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Abstracts from the 3rd ECFG

Abstract

Abstracts from the European Congress on Fungal Genetics #3, held March 27-30, 1996, Munster, Germany

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Lecture Abstracts

Hyphal Growth and Development

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Growth by means of apically extending hyphae provides fungi with a certain mobility to locate organic resources, permits penetration of solid substrata, and enables formation of multihyphal structures. Plants are totally dependent on these hypha-based fungal activities, both for their degradation and nutrition, although pathological interactions have also developed.
Fundamental to hyphal growth is extension of the wall at the growing

apex (cellular morphogenesis). Apart from the apical cytoskeleton, plastic deformation and subsequent hardening of the wall play important roles (steady-state growth theory). The inside to outside growth of the wall also leads to efficient translocation of proteins over the nascent wall at the apex (bulk-flow hypothesis).
Among secreted proteins are extracellular enzymes and wall proteins, such as hydrophobins. Hydrophobins display self-assembly into amphipathic films when confronted with a hydrophilic-hydrophobic interface (molecular morphogenesis). In this way, hydrophobins are thought to be involved in, for instance, formation of aerial hyphae, dissemination of spores, formation of fruit bodies, and numerous interactions between fungi and plants. Hydrophobins may therefore be central to evolution of both fungi and plants. Hydrophobin-based activities of fungi also inspire industrial applications.

Fungal Genetics: From Fundamental Research to Biotechnology
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Fungal genetics is a rather young discipline in science as compared to biotechnology which is, albeit unconciously, correlated with begin of human civilization. It was at first almost exclusively devoted to fundamental research and came only during the last two decades in close relation to biotechnology, when it appeared meaningful to apply chromosomal genetics in a concerted manner to improve the production or transformation capacities of industrial fungi.
A landmark in the young relation between fungal genetics and biotechnology was the discovery of fungal plasmids and its relationship to mitochondrial DNA. Thus it became possible to incooperate also the fungi in the concept of genetic engineering, previously established for prokaryotes. After a short historical survey I will present trends in fundamental research of chromosomal and extrachromosomal genetics, involving breeding systems, recombination, gene expression, extrachromsomal genetic elements and genetic engineering. This will be followed by trends in

biotechnology and in conclusion with the future development of fungal genetics.

Molecular Genetics of Vegetative Incompatibility in the Filamentous Fungus *Podospora anserina*

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Vegetative incompatibility is common to filamentous fungi. This process prevents the formation of heterokaryons between strains which are non-isogenic for *het* loci. In most cases the filaments of incompatible strains can fuse but a necrotic reaction rapidly destroys the heterokaryotic cells. Cloning genes involved in vegetative incompatibility has now been initiated in several species and some genes have been characterized providing the basis to understand their function and the mechanisms that are induced after the coexpression of incompatible *het genes.*

In *Podospora anserina* we have cloned genes from several *het* loci. Comparison of differents alleles from the same locus provide evidence that incompatibility might be the consequence of the evolutionary divergence in natural populations, of genes that have a basic function in cell physiology.

From sequence data and mutant phenotypes, it appears that some *het* genes, but also *mod* genes, the mutations of which suppress or attenuate incompatibility, encode proteins that are involved in signal transduction essential for some differentiation stages.

These results will be discussed with reference to the role of vegetative incompatibility in natural populations of fungi.

Sexual Development in *Podospora anserina:* Reproductive Structures, Mating Types and Peroxisomes

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Two interesting developmental problems were brought to light during the study of sexual reproduction in *P. anserina.*

The *mat+ and mat-* mating types were shown to control a recognition process not only during fertilization, but also at a later stage, when reproductive nuclei which have devided in a common cytoplasm form a *mat+/mat-* pair within the ascogenous hyphae (1). We found that mutations in the single *mat+* gene, *FPR1,* and in two of the three *mat- genes, FMR1* and *SMR2,* inpaired the formation of *mat+lmat-* dicaryons and led to production of uniparental progeny. These arose from homocaryotic ascogenous hyphae with one or a pair of *mat* mutant nuclei. In contrast, mutations in *SMR1* caused sterility. Complementation studies clarified the role played by each *mat* gene. The absence of internuclear complementation in *SMR2* mutants indicated that the SMR2 protein returns to the nucleus in which the gene has been transcribed. Thus, *SMR2* may control *mat-* identity. Opposite data suggest that *FMR1* is not the main determinant of identity, but acts as an enhancer of *SMR2.* In agreement with that, SMR2 and FMR 1 proteins were shown to interact in the yeast two-hybrid system. The sterility of the *SMR1* mutants and their internuclear complementation suggest a different function for *SMR1,* which might act either prior to or after *FMR1* and *SMR2.* As it can be present in either *mat+* or *mat-* nuclei, or in both, it is not a *bona fide* mating type gene. Identification of the *mat* target genes will help to elucidate how *mat* genes ensure recognition between nuclei.

A link between sexual development and peroxisomes was suggested by the demonstration that the *car1* gene, involved in caryogamy, encodes a peroxisomal protein (2). The analysis of revertants has identified new genes involved in sexual development and fatty acid metabolism. These should clarify the role of peroxisomes in the sexual cycle.

In an attempt to discover new developmental pathways, we have cloned and are sequencing three genes involved in the formation of the male gametes and/or the female organs.

