathic helices. To investigate this hypothesis, we have constructed a series of mutant derivatives of TOM22 in which varying numbers of the negative charges have been neutralized. These mutant

constructs have been transformed into a sheltered heterokaryon bearing a *tom22::hygromycinB* disruption in one nucleus and assessed for their ability to rescue the inviable disruption-containing component. Constructs with up to 11 negative charges removed show little difference from the wild-type gene in their ability to rescue the *tom22* disruption nucleus. Isolates with 15 negative charges removed can also rescue, though with reduced efficiency. We are currently investigating the ability of mitochondria, isolated from strains containing mutant constructs, to import mitochondrial precursor proteins. So far we have shown that strains containing TOM22, in which six negative charges have been removed, are able to import mitochondrial precursors with normal efficiency. Our results suggest that TOM22 receptor function does not depend simply on its highly negatively charged nature.

36. The *Aspergillus nidulans sconB* sulphur regulatory gene encodes a protein with WD40 repeats and F-box.

Renata Natorff, Malgorzata Piotrowska and Andrzej Paszewski, Institute of Biochemistry and Biophysics, Warsaw, Poland.

The *Aspergillus nidulans sconB* sulphur regulatory gene has been cloned by complementation with a cosmid gene library. The 2081 bp long open reading frame contains one 47 bp intron. The *sconB* gene encodes a polypeptide of 678 amino acids which belongs to the WD40 family of highly conserved eukaryotic regulatory proteins. The SCONB protein contains seven WD40 repeats spanning the C-terminal half of the protein. The SCONB protein shows 56% identity to the SCON2 protein of *N. crassa* and 44% to *S. cerevisiae* essential Met3O protein; both being involved in the regulation of sulphur metabolism. In addition, SCONB possesses the F-box, a recently discovered structural motif (Bai *et al* 1996, Cell, 86, 263-274) found in a number of proteins including the yeast CDC4. In the latter F-box serves as a target for binding Skp1p - yeast homolog of *A. nidulans* SCONC protein. This suggests that the SCONB and SCONC proteins may interact.

In the wild type, the *sconB* transcript of 2.8 kb is present at approximately the same level under all sulphur conditions tested, except for sulphur limitation, where it is up-regulated. It is present in all four *scon*$^{-}$ strains.

The *sconB2* mutation is complemented by the heterologous *N. crassa scon2*$^{+}$ gene. Interestingly, the *sconB*$^{+}$ gene transforms the *sconC* mutant to the wild type phenotype, but not *vice versa.*

37. Developmental regulation of catalases in *Aspergillus nidulans*

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A general hypothesis to explain microbial cell differentiation as a response to hyperoxidant states was derived from studies on sporulation in *Neurospora crassa.* Since catalases are ubiquitous enzymes that are central to cellular antioxidant responses, we have now approached this

hypothesis by studying the function and regulation of catalases during *A. nidulans* asexual sporulation (conidiation). We have found two catalases in this fungus encoded by the *catA* and *catB* genes, whose predicted polypeptides are as similar between them, as they are to *E. coli* HPII catalase. The *catA* and *catB* expression is differentially regulated during growth and development. The *catA* mRNA and protein appear during sporulation and are accumulated in both, sexual and asexual spores independently of the *brlA* regulatory gene, in a process that involves transcriptional and translational controls. In contrast, the *catB* mRNA and protein are very low in spores, accumulate in mature hyphae throughout conidiation and in response to H_20_2, both catalases can offer protection against H_20_2 at different stages of the life cycle. Although the increase in CatB activity and the appearance of CatA during sporulation is consistent with the occurrence of oxidative stress during development, our data indicate the operation of efficient alternative pathways for H_20_2 detoxification.

38. The *alcA* gene in *A. nidulans:* a full characterization of synergistical activation and carbon catabolte repression.

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In the ascomycete *A. nidulans,* ethanol can be used as sole carbon source by the action of two enzymes: alcohol dehydrogenase I (ADHI) encoded by *alcA* and aldehyde dehydrogenase (aldDH) encoded by *aldA.* The system is subject to two regulatory mechanisms consisting in specific induction mediated by the transcriptional activator AlcR and the carbon catabolite repression governed by the general repressor CreA. The *alcA* gene is one of the strongest inducible genes in *A. nidulans.* It is the most widely used for proteins overexpression in filamentous fungi both for fundamental research and applied aspects in biotechnology. A complete molecular characterization of AlcR and CreA binding sites have been carried out by gel band shift analysis.The role and the importance of each AlcR and CreA binding sites have been determinated by mutagenesis and deletion experiments. There are two types of AlcR binding sites: direct and inverted repeats which were shown to be functional *in vitro* and *in vivo.* We have shown that there is a strong synergistic activation of *alcA* transcription related to the three clustered AlcR specific sites, which positions are crucial in the activation process. Disruption of the CreA targets led to a totally derepressed *alcA* promoter which is associated to an overexpressed *alcA* gene transcription. The close proximity of the AlcR and CreA binding sites in the *alcA* promoter suggest a direct mechanism of competition between the two regulatory proteins as shown for the *alcR* promoter.

39. *SconC,* an *Aspergillus nidulans* sulphur metabolism regulatory gene is homolog of yeast *SKPI* essential gene.

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SconC, one of the four regulatory genes *(A, B,* C, D) involved in controlling sulphur amino acid metabolism, was cloned and sequenced. The 669 bp open reading frame is interrupted by three short introns. It encodes a protein of 163 amino acids. The SCONC protein sequence predicts glycosylation, phosphorylation (ATP/GTP binding site: P-loop) and the PEST sequence common in rapidly degraded proteins. The SCONC protein shares 51 % identity with yeast Skp1p, a protein of central importance to a number of cellular processes, e.g. cell cycle (Bai *et al* Cell, 86, 263-274, 1996). *SconC* mRNA is expressed on the same level in the wild type grown on MM and in the presence of methionine. Interestingly, *sconC* mRNA is present in the *sconC3* and in *sconB2* mutants, but not in the *sconD6.* This may indicate that these two regulatory genes act in a cascade. Point mutations in highly conserved regions were identified in the *sconC3 and sconC1* mutants relieved of methionine repression.

40. Identification of a regulatory gene in *Ustilago maydis* that affects the expression of genes regulated by the *b* locus.

Claudia Quadbeck-Seeger, Joerg Kaemper and Regine Kahmann, Institut fuer Genetik, Munchen, Germany.

One of the genes regulated by the *b* locus of *U. maydis* is *egl1*, a gene encoding an endoglucanase. *egl1* is expressed in the dikaryon during the filamentous phase when an active bE/bW heterodimer is present in the cell, but not in haploid yeast like growing cells. Since *egl1* expression can easily be monitored in a plate-assay, we used the gene as a reporter to screen for mutants that express *egl1* in haploid cells. One such mutant was found. Northern analysis showed that the mutation leads to constitutive expression of several *b*-dependent genes. This indicates that the mutation has affected a gene with a more general regulatory function. Assays in planta showed that the mutation does not attenuate pathogenicity. Interestingly, however, formation of spores was abolished. Complementation with a cosmid library led to the identification of the gene, *rum1* (regulator *Ustilago maydis* 1), affected in the mutant. We will discuss how this negative regulator fits into our current scheme on gene regulation exerted by the bE/bW heterodimer.

41. lnterspecies sequence and regulatory comparisons of amidase genes in *Aspergillus* species.

Julie A. Sharp, Sarah J. Bugg, Meryrl A. Davis, Michael J. Hynes. University of Melbourne, Australia.

The *amdS* gene of *Aspergillus nidulans* encodes an amidase enzyme involved in. acetamide hydroiysis. *amdS* mRNA is regulated at the transcriptional level by seven independent regulatory pathways in response to environmental stimuli. This elaborate display of regulatory control makes this system an excellent model for the study of gene regulation. Sequences with homology to the *A. nidulans amdS* gene have been cloned and sequenced from a variety of *Aspergillus* species identify conserved cis-acting regulatory elements. The *amdS* genes isolated from *Aspergillus oryzae, Aspergillus unguis* and two *amdS* genes from *Aspergillus ustus* have been

sequenced. Comparison of the 5' untranslated regulatory regions reveal sequence divergence between the species whilst small scattered regions of sequence conservation can be identified as potentially important regulatory elements. Functional studies suggest these elements specific DNA binding sites for some of the known regulatory proteins. In an attempt to isolate *amdS* genes from other *Aspergillus* species a new amidase gene with different substrate specificities was isolated. The *A. nidulans* homologue was subsequently cloned by homology and sequenced. Functional studies have been used to investigate substrate specificity and regutatory control. Comparison of exon conservation and intron position between the two amidase genes suggests they are paralogues.

42. Nitrogen regulation and the *tamA* gene of *Aspergillus nidulans.*

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In *Aspergillus nidulans,* the expression of many genes involved in nitrogen utilization is increased in the absence of easily metabolized nitrogen sources such as glutamine. This activation is mediated by the positively acting AreA protein, which binds to 5'GATA3' sequences in the promoters of regulated genes and activates transcription under nitrogen limiting conditions. The *tamA* gene in *A. nidulans* may also play a role in nitrogen regulation. *tamA* mutants have been identified which have low levels of a number of nitrogen metabolic enzymes. Mutants are also resistant to the toxic ammonium analogue methylammonium. The *tamA* gene has been cloned, and sequence analysis revealed a number of features suggestive of regulatory function. The predicted protein contains a Zn(II)2Cys6 zinc cluster motif similar to others known to bind DNA, and downstream regions characteristic of the Zn(II)2Cys6 cluster family. TamA shows strongest homology to UGA35, a nitrogen regulatory protein in *Saccharomyces cerevisiae.* The importance of these regions to TamA function is being investigated. PCR mutagenesis has been used to demonstrate that the zinc cluster motif is not required for TamA function. Substitution of the crucial fourth cysteine residue in the motif did not prevent an altered *tamA* construct from complementing a *tamA* mutant strain in cotransforrnation experiments. An internal deletion has demonstrated that sequences towards the C-terminal of the protein are required for function. Sequence changes in a range of *tamA* mutants have been determined and further implicate the C-terminal region as critical in the function of the TamA protein.

43. Preliminary characterization of the iso-orotate Decarboxylase (IDCase) activity of *Neurospora crassa*.

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Many microorganisms have developed metabolic pathways that allow them to operate efficiently in the presence in a wide variety of nutrients. An example of such a pathway is the thymidine

salvage pathway found in some species of fungi. This pathway allows fungi to convert thymine nucleotides into uracil nucleotides. One of the enzymes this pathway is iso-orotate decarboxylase (IDCase). We have developed a sensitive, specific radioactivity-based assay which is capable of easily and accurately measuring IDCase activity even in the most impure cell lysate. Our assays have shown that IDCase levels in *N. crassa* may be subject to induction dependent upon medium nitrogen source. We have observed that the specific activity of IDCase in wild type *N. crassa* increases by 2-3 fold when this strain is grown on Westergaard's medium, a minimal medium containing nitrate as the sole nitrogen source, versus Vogel's medium, a standard minimal medium containing ammonium salts. We have also shown that IDCase levels are increase 8-10 fold in strains which contain the *uc-1* Mutation which may influence the production of the enzymes of the thymidine salvage pathway. The mechanism behind this increased production of IDCase remains unclear.

44. Characterization of a *Neurospora crassa* gene with sequence and functional homology to *Aspergillus nidulans flbD.*

Wei-Chiang Shen, Jenny Wieser, Thomas H. Adams and Daniel J. Ebbole. Texas A&M University.

The *flbD* gene regulates conidiation in *A. nidulans. flbD* contains a myb-like DNA-binding domain and is thought to function as a transcription factor. *flbD* acts in concert with other developmental regulators to activate expression of the key regulator of conidial morphogenesis in *A. nidulans, brlA.* We identified a gene from *N. crassa* with sequence similarity to the DNA-binding domain of *flbD* . The *N. crassa* gene complemented the conidiation defect of an *A. nidulans flbD* mutant. Furthermore, induced expression of the cDNA clone of the *N. crassa* gene using the *alcA* promoter induced conidiation in submerged cultures of *A. nidulans.* Thus, the *N. crassa* gene appears to be a functional homologue of *A. nidulans flbD.* A *N. crassa* mutant was produced by deletion of the gene. The growth rate of the mutants was indistinguishable from wild type and macroconidiation, microconidiation, and ascospore formation was normal. This finding suggests that the function of the gene in *N. crassa* is subtle or redundant under laboratory conditions, or that the gene plays no role in development in *N. crassa.* We have demonstrated that a *N. crassa* gene can complement a gene involved in conidiation in *A. nidulans.* The strategy of direct complementation could be useful in isolation of additional genes of *N. crassa* with homology to developmental regulators of *A. nidulans.* Such studies would help address the question of whether the regulatory strategies governing initiation of conidiation are fundamentally different or similar between these fungi.

45. Identification of differentially expressed genes during life cycle of *Microbotryum violaceum*.

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Microbotryum violaceum is a basidiomycete fungus whose hosts are Plant species in the Caryophyllaceae. Development of the fungus was studied using mRNA Differential Display.

Three cell types occur during the life cycle of *M. violaceum*: haploid sporidial cells (of opposite mating-type), dikaryotic hyphae and diploid teliospores. Of these, only dikaryotic hyphal stage is infectious. Differential display, provided us four candidate fragments representing transcripts expressed in the dikaryotic hyphal stage, but not in the haploid stage; one of these was also expressed in mated haploid cells. Differential expression of these fragments was confirmed by Northern blotting. These partial cDNAs were cloned and sequenced, then the RACE (Rapid Amplification of cDNA End) technique was used to recover full length cDNA. Two fragments produced by RACE (a 1.6 Kb and a 1.4 Kb fragment) were cloned and sequenced. The gene (C34) transcribed in both mating and hyphal stages had a homeo-domain box in the largest open reading frame. Southern hybridization detected C34 sequences in haploid sporidia of both mating type and homologous sequences were detected by Southems for both *Ustilago maydis* and *Saccharomyces cerevisiae*. Functional studies of these genes are currently underway. This work shows that the combination of mRNA differential display and RACE provide an efficient way to identify genes involved in different developmental stages in fungi, an approach that should be widely applicable to research on other organisms.