1- Zickler *et al.* (1995) Genetics 140, 493-503. 2- Berteaux-Lecellier *et al.* (1995) Cell 81, 1043-1051

Control of Sexual Development *in Schizophyllum commune* by the B Mating Type Locus

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The multiallelic B mating type locus that controls nuclear migration and pseudoclamp fusion encodes a pheromone receptor system. Analysis of different alleles of the pheromone receptor gene revealed putative binding sites for intracellular factors involved in signal transduction and adaptation. They are conserved between receptors of different specificity. Putative extracellular pheromone binding sites display sequence diversity between different specificities.
The pheromones encoded by three genes of the locus B l show no amino acid sequence similarity. However, all three genes were active in transformation of a B 2 recipient strain. Transformation with one of the pheromone genes, bap1(l), induced B-regulated development in strains with different specificities indicating promiscuous binding of pheromones. This makes the multiallelic pheromone receptor system of *S. commune* an ideal system to investigate specificity of ligand-receptor interaction.
Activation of the pheromone receptor by binding of a compatible pheromone triggers nuclear migration. The genes transcriptionally regulated by the pheromone response are investigated using the differential display technique.

***Aspergillus* Conidiation**

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The first genetic investigations of conidiation concentrated on its

most conspicuous stages, for the obvious reason that the resulting mutants were easy to identify and interpret. However, this proved to be a fruitful strategy, since mutants with the most conspicuous phenotypes did indeed turn out to be in genes with key roles in development. Only a small number of genes were identified by this route: what proportion of conidiation genes (whatever they may be) have been discovered? Biochemistry suggest only a minority. Subsequent genetic studies have picked on a limited number of new mutants with lesser developmental effects, while the biochemical route has led to further relevant genes, some with identifiable phenotypic effects, but some with none.

Extensions of the original studies in two directions ask, on the one hand, what leads to initiation of conidiation, and on the other, what determines the character of the resulting conidia? The nature of conidia is problematic because their properties are mainly negative: the function of a spore is prolonged inactivity. So far, mutation studies have yielded spore-defective mutants which are difficult to study because of their indistinct phenotypes, while biochemistry has revealed clusters of genes, the only one of which to be dissected in detail remains of unknown function.

However the initiation of conidiation is a much more open field, the main block to its study in the past being definition of a narrow enough area to allow any sense of progress. For the fungus, the alternatives to conidiation are either the even more complex (and interesting) process of sexual reproduction., which has been under study for some time, or the production of aerial mycelium, a field which was, for historical reasons, unpalatable to Aspergillus Geneticists. Now, this nettle has also been gasped, and is bearing interesting fruit.

Signal Transduction Pathways Controlling Multicellular Development in *Dictyostelium*

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Dictyostelium discoideum grows as single-celled vegetative amoebae. Multicellular development is initiated by starvation. Up to 105 cells chemotactically agregate to form a mound structure that then undergoes cell type differention and morphogenesis to produce a mature fruiting body. Aggregation in *Dictyostelium is* mediated by extracellular cAMP that interacts with G protein-coupled serpentine receptors. This initiates a variety of intracellular signaling pathways that include the activation of adenylyl cyclase and the relay of the signal, activation of guanylyl, cyclase that regulates chemotactic movement, and the activation of aggregation-stage gene expression. Upon formation of the mound, there is a developmental switch in which the cAMP signal changes from nanomolar oscillatory pulses to a high micromolar, more continuous, signal. This results in the adaptation of the aggregation-stage pathways and the induction of a signaling pathway regulated by the same receptors but is G protein independent. The rise in extracellular cAMP activates the transcription factor GBF that induces a developmental transcription cascade that induces the expression of a variety of genes, including additional G protein subunits, receptors, other transcription factors, and a ras/ras-GAP complex. These gene products are then required for both morphogenesis and subsequent cell-type differentiation. Molecular and genetic analysis has identified nine distinct developmentally regulated heterotrimeric G protein subunits, four cAMP receptors, components of multiple MAP kinase cascades, a variety of other kinases controlled through signal transduction pathways including cAMP-dependent protein kinase, and pathways differentially controlled by protein tyrosine phosphorylation that play central roles in regulating development. How these diverse signal transduction pathways are integrated to control various aspects of multicellular development will be described. The discusion will concentrate on how cAMP receptors differentially mediate G protein-dependent and independent pathways to control development.

N-glycosylation in Aspergillus: Consequences for Heterologous Gene Expression

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The emerging field of glycobiology has had a significant impact on the production of recombinant proteins. The post-translational modification of proteins by the addition of sugar residues can significantly affect protein stability, conformation and functional activity. Glycosylation also plays an important role in cell-cell and intracellular protein targeting, These factors can have important effects on the commercial development of recombinant products, particularly in the health care and pharmaceutical industries. During the recombinant production of therapeutic proteins, the composition and types of oligosaccharides can be very sensitive to host cells and culture conditions. Heterogeneity in chain composition, chain structure and the utilization of alternate glycosylation sites is common during the recombinant production of therapeutic proteins and may, as a result, affect such factors as serum half-life, antigenicity, resistance to proteases and biological activity. To obtain stable and functional recombinant proteins, methods must be developed which yield glycoproteins having highly uniform carbohydrate moieties. This could be achieved by post-purification processing of recombinant proteins with enzymes which modify the carbohydrate moieties in a highly specific and predictable manner. Alternately the custom production of recombinant glycoproteins could also be achieved *in vivo* through the genetic alteration of the glycosylation pathway in the host expression system. This method, while more difficult to develop, would provide a more effective and lasting means to produce glycoproteins with specific glycosylation patterns. Our objective is to characterize the complete set of carbohydrate processing enzymes produced by the filamentous fungus *Aspergillus* in order to tailor the glycosylation pattern of recombinant therapeutic proteins produced in this fungus. The downstream products of such N-glycan remodeling research will

include highly uniform glycoforms for pharmaceutical drugs. In this paper, we report the isolation and cloning of a novel cytosolic mannosidase from *Aspergillus nidulans.* Sequence analysis revealed a coding region of 3383bp containing three short introns. The deduced amino acid sequence of the *A. nidulans* mannosidase gene showed considerable homology to both the rat cytosolic/ER and yeast vacuolar mannosidases and, in common with these enzymes, did not contain a recognizable transmembrane domain. Phylogenetic analysis indicated that these cytosolic enzymes form a closely related group (Group III) which is distinct from both the mannosidase I enzymes (Group I) and the mannosidase II/ lysosomal mannosidase (Group II) enzymes.

Genetic Regulation of Nitrogen Metabolism

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In Neurospora crassa, expression of many structural genes for nitrogen catabolic enzymes and permeases is regulated by nitrogen repression and by pathway-specific induction. Transcription of the *nit-3* gene is highly regulated by NIT2, a global, positive-acting regulatory protein and by NIT4, a minor control protein that mediates nitrate induction. NIT2 is a DNA-binding protein which recognizes sites containing GATA core sequences; NIT4 has a GAL4-like Cys6/Zn2 DNA binding motif and binds to an 8 bp palindromic sequence TCCGCGGA and closely related sequences. Three NIT2 and two NIT4 binding sites occur in the *nit-3* promoter, most as a cluster of sites at approximately -1,000 bp. All of the binding sites contribute to *nit-3* gene expression, but one NIT2 and one NIT4 site which lie immediately adjacent are of primary importance.
NMR is a negative-acting regulatory protein which functions in nitrogen repression; *nmr* mutants are largely insensitive to nitrogen repression. A specific NMR-NIT2 protein-protein interaction has been identified with the yeast 2-hybrid system and by direct in vitro

protein binding studies. NMR binds to two distinct regions of NIT2, one an -helical segment in the DNA.binding domain, the second approximately 12 amino acids at the carboxyl terminus which also appear to form an -helix. Amino acid substitutions in either of these regions result in nitrogen derepression and in the loss of the ability to bind to NMR.

Molecular Regulation of the Penicillin Biosynthesis in *Aspergillus nidulans*

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The secondary metabolite penicillin is produced by some filamentous fungi only, most notably by *Aspergillus nidulans* and *Penicillium chrysogenum.* Starting from the three amino acid precursors, 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. In *A. nidulans, acvA and ipnA* are bidirectionally transcribed. The *aat* gene lies 825 bps downstream of *ipnA.* Several regulatory circuits affecting the regulation of these penicillin biosynthesis genes have been identified. A moving window analysis of the 872-bp intergenic region between *acvA and ipnA* using reporter gene fusions indicated that the divergently orientated promoters are, at least in part, physically overlapping and share common regulatory elements. Removal of nucleotides -353 to -432 upstream of the *acvA* gene led to a 10-fold increase of *acvA-uidA* expression and simultaneously, to a reduction of *ipnA-lacZ* expression to about 30 %. Band shift assays and methyl interference analysis using partially purified protein extracts revealed that a short DNA element within this region was specifically bound by a protein (complex) which we designated PENR, for penicillin regulator. Deletion of 4 bps within the identified protein binding site caused the same contrary effects on *acvA and ipnA* expression, as observed for all of the deletion clones

lacking nts -353 to -432. In addition, band shift assays and mutagenesis experiments led to the identification of a functional DNA element in the *aat* promoter region which was specifically bound by the same PENR protein (complex) as the intergenic region between *acvA and ipnA*. Hence, PENR seems to be involved in the regulation of all penicillin biosynthesis genes. Furthermore, the promoter regions of the corresponding genes of the -lactam producing fungi *Penicillium ch sogenum and Acremonium chrysogenum* also diluted the complex formed of the *A. nidulans* probes with PENR *in vitro,* suggesting that these -lactam biosynthesis genes are regulated by analogous DNA elements and proteins.