Mycorrhizae

46. Molecular cloning and characterization of phosphate transporters from arbuscular mycorrhizal associations.

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Arbuscular mycorrhizal (AM) fungi are soil borne fungi that form mutualistic associations with the majority of species of land plants. The fungi are obligate symbionts and colonize the cortex of the plant root in order to obtain carbon from their plant hosts. The association is also beneficial for the plant as the fungi assist the plant with the aquisition of phosphate and other mineral nutrients from the soil. Our aim is to investigate the molecular mechanisms underlying phosphate transport in the symbiosis. Towards this goal we have isolated cDNA clones encoding phosphate transporters from roots of *M. truncatula* colonized with the AM fungus *Glomus versiforme*. cDNA clones from both the fungus and the plant were obtained and both encode proteins that are predicted to contain 12 membrane spanning domains, a secondary structure that is typical of membrane transporters from prokaryotes and eukaryotes. The cDNAs were expressed in yeast where they were able to complement a yeast phosphate transport mutant, therefore indicating that the encoded proteins are functional phosphate transporters. Phosphate transporters have been cloned previously from *Neurospora* and *Saccharomyces*. The *Glomus* transporter shares 48% identity at the amino acid level with the *PHO84* gene product from *Saccharomyces* and 45% with the *PHO-5* gene product from *Neurospora*. The *Glomus* and *Medicago* proteins are less similar and share approximately 37% amino acid identity. The *Glomus* phosphate transporter gene is expressed in the external hyphae which extend out of the root into the soil, a location which is consistent with a role in phosphate uptake in the

mycorrhizal symbiosis. The expression of the *Medicago* transporter gene is currently under investigation.

47. Development of DNA markers for the identification of VA mycorrhizal fungal strains.

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AM mycorrhizal funai are obligate symbiotes of higher plants. Although they are thought to be plant parasites it is clear that they have a mutualistic relationship with their host. The plant benefits because the fungi can provide a better supply of inorganic nutrients than the plant can acquire on its own. AM fungi have been difficult to study because of their requirement for a host plant for growth. Consequently specific host fungal associations have not been easily substantiated. In order to more closely examine this and other fungal/host relationships we are developing DNA based detection methods which use the DNA Polymerase Chain Reaction (PCR) to detect the presence of specific AM species in field plant and soil samples. Random amplification of polymorphic DNA analysis was used to generate DNA fragments that are unique to isolates of several arbuscular mycorrhizal fungi. Sequence analysis of these fragments allowed generation of primer pairs, and subsequent specific identification of these genomes even in the presence of competing genomic DNA's. This approach can be used to specifically detect the presence of these fungi in mixtures of spores or infected roots.

48. Cloning of differentially expressed novel transcription factor like gene from early stages of ectomycorrhizal interaction of *Laccaria bicolor* and *Pinus* resinosa.

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We have cloned several cDNA clones that are differentially expressed from ectomycorrhizal fungus *Laccaria bicolor* in response to its host red pine. DDRT-PCR has been used to identify and isolate partial cDNA clones from *L. bicolor*, after various time points of interaction with red pine seedlings, in an *in vitro* system we have developed in our lab. Full length cDNAs of these differentially expressed clones were obtained using a combination of novel 5' and 3' RACE technique. The differential expression of these genes in response to red pine seedlings has been confirmed through Northerin blot analysis of RNA samples isolated from various time points of interaction between the *L. bicolor* and red pine seedlings. Genbank data base searches have indicated that one of the clones isolated from very early stage of interaction belongs to a novel class of transcription factors. It is now possible to isolate symbiosis related genes from ectomycorrhizal interactions using the DDRT-PCR methods. Details of this novel transcription factor and importance of its expression in the very early stages of ectomycorrhizal interaction between *L. bicolor* and red pine will be presented.

49. Signalling in the ectomycorrhizal symbiosis. The tryptophan betaine, hypaphorine, produced by *Pisolithus tinctorius* stimulates the expression of an auxin-regulated gene in roots of *Eucalyptus globulus.*

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The development of the ectomycorrhizal association between *Eucalyptus globulus* and *Pisolithus tinctorius* induces dramatic changes in gene expression in both symbionts.

Hypaphorine, the betaine of tryptophan, is the major indolic compound which accumulates in *P. tinctorius* hyphae. Hypaphorine is accumulated in *P. tinctorius* tissues as soon as hyphae are in contact with host plant root surface. Such accumulation is controlled by specific root diffusible molecules, it could be induced through a membrane, but non-host plants are inactive.

The fungal hypaphorine had no IAA like activity on *E. globulus* root elongation and ramification, instead, a strong reduction of root hairs elongation was recorded. Furthermore the up-regulation of the level of *EgPar* transcripts was observed in roots incubated in the presence of either Pisolithus acellular extracts or hypaphorine. The *EgPar* gene expressed at a low level in roots and shoots of eucalypt seedling is amongst the identified symbiosis-regulated genes, the steady-state level of *EgPar* transcripts being drastically up-regulated in roots during the early stages of ectomycorrhiza development. It shows a high homology with auxin-induced genes from tobacco and *Arabidopsis*.

The latter data indicates that the fungal hypaphorine is able to trigger gene expression of the host plant and may act as an auxin derivative in eucalypt roots. This is the first report of an alteration of the host plant gene expression by a diffusible signal from an ectomycorrhizal fungus.

50. Phylogeny and population structure of the asexual mycorrhizal fungus *Cenococcum geophilum* Fr.

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The phylogenetic relationship of the asexual mycorrhizal fungus *Cenococcum geophilum* Fr. among ascomycetes was examined by phylogenetic analysis of nucleotide sequence data from the 18S ribosomal RNA genic region. A specific focus of this study was to test the hypothesis that the genus *Elaphomyces* is the closest sexual relative of *Cenococcum geophilum*. DNA parsimony and distance analysis of the sequence data separated *C. geophilum* and *Elaphomyces* on distant clades when 44 additional genera of ascomycetes were included in the phylogenetic analyses. *Cenococcum* was positioned as a basal, intermediate lineage between the two Loculoascomycete orders, the Pleosporales and the Dothidiales, and strongly supported *Elaphomyces* to be of Plectomycete origin. Among the sexual Ascomycetes examined no close sexual relative to *C. geophilum* was identified. Currently, we are examining the hypothesis that natural populations of *C. geophilum* are clonal and not recombining. Ten polymorphic loci

present in two population sites have been identified by a PCR-SSCP strategy. Population genetic analysis of the multilocus genotypes obtained will measure levels of effective linkage between the loci (linkage disequilibrium) and determine if *C. geophilum* has a clonal or recombining population structure.

51. Rapid typing of *Tuber borchii* mycorrhizae by PCR amplification with specific primers.

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Truffles are ascomycetous fungi that form ectomycorrhizae with the roots of trees such as oak, poplar, willow and hazel, and some shrubs such as *Cistus*. Truffle fruitbodies are usually identified on the basis of the structure of the peridium and gleba, the size and shape of their spores and asci, and wall ornamentation. However, all these features are lost during the symbiotic phase. Identification of truffles during their symbiotic phase is a main step in applied research on species of commercial value. We are therefore developing molecular typing methods based on PCR amplification.

DNA isolated from fruitbodies, mycelia and mycorrhizae of *T. borchii* was amplified with the universal primers pair ITS1/ITS4. RFLP analysis of the amplified ITS region of the ribosomal genes allowed discrimination of *T. borchii* from other truffle species on all DNA sources. The ITS region of *T. borchii* was then cloned and sequenced to design specific primers. The primers pair TBA/TBB was shown to be specific for this species after testing several fungal and plant species. Interestingly, they also amplified DNA from ancient herbarium samples. In conclusion, we have developed quick and reliable methods to identify *T. borchii* during all phases of the life cycle. The next step will be the construction of primers specific for *T. magnatum,* a species of extreme economic value.

52. Carbon allocation in ectomycorrhiza: Identification and first characterisation of monosaccharide transporters. U. Nehls, A. Wiese, R. Hampp, Universitat Tubingen, Physiol. Okol. der Pflanzen, Auf der Morgenstelle 1, D 72076 Tubingen, Germany

An ectomycorrhiza is a symbiotic organ formed between some soil fungi and the fine roots of woody plants. One important feature of ectomycorrhizal function is the conversion of plant carbohydrates into amino acids by the fungus, and their reimport into the plant. The main transport form of carbohydrates in plants, sucrose, cannot be utilized directly by ectomycorrhizal fungi. We thus assume that a plant apoplastic acid invertase cleaves sucrose to hexoses, and both, plant cortical cells and fungal hyphae compete for hexose uptake.

In this study, primers, designed against conserved regions of known fungal and plant monosaccharide transporters, were used to amplify cDNA fragments from *P.abies/ A. muscaria*

mycorrhiza. These PCR fragments in turn, were used to isolate one fungal and one plant full length cDNA clone from a cDNA library. The deduced protein sequence of the *P. abies* cDNA (PaMST-1) revealed high homology to a *Saccharum* H+/monosaccharide transporter. PaMST-1 was mainly expressed in stem and roots. Its expression was slightly reduced in roots by ectomycorrhiza formation. The *A. muscaria* transporter (AmMST-1) gene codes for an open reading frame showing best homology to a *N. crassa* H+/monosaccharide transporter. The AmMST-1 gene was expressed at a basal level in all fungal hyphe. Nevertheless, its expression was significantly enhanced in symbiosis. An enhancement of AmMST-1 expression, comparable to that found in mycorrhiza, was also obtained in mycelia grown in suspension culture at increased glucose concentrations. This could be used to estimate a threshold concentration of monosaccharides at the root/fungus interface of ectomycorrhiza.

Molecular and functional diversity of ericoid mycorrhizal fungi.

Perotto S. and Paola Bonfante, Dipartimento di Biologia Vegetale and CSMT- CNR, V.le Mattioli 25, 10125 Torino, Italy
Ericaeous plants are widespread on the globe and colonize acidic substrates ranging from and sandy soils to humid mor-humus substrates. Our aims are to investigate whether this wide variety of environments also corresponds to genetic and functional diversity of the associated fungal symbionts. We have investigated the nuclear rDNA genes (ITS and 18S regions) of several ericoid isolates collected worldwide, in order to investigate their relationships. The results obtained so far suggest that the number of fungal species known to form ericoid mycorrhiza is probably greater than previously thought. An interesting feature revealed by amplification with universal primers and sequencing of the amplified DNA fragments is the occurrence, in the 18S rDNA of most ericoid isolates, of several Group I introns, an event rarely reported in fungi and in some algae. For several isolates, insertion of these elements at specific positions in the 18S genes is optional in different rDNA repeats, Moreover, specific insertions can be present or absent in isolates belonging to the same species and geographically very. In conclusion, the occurrence of Group I introns in the rDNA genes further increases the genetic diversity already observed in ericoid fungi with other methods (Perotto et at, 1996). The behaviour of these introns in ericoid fungi seems to be quite similar to the situation observed in lichen-forming fungi (Gargas et al, 1995), another symbiotic group of fungi. However, the functional significance of Group I introns for both symbiosis remains to be elucidated.
Gargas A, DePriest PT, Taylor J-W. Mol Biol Evol, 12: 208-215, 1995
Perotto S, Actis-Perino E, Peruginin I, Bonfante P. Mol Ecol, 5: 123-131, 1996

Update on Pathogenicity Factors

61. Identification of pea pathogenicity (PEP) genes on dispensable chromosome in *Nectria haematococca.*

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Previous research suggests that genes (*PDA*) for detoxifying the pea phytoalexin pisatin and other pea pathogenicity (*PEP*) genes are located on dispensable chromosomes in *N. haematococca.* Recent work indicates that the cosmid clone 55-D-8, derived from the 1.6-Mb dispensable chromosome of *N. haematococca,* contains *PEP* genes as well as the disrupted PDA1 locus. Portions of cosmid 55-D-8 have been used as hybridization probes to screen a cDNA library constructed from mRNA isolated from infected pea tissue collected two days after inoculation. Four transcripts were identified in this screening. No homologs have been found in sequence databases for the four transcripts. A 5.3 kb *Xho*I/*Not*I fragment encoding cDNAl and cDNA2 is capable of converting a dispensable chromosome-deficient, Pda$^-$, nonpathogenic isolate 94-6-1 to pathogenicity on pea. Furthermore, in preliminary results, a 2.9 kb *Not*I/*Bgl*II fragment encoding only the cDNAl transcript is sufficient to convert 94-6-1 to pathogenicity. These results indicate that genes affecting the virulence of *N. haematococca* on pea *(PDA1* and one or more PEP genes) are linked on one of the dispensable chromosomes of this broad host range fungus.

62. A multifunctional peptide synthetase involved in pathogenesis of *Cochliobolus heterostrophus* to corn.

Shun-Wen Lu, O.C. Yoder and B. G. Turgeon, Cornell Univ., Ithaca NY

Peptide synthetases are large multifunctional enzymes responsible for the non-ribosomal synthesis of a structurally diverse family of bioactive peptides, including antibiotice, toxins and immunosuppressants. We have partially cloned a gene (*CPS1*) from the corn pathogen *C. heterostrophus* that appears to encode a cyclic peptide synthetase required for pathogenesis. A *cps1*$^-$ mutant, identified in a screen of REMI transformants, appears morphologically and developmentally identical to wild type but is 60% less virulent to corn. Genetic analysis of the mutant, as well as site specificdisruption of the gene in wild type, confirmed that a single tagged mutation was responsible for the mutant phenotype. Sequencing of DNA flanking the REMI vector insertion site and translation reveals an open reading frame (>4.5 kb) with similarity to SafB, of the multifunctional enzymes catalyzing the biosynthesis of the cyclic peptide antibiotic safamycin Mx1 produced by the bacterium *Myxococcus xanthus.* A second ORF (1.1 kb; *TEA1*), encoding a thioesterase (proposed function: termination of non-ribosomal peptide synthesis), was identified 5' of *CPS1*. These results suggest that the two genes are part of a cluster controlling biosynthesis of a peptide (as yet unidentified) required for fungal pathogenesis.

63. Identification and mapping of *Les2,* a gene controlling lesion size in *Cochliobolus heterostrophus*.

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A mutant strain of *Cochliobolus heterostrophus* that produces significantly smaller disease lesions on corn leaves than does wild-type was obtained by ultraviolet light mutagenesis. In progeny of crosses between the mutant and a near-isogenic wild-type strain, all ten of ten complete tetrads segregated 4:4 (mutant pathogenicity:wild-type pathogenicity), and random spores segregated 47:45, suggesting a single mutated locus. Intercrosses between this mutant and six other lesion-deficient mutants of *C. heterostrophus* indicated that the locus had not been identified previously. The locus has been named *Les2* because of its lesion-deficient phenotype. The mutant retains normal growth on media, and normal abilities to germinate, attach, form appressoria and penetrate corn leaves, which suggests that *Les2* is needed for lesion production only after the fungus enters the corn leaves. *Les2* is now being mapped by using AFLP (amplified fragment length polymorphism); tetrad analysis indicates that AFLP is a highly reliable and reproducible method for detection of molecular markers in this fungus. Using bulked-segregant analysis, eighteen AFLP markers have been identified which may be closely-linked to *Les2*.

64. Isolation of the cultivar specificity gene *AVR1-MARA* of *Magnaporthe grisea*.

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AVR1-MARA is a stable avirulence gene of *Magnaporthe grisea* that elicits a resistant response in the rice cultivar Maratelli. To address the question of how this gene acts and why it is maintained in the genome, we are trying to clone *AVR1-MARA* using a map-based approach. We initiated a chromosome walk from molecular marker 12B5 located on chromosome 2b in the RFLP map of Sweigard et al (1). We have isolated the virulent locus *avrl-MARA* and portions of the avirulent locus (2). However, parts of the *AVR1-MARA* locus appears to be unclonable in *E. coli*, as these sequences are not present in any of the lambda, cosmid and BAC libraries that we constructed. The *AVR1-MARA* locus spans approximately 55 kb as defined by recombination breakpoints. There are two regions of 14 kb and 25 kb present in the the avirulent strain that are absent in the virulent strain.

To address whether the gene is essential, and to localize it further, we constructed 3 plasmids to disrupt parts or all of the locus by transformation-mediated gene disruption. We have deleted the entire locus in the aviruient strain 4224-7-8, which rendered the transformed strain virulent on rice cultivar Maratelli. We are currently using the partial locus disruption plasmids, to determine which half of the locus contains *AVR1-MARA.* We have also used UV mutagenesis to isolate virulent mutants and to increase the virulence of a low virulence mutant we obtained. These results, as well as our latest efforts in the cloning of *the AVR1-MARA* gene, will be presented.

1.Sweigard, J. A., B. Valent, M. J. Orbach, A. M. Walter, A. Rafalski, and F.G. Chumley. 1993. Genetic map of the rice blast fungus *Magnaporthe grisea.* in: Genetic Maps, 6th Ed. CSHL Press. Cold Spring Harbor, NY.

2. Mandel, M.A., V.W. Crouch, T.M. Harper, and M.J. Orbach. 1997. Physical mapping of the *Magnaporthe grisea* AVR1-MARA gene reveals the virulent allele contains two deletions. (accepted Molecular PlantMicrobe Interactions)

65. Molecular studies on the cell wall degrading enzymes from *Botrytis cinerea* I: Characterisation of

the endopolygalacturonase gene family.

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In order to develop an effective control strategy for the plant pathogenic fungus *Botrytis cinerea*, insight is required in the mechanisms of infection employed by the fungus and the roles played by various factors. Several reports have clearly shown that pectinolytic enzymes, excreted by the fungus, play an important role in the infection process by degrading the plant cell wall. Therefore, we started a molecular genetic study in order to identify and characterise these enzymes and to elucidate their roles in the process of pathogenicity.

Genes encoding pectinolytic enzymes were isolated via heterologous hybridisation, using DNA-probes derived from several *Aspergilli.* Six members of a endopolygalacturonase gene family were isolated. *Bcpga1* and *Bcpga2* code for enzymes with an alkaline pI and are highly homologous to the PGs of the related fungus *Sclerotinia sclerotiorum.* The other four *pga* genes code for enzymes with an acidic pI. Interestingly, *Bcpga3* and *Bcpga6* show significant homology to the PG encoding gene of the root pathogenic fungus *Fusarium moniliforme.* In addition, we characterised the *Bcpel1* gene, encoding a pectin lyase, which displays significant homology to *pelA* of *A. niger*. Recently, we identified Lambda phages hybridising to *Aspergilli* genes coding for pectin methyl esterase, exopolygalacturonase and pectate lyase. Cloning and characterisation of these genes is in progress. Regulation of expression of the isolated genes is studied in time course experiments using various growth conditions in flask cultures. Obtained results will be presented and possible roles of pectinolytic enzymes in pathogenicity will be discussed.

66. Suicide-Substrate Selection of Genes that Regulate Plant Cell Wall Breakdown in *Cochliobolus sativus.*

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The fungus *Cochliobolus sativus* causes plant disease on cultivated barley and related crops. Infection occurs through production of differentiated cell-types initiated by vegetative propagating hyphae. Cells, specialized in invasion, produce a complex set of enzymes that depolymerize plant cell walls during the penetration process and require multiple activities that overlap functionally. We are interested in identifiing genes involved in the induction phase of these enzymatic systems. To address the problems associated with redundant substrate-enzyme interactions, we tested a novel suicide selection approach to reveal pectn-degrading induction loci. The idea is simple: Lethal-substrates are synthesized by covalently linking fungicide molecules to substrate fragments; wild-type strains degrade the substrate releasing the fungicide and die. Mutants that fail to degrade lethal-substrates survive, probably because they fail to recognize the substrate, are unable to produce inducer molecules via positional isomerization, properly transduce the activation signal or activate transcription of gene expression. Suicide substrates prepared by covalently bonding hygromycin B to pectin (HY-Pectin) or xylan (HY-xylan) fragments function as predicted, they need to be enzymatically degraded before they are able to arrest vegetative growth. Moreover, a significant number of mutants that survive HY-pectin suicide selection have been isolated and one class is unable to assimilate other polysaccharides (i.e., cellulose and xylan) as well.

67. New approaches to identify pathogenicity genes of *Botrytis cinerea*.

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By definition, a prerequisite for pathogenicity factors is, that they are expressed during penetration and invasion of the host plant. Examples of such factors could be genes coding for extracellular hydrolases (cutinase, pectolytic enzymes), toxins or other, as yet unidentified, genes. We are studying the *B. cinerea* (grey mould)-tomato interaction. The aim is to isolate fungal genes expressed during infection. This is carried out in a non-biassed approach using two methods. The role of isolated genes in pathogenicity needs to be evaluated by disruption. For a Differential Screening, poly(A)$^+$ RNA was isolated from *B. cinerea* grown *in vitro* and a tomato-*B. cinerea* interaction, reverse transcribed into cDNA in the presence of -^{32}P dATP and hybridised to duplicate filters of a genomic library of *B. cinerea*. Since the fungal poly(A)$^+$ RNA in the interaction sample only represents 3%, the *in vitro* probe was compensated. One phage which hybridized differentially after the first and second screening was characterised in detail. The gene appeared to encode polyubiquitin. The structure of the gene is similar to other ubiquitin genes and consists of four repeats in a head-to-tail arrangement with intervening sequences. By DDRT-PCR, the fungal expression *in planta* was compared with the expression of *B. cinerea in vitro*, uninfected tomato and *P. infestans*- and TNV-infected tomato as controls. Five *B. cinerea* cDNAs were isolated and sequenced but no homology was found in the database. Results will be presented on further characterisation of polyubiquitin and the DDRT-PCR fragments.

68. Molecular and biochemical analyses of saponin detoxiefication by the phytopathogenic fungus *Botrytis cinerea*.

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Botrytis cinerea is the causal agent of "grey mould" diseases of many economically important fruits, vegetables and flowers. Our main interest are the mechanisms of virulence and pathogenesis during the interaction ptocess between *B. cinerea* and its hosts. Since the detoxification of saponins - preformed fungi-toxic compounds involved in plant defense against pathogens - has been shown to determine host-specificity in the *Gaeumannomyces-Avena* pathosystem, we started to investigate the importance of saponin-detoxification in the interaction of *B. cinerea* with its hosts, especially tomato.

B. cinerea has been described to detoxify -tomatine - a saponin from tomato - by deglycosylation. A tomatinase deficient field isolate showed enhanced sensitivity towards -tomatine in in-vitro assays and reduced virulence on tomato leaves. By screening a genomic library of *B. cinerea* using the *Septoria* tomatinase cDNA we were able to clone a genomic fragment carrying an ORF with signficant homology to family 3 glycosidases on both DNA and amino acid level. Targeted gene replacement showed the loss of avenacinase activity in three transformants. Detoxification of other structurally related saponins is not affected, suggesting a high substrate specificity of the enzyme. The potential role of this avenacinase-like enzyme in pathogenicity will be discussed.

69. Analysis of pectinase genes in *Cochliobolus carbonum*.

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The maize pathogen *Cochliobolus carbonum* secretes a large number of plant-cell-wall-degrading enzymes when grown with maize cells walls as the sole carbon source. Although pectin constitutes less than ten percent of the maize cell wall, at least three pectin-degrading enzymes are produced by the fungus. Genes encoding an endo-polygalacturonase (PGN1), an exo-polygalacturonase (PGX1) and a pectin methylesterase (PME1) have been cloned and used to create strains containing deletions of each gene. These deletions, singly and in combination, are being examined for their effects on the growth of the fungus in vitro on pectin and on the ability of the fungus to infect maize.

70. The effects on pathogenicity of tomato of heterologous expression of a $_2$-tomatinase gene in *Nectria haematococca* MPVI and disruption of this gene in *Colletotrichum coccodes* and *Septoria lycopersici.*

Robert W. Sandrock* and Hans VanEtten. University of Arizona, Tucson, * present address, Cornell University.

The presence of the antimicrobial compound -tomatine has been proposed to function as a chemical barrier in tomato tissue to potential pathogens. However, most successful pathogens of tomato are tolerant of this glycoalkaloid. A gene encoding a P-1,2-D glucosidase, called $_2$-tomatinase, which detoxifies - tomatine to $_2$-tomatine, has been cloned from the fungal tomato pathogen *Septoria lycopersici.* In this study, a portion of a $_2$-tomatinase homologue that possesses 90% similarity to the *S. lycopersici* $_2$-tomatinase amino acid sequence was cloned from the tomato pathogen *Colletotrichum coccodes.* Unlike *S. lycopersici, C. coccodes* degrades -tomatine to the aglycone tomatidine. Transformation-mediated gene disruption was utilized to create $_2$-tomatinase-deficient mutants of both these pathogens in order to evaluate the importance of this enzyme in pathogenicity. Gene disruption of the *C. coccodes* $_2$-tomatinase homologue resulted in a loss of $_2$-tomatinase activity but these mutants retained their tolerance to -tomatine and their abilities to degrade -tomatine to tomatidine. The gene-disrupted mutants also were still able to parasitize green tomato fruit, an organ containing high levels of -tomatine. Gene disruption of the *S. lycopersici* $_2$-tomatinase gene resulted in both a loss of $_2$-tomatinase activity and a loss of tolerance to -tomatine. Preliminary pathogenicity tests with this mutant were inconclusive. Expression of the *S. lycopersici* gene in the pea pathogen N. *haematococca* MPVI, a fungus that lacks $_2$-tomatinase activity, increased its tolerance to -tomatine *in vitro* and its ability to form lesions on green tomato fruit.

71. Gene expression of the blackleg fungus *Leptosphaeria maculans* in the presence of alkenyl glucosinolates.

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Blackleg caused by the ascomycete *Leptosphaeria maculans* is the most economically important disease of oilseed Brassicas worldwide. Generally Indian mustard *(B. juncea)* is resistant to the blackleg fungus and consequently has been used as a source of resistance in canola *(B. napus)* breeding strategies. Indian mustard has high levels of alkenyl glucosinolates, sulphur-containing compounds that give mustard a pungent taste. Upon wounding of the plant, these compounds are cleaved by the plant enzyme myrosinase into glucose and gases such as isothiocyanates, which are toxic to many organisms including *L. maculans.*

Recently blackleg isolates that can attack some Indian mustard varieties have been found in Australia. These isolates may be able to tolerate or detoxify hydrolysis products of glucosinolates in the plant. We using differential display to identlfv genes expressed by *L. maculans* isolates in the presence of glucosinolates. Such genes may be involved in detoxification of glucosinolates or evasion of their effects. A 1 kb mRNA is expressed only in the presence of 2-propenyl glucosinolate (5 and 10 *u*g/ml) and myrosinase (5 *u*g/ml). This message has a high degree of'sequence similarity to a yeast 5S ribosomal DNA binding protein, which is transcribed at high levels as a response to growth in high concentrations of glucose. Other differentially expressed genes of *L. maculans* are being sought.

72. Conversion of the soybean pathogen *Colletotrichum destructivum* to a pathogen of alfalfa by introduction of the *MAK1* locus of *Nectria haematococca.*

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The soybean pathogen *Colletotrichum destructivum (Glomerella glycines)* can be isolated from anthracnose lesions caused by the alfalfa pathogen *Colletotrichum trifollii,* but is not known to be an important or aggressive pathogen of alfalfa. An examination of its ability to detoxify the major phytoalexin produced by alfalfa, medicarpin, shows that *C. destructivum* is able to slowly detoxify medicarpin by 6a hydroxylation, whereas C. *trifollii* and other alfalfa pathogens are able to metabolize medicarpin rapidly and by more than one pathway. In an attempt to determine whether the ability to detoxify medicarpin is important in the ability to cause disease in alfalfa we transformed an isolate of *C. destructivum* with the *Nectria haematococca MAK1* gene. The *MAK1* gene encodes the ability to detoxify medicarpin and the closely related compound maackiain by la hydroxylation. *MAK1* was transformed into *C. destructivum* as either a genomic or cDNA clone and in both cases under control of its native promoter. Transformants of *C. destructivum* containing genomic copies of *MAK1* metabolize maackiain at 150 times the wild type rate, and those with the cDNA clone at 50 times wild type. Virulence tests *in planta* show an increase in pathogenicity of the transformants as measured by the number and extent of lesions, and by an increase in the amount of stem damping-off. These results are consistent with the hypothesis that detoxification of phytoalexins is an important function in the virulence of fungal pathogens.

73. Withdrawn

74. *PTH11*, a gene required for surface recognition by *Magnaporthe grisea.*

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PTHII was identified twice in a mutant hunt based on restriction enzyme mediated insertional (REMI) mutagenesis. The integration events leading to both mutants were more complex than typical REMI insertions. *pth11-1* contains a deletion of greater than 10 kb at the vector insertion site. *pthll-2* resulted from the apparent *in vivo* ligation of mitochondrial DNA with the integrating vector, followed by integration into *PTH11*. *pth11*$^{-}$ mutants form few appressoria both on hydrophobic surfaces *in vitro* and on plant surfaces and therefore the mutant causes very few disease lesions. These mutants form wild-type levels of appressoria on other surfaces, including cellulose acetate. Therefore we conclude that *PTH11* is not required for appressoria formation *per se,* but rather for surface recognition that leads to appressoria formation. *PTH11* encodes a 68 kD protein and lacks significant homology to any known protein. Hydropathy plots of Pthllp suggest seven transmembrane domains and a long hydrophilic C-terminal tail. *pth11*$^{-}$ mutants can be rescued by cAMP and IBMX suggesting that PTH11 is upstream of a cAMP-

dependent pathway required for appressoria formation.

75. Cloning, molecular characterization and disruption of chitin synthease genes in Wangiella.

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Polymerase chain reaction technologies were used to clone four chitin synthase (CHS) structural genes from the phaeohyphomycotic agent W. *dermatitidis*, which were named *WdCHS1*, *WdCHS2, WdCHS3* and *WdCHS4.* Molecular characterizations showed that WdC-HSl and *WdCHS2* are most similar to *CHS2* and *CHS1* respectively of *S. cerevisiae,* whereas *WdCHS4* is a homolog of the *CAL1/CSD2* gene that encodes the Chs3 isozyme in that species. In contrast, *WdCHS3* has no homolog in *S. cerevisiae.* RT-PCR experiments confirmed that all four *WdCHS* genes are transcribed during logarithmic yeast reproductive growth at both 25 C and 37 C. However, northern analyses of total RNA from cells cultured under a variety of conditions showed that only *WdCHS3* is overexpressed to any extent and on when cells are subjected to stress conditions, including shifts of cells from 20- 25 C to 37 C. This result suggests that *WdCHS3* overexpression is virulence factor that enriches the cell walls of *W. dermatitidis* with chitin during infection. Support for this hypothesis has been obtained by disrupting *WdCHS3* in the wild-type background, showing that the mutant strain grow normally at 25 C and 37 C, but has reduced activity associated with it membranes, and has reduced virulence when tested in mouse models. Studies are in progress to evaluate the virulence of the three other single, three double and even one triple *wdchs* disruption strains we have derived.