Efficient Production of Secreted Proteins by Aspergillus: Progress, Limitations and Prospects

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Aspergilli, such as *A. niger* and *A. oryzae,* are widely used for the commercial production of a variety of extracelluar enzymes due to their ability to secrete large amounts of proteins and their status as "GRAS organisims". To achieve high levels of fungal-enzyme production long-lasting classical strain-improvenient programs have been used.
The application of modern molecular-geiietic techniques, however, has greatly accelerated fungal strain improvement and has created new opportunities for the production of fungal enzymes, but also of non-fungal enzymes and phamraceutical proteins. In the last years all the "basic tools" have been developed, which are required for (molecular)-genetic analysis of *A. niger* and construction of high production strains. To prevent degradation of nonfungal proteins,

several protease-deficient niutants of *A. niger* have also been isolated. In addition, strategies have been developed by which efficient production of fungal and non-fungal proteins can be achieved. These strategies are based on the use of well characterised strong, constitutive or regulated gene expression signals and the generation of multicopy strains. Furthermore a gene-fusion strategy has been developed which improves the production of mature non-fungal proteins. Although substantial progress has been made, systematic studies on protein production have identified clear limitations that restrict the production yields of fungal and especially of some non-fungal proteins by *Aspergillus*.
In the lecture the progress, limitations and prospects for efficient production of secreted proteins bv *Aspergillus* will be discussed and illustrated with some examples of our systematic study on the production of fungal and non-fungal proteins in *Aspergillus,* such as *A. niger* glucoamylase, plant *Cyamopsis tetragonoloba* - galactosidase, human Interleukin 6 and antibody fragments.

Mating and the Requirement for Allelic Pairing

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A puzzling phenomenon seen in ascomycetous fungi is that of "ascus dominant" mutations. A classic example of this is the mutation *Round spore* in *Neurospora crassa*. In crosses heterozygous for the mutant allele, all of the ascospores, including the four that contain the wild type allele, are “round” (spherical) rather than having the spindle-shaped spores typical of the species. The simple ad hoc explanation of ascus dominance is that the mutant makes a toxic or inhibitory product that interferes with the formation or function of the wild type product - that is, the dominant mode of expression of the mutant allele is due to negative complementation in the zygote. The effects on the zygote are somehow carried into the ascospores.

Our study of an ascus-dominant mutation called *Asm-1* suggests a different mechanism. *Asm-1* (Ascospore maturation) has both a vegetative phenotype and a sexual phase phenotype. When crossed to wild type or its equivalent, *Asm-1* causes all spores in almost all asci to fail to mature; they remain very small, white, and inviable. The significant point is that *Asm-1* is a deletion mutant, and cannot be making a toxic product. We have found that when a normal allele, *Asm-l+,* reintegrated into the genome in a defined but ectopic location, corrects the vegetative phenotype of the deletion mutant, but completely fails to correct the ascus-dominant failure of spore maturation. However, when two *Asm-1* strains of opposite mating type are both subjected to re-insertion of *Asm-l+* at the same "wrong" location, mature black ascospores are produced. Our interpretation of this and related experiments is that, not only is *Asm*1+ function needed for ascospore maturation, but the wild type allele must be paired with its homolog in the zygote for it to be correctly expressed during later development. A phenomenon analogous to this occurs in the diploid somatic cells of certain Drosophila mutants, and is known as *transvection.* We suggest that Asm-1 acts in a way formally similar or identical to transvection, and speculate that other ascus-dominant mutations like *Round spore* may exert their effects by transvection.

Fungal Avirulence Genes, Major Players in the Gene-for-gene Game

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The interaction between the biotrophic fungal pathogen

Cladosporium fulvum and tomato complies with the gene-for-gene model. Resistance, expressed as a hypersensitive response (HR) followed by other defence responses, is based on recognition of products of avirulence genes from *C. fulvum* (race-specific elicitors) by receptors (putative products of resistance genes) in the host plant tomato. The AVR9 elicitor is a 28 arnino acid (aa) peptide and the AVR4 elicitor a 106 aa peptide which induces HR in tomato plants carrying the complementary resistance genes *CJ9* and *Cf4*, respectively. The 3-D structure of the AVR9 peptide, as determined by 1H NMR, revealed that AVR9 belongs to a family of peptides with a cystine knot motif. This motif occurs in channel blockers, peptidase inhibitors and growth factors. The CJ9 resistance gene encodes a membrane-anchored extracellular glycoprotein which contains leucine-rich repeats (LRRs). 125I labeled AVR9 peptide shows the same affinity for plasma membranes of Cf9+ and Cf9- tomato leaves. Membranes of solanaceous plants tested so far all contain homologs of the *Cf9* gene and show similar affinities for AVR9. It is assumed that for induction of HR, at least two plant proteins (presumably CF9 and one of his homologs) interact directly or indirectly with the AVR9 peptide which possibly initiates modulation and dimerisation of the receptor, and activation of various other proteins involved in downstream events eventually leading to HR. We have created several mutants of the *Avr*9 gene, expressed them in the potato virus X (PVX) expression system and tested their biological activity on Cf9 genotypes of tomato. A positive correlation was obseverd between the biological activity of the mutant AVR9 peptides and their affinity for tomato plasma membranes. Recent results on structure and biological activity of AVR4 peptides encoded by aviruient and virulent alleles of the Avr4 gene (based on expression studies in PVX) will also be presented.

Engineering Mycoviruses to Understand and Alter Fungushost Pathogenic Interactions

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Interactions between the fungal pathogen *Cryphonectria parasitica* and its plant host are significantly altered by virulence-attenuating mycoviruses of the genus hypovirus, thus providing a unique paradigm for examining mechanisms that drive fungal pathogenic processes. The utility of this system has been validated by recent observations that hypovirus infection attenuates fungal virulence by disrupting a key fungal G-protein (CPG-1) linked signal transduction pathway. We now report that hypovirus infection and CPG-1 transgenic suppression elevate cAMP levels three to five fold. This result is consistent with the prediction that *C. parasitica* CPG-1, like mammalian Gi subunits, function to negatively regulate adenylyl cyclase. Fungal genes that are specifically regulated through the CPG-1 pathway were recently identified with the aid of mRNA differential display. It was also possible to mimic the effect of both hypovirus infection and CPG-1 transgenic suppression on the expression of these genes by drug-induced elevation of cAMP levels. These results identify G-protein-regulated cAMP accumulation as a determinant of hypovirus-mediated alteration of fungal gene expression and virulence.
The recent development of an infectious cDNA copy of a hypovirus RNA has provided the ability to genetically modify hypoviruses that confer specific phenotypic traits on their fungal host and to generate "engineered" hypovirulent fungal strains with enhanced biocontrol potential. The development of a hypovirus transfection system has allowed the introduction of infectious hypovirus synthetic RNA transcripts into fungal pathogens other than *C. parasitica.* Moreover, these infections produced profound phenotypic changes, including hypovirulence. Combined, these basic and technical advances have provided new opportunities for broadening the potential application of hypoviruses for purposes of understanding and controlling fungal pathogenesis.

Increasing Fungal Pathogenicity Towards Insects

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We have developed molecular biology methods to elucidate pathogenic processes in the deuteromycete entomopathogen *Metarhizium anisopliae* and have cloned 53 cDNAs which are specifically expressed when the fungus is induced by physical and/or chemical stimuli to alter its saprobic growth habit, develop a specialized infection structure (the appressorium) and attack its insect host. We are testing the involvement of selected genes in pre-penetration growth and development (e.g. genes encoding protein kinases), cuticle penetration (protease) and post-penetration subduing of the host (toxins). In addition to gene disruption studies (for those activities which exists as only one or two gene copies) we are constitutively overexpressing genes in *Metarhizium* as possible means of speeding infection. We are also using *Metarhizium* genes to transform a weaker entomopathogen, *Aschersonia aleyrodis* and determine the effects on pathogenicity.
These experiments are allowing us to assess the genetic and biochemical factors which regulate/limit the degree of fungal pathogenicity to insects and provide new and important resources for manipulating microbial-plant pest interactions and for engineering advanced biopesticides.

Mechanisms of Resistance to Azole Antifungal Agents in the Human Fungal Pathogen *Candida albicans*

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Oropharyngeal candidiasis (OPC) caused by the human pathogen *Candida albicans* is a common infection in patients with the acquired immunodefficiency syndrome (AIDS). Azole antifungal agents, and

especially fluconazole, have been used widely to treat OPC in hospitals. An increasing number of cases of clinical resistance against this antifungal correlating with *in vitro* resistance have been reported recently. Our goal is to investigate the mechanisms of resistance to azole antifungal agents at the molecular level in *C. albicans* clinical isolates. We have shown in a recent study [1] that sequential fluconazole resistant yeast isolates from patients failed to accumulate [3 H]-fluconazole. This phenomenon was linked to an increase of mRNA for a recently cloned (ATP Binding Cassette) ABC-transporter gene called *CDR1* and of mRNA of a gene conferring benomyl resistance (*BENr*, the product of which belongs to the class of Major Facilitator multidrug efflux transporters. Therefore, in *C. albicans* clinical isolates, different multidrug transporters can be involved in the appearance of resistance to fluconazole and potentially to other azole derivatives such as itraconazole and ketoconazole. In fact, a mutant of the model yeast *Saccharomyces cerevisiae* lacking the ABC-transporter Stsl or C. *albicans* mutants lacking the ABC-transporter Cdr1 were hypersusceptible to the azole derivatives fluconazole, itraconazole and ketoconazole, thus showing that indeed these ABC-transporters can use these compounds as substrates. Surprisingly, the disruption of the *BENr* gene in *C. albicans* did not result in hypersusceptibility to azole antifungal agents. Other factors that could render *C. albicans* clinical isolates resistant to azole derivatives have been also characterized more recently in our laboratory: i) New multidrug transporters genes have been isolated, but their involvement in the resistance of clinical isolates to azole derivatives is still not clear. ii) Changes in the affinity of azole derivatives to their cellular target, i.e. a cytochrome P450 (*14DM*), have been also observed using a system enabling an expression screening of different *14DM* genes. Specific mutations in the *14DM* gene coding region altering the affinity of 14DM to azole derivatives have been mapped by this method.

[1] Sanglard, D., Kuchler, K., Ischer, F., Pagani, J.-L., Monod, M. and J. Bille (1995). Antimicrob. Agents Chemother. 39: 2378-2386.

Mechanisms of Fungal Pathogenesis

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It has long been hypothesized that fungal pathogens produce molecules which condition host tissues for colonization, and that other fungal molecules function to elicit resistance responses in cells of potential hosts. In recent years direct evidence in support of both types of molecule has been obtained. Thus, it can be predicted that the occurrence of disease is determined by the balance between susceptibility-producing vs. resistance-producing molecules in any given fungus/host interaction. The goal of this lecture is to briefly survey fungal factors known with reasonable certainty to be involved in fungal pathogenesis, then focus on a particular case study from our own laboratory, and finally indulge in the conjecture that future investigations will reveal common mechanisms of pathogenesis for fungal pathogens of both plants and animals.