76. Characterization of a gene for export of the toxic compound, sirodesmin, from the phytopathogenic fungus *Leptosphaeria maculans*.

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Leptosphaeria maculans is a heterothallic ascomycete that causes blackleg disease in many cruciferous plants. The fungus produces several non-selective toxins that contain a epipolythiodioxopiperazine ring and are called sirodesmins. A report of a yeast gene, *STS*1, whose overexpression conferred resistance to the related toxin sporidesmin inspired us to search for a similar gene in *L. maculans.* The *STS*1 gene is a member of the highly conserved family of translocases known as ABC transporters. Proteins of this family are frequently involved in antibiotic export and are characterized by the presence of two ATP binding cassettes, containing Walker A and Walker B motifs, and 12 transmembrane helices. In the belief that a similar protein may be involved in the export of sirodesmin from *L. maculans*, we used degenerate oligonucleotides encoding amino acids from Walker A & B to amplify genomic DNA from the fungus. Two PCR products were identified with high homology to ABC transporters and we have characterized the genes from which they were derived. We have transformed *L. maculans* with a construct for disruption of one of the genes and obtained a high percentage of

transformants that apparently no longer secrete sirodesmins into the medium. Our results will be presented.

77. The biological role of the hydrophobin cerato-ulmin in the life history *of Ophiostoma ulmi* and *0. novo-ulmi.*

B. Temple, W. E. Hintz, L. Bernier, and P. A. Horgen; Universities of Toronto, Victoria and Laval .

The causal agents of Dutch elm disease, *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* (Brasier, 1991) have been responsible for close to 80% elm mortality in the Northern hemisphere. One of most readily noticeable differences between the aggressive *O. novo-ulmi* and the less aggressive *O. ulmi* is the differential production of a protein called cerato-ulmin (CU), which is synthesized in large amounts by *O. novo-ulmi*. This small protein belongs to a class of molecules known as hydrophobins and is able to cause wilting in elm seedlings exposed to CU *in vitro.* Our, results suggested that CU is not a major wilt toxin. Over-expression of a copy of the *cu* gene from an aggressive *O. novo-ulmi* isolate in a nonaggressive isolate of *0. ulmi* did not alter the virulence of the fungus on 3 year old elm seedlings. The enhanced production of CU in the *cu* overexpressing transformant resulted in a dramatic phenotype change compared to the wild type. The role of CU in parasitic fitness is evaluated. Our data indicate that CU can be considered a parasitic fitness factor that may be a mediating factor in adhesion of infectious propagules to the bark beetle vector, or functions to increase resistance of infectious propagules to environmental stress.

78. Expression of the Host-Selective Toxic Protein, ToxA, in *Escherichia coli*.

Robert P. Tuori and Lynda M. Ciuffetti. Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97330.

ToxA is a 13.2 kD host-selective toxic protein produced by isolates of the wheat pathogen *Pyrenophora tritici-repentis*. It has been shown to be a primary determinant of pathogenicty of this fungus on susceptible wheat cultivars. The ToxA gene encodes two polypeptide domains, an N-terminal anionic domain of unknown function and destiny, and a C-terminal cationic domain which is the mature, secreted ToxA protein. In order to initate a study of the processing of the ToxA proprotein, the expression of various constructs of the gene was attempted in *E. coli*. Three constructs were initially designed and expressed in the Promega PinPoint system: 1, The N-domain, which encodes a predicted 4.5 kD anionic peptide (N-FP); 2, The *ToxA* or C-domain (C-FP); And 3, the entire ORF minus the signal peptide region, which encodes a 17.5 kD protein (NC-FP). Cloning into the multiple cloning site of the PinPoint vector results in the construst of interest being expressed as a fusion protein attached to an N-terminal 14 kD biotinylated protein tag. *E. coli* expressing C-FP and NC-FP both produce abundant amounts of the appropriate sized fusion protein, however, much of this appears to be insoluble. *E. coli* expressing N-FP produce

scant amounts of fusion protein with cultures multipling very slowly after induction. In all three cases it has been difficult to achieve efficient proteolytic cleavage of the fusion protein with factor Xa protease. Currently, the NC-FP construct is being expressed in a His-tag vector with a TEV protease recognition site separating the tag and toxin sequences.

79. Genetics of resistance and virulence in the *Hordeum vulgare:Cochliobolus sativus* pathosystem.

Majda Valjavec-Gratian and Brian Steffenson, Department of Plant Pathology, North Dakota State University, Fargo, ND 58105.

The perfect stage of *Cochliobolus sativus,* a heterothallic ascomycete, is rarely found in nature, but it can be reliably produced in culture. The objective of this study was to examine the genetics of host specific virulence in *C. sativus* and to complement it with an examination of host specific resistance in barley. A cross was made between fungal isolate ND90Pr (which exhibits high virulence on barley genotype Bowman and low virulence on genotype ND 5883) and ND93-1 (which exhibits low virulence on both genotypes). For hybridization, barley seed was used as a substrate. Seed, amended with conidia of both isolates was incubated in petri plates on Sach's nutrient agar. One hundred and three ascospores were obtained from the cross, and the virulence of each single ascospore culture was evaluated on the barley genotypes Bowman and ND 5883. The fungal progeny could be clearly differentiated into two phenotypic groups based on their infection responses on Bowman. One group exhibited the high virulence character of isolate ND90Pr and the other the low virulence character of isolate ND93-1. The ascospore population segregated 48:55 for low virulence:high virulence on Bowman, indicating the presence of a single virulence gene in isolate ND90Pr. Barley progeny segregated approximately 3:1 for resistance:susceptibility to isolate ND90Pr, indicating that ND 5883 carries a single gene for resistance. These data demonstrate that both virulence in the pathogen and resistance in the host are under monogenic control in this specific host genotype:fungal isolate combination.

80. The role of arginine biosynthetic enzymes in the pathogenicity of *Venturia inaequalis.*

Sally P Wither and Keith Johnstone, Department of Plant Sciences, University of Cambridge, UK.

Venturia inaequalis is the cause of apple scab and is the major pathogen of apples world-wide. The aim of this study is to establish at the molecular level whether arginine biosynthetic enzymes are required for pathogenicity. An existing, argininosuccinate lyase (ASL) *V. inaequalis* mutant (1) was chosen for further study.

The ASL mutant was shown to be non-pathocyenic on apple seedling. An enzyme assay has been established for ASL and used to compare the enzyme activity of the wild type and mutant. The phenotype is also beina verified by western blot analysis using, chick ASL antibodies.

Prgaress in this work and in the construction and screening, of a cDNA library for the ASL gene will be reported,

(1) Boone D.M., Stauffer M.A. and Keitt G.W. (1956) Am J Bot **43:** 199-204.

81. Application of differential display RT-PCR to analyse fungal gene expression in a plant-pathogen interaction.

Jan A.L. van Kan, Ernesto P. Benito & Theo W. Prins, Dept. of Phytopathology, Wageningen Agricultural University, PO Box 8025, 6700 EE Wageningen, the Netherlands.

Establishment of a plant-pathogen interaction involves differential gene expression in both organisms. In order to isolate *Botrytis cinerea* genes whose expression is induced during an interaction with tomato, a comparative analysis of its expression pattern *in planta* with its expression pattern during *in vitro* culture was performed by differential display of mRNA. Fungal genes induced *in planta* were distinguished from plant defense genes induced in response to the pathogen, by including in the comparison expression patterns of healthy tomato plants and of tomato plants infected with two different pathogens, *(Phytophthora infestans* or Tobacco Necrosis Virus). Using a limited set of primer combinations three *B. cinerea cDNAs, Bipi2, Bipi5* and *Bipi47,* were isolated. Northern blot hybridization showed that *Bipi2* and *Bipi5* mRNAs accumulated at detectable levels only at late time points during the interaction. *Bipi47* detected two different mRNAs, both corresponding to genes expressed at a high level during the entire interaction. None of the cDNAs displayed homology to sequences in the database. These results show that the differential display procedure possesses enough sensitivity to be applied to the detection of fungal genes during early stages of a plant-pathogen interaction. Some of the genes isolated by this method may be involved in pathogenicity of *B. cinerea.*

82. Cutinase A of *Botrytis cinerea* is not essential for infection of tomato fruits and gerbera flowers. Jan van Kan, L. Wagemakers, J.W. van 't Klooster, D. Dees, C.J.B. van der Vlugt-Bergmans. Dept. of Phytopathology, Wageningen Agricultural University, PO Box 8025, 6700 EE Wageningen, the Netherlands.

Cutinase was proposed to play an early role in the infection of host tissues by *Botrytis cinerea.* The enzyme was purified, characterized and amino acid sequences were used to design primers for PCR based gene cloning from strain SAS56. Expression of the cutinase (*cutA*) gene during the infection of gerbera flowers and tomato fruits was studied, using a *cutA* promoter-GUS construct, transformed into *B. cinerea.* During germination and penetration of host tissue, fungal structures demonstrated high GUS activity, indicating that the *cutA* promoter is active *in planta.* During *in vitro* growth on water agar, GUS staining was observed, indicating that substrate induction is not essential. The addition of a cutin monomer to the agar resulted in a much more intense GUS staining. *In vitro,* cutinase expression is subject to catabolite repression. To study the necessity of cutinase A for penetration of host tissue by *B. cinerea, cutA*-deficient mutants were constructed by means of gene disruption. Pathogenicity of these mutants was tested on tomato fruits and gerbera flowers. The ability of the mutants to penetrate and cause infection was

not altered. Infection structures produced by the mutant did not differ morphologically from the wild type. We conclude that cutinase A is not an essential pathogenicity factor for *B. cinerea.*

83. Reduced pathogenicity strain of *Colletotrichum gloeosporioides* obtained by REMI.

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Colletotrichum gloeosporioides is the main postharvest pathogen attacking avocado and other subtropical fruits. In the past it was found that a mutant of *C. magna* strain, lacking the capability of secreting pectate lyase (PL) was non pathogenic on avocado fruit. Furthermore, antibodies developed against PL of *C. gloeosporioides* were able to inhibit symptom development by the pathogen on avocado fruits. Both experiments suggested the importance of pectate lyase during symptom development on fruits. We have used restriction enzyme mediated integration (REMI) to obtain tagged mutants with reduced pathogenicity as a possible tool to demonstrate the importance of pectate lyase during Colletotrichum pathogenicity. The *hph* gene was subcloned into pGEM-7Z and following restriction with HindIII was transformed into *C. gloeosporioides* protoplasts. Transformants were screened for pathogenicity in 24h assay, by flesh inoculation and on long term assay by peel inoculation. From 300 screened transformants one reduced pathogenic strain was found. The presence of *C. gloeosporioides pel* gene in the mutant was determined by Southern blots analysis. Further analysis of the mutant will be discussed in order to demonstrate the involvement of PL during pathogenicity of *C. gloeosporioides* in avocado fruits.

84. *Ecm1:* a locus affecting extracellular matrix production and lesion size in *Cochliobolus heterostrophus.*

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A mutant of *Cochliobolus heterostrophus* lacking the outer layer of extracellular matrix around its germ tubes was obtained by mutagenizing protoplasts. The mutant not only lacks the outer matrix, but also produces much smaller lesions on corn leaves than non-mutant strains; mutant lesions average 1. 9 mm^2 compared to 6.6 mm^2 for non-mutant lesions. Genetic analysis demonstrated that the failure to produce the outer matrix cosegregates with the reduced lesion size, which suggests that the two traits are controlled by the same locus. This locus has been named Ecm1 (extracellular matrix deficient). The mutant retains normal growth on media and normal abilities to germinate, attach, form appressoria, and penetrate corn leaves. This indicates that the outer matrix is not necessary for infection prior to entrance of the fungus into the leaf and that the pathogenicity defect occurs after penetration. Seven markers linked to *Ecml* were found by analysis of amplified fragment length polymorphisms (AFLP). *Ecml* maps to chromosome 4; the closest marker to *Ecml* is 5 cM, which is estimated to be about 115 kb. These results should enable the cloning of *Ecml* by mapbased methods.

Fungal Melanin

85. Isolation of an *Aspergillus fumigatus* mutant strain with reduced virulence.

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[4]Botanisches Institut, Universitat,Munchen, 80638 Munchen, FRG.

Aspergillus fumigatus is an important pathogen of the immunocompromised host causing pneumonia and invasive disseminated disease with high mortality. Since the survival of conidia in the host is prerequisite for establishing disease, we have been attempting to identify factors which are associated with conidia and, simultaneously, of importance for infection. Therefore, *A. fumigatus* mutant strains were isolated exhibiting altered conidia pigmentation. One mutant strain produced white conidia (white, W) and apparently lacked the pigment. Scanning electron microscopy revealed that conidia of the W mutant differed in their cell wall morphology from those of the wild type (WT). Luminol-dependent chemiluminescence was ten-fold higher when human neutrophils or monocytes were challenged with W conidia compared with WT conidia. Furthermore, W conidia were more susceptible to killing by oxidants in *vitro.* W conidia were also more efficiently damaged by human monocytes in *vitro* than WT conidia. ln a murine animal model, compared with the wild-type, conidia of the W mutant strain showed reduced virulence. These results suggest that pathways related to conidial pigmentation contribute to pathogenicity of *A. fumigatus.*

86. Fungal reactive armor: Redox buffering by melanin and Fe(II) *Cryptococcus neoformans*.

Eric S. Jacobson, McGuire Veterans Affairs Medical Center and Virginia Commonwealth University, Richmond, VA.

Melanin is an fungal extracellular redox buffer which, in principle, can neutralize antimicrobial oxidants generated by immunologic effector cells, but ist source Of reducing equivalents is not known. We wondered whether Fe(II) generated by the external ferric reductase of fungi might have the physiologic fimction of reducing fungal melanin and thereby promoting pathogenesis. Exposure to micromolar Fe(II) decreased the open circuit potential of a melanin film electrode from 0.00 V to -0.10 V, relative to a silver-silver chloride standard, and decreased the area of the cyclic voltammetric reduction wave by 50%, indicating reduction. Moreover, exposure to FE(II) increased electrochemical buffering by 44%, while exposure to millimolar dithionite reduced the film but did not increase buffering. The ratio of the amount of bound iron to the amount of the incremental increase in the following oxidation wave was approximately 1.0, suggesting that

bound iron participates in buffefing. Light absorption by melanin suspensions or suspended, melanized *C. neoformans* was decreased 14% and 8%, respectively, by treatment with Fe(II), consistent with reduction of melanin. Cultures of *C. neoformans* grown in solubilized 1 mM Fe(III) generated 160 *u*M FE(II) in cultural supernatant. We infer that Fe(II) can reduce melanin under physiologic conditions; moreover, it binds to melanin and cooperatively increases redox buffering. The data support a model for physiologic redox cycling of fungal melanin, whereby electrons exported by the yeast to form extracellular FE(III) maintain the reducing capacity of the extracellular redox buffer.

87. The genetics of *Gaeumannomyces graminis* with particular reference to pigment production and pathogenicity.

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Pigmented hyphae of the take-all fungus *Gaeumannomyces graminis (Gg)* are observed both on infected host tissue and when the fungus is grown *in vitro.* The pigment has previously been partially purified and has properties characteristic of melanin. Growth of isolates in the presence of inhibitors of melanin biosynthesis such as tricyclazole and pyroquilon, give rise to characteristic changes in hyphal pigmentation, but did not affect growth rate. We are using two approaches to determine whether the pigment is necessary for pathogenicity and survival as a saprophyte. An albino and several pale mutants have been isolated following irradiation of protoplasts with ultra-violet light. Results suggest that melanin production is essential for pathogenicity in *Gg.* The second approach is to disrupt genes involved in melanin biosynthesis. A region of ~2.7kb has been subcloned and sequenced. Transformation mediated gene disruption of *Gg* will ultimately confirm the function and importance of this cloned DNA.

88. Construction of cDNA library and screening of genes that expressed specifically during appressorium formation of *Colletotrichum lagenarium*.

I. Kuroda, Y. Takano[1], I. Furusawa[1], O. Horino and Y. Kubo. Kyoto Prefectural University and [1]Kyoto University, Kyoto, Japan .

Infection by *Colletotrichum lagenarium* requires appressorium differentiation. To identify genes that express preferentially during germination and appressorium formation, we constructed cDNA library and performed differential cDNA screening. Poly(A)+ RNA from appressorium-forming conidia incubated 6 hours was used to construct a directional cDNA library in LambdaGEM4. To identify specific cDNAs in appressorium-forming conidia, 12,000 cDNA clones from this cDNA library were applied to differential screening. In first screening, 125 cDNAs which did not hybridize to cDNA probes synthesized from poly(A)+ RNA of growing vegetative hyphae were selected. As second screening, 32 cDNAs which preferentially

hybridized to cDNA probes synthesized from poly (A)+ RNA of appressorium-forming conidia at 6 hours were selected. Finally, some cDNAs were confirmed to be preferentially expressed during appressorium formation by RNA blot analysis. Two of these clones had strong signal were sequenced. One clone had high percentage of agreement with *grg-1* glucose-repressive gene in *Neurospora crassa*, and the other had with ClpB the heat shock protein in *E. coli*.

89. Molecular phylogeny of graminicolous species of *Heiminthosporium* sensu lato, *Bipolaris, Curvularia, Drechslera* and *Exserohilum*.

Kiminori Shimizu, Chihiro Tanaka and Mtsuya Tsuda. Pesticide Research Institute, Faculty of Agriculture, Kyoto University, Kyoto 606-01, Japan.

Some graminicolous plant pathogenic fungi, *Bipolaris, Curvularia, Drechslera* and *Exserohilum* had been classified in *Helminthosporium sensu lato.* They are now treated as separate genera mentioned above. These fungi have melanin with 1,8-dihydroxynaphthalene as a precursor. We cloned and sequenced the *Bipolaris maydis* gene coding for the reductase gene involved in melanin biosynthesis *(Brn1).* The *Brn1* gene contains one open reading frame, consisting of 3 exons separated by two introns, and the predicted Brnl polypeptide consists of 267 amino acids. This gene was contained only one copy per genome in *B. maydis.* By using this gene, we attempt to elucidate the taxonomical relationships in so-called graminicolous *Helminthosporium species.* The *Brn1* gene of some selected members in four genera was amplified with partial sequences derived from that of *B. maydis* as primers in PCR and sequenced. Alignment of these sequences showed high similarities to each other through the entire sequence but intron regions. Maximum parsimony analysis using these sequences constructed four clusters. The cladogram well reflected the differentiation in this gene within these four genera. However, relationships within these four genera differ from those based on morphological characters.

90. The temporal transcriptional pattern of three melanin biosynthesis genes, PKS1, SCD1, and THR1 in appressorium-differentiating and non-differentiating conidia of *Colletotrichum lagenarium* .

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A phytopathogenic fungus, *Colletotrichum lagenarium* produces melanized appressoria that display temperature-sensitive differentiation. Conidia incubated in water at 24 C germinated and germ tubes differentiated into melanized appressoria. On the other hand, conidia in water at 32 C germinated and elongated germ tubes without appressorium differentiation. Conidia in 0.1% yeast extract solution at 32 C germinated and developed into vegetative hyphae. Here, we investigated the temporal transcriptional pattern of cloned melanin biosynthesis genes, PKS1,

SCD1, and THR1 in these differentiating and non differentiating conidia. During appressorium differentiation, de novo transcripts of the three melanin biosynthesis genes accumulated by 1-2 h after the start of conidial incubation at 24 C and began to decrease at 6 h. In conidia germinating in water at 32 C, the transcriptional pattern of that in appressorium-forming conidia, although no appressoria were formed. However, in conidia in 0.1% yeast extract solution at 32C, transcripts of the three melanin biosynthesis genes hardly accumulated.

91. Purification and analysis of expression pattern of melanin biosynthetic enzymes, scytalone dehydratase and 1,3,8-trihydroxynaphthalene reductase in *Colletotrichum lagenarium*.

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Melanin biosynthesis of *Colletotrichum lagenarium* is essential for appressorial penetration of its host plants. In the melanin biosynthetic pathway, scytalone dehydratase and 1,3,8-trihydroxynaphthalene reductase catalyze the conversion of scytalone to 1,3,8-trihydroxynaphthalene and 1,3,8-trihydroxynaphthalene to vermelone, respectively. Previously, we cloned SCD1 gene coding for scytalone dehydratase and THR1 gene coding for 1,3,8-trihydroxynaphthalene reductase. In this study, the heterologuous expression vectors were constructed and the recombinant enzymes were purified. Polyclonal antibodies against purified recombinant scytalone dehydratase and 1,3,8-trihydroxynaphthalene reductase were prepared. By western blot analysis, scytalone dehydratase and 1,3,8-trihydroxynaphthalene were detected in melanized mycelia of *C. lagenarium.* In *C. lagenarium*, appressorial differenciation and melanization proceed synchronously. At present, we are investigating expression patterns of these enzymes during appressorium differentiation.

Medical Mycology

92. Effect of sub-Minimal Inhibitory Concentrations (sub-MIC) of antifungal tioconazole on expression of esterase of *Trichophyton rubrum.*

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T. rubrum is a filamentous fungus that has the capacity to invade keratinized tissue (skin, hair and nails) of humans to produce infections. Tioconazole, one of the antifungals used to control *T. rubrum*, acts by inhibiting fungal ergosterol biosynthesis. In order to determine the relationship between resistance to tioconazole and esterases expression in this fungus, we isolated several tioconazole-resistant mutants by treating with UV light, an isolate from a patient from the University Hospital. The original isolate (MIC=0.5 mg/ml) and the resistant mutants (MIC=1.0

to 2.5mg/ml) were cultivated on Sabouraud liquid medium with and without tioconazole concentrations below the MIC (sub-MICs) of each strain. After the extration of intracellular total protein the esterase eletrophoretic pattern was developed with -naphthyl acetate. All strains cultivated on Sabouraud medium containing tioconazole (original isolate and resistant mutants) presented 5 bands, whereas those cultivated on Sabouraud medium alone presented only 2 clarly visible bands. Furthermore, the visualization of these 3 extra bands was found to depend on antifungal concentration. Although the physiological role of this esterase is not clear, its overexpression may be a response to the cellular stress caused by the presence of the antifungal, even in the resistant mutants, or may even play a role in cellular drug detoxification.

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93. Cloning and Characterization of a Putative Enolase from *Pneumocystis carinii*.

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The glycolytic enzyme enolase is one of the most highly abundant proteins expressed in fungi and has also been shown to be an immunodominant cell wall-associated antigen of the pathogenic fungus, *Candida albicans*. To investigate the expression and antigenicity of enolase in the opportunistic pathogen *Pneumocystis carinii*, the genomic and cDNA enolase were cloned and characterized. The genomic enolase clone was hybridized to restriction enzyme digested DNA, revealing the presence of a single *P. carinii* enolase gene. Hybridization of the enolase clone to electrophoretic karyotypes localized the gene to a 550 kb chromosome of the protoype form of *P. carinii*. The level of expression of *P. carinii* enolase mRNA was examined under a variety of growth conditions. Northern analysis identified a 1.4 kb transcript which was expressed at constant levels in either starvation or high glucose conditions. Sequence analysis of the enolase cDNA identified a 1350 bp continuous open reading frame. The predicted protein is 433 amino acid residues in length and shows extensive homology to other fungal enolase proteins, including *C. albicans* (87%), *Aspergillus oryzae* (88%) and *Saccharomyces cerevisiae* (85%). The active site and conformation metal ion-binding site residues are conserved in the predicted *P.carinii* enolase protein. Experiments are currently underway to purify the enolase fusion protein for immunoblot analysis with immune sera from both *P. carinii* and *C. albicans*. In addition, the enzymatic activity and subcellular localization of the enolase protein will be determined.

94. Disruption of Mitochondrial Ultrastructure and Function Induced by Exogenous Sphinganine in *Neurospora crassa*.

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Sphinganine, a sphingoid base found concentrated in mammalian epidermis, may serve as a natural antifungal barrier, preventing infection by pathogenic fungi. *Neurospora crassa* is being

used as a model to study the effects of sphinganine in fungal pathogens. In Neurospora, exogenous sphinganine is metabolized into complex sphingolipids, such as ceramides and cerebrosides over a 1 hr period, suggesting that the lipid is being internalized. However, only sphinganine is associated with mitochondrial fractions during this time. Sphinganine appears to have a dramatic effect on mitochondrial structure and function. Transmission electron microscopy demonstrates that the disruption of ultrastructure is both a time- and concentration-dependent phenomenon. Exposure to 100 *u*M sphinganine for 1 hr completely disrupts the outer membranes of a majority of mitochondria. Analysis also shows that short-term exposure to subinhibitory concentrations of sphinganine causes swelling of mitochondria, while exposure to increasing concentrations of the lipid results in the complete disruption of outer membranes. The presence of cytoplasmic lamellar and mesosome-like structures near the plasma membrane, and enlarged vacuolar bodies, further indicate the deterioration of cellular vitality. The changes observed in mitochondrial ultrastructure appear to coincide with a loss of mitochondrial function. Using the fluorescent dye Rhodamine 123, it was observed that germinating cells treated with 100 *u*M sphinganine exhibit a severe decline in electrogenic mitochondrial activity. These results demonstrate that the loss of cell growth and viability observed in sphinganine-treated Neurospora may be due in part to the disruption of mitochondrial morphology and activity.

95. The Role of Multidrug Efflux Transporters in Antifungal Drug Susceptibility.

Yi Li and Chuck Staben. T.H. Morgan School of Biological Sciences, University of Kentucky.

Fungi carefully control influx and efflux of small molecules, including antifungal drugs, toxins, and mating factors. Fungi apparently have 2 main classes of drug efflux transporters: ABC (ATP-binding cassette) proteins and MFS (major facilitator superfamily) transporters. These transporters endow some wild type fungi with inherent resistance to many inhibitory drugs. Alterations in activity or specificity of efflux appears to be a common method of acquiring drug resistance. Genetic characterization

of the *Saccharomyces cerevisiae SGE1* gene indicates that its product mediates resistance to pentamidine and other structurally related inhibitory compounds, including G418, gentian violet, and ethidium bromide. Sge1p appears to be a typical transmembrane efflux protein of the MFS class that utilizes the proton gradient to transport its substrates. The activity of this exporter is modulated by environmental conditions, being much lower under respiratory conditions than during fermentative growth. *S. cerevisiae* and other fungi, including *Candida albicans*, have multiple members of the export protein families that have overlapping specificities. MFS efflux transporters also function in export of toxins associated with virulence in plant pathogens. Manipulating the transport capacity of fungi may be an effective means of modulating virulence or increasing drug susceptibility. Ability to manipulate the activities of these pumps relies upon a molecular understanding of pump function.

96. Isolation of a *Cryptococcus neoformans* gene that encodes a protein which elicits a delayed-type hypersensitivity response in mice.

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Control of cryptococcal infection is critically dependent on T cell-mediated immune responses but little is known about the antigens which induce this activity. In mice immunized with cryptococcal extracts from a virulent encapsulated strain, delayed-type hypersensitivity (DTH), measured as footpad swelling, has been used as a marker for T cell response. A 20 kDa protein isolated from the culture filtrate of an acapsular mutant of *C. neoformans* (cap-67; from strain B-3501, serotype D, mating type) was found to stimulate DTH in this model.

We took a reverse genetic approach in order to clone the gene encoding this protein. Following gel purification of the protein, 20 amino acid residues were sequenced from the N-terminus and 20, 1 1 , and 6 residues were obtained from peptides produced by endoproteinase LysC cleavage. Degenerate oligonucleotides were designed for the N-terminal peptide and two of the three internal peptides for use in RT-PCR. Using oligonucleotides corresponding to the N-terminal sequence and one of the internal peptides, a 778 bp fragment was amplified. Translation of the sequence of this fragment revealed it contains sequences corresponding to amino acids adjacent to those in the peptides used for oligo design, as well as to one of the other internal peptides. Analysis of the genomic sequence and its expression will be presented.

Further work involvng the production of recombinant protein to assess the the vaccine potential of this protein is now possible.

97. Development ot sulfonamide resistance as a selectable marker for the transformation of *Pneumocystis carinii* (*Pc*).

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An initial step critical in the development of a transformation system for the opportunistic pathogen, *P. carinii*, is the availability of a selectable marker allowing positive selection for transformed organisms. This is especially important given the inability to sustain long term *in vitro* culture of the organism and the reliance on immunosuppressed animals as a source of organisms. We have explored the use of many conventional markers in Pc. Tested in an *in vitro* cytotoxicity assay, Pc appeared resistant to G418, hygromycin and bialophos at levels up to 10 *u*g/ml. Pc is extremely sensitive to sulfonamides both *in vivo* and *in vitro.* The gene encoding dihydropteroate synthase (DHPS), the target of sulfa, has previously been cloned from Pc (i.e. Folic Acid Synthesis [FAS] gene). Sulfa resistance in *Plasmodium falciparum* and many bacteria has been associated with a few well characterized mutations at positions flanking the presumptive catalytic site of DHPS. We introduced two mutations (T-> F at residue 517 and G -

>S at residue 687) associated with high level of sulfa resistance into the DHPS cDNA of Pc by site directed mutagenesis. The mutant FAS cDNA was cloned downstream of a Pc promoter and used to transform *S. cerevisiae.* The resultant yeast transformants had increased MICs to sulfadiazine compared to the parental yeast strain or yeast transformed with the identical plasmid construct containing the wild type FAS cDNA. Biochemical characterization of the recombinant enzyme is currently underway. The activity of sulfas against Pc in *in vivo* and *in vitro* model systems will allow development of a transformation system with selection for transformed organisms in short term *in vitro* culture and in the immunosuppressed rat model.