A CASE STUDY: The *Tox1* locus of the haploid, filamentous ascomycete *Cochliobolus heterostrophus* determines the essential difference between the two forms of the fungus found in nature. Race T and race 0 are pathogenic to maize, but race T is distinguished by its extreme virulence to maize with Texas (T) male sterile cytoplasm, and by its ability to produce T-toxin, a family of linear polyketides which specifically affect T-cytoplasm. When the two races are crossed, only parental type progeny segregate, in a 1:1 ratio, thereby defining the single locus *Tox1* which controls T-toxin production and high virulence. We have investigated the molecular nature of *Tox1* to evaluate the role of T-toxin in pathogenesis and to determine the genetic mechanism underlying the evolution of a new pathogenic race. Insertional mutagenesis allowed the cloning and sequencing of three genes at the *Tox1* locus: *PKS1, DEC1,* and *RED1* encode a polyketide synthase (PKS1), a decarboxylase (DEC1), and a reductase (RED1), respectively. Site specific gene disruption revealed that *PKS1 and DEC1* are required for production of T-toxin and high virulence, whereas *RED1* is associated with no apparent

phenotype. We propose that PKS1 is needed to construct the T-toxin polyketide carbon chain and that DEC1 activates the molecule by removing the terminal carboxyl group. Surprisingly, although *PKS1* and *DEC1* are linked to *Tox1,* genetic and physical analyses have revealed that they are not linked to each other, and indeed are on two different chromosomes! The resolution of this apparent paradox is that *Tox1* maps to the breakpoints of chromosomes which are reciprocally translocated in race T with respect to race 0. Thus, *PKS1* is at a locus now called *Tox1A* on one of the translocated chromosomes, and *DEC1* and *RED1* are at a locus *(Tox1B)* on the other translocated chromosome. These three genes are found only in race T, and not in race 0 or in any other fungus examined to date. The data suggest an explanation for how race T evolved, ie., the *Tox1* locus was not inherited by race T from an ancestral strain but rather was transferred horizontally to *C. heterostrophus* from an alien organism; after insertion of this heterologous DNA into the genome, a reciprocal translocation occurred, resulting in "*Tox1*" residing on two different chromosomes.

Poster Abstracts, Differentiation

Neurospora crassa Has at Least Two Complex-type Chitin Synthases

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In *Saccharomyces cerevisiae,* most of the cellular chitin is produced by chitin synthase III, which requires the product encoded by the *CSD2/CAL1/DIT101/KTI2* gene. We have identified, isolated and structurally characterized a *CSD2/CAL1/DIT101/KTI2* homologue in the filamentous ascomycete *Neurospora crassa* and have used a "reverse genetics" approach to determine its role *in vivo.* The yeast gene was used as a heterologous probe for the isolation of a *N. crassa* gene (designated *chs4)* encoding a polypeptide belonging to the class of chitin syntheses, which we have designated class IV. The predicted polypeptide encoded by this gene is highly similar to those of *S. cerevisiae and Candida albicans. N. crassa* strains in which *chs-4* had been inactivated by the Repeat-Induced Point mutations (RIP) process grew and developed in a normal manner under standard growth conditions. However, when grown in the presence of sorbose (a carbon source which induces morphological changes accompanied by elevated chitin content), chitin levels in the *chs-4*RIP strain were significantly lower than those observed in the wild type. We suggest that CHS4 may serve as an auxiliary enzyme in *N. crassa* and that in contrast to yeasts, provide evidence for the presence of at least two complex-type chitin syntheses.

Vegetative Incompatibility in *Podospora anserina* Identification of Proteins Involved in Cell Death

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In the filamentous fungus *Podospora anserina,* the coexistence in one cell of two allelic or non-allelic incompatible *het* genes leads to cell lysis. The avaibility of an incompatible thennosensitive strain, bearing two non-allelic genes *(het-R* and *het-V),* led to the display of transcriptional and/or translational regulation of genetic expression and an increase of the proteolytic activity in cells undergoing cell death.
Cloning genes under transcriptional regulation was initiated. A cDNA library was enriched in sequences preferentially expressed in cells undergoing lysis. Part of this library has been used to screen a *P. anserina* genomic library. Expression of the first two genes identified, *lyt1* and *lyt2,* has been determined in other genetic backgrounds. No expression of *lyt1* is observed when the lytic reaction is suppressed by mutation in specific genes *(mod* genes). Under the same conditions, *lyt2* is expressed. The two genes might play different roles in the pathway leading to cell death. Expression of *lyt1* is also observed when two other non-allelic genes *(het-C and het-E)* are responsible for incompatibility. Under the same conditions, *lyt2* is not expressed. Some results obtained from the *het-R/het-* V incompatible reaction may be extrapolated to another non-allelic incompatible reaction. These different reactions exhibit common steps leading to cell death as it was suggested by the existence of common suppressor genes. *lyt1* product is involved in one of these common steps. Consequences of inactivation of *lyt* genes and of their constitutive expression are under investigation.
Cloning *P. anserina* proteases involved in the incompatibilty reaction was also initiated. Previous results showed that aspartylproteases were implicated, the most active protease being protease C. Their activities are absent when incompatibility is suppressed by mutation in *modA* gene. Thus we initiated cloning of the corresponding genes. *papA* gene encoding a *P. anserina* aspartylprotease has been cloned by heterologous hybridization with the major aspartylprotease

encoding gene of *Cryphonectria parasitica.* This gene has been inactivated. Comparative analysis of wild type strain and papa-inactivated strains by FPLC has allowed papa gene product identification. It doesn't correspond to protease C. No macroscopic modification of the incompatible reaction has been observed as a consequence of *papA* inactivation. Another aspartylprotease encoding gene has been isolated by low stringency hybridization with *papA.* Its sequence is in progress. Partial purification of protease C has been undertaken to establish a microsequence and to clone the corresponding gene.