98. Nuclear Proteins Involved in Dimorphic Growth of *Candida aibicans.*

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Candida albicans is the most prevalent of the human pathogenic yeasts. The *Candidas* are dimorphic in that they display one or more alternative growth forms. It is generally accepted that alternative growth forms are important for tissue invasion and immune-avoidance during infections. We have been characterizing growth-form-specific transcripts of C. *albicans* using ddRT-PCR to dissect this fundamental differentiation process. Differentially expressed mRNAs are characterized for sequence and time of expression during morphogenesis. We have identified two cDNAs for transcripts which are apparently unique to hyphal cells. Both cDNAs appear to encode proteins homologous to known nuclear proteins. One transcript (Caml) is expressed only transiently during early germ tube formation. This transcript is most similar to a *Saccharomyces cerevisiae* ORF related to the yeast nuclear transport protein NIP80. The other CDNA (Cam3) encodes a polypeptide most similiar to a uracil phosphoribosyl transferase. Portions of the gene align with a protein which interacts with Sin3, a general transcriptional regulator in *S. cerevisiae.* Further characterization of the genes for these mRNAs is under way. These genes will be analyzed for phase-specific upstream promoter sequences as well as the effects of gene disruption and constitutive expression on growth form.

Secondary Metabolism

109. *Aspergillus flavus* mutant strain 241, blocked in aflatoxin biosynthesis, does not accumulate *aflR* transcript.

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Eleven non-allelic aflatoxin biosynthesis mutants in *A. flavus* have been mapped to linkage groups (LG), 10 to LG VII, which includes genes in the aflatoxin biosynthetic cluster, and one in strain 241 to LG 11. The absence of a colored intermediate in strain 241 and the physical separation of its mutation from the biosynthetic cluster, suggest that the mutated gene may be regulatory in nature. An analysis of the RNA transcripts from strain 241 *(tan, afl-4, pdx6* indicates that *aflR* message is absent from this strain. Genetic analysis demonstrated that the

mutation in 241 is not due to a non-functional copy of *aflR*. Transformants containing an *aflR*::GUS construct failed to exhibit -glucuronidase activity, indicating that the *aflR* promoter is not activated in this strain. Transformants provided with *aflR* message, by a construct expressing *aflR* via a heterologous promoter, were restored in both *aflR* transcript and aflatoxin biosynthesis. These data allow us to putatively identify the *afl-4* mutation as an upstream regulatory gene controlling the transcriptional activity of *aflR* Strain 241 exhibits no abnormalities in development or morphology, thus distinguishing it from developmental mutants which have been shown to also be affected in aflatoxin biosynthesis. A cosmid genomic library is being screened in strain 241, and progress in the cloning of the regulatory gene mutated in strain 241 by complementation will be presented.

110. A visual screen to detect *Aspergillus nidulans* mutants defective in *aflR* regulation.

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Aspergillus nidulans possesses a 60 kb gene cluster containing 25 coregulated transcripts involved in the biosynthesis of the mycotoxin sterigmatocystin (ST). The sixth gene in the cluster, *aflR*, is a positively acting transcription factor required for the expression of most, if not all, of the remaining 24 cluster genes (called stc genes for sterigmatocystin cluster). aflR activity can be visually assessed in *A. nidulans* TSS40 *(wA, methGl, biA1; stcE::argB)* because a *stcE* mutation results in the accumulation of norsolorinic acid (NOR, an orange colored intermediate in the ST biosynthetic pathway) rather than ST. Chemical mutagenesis of TSS40 has resulted in ~100 mutants unable to produce NOR. Genetic analysis of the mutants show that some contain mutations that are linked and some unlinked to the ST cluster. The linked mutations could represent lesions in one of four cluster genes required to produce NOR *(aflR, stcA, stcJ* and *stcK)* whereas the unlinked mutations potentially represent an *aflR trans*-acting regulatory factor. We are characterizing mutants in each class through genetic complementation and isolation of the gene(s) involved in the defect.

111. Development of a vector for introducing a deletion in the aflatoxin blosynthetic pathway of *Aspergillus parasiticus* and *A. flavus.*

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Transformation of *A. parasiticus* RHNL with a *norA* disruption construct fortuitously gave rise to a transformant that was found to have undergone an approximate 6 kb deletion of a region of the aflatoxin biosynthetic pathway. The deletion event resulted in the partial and complete loss of the known aflatoxin biosynthetic genes *avnA*, *ver-1*, and *orf2* (a homolog of *A. nidulans stcS)* respectively. Due to the loss of function of the *avnA* gene in the deletion mutant, it was unable to further metabolize averantin, and therefore this metabolite was the main polyketide found in the product mixture. However, besides averantin, methylaverantin, represented 16% of the recovered product mixture. Utilizing PCR techniques a vector has been constructed that was used to

introduce this deletion into A. *parasiticus* or *A. flavus* via homologous recombination. One area of intense interest has been in the development of biological control agents to reduce levels of aflatoxin contamination in field crops. *A. flavus* strains in which the aflatoxin pathway deletion has been introduced should make for excellent biocontrol agents with respect to control of aflatoxin contamination of susceptible crops.

112. Characterization of a gene involved in singlet oxygen resistance in *Cercospora nicotianae.*

M. Ehrenshaft, A.E. Jenns, and M.E. Daub. NC State University.

Singlet oxygen is an extremely toxic form of activated oxygen against which cells have few effective defense mechanisms. Photoactivated compounds that produce singlet oxygen (photosensitizers) are of diverse chemical structure and are ubiquitous in nature. Numerous *Cercospora* species synthesize cercosporin, a polyketide photosensitizer, which is toxic at micromolar concentrations to many other fungi, most bacteria, virtually all plants and even mice. *Cercospora* fungi, however, can accumulate up to 1 mM cercosporin in culture without measurable effect, and are also resistant to other, diverse singlet oxygen-generating photosensitizers. Using functional complementation of mutants sensitive to cercosporin and other photosensitizers, we have isolated a clone that appears to confer singlet oxygen resistance to *C. nicotianae.* Sequence analysis revealed an open reading frame encoding a protein of 343 amino acid residues. Data base searches found several protein sequences with 55-75% homology to this predicted polypeptide. None of the homologous sequences have a known function. Two are from higher plant expression libraries while the rest are open reading frames derived from large-scale sequencing projects. Studies are underway to determine if our gene is regulated or expressed constitutively and if it can be expressed in other organisms to confer cercosporin and/or singlet oxygen resistance.

113. Effects of deletions in the *aflR* promoter on overproduction of averantin by *Aspergillus parisiticus*.

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The gene, *aflR*, encodes a CYS_6-zinc clusters sequence-specific, DNA-binding protein that appears to activate all of the genes in the aflatoxin biosynthesis gene cluster. Previously, we found that A. *parasiticus* containing extra copies of *aflR* gave reddish-orange pigmented colonies resulting mainly from nine-fold higher yields of averantin and other pigmented metabolites. We hypothesized that this overproduction resulted from self-activation by AFLR. We, therefore, prepared for transformation of A. *parasiticus niaD*-containing plasmids where the region from -220 to -12 bp upstream of the *aflR* translation start site was mutated using overlap extension PCR. Transformants in which this entire region was deleted had normal levels of metabolite

production, indicating that they contained an intact copy of *aflR* that was not affected by the mutant copy. When only a portion of the palindrome, TTAGGCCTAA, identified previously as an AFLR autoregulatory site, was deleted, clones with this altered copy of *aflR* had one-fourth the level of averantin as did clones with an extra copy of intact *aflR*, but still almost 2-fold more than the untransformed strain. Clones in which two other dyad symmetric sites were deleted gave averantin yields equal to those of wild-type transformants, suggesting that these sites are not involved in autoregulation of *aflR* expression.

114. Role of polyamines in sporulation and mycotoxin formation in *Aspergillus nidulans*.

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The importance of polyamines during cell growth and differentiation in eucaryotes has been substantiated, although their precise mode of action remains unknown. In A. *nidulans* there occurs an increase in the levels of the enzymes involved in polyamine synthesis during spore germination . Similar results have been observed during cell differentiation in other fungal systems. Addition of the ornithine decarboxylase (ODC) competitive inhibitor diaminobutanone (DAB), leads to the inhibition of differentiation in these systems

Previously we have decribed (Fung. Gen. Biol., in press) that DAB inhibits both sporulation and aflatoxin biosynthesis in *Aspergillus parasiticus.* With these data we have proceeded to analyze the probable role of polyamines in sporulation and sterigmatocystin biosynthesis in *A. nidulans* FGSC A26, and *brlA1*$^{-}$, and *puA*$^{-}$ (ODC^{-}) mutants. We observed that blocking the synthesis of polyamines by the ODC competitive inhibitor DAB, inhibited sporulation of the wild type strain, and the formation of aerial mycelium in brlA^{-}. Growth of *puA* with limiting concentrations of putrescine did not inhibit vegetative growth, but severely affected sporulation. Under all these conditions, synthesis of sterigmatocystin was inhibited, and addition of putrescine restored the normal phenotype in all cases. We then proceeded to analyze the effect of DAB on the transcription of *brla* in *A. nidulans.* It was observed that DAB inhibited sporulation and sterigmatocystin formation. RNA was purified from both cultures and hybridized under stringent conditions with a *brlA* fragment from pBS2.5. The results showed that accumulation of the *brlA* transcript, was inhibited when the fungus was grown in the presence of DAB. According to these results we may suggest that polyamines play a role in sporulation and the regulation of mycotoxin formation in *A. nidulans,* at a step previous to the execution point of *brlA.*

115. Cometabolic degradation of methyl tert-butyl ether (MTBE) by the filamentous fungus Graphium after growth on gaseous n-alkanes and diethyl ether.

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Ether-bonded compounds are in general recalcitrant to biological transformation. In this study we demonstrate that the filamentous fungus *Graphium* sp. can utilize diethyl ether as a growth-supporting substrate, and that the gasoline oxygenate methyl tert-butyl ether (MTBE) can be rapidly degraded by mycelia grown on diethyl ether or gaseous n-alkanes. *Graphium* is one of the few eukaryotic organisms known to grown on gaseous n-alkanes (ethane, propane, n-butane); the initial oxidation of these substrates is thought to be catalyzed by an inducible cytochrome P450 enzyme. Several observations lead us to conclude that in *Graphium* gaseous n-alkanes, MTBE, and diethyl ether are oxidized by the same enzyme, a putative cytochrome P450, and that MTBE oxidation is a cometabolic process. First, acetylene and other unsaturated gases are potent inhibitors of growth on both n-alkanes and diethyl ether, and also inhibit MTBE degradation in n-alkane- and diethyl ether-grown mycelia. Second, MTBE and n-butane (or diethyl ether) are competitive substrates, although the presence of a growth-supporting substrate is required for sustained MTBE degradation. In contrast, MTBE is not degradad by mycelia grown on non-specific media. The maximal rate of MTBE degradation we have observed is ~10nmoles MTBE/h/mg dry weight. We have detected two major products of MTBE degradation, tert-butyl formate (TBF) and tert-butyl alcohol (TBA). Kinetic studies demonstrate that TBF is the first detectable product of MTBE oxidation and that TBF is hydrolyzed both biotically and abiotically to yield TBA. Our studies suggest a potential role for n-alkane-utilizing fungi in the bioremediation of MTBE and diethyl ether-containing solvent contamination.

116. Regulation of Mycotoxin Biosyntheis in *Aspergillus* spp.

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Several species of the fungal genus *Aspergillus*, including *Aspergillus nidulans*, produce sterigmatocystin (ST) which also serves as the penultimate precursor in the aflatoxin (AF) biosynthetic pathway. Because AF and ST are among the most toxic, carcinogenic, and mutagenic compounds produced in nature, contamination of commercial food crops by these toxins results in significant health and economic losses. We are interested in finding means by which toxin synthesis is controlled during the fungal lifecycle and have found that *flbA* and *fluG*, two genes required for asexual sporulation in A. *nidulans,* are also needed for ST biosynthesis. In addition, overexpression of *flbA* in submerged culture causes both activation of conidiation and ST production. We have shown that FlbA functions in negatively regulating FadA, the alpha subunit of a heterotrimeric G-protein that functions in stimulating a proliferative growth pathway. Thus, a common link between sporulation and ST biosynthesis is a requirement for endogenous growth control. We have found that this requirement for growth control in secondary metabolism is conserved in the closely related A. *parasiticus* AF pathway. Recently, we have identified and begun characterizing several putative A. *parasiticus flbA* homologues.

117. DNA polymorphisms in sterigmatocystin biosynthetic pathway genes of *Aspergillus nidulans, A. rugulovalvus* and a related taxon.

Maren Klich, Ed Mullaney and Catherine Daly, USDA/ARS/SRRC, New Orleans LA.

Stergimatocystin is a mycotoxin blosynthetically similar to aflatoxin. Sterigmatocystin is produced by over 20 *Aspergillus* species. Several of these species have teleomorphs in the genus *Emericella*, including an as yet poorly described taxon which has morphological affinities with *A. nidulans* and *A. rugulovalvus* which both also have sexual states in *Emericella.* One strain of this new taxon is a patent strain improperly described as *A. nidulans var. roseus.* Strains of all three of these taxa have been isolated from cotton field soils in the desert southwest of the US, indicating that the species have ecological similarities. Morphological and physiological studies have shown that this new taxon is more closely related to *A. rugulovalvus* than *A. nidulans.* To help establish the molecular affinities of this new taxon in the important area of mycotoxin biosynthesis, we compared RFLPs of total DNA of a number of strains of each taxon utilizing previously cloned genes from the aflatoxin/sterigmatocystin biosynthetic pathway.

118. Regulation of iron homeostasis in *Ustilago maydis* .

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Regulation of cellular iron homeostasis requires a careful balance between uptake of adequate iron for metabolism and restriction of iron uptake such that deleterious iron-catalyzed free radicals are not generated in vivo. In response to iron starvation *Ustilago* maydis produces the cyclic peptide siderophores ferrichrome and ferrichrome A (Budde, A. D. and S. A. Leong, 1989, Mycopathologia 108:125-133). Two clustered genes, *sid1* and *sid2*, are required for ferrichrome siderophore biosynthesis. *sid1* encodes ornithine-N^5-oxygenase (Mei, B., Budde, A. and S. A. Leong, 1993, Proc. Natl. Acad. Sci. U. S. A. 90:903-907) while *sid2* encodes a ferrichrome peptide synthetase.

Regulation of *sid1* has been studied by GUS reporter analysis and in vitro electrophoretic gel mobility shift analysis (An, Z., Mei, B., Yuan, W. M. and S. A. Leong, In Press, EMBO J). As expected, GUS activity was regulated by the availability of iron. Sequences required for iron-mediated regulation of *sid1* were identified by deletion and site-directed mutagenesis. Two GATA motifs located 1.6 kb upstream of the transcription initiation site were required to mediate repression of *sid1*. Previous studies have led to the cloning of *urbs1,* a putative repressor of *sid1* (Voisard, D., Wang, J., McEvoy, J., Xu, P. and S. A. Leong, 1993, Mol. Cell. Biol. 13:7091-7100). Electrophoretic gel mobility shift analysis using these motifs as well as GAT mutant derivatives as DNA probes indicated that Urbs1 binds specifically to the GATA sequences of *sid1*. Mutation of the C-terminal finger motif but not the N-terminal finger motif of Urbs1 significantly reduced DNA binding activity. Efforts are underway to purify Urbs1 from *U.*

maydis and to determine how Urbs1 senses intracellular levels of iron in cells. In vitro DNA binding and in vivo reporter gene analysis has revealed that siderophores are not corepressors of Urbs1. As with the bacterial homologue Fur, we anticipate that iron may interact directly with Urbs1 to mediate repression of target genes.

119. A CCAAT binding protein (PENR1) regulates all *Aspergillus nidulans* structural genes required for biosynthesis of the secondary metabolite penicillin.

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The -lactam antibiotic penicillin is a classical secondary metabolite produced by some filamentous fungi, e. g. by *Aspergillus nidulans.* The penicillin biosynthesis is catalysed by three enzymes which are encoded by the following three genes: *acvA (pcbAB), ipnA (pcbc)* and *aat (penDE).* The genes are organised into a gene cluster. *AcvA* and *ipnA* are bidirectionally oriented, separated by an intergenic region of 872 bp. The *aat* gene lies 825 bp downstream of *ipnA.* Promoter analysis using deletion constructs, band shift and methyl interference analyses led to the identification of two CCAAT containing DNA elements, one of which is located between *acvA* and ipnA, and the other one in the *aat* promoter region. These elements were specifically bound by a protein (complex) designated PENR1, for penicillin regulator. In addition to the CCAAT sequence, some of the neighbouring nucleotides were also required for binding of PENR1. Specific deletion of the PENR1 binding site between *acvA* and *ipnA* led to a 10-fold increase of *acvA* expression and, simultaneously, to a reduction of *ipnA* expression to about 30%. Mutagenesis of the CCAAT core sequence in the *aat* promoter resulted in a four-fold reduction of *aat-lacZ* expression. Hence, PENR1 represents a major regulatory protein involved in the regulation of all penicillin biosynthesis genes. Formally, the CCAAT sites mediate a negative effect on *acvA,* but positive effects on both *ipnA* and *aat* expression.

Then Bergh et al. (1996) J Bacteriol 178:3908; Litzka et al. (1996) EJB 238:675

120. Biodegradation of the polyketide toxin cercosporin.

Thomas K. Mitchell and Margaret E. Daub, North Carolina State University, Raleigh, N.C.

Cercosporin is a photoactivated polyketide toxin synthesized by many species in the genus *Cercospora.* Cercosporin produces singlet oxygen when photoactivated, and production of cercosporin is highly correlated with the ability of these pathogens to cause disease on a wide range of hosts. The only resistance known to cercosporin is restricted to the fungi that produce it. In an attempt to identify genes which may have utility in engineering resistant plants, we have searched for bacteria able to degrade the toxin. We screened 160 isolates of bacteria representing 11 genera. Isolates from 4 genera produced cleared zones when grown on solid media containing cercosporin (which is normally red in color), suggesting that they are able to degrade cercosporin. Degradation of cercosporin was confirmed by toxin extraction from bacterial cultures grown in cercosporin containing medium. A pigmented compound extracted from liquid

cultures containing cercosporin has been isolated from thin layer chromatography plates and is believed to be the breakdown product of cercosporin. This compound and the kinetics of its production are currently being characterized. Isolation of the gene necessary for the detoxification of cercosporin is proceeding. Once isolated, we intend to mobilize it into tobacco tissue to create plants capable of degrading cercosporin, rendering them resistant to *Cercospora* diseases.

121. Rapid prescreen for antitumor activity with mutant *B. subtilis* assay.

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In studies aimed at discovering new compounds with biological activity from microorganisms the choice of a suitable prescreen is important to eliminate those species with little or no activity and focus detailed studies on potentially promising isolates. Mechanism based screens are best used as leads to identify species with bioactive compounds. In our research, mushrooms and filamentous fungi from diverse sources were examined as novel sources of antiumor substances. A microbial prescreen with supersensitive mutant strains of *Bacillus subtilis* was successful in screening large numbers of cultures. The assay was highly specific and selective for cytotoxic activity. This was confirmed with in virto mammalian cell toxicity assays on murine cell lines. The mutant assay was easy to perform and results were readily available within 24-hours.

122. Aflatoxin production in yeast.

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Aflatoxins are polyketide-derived secondary metabolites, produced by *Aspergillus parasiticus* and *Aspergillus flavus*, that are highly toxic, mutagenic and carcinogenic in animals and are suspected carcinogens in humans. The final enzymatic step in the aflatoxin biosynthetic pathway is the conversion of O-methylsterigmatocystin (OMST) to aflatoxin by an oxidoreductase. Transformants of *A. flavus* strain 649WAF2 containing a 3.3 kb genomic DNA fragment and the aflatoxin biosynthesis regulatory gene *aflR* converted exogenously supplied O-methylsterigmatocystin to aflatoxin Bl. A gene, *Ord1*, corresponding to a transcript of about 2 kb was identified within the 3.3 kb DNA fragment. The promoter region contained a putative AFLR binding site and a TATA sequence. The nucleotide sequence of the gene revealed an open reading frame encoding a protein of 528 amino acids with a deduced molecular mass of 60.2 kDa. The gene contained six introns and seven exons. *Ordl* encodes a cytochrome P450-type monooxygenase, and the gene was assigned to a new P450 gene family named CYP64. Heterologous expression of the *ordl* open reading frame in *Saccharomyces cerevisiae* under the transcriptional control of the yeast galactose-inducible gall promoter, resulted in the ability to convert O-methylsterigmatocystin to aflatoxin Bl. The data indicate that *Ord1* is sufficient to accomplish the last step of aflatoxin biosynthesis.

123. Analysis of a *Gibberella fujikuroi* mutant deficient in a hydroxylation step of fumonisin biosynthesis.

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Gibberella fujikuroi mating population A is a pathogen of maize and sorghum and produces a family of toxins known as fumonisins that are associated with a number of mycotoxicoses. The B-type fumonisins consist of a 20-carbon-long backbone with two tricarboxylic acid moieties and one amino, two methyl, one to three hydroxyl groups. We have employed UV mutagenesis to study the genetics of fumonisin biosynthesis in *G. fujikuroi*. Three hundred forty-three strains derived from conidia that survived UV exposure were analyzed for their ability to produce fumonisins. Nine of the survivors failed to produce fumonisins and one (uv26) only produced fumonisin B_3, which lacks the hydroxyl group at carbon atom 5 (C-5). Wild-type strains of the fungus produce fumonisins B_1 and B_2, which are C-5 hydroxylated, as well as B_3. Genetic analyses revealed that the mutation segregates as a single locus and is linked to the *fum2* and *fum3* loci (previously identified in natural variants), the RAPD marker OPA16, and a putative aldehyde dehydrogenase gene on chromosome 1 of *G. fujikuroi*. In addition, *fum3*$^-$ and the uv26 mutation may be allelic because both confer the same fumonisin phenotype and no recombinant progeny were recovered from a cross between strains carrying these two markers. Currently, we are attempting to complement the *uv26* mutation by transformation with cosmid libraries constructed with DNA from wild-type strains of *G. fujikuroi*.

124. Characterizing the interaction of *Aspergillus parasticus* and its host, peanut.

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We hypothesize that there are specific factors in resistant lines of peanut that repress the invasion of *Aspergillus parasiticus* into the peanut pod and the synthesis of aflatoxin in the pod. The timing of induction and shutdown of expression of these resistance factors may be responsible for the resistance breakdown observed under certain field conditions (most notably drought and high temperatures). We seek to identify the natural resistance mechanisms present in the developing peanut pod that are important to limiting fungal growth and aflatoxin production initially by detecting the timing and location of known resistance genes in infected pods. Several peanut lines are now available that appear to have differing resistance mechanisms as observed in the field. As yet uncharacterized resistance mechanisms affecting aflatoxin production and infection can be detected by use of genetically engineered strains of A. *parastiicus* and by observation of infection at the cellular level. Once resistance mechanisms have been identified that are effective, their expression may be optimized through genetic engineering so that the resistance is protective over a longer period of time.

125. Isolation and Regulation of Genes of the Gibberellin Biosynthesis Pathway in *Gibberella fujikuroi*.

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The rice pathogen *Gibberella fujikuroi* accumulates large amounts of gibberellic acid (GA_3) and some other gibberellins which induce the superelongation disease on infected rice seedlings. The biosynthetic pathway for gibberellins has been established from the identification of intermediates and by using mutants affected in the gibberellin biosynthesis. However, genetics of gibberellin formation and regulation of this biosynthetic pathway are not well understood until now. Therefore, we first isolated some of the versatile genes of the central isoprenoid pathway which are involved in the biosynthesis of sterols, carotenoids and gibberellins. So far, the genes coding for HMG-COA reductase, FPP- and GGPP syntheses were isolated and characterized. The sequence comparison with analogous genes of other fungi and plants showed that the isoprenoid pathway must be highly conserved.

Beside those genes from the central terpenoid pathway, we are trying to isolate the most important genes from the gibberellin-specific part of the pathway such as the kaurene synthetase-. the kaurene oxidase-and the C20-oxidase genes. On the basis of sequence alignment of the recently isolated corresponding plant genes, we designed specific PCR-primers in order to amplify, parts of the corresponding *Gibberella* genes. Additionally, REMI mutagenesis and differential cDNA screening were used to isolate structural or regulatory genes of the gibberellin pathway. Furthermore, in order to understand the nitrogen and carbon repression of gibberellin formation on molecular level, the corresponding major regulatory genes, *areA, nmr* (nitrogen) and *creA* (carbon) were isolated. Since we could show by Northern analyses that the recently isolated genes are expressed constitutively, the target genes for both of the different regulation types should be in the more specific part of the gibberellin pathway e.g. in the kaurene synthetase gene coding the formation of the first intermediate with the specific gibberellin sceleton.

126. Plasmid tagging of paxilline biosynthetic genes in *Penicillium pasilli*.

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Penicillium paxilli is a filamentous fungi that produces large quantities of the tremorgenic mycotoxin, paxilline, in submerged cultures. This compound is of particular interest as it is an important intermediate in the biosynthesis of several other mycotoxins, including lolitrem B, that is produced by *Acremonium* endophytes in association with perennial ryegrass. We are interested in cloning some of the genes involved in paxilline biosynthesis with the aim of understanding the regulation of this pathway in both *P. paxilli* and A. *lolii.*

One of the strategies we are using to clone genes involved in paxilline biosynthesis is that of pAN7-1 plasmid tagging (Itoh *et al.* 1994; Itoh and Scott 1994). Using a monoclonal based ELISA assay for the detection of paxilline, 600 single-spore purified pAN7-1 transformants of *P. paxilli* were screened for paxilline production and one Pax$^-$ mutant, YI-20, was identified. Molecular analysis of this mutant showed that pAN7-1 had integrated at a single site but was present in 4 - 6 copies in tandem array. Rescue of sequences flanking the point of integration and subsequent molecular analysis revealed that YI-20 consisted of an extensive deletion and a chromosomal rearrangement between chromosomes V and VI. Gene disruptions at the chromosome V locus resulted in the generation of two additional Pax$^-$ mutants CY-2 and CY-102. Both of these new mutants were the result of single crossovers and had deletions extending beyond the mapped region but did not have chromosomal rearrangements. The extent of the deletion in these three Pax$^-$ mutants is currently being mapped by chromosome walking.

127. Aflatoxin biosynthetic pathway gene cluster.

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Aflatoxins, secondary metabolites of *Aspergillus flavus* and *A. parasiticus,* are furanocoumarins which are toxic and extremely carcinogenic in animal systems. A comprehensive and cooperative research effort has resulted in important breakthroughs in understanding of the molecular regulation of aflatoxin biosynthesis, A "gene cluster" of about 70 kilobases in size has been revealed upon which resides a DNA-binding protein thought to function in aflatoxin pathway regulation. In addition, several aflatoxin pathway genes encoding P450 monooxygenases, dehydrogenases, 0-methyltransferases, polyketide synthase and fatty acid synthase, and some yet uncharacterized open reading frames have been localized on the gene cluster. Recent studies have revealed that there are several additional transcripts beyond the 70 kb cluster; their specific involvement in aflatoxin biosynthesis is being examined. A similar cluster has been characterized from *A. nidulans; A. nidulans* produces sterigmatocystin (ST) as end product, which is the penultimate precursor of aflatoxin. The genes and the newly identified transcripts involved in aflatoxin and sterigmatocystin biosynthetic gene cluster in *A. parasiticus, A. flavus,* and in *A. nidulans* will be discussed.

128. Investigation of the intergenic promoter region of the ferrichrome siderophore biosynthesis Gene Cluster in *Ustilago maydis.*

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Two genes required for ferrichrome siderophore biosynthesis in *Ustilago* have been cloned and characterized. *sidl* encodes ornithine-N^5oxygenase, while *sid2* encodes a ferrichrome peptide synthetase. The two *sid* genes are divergently transcribed from a 4.5 kb intergenic region. Urbs1, a GATA-family transcription factor, regulates *sid* gene expression under different iron conditions. Multiple GATA motifs have been identified in the intergenic region by DNA sequence analysis, one pair of GATA sequences in the near center of the intergenic region was shown to be crucial for the iron-mediated regulation of *sid1* expression. It is hypothesized that *sid1* and *sid2* expression may be divergently governed by the same promoter region. A twin reporter system will be exploited to test this hypothesis. LacZ and GUS reporter genes will be translationally fused in opposite orientation with the intergenic region in a self-replicating vector ans site directed mutagenesis will be performed to identify important cis elements. To eliminate copy number efects, the key dual reporter constructs will be further tested at single copy in *U. maydis* by gene replacement. The extent of the *sid* gene cluster and its coregulation will be assessed by Northern hybridization analysis of RNA extracted from cells grown in low and high iron media.