Isolation and Microscopic Characterization of Oleate Non-utilizing Mutants in *Aspergillus nidulans.*

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Despite the limited information on the role of microbodies in filamentous fungi, recent studies have stressed the importance of these organelles. The final enzyme of the penicillin biosynthesis pathway has been located in microbodies of *Penicillium chrysogenum.* More recently, peroxisomes have been shown to be required during karyogamy in *Podospora anserina.* Isolation of peroxisomes biogenesis mutants in *A. nidulans,* could help in further improvements in antibiotic production as well as in determine the role of these organelles in the metabolism of filamentous fungi.
In order to isolate peroxisomes mutants in *A.nidulans,* we used a new positive method of selection recently utilized in *Saccharomyces cerevisiae.* This procedure is based in the lethality of H2O2 during the -oxidation of fatty acids in the presence of the catalase inhibitor 3-aminotriazole. Cells that do not accumulate H,O, as a result of, either a non-functional -oxidation system or ill-assembled peroxisomes are able to grow.
Using UV-light mutagenesis, 400 stable mutants resistant to 3-AT

were obtained. They were checked for their inability to grow in minimal oleic acid medium and their ability to grow in minimal medium plus glycerol or maltose. A total of 40 oleate non-utilizing (*olu*) mutants were isolated.
Complementation analyses through heterokaryons indicated that the *olu* mutants fall into 5 complementation groups. Preliminary characterization studies pointed out their inability to grow in acetate as sole carbon source, suggesting that the *olu* mutants do not seem to carry any lession in the -oxidation pathway. Since the selection method used in this work yielded peroxisome assembly mutants in *S.cerevisiae,* it is possible that lessions in our *olu* mutants are concerned with the biogenesis of peroxisomes. Electron microscopy studies in the wild-type and *olu* mutants are in progress and results will be presented.

Chemical Characterization of SC3 Hydrophobin

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The Class I hydrophobin SC3 of *Schizophyllum commune* self-assembles at hydrophobic/hydrophilic interfaces into a 10 nm, highly insoluble amphipathic film, thereby changing the wettability of surfaces. To understand the mechanism underlying the process of self-assemblage and to explore various technical/medical applications a chemical and structural characterization of SC3 is required.
The secreted protein contains 100-101 a.a. with 8 cysteines located in a characteristic pattern. Amino acid composition analysis agreed well with that predicted. A Cys-count analysis using iodoacetate/iodoacetamide mixtures confirmed the presence of 8 Cys residues per molecule. Since SC3 was found not to contain any free SH groups it is likely that all Cys groups are involved in disulphide

bridges.
SC3 is a glycoprotein binding ConA. TLC and GLC analysis after acid methanolysis showed the presence of 22-24 residues mannose per molecule, assuming a mass of 9.8 kD for the polypeptide. On the other hand, mass spectrometry showed an average mass of 14.2 kD, implicating 27 mannose residues assuming no other post-translational attachments. The mannose residues are likely linked to serine and threonine res. (25 in total) since no putative N-glycosylation site is present. They are mainly located at the hydrophilic side of the SC3 film after self-assembly as shown by XPS measurements.
Isoelectrofocusing revealed several isoforms: a major form with pI 5.4 (77 %), and minor forms with pl 4.9 (14 %) and pI 4.7 (1.5 %). The calculated pl of the unmodified protein is 6.8, indicating that SC3 contains acidic modifications.

The Promoter Region of the *nii*A-*nia*D Gene Cluster of *Aspergillus nidulans* contains a Meiosis-specific Recombination Initiation Site

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Genetic markers typically show a Mendelian segregation (2:2) in meiosis, but with a low frequency deviations are observed which, as a rule, affect only a single locus as adjacent loci usually segregate normally. The aberrant segregations 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 probably reflects the chromosomal search for homology during meiotic prophase 1, prior to pairing and subsequent disjunction of the homologues. The mechanistic details of the detection of DNA homology between chromosomes are not thoroughly understood, but current models incorporate the formation

of a heteroduplex tract between strands of nonsister chromatids that may include one or more mismatches and the physical linkage of the homologues by Holliday junctions. Aberrant segregations may originate in the repair of mismatches in the heteroduplex tract.
One characteristic of gene conversion is that the position of a marker within a locus is of strong influence on the frequency of its conversion. Typically a polarity of gene conversion is observed: markers on one side of the locus show a high gene conversion frequency in comparison with markers on the other side. This was observed genetically in many organisms, but practically all of the data correlating gene conversion frequency and physical position of a marker have been generated in the yeast *Saccharomyces cerevisiae.* Polarity of gene conversion is interpreted as a consequence of fixed initiation sites of heteroduplex formation and distance-dependent resolution. Markers located close to the initiation site have a higher probability to be included in the heteroduplex tract and thus to be converted. The initiation sites in the *ARG4* and *HIS4* genes of yeast (Petes *et al,* (1991) The Molecular and Cellular Biology of the Yeast *Saccharomyces,* Vol.1) and the *niiA-niaD* gene cluster of *A. nidulans (*Thijs *et al*. MGG (1995) **247** 343-350) are located in the promoter regions of these genes.
We have taken advantage of the excellent possibilities for molecular manipulation in *A. nidulans* and introduced a series of silent point mutations in the *nii*A-*nia*D gene cluster by *in vitro* mutagenesis. These mutations alter restriction sites to allow detection by restriction enzyme analysis. Data from two-point crosses show that meiotic recombination events leading to functional restoration of the *niiA* and *niaD* genes are initiated in the promoter region of the cluster. The recombination tracts do not appear to cover the length of the genes and mutations included in a tract are always co-converted. The initiation site located in the promoter region is meiosis-specific; the analysis of mitotic recombination events indicate there is no specific initiation site. Implications for the mechanism of initiation and resolution of recombination intermediates will be discussed.

Mutants in Development and Differentiation of *Coprinus cinereus*

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During life cycle, the Homobasidiomycete *Coprinus cineretis* switches from monokaryotic to dikaryotic cell growth in order to accomplish fruitbody development and sexual reproduction. Starting from a germinating haploid spore the monokaryotic mycelium is formed. It is uninucleate, has simple septa and produces asexual propagules, the so called oidiospores, on aerial hyphae. If two compatible monokaryons mate a dikaryon with two genetically different nuclei per cell and with clamp cells at each septum is formed. The sexual dikaryon represses oidiospore formation but may develop into fruitbodies where karyogamy and meiosis occur.
Asexual and sexual differentiation in *Coprinus cinereus* is controlled by the two mating type loci, *A* and *B*. Specific mutations in *A* and *B* can transform a monokaryon into a homokaryon that partly behaves like dikaryons. *AmutBmut* homokaryons are sexually fertile, form clamp cells and fruitbodies. Unlike dikaryons and similar to monokaryons, *AmutBmut* strains still produce oidiospores.
In our current project we use an *AmutBmut* strain of *C. cinereus* to study developmental processes in a homokaryotic background. Two different approaches were choosen to generate mutants in development. Oidiospores were either UV irradiated and or subjected to restriction enzyme-mediated DNA integration (REMI) by transformation. About 1200 UV treated colonies and 7500 REMI transformants were screened for both changes in oidiation and defects in fruitbody development. A range of specific mutations either in oidiation or in stages of fruitbody development were obtained.

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Light Mediated Development in *Aspergillus giganteus*

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Conidiation in the filamentous fungus *Aspergillus giganieus,* in common with many other fungi, is influenced by light. In this organism, two forms of conidiophore are produced in the light, short (<2mm) and long (2-20mm), while only short conidiophores are formed in the dark. In addition the long conidiophores developed in the light display positive phototropism.
To investigate the role of light on conidiophore development, conidia were mutated and resulting colonies screened by eye for altered conidiation phenotypes when grown in dark and light conditions. A number of mutant strains have been isolated, including white, yellow and pink conidial colour variants, and mutants similar in phenotype to the *wetA* and *abaA* developmental mutants of *A. nidulans.* In addition, four classes of mutants altered in their light response have been picked up;-

DARKGROWN	LIGHTGROWN
short only	very tall only
short+tall	short+tall
short only	short only
short only	aconidial tall only

We suggest that there is a balance between conidiophore elongation and conidial head formation, and that light promotes elongation and delays head formation. Mutants totally insensitive to light should have only short conidial heads and show no phototropism.
We have also isolated auxotrophic mutants suitable for use as transformation recipients and are using PCR and hybridisation approaches to identify the *brlA* gene.

Comparison of pheromone receptor genes in *Schizophyllum commune.*

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The mating type locus B l of *S. commune* contains a pheromone receptor and putative pheromone genes. The pheromone receptor B l shows homology to other pheromone receptors such as Ste2 and Ste3 of *Saccharomyces cerevisiae* and pral and pra2 of *Ustilago maydis.* It also contains sites conserved among other G protein-linked receptors of the seven transmembrane domain family. The fact, that the mating system in *S. commune* provides nine different allelic specificities at the mating type locus B makes it a model system to investigate ligand interaction between different allelic specificities of pheromones and receptors.
To approach the mechanism of selective pheromone binding we constructed chimeric receptors of the two allelic genes *bar1* and *bar2 by* exchanging the extracellular loops which are known to be involved in pheromone binding in other receptors.

Mutations Causing Chromosomal Aneuploidy *in Aspergillus nidulans*

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When a eukaryotic cell divides, a complete set of chromosomes must be segregated faithfully to each of two daughter cells. Errors in segregation result in aneuploidy, which often causes cell death and is implicated in oncogenesis and human birth defects.
We are using *Aspergillus nidulans* as a model system to identify proteins that monitor chromosome stability and ensure accurate partitioning of the genetic material. Conditional-lethal, heat-sensitive (42) mutants were assayed at sub-restrictive temperature