Citric Acid Fermentation and Ageing

129. A spontaneous rise of intracellular cAMP levels in *Aspergillus niger*.

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A spontaneous rise in intracellular cAMP levels was observed in the early stages of *Aspegillus niger* growth under high citric acid yielding conditions. The amount of cAMP formed was found to be dependent on initial sucrose concentration in the medium. Under higher sucrose conditions the peaks appeared earlier and were higher, while in lower sucrose media flattened peaks were observed later in fermentation. Since in media with a higher sucrose concentrations intracellular citric acid has started to accumulate earlier and more rapidly, it might be that cAMP synthesis is triggered by intracellular acidification, caused by dissociation of citric acid. Stimulation of cAMP synthesis by acidification was supported by the fact that addition of azide to the fermentation broth caused an imediate rise in cAMP levels. However, no spontaneous rise in cAMP concentration could be detected, if the cells were grown in continuously illuminated cultures, suggesting that *A. niger* phosphodiesterase might be photoregulated. More evidence for the light activated PDE was obtained by morphological studies under the light and dark conditions in the presence of cAMP or dbcAMP, and it was additionally supported by experiments where specific phosphodiesterase inhibitors were tested.

130. Citric acid production by solid state fermentation.

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Citric acid is a commercially important product that has been obtained by submerged fermentation of glucose or sucrose by *Aspergillus niger*. This work was undertaken to determine the potential of food processing solid residues as a substrate for citric acid production by solid state fermentation using *Aspergillus niger.* Yields of citric acid varied considerably and were found to depend significantly on the strain of *Aspergillus niger* used and the following factors: the type of raw material fermented, the initial moisture content of the substrate, the amount of methyl alcohol present, and the fermentation time and temperature. Under favorable conditions, yields from more than 50 to nearly 90% were obtained on the basis of the amount of carbohydrate consumed. The results of this study indicate that food processing solid residues can serve as a low-cost substrate for citric acid production by solid state fermentation using *Aspergillus niger.*

131. Genetic improvement of industrial microorganisms: induction of further citric acid fermentation mutants of *Aspergillus niger* with the help of Gamma-rays.

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Citric acid is an organic acid of diverse economic use. The food and beverage industries use this acid extensively as a food additive. The present annual global requirement is nearly 4 lakh MTons which is produced almost entirely by fermentation of molasses sugar with selected strains of *Aspergillus niger.* Mutagenic techniques have been successfully employed for the improvement of *Aspergillus niger* strains. With the help of gamma radiations several high citric acid producing strains of *Aspergillus niger* were induced at our laboratory. These strains were found to yield 3-5 times more of citric acid from cane molasses than their parent strain (CA16). The induction of further mutation for higher yield of citric acid has been attempted using mutant strain, 14/20 as the parent. Three of the new isolates were selected for further test under semi-pilot scale studies, Of these, mutant strain no. 318 seems to be the most promising one which yields 88.5% citric acid in relation to sugar supplied.

132. Cloning and sequencing of cDNAs encoding citrate synthase and $NADP^+$ -specific isocitrate dehydrogenase from *Aspergilus niger*.

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The cDNAs encoding citrate synthase (CS) and $NADP^+$ specific isocitrate dehydrogenase (ICDH) from *Aspergillus niger* WU-2223L, a citric acid-producing strains were cloned by using

designed oligonucleotide primers. The CS cDNA encodes 475 amino acid residues, and shows high homology with those of *Neurospora crassa* and *Saccharomyces cerevisiae.* The ICDH cDNA encodes 497 amino acid residues, and shows high homology with mitochondrial type ICDH of *S. cerevisiae.* The chromosomal gene encoding ICDH containing seven introns was also cloned and sequenced.

133. Metabolic engineering of the glycolytic pathway in *Aspergillus niger.*

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Genetic engineering of *A. niger* primary metabolism is employed to improve citric acid production by this fungus. The aim in such an approach would be to increase the metabolic flux through the pathway leading to citric acid formation. One aspect of this strategy is to decrease the fluxes through branches of the main pathway resulting in decreased formation of by-products, such as gluconic acid and oxalic acid. We have isolated a mutant that lacks an active glucose oxidase and hence does not produce gluconic acid from glucose. Another aspect is direct increase of the flux through the main pathway, for example, by overproduction of the enzymes involved. For genetic engineering of glycolysis we have isolated the genes encoding the glycolytic enzymes hexokinase, glucokinase, phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and pyruvate kinase (PKI). To date hexose phosphorylation has not been studied in detail in *A. niger.* We have recently found that *A. niger* possesses a hexokinase and a glucokinase. Moderate overexpression of PFK and PKI, either individually or simultaneously, did not increase citric acid production by the fungus significantly. Product yields (g citric acid formed per g glucose consumed) of the transformants were comparable to a wild-type strain making changes in by-product formation unlikely. Furthermore, no significant changes in the activities of other enzymes in the pathway or in the concentrations of intermediary metabolites were found. However, in strains overexpressing PFK the level of fructose-2,6-bisphosphate (F2,6BP), a positive allosteric effector of PFK, was reduced almost two-fold compared to the wild-type strain. Simulation experiments with purified PFK showed that such a reduction in the F2,6BP level could decrease the in vivo activity of PFK significantly. Thus, by decreasing the F2,6BP level *A. niger* seems to adapt to overexpression of PFK.

Neurospora

134. Calcineurin of *Neurospora crassa,* Essential for Hyphal Growth, Morphology and the Apical $Ca2^{+}$ -Gradient - an Inducible Loss of Function Analysis.

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The function of *Neurospora crassa* calcineurin was investigated in *N. crassa* strains transformed with a construct for the inducible expression of the catalytic subunit of calcineurin (CNA) antisense-RNA. No detectable *cna-1* mRNA, reduced levels of immunodetectable CNA1 protein and decreased calcineurin specific enzyme activity, on induction medium only, were evidence that a conditional reduction of the target function had been achieved in the isolated antisense-transformants. In these transformants induction conditions procured a growth arrest which occurred after extensive hyphal branching, changes of hyphal morphology and concomitant loss of the distinctive tip-high $Ca2^{+}$-gradient typical for growing wild-type hyphae. The phenotype of the loss of function mutants' created by antisense-technique indicated that the *cna-1* gene of *N. crassa,* coding for the catalytic subunit of calcineurin, is an essential gene. In vitro inhibiton of *N. crassa* calcineurin by the complex of CsA and cyclophilin20 and increased sensitivity of the induced transformants to the calcineurin specific drugs, cyclosporin (CsA) and FK506, were evidence that the drugs act in *N. crassa,* as in *Saccharomyces cerevisiae* and T-cells, by inactivating calcineurin. Consistently, the exposure of growing wild-type mycelium to CsA or FK506, respectively, led to a phenotype very similar to that of the *cna-1* antisense-mutants, i.e. the disturbance of the $Ca2^{+}$-gradient, hyperbranching, altered hyphal morphology and ultimately to growth arrest. Together these data indicate for the first time a crucial role of calcineurin in the precise regulation of apical growth, a common form of cellular proliferation.

135. Characterization of a translocation in the mutant, SS-656, of *Neurospora crassa.*

Amy Richardson, Wavne A. Krissinger, and Sara Neville Bennett. Georgia Southern University.

SS-656, an osmotic-sensitive mutant of *N. crassa,* was isolated in the Georgia Southern Neurospora Genetics Laboratory following UV irradiation of wild type conidia. The morphology of SS-656 resembled that of the wild type strain and differed from the altered morphology of typical osmotic sensitive mutants. Crosses to the *alcoy* tester strain to begin mapping the osmotic sensitivity of the mutant produced conflicting results which suggested the possibility of a translocation. Analysis of a cross of SS-656 to the tester strain *fluffy* (OR) indicated 21 % of shot ascospores were white rather than the normal black, supporting the presence of a translocation. A total of 220 unordered tetrads from the same cross produced black to white ascospore ratios of 8:0 (58.2%); 6:2 (15.5%); 4:4 (23.6%); 2:6 (1.8%); 0:8 (9%) which further supported the presence of a translocation. When the progeny were crossed to wild type, 50.6% of the progeny produced perithecia but shot few to no ascospores and were classed as barren. Examination of the culture tubes of the fertile progeny showed an abundance of white ascospores from 11 of the crosses. Four of the 11 were examined for numbers of white ascospores. One produced a normal number of white ascospores. The other three, two of which were not osmotic-sensitive, produced 25.4%, 20.1%, and 20.5% white ascospores among those shot, indicating that the translocation went through the cross and segregated from the osmotic-sensitive trait. Crosses of SS-656 with *multicent,* a tester strain useful for mapping translocations, have been made.

136. Analysis of clock-controlled conidiation in *Neurospora crassa.*

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A circadian biological clock controls several aspects of growth and development in *Neurospora crassa*, including the timing of the initiation of conidiogenesis. In addition to previously isolated clock-controlled genes, *ccg-1 (grg-1)* and *eas (ccg-2)*, differential screening using 4 cDNA libraries representing different times of day uncovered 6 new clock-controlled genes. Transcripts from each gene cycle in abundance, with peak accumulation occurring in the late-night to early-morning (Bell-Pedersen et al. 1996. PNAS 93: 13096). Sequence analysis revealed that *ccg-7* encodes glyceraldehyde 3-phosphate dehydrogenase (a glycolytic enzyme), *ccg-12* to be the *N. crassa* copper metallothionein gene *(cmt)*, and the others encode novel genes. Considering the *ccg-4*, *ccg-7* and *cmt (ccg-12)* transcripts are not induced by light or conidial development, their function may be distinct from conidiation. Disruption of *ccg-6* and *ccg-9* by RIP results in strains which possess morphologically abnormal conidia. Additionally, these strains display a loss of clock-controlled conidial banding on race tubes. In both strains, however, the clock functions normally as shown by cycling of the FRQ protein, a central clock component. This suggests that CCG-6 and CCG-9 might work as messengers transducing a time-of-day signal from the oscillator to an overt rhythm, i.e. circadian conidiation. Our preliminary data indicates that cycling of the *ccg-4, ccg-6, ccg-7* and *ccg-12* transcripts is severely diminished in the *ccg-9*^{RIP} strain, consistent with a requirement for the *ccg-9* product in circadian regulation of these genes.

137. Cloning of the last iron-sulfur protein and mapping of complex I genes of *Neurospora.*

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Complex I is a component of the mitochondrial respiratory chain, containing more than 30 polypeptide subunits, that transfers electrons from NADH to ubiquinone through a series of protein-linked prosthetic groups, FMN and [Fe-S] clusters. Prokaryotes contain enzymes similar to complex I, called NDH-1, but of simpler composition. Their subunits are homologous of the seven subunits of complex I that are coded by mitochondria and of further seven nuclear-coded subunits. Of these, a [Fe-S] protein, named PSST in bovine, was never identified in *Neurospora crassa*. Based on conserved amino acid sequences of the protein, we designed degenerated primers and synthesised a DNA probe by PCR. This probe was used in the screening of a cDNA library and the *Neurospora* PSST homologue was cloned. We will present further characterisation of the gene and of the protein. Furthermore, the chromosomal location of several nuclear genes coding for complex I subunits, that have been achieved through RFLP mapping, will be indicated.

138. Analysis of *het-C* heterokaryon incompatibility in *Neurospora crassa*.

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Fusion of hyphae from genetically distinct fungal individuals (heterokaryosis) during growth results in the formation of heterokaryons, hyphae which contain genetically different nuclei but share a common cytoplasm. Fungi have heterokaryon (or vegetative) incompatibility systems that recognise specific genetic differences between self and nonself and limit heterokaryon formation. In *Neurospora crassa*, at least 10 loci, called *het* loci, in addition to the mating type locus, elicit heterokaryon incompatibility.

The *het-C* locus is the best characterized of the *het* genes. Inhibited growth resulting from *het-C* incompatibility is observed in forced heterokaryons between individuals of different *het-C* genotype and in partial duplications heterozygous for *het-C.* The *het-C* gene encodes a 966 amino acid polypeptide which contains an Nterminal signal sequence motif, a central leucine zipper-like coiled-coil motif and a C-terminal glycine-rich region which shows similarity to extracellular structural proteins. Deletion constructs used in transformation assays indicate that the signal peptide is not required for incompatibility function. We are analysing the subcellular localization of the HET-C protein to provide insight into the molecular mechanisms of *het-C* incompatibility.

139. Arginine-specific translational regulation of *Neurospora crassa arg-2* is associated with ribosome arrest at an upstream open reading frame in the mRNA.

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An upstream open reading frame (uORF) in the *N. crassa arg-2* mRNA has a role in negative translational regulation by Arg. We used a primer extension inhibition (toeprint) assay to examine *arg-2* uORF-mediated translational regulation in a homologous cell-free translation system. Wild-type and mutant *arg-2* uORF sequences were placed upstream of the luciferase polypeptide coding region and the distribution of the translational machinery on these arg-2ĐLUC RNA constructs examined. In analyses of RNA containing uORF sequences, prematurely terminated reverse-transcription products were found 16 nt distal from the uORF start codon, 13 nt distal from the uORF termination codon and 16 nt distal from the LUC start codon. The appearance of these toeprint signals depended on the coding sequence and coding capacity of the uORF; their appearance also was affected by translational inhibitors such as puromycin. These toeprint sites appear to correspond to ribosomes positioned on RNA with initiation codons at their P sites and termination codons at their A sites, respectively. When surplus Arg was added to translation reactions, a marked increase in the intensity of the toeprint signal at the wild-type uORF termination codon was observed, and an additional signal appeared 21-30 nt upstream of this codon. A decrease in the toeprint signal at the luciferase initiation codon was also observed. These results suggest that an Arg-mediated increase in ribosomal arrest at the uORF termination codon causes a block in the movement of ribosomes and reduces ribosome loading at the downstream luciferase initiation codon. uORF-mediated ribosomal arrest appears central to the Arg-specific translational regulation observed in vitro.

140. Expressing Zeamatin in *Neurospora crassa.*

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We have investigated the ability of a fungus, *Neurospora crassa*, to secrete zeamatin, a protein produced by *Zea mays.* Heterologous expression of plant genes by *N. crassa* may serve as an important alternative for producing plant proteins difficult to isolate or to obtain in useful amounts by other means. Zeamatin was expressed as a fusion protein with glucoamylase, an extracellular hydrolase induced by *N. crassa* upon depletion of glucose from the culture medium. A *kex-2* protease site was engineered at the N-terminus of the zeamatin cDNA, allowing cleavage of chimeric product in the secretary pathway. The modified zeamatin cDNA was inserted into a plasmid (pGE) containing the glucoamylase promoter, a truncated glucoamylase open reading frame and the glucoamylase terminator sequence, to form the plasmid pGEZ. *N. crassa* strains were co-transformed with pGEZ and a plasmid containing selectable markers for hygromycin resistance (pMP6) or to restore histidine *(his-3)* autotrophy. Transformed strains of *N. crassa* were screened by polymerase chain reaction analysis for the pGEZ insert using zeamatin-specific primers. Colonies positive for the zeamatin gene were grown in liquid culture and induced for glucoamylase and zeamatin production. Concentrated culture media were tested for the presence of secreted zeamatin protein using western blot analysis with an anti-zeamatin polyclonal antibody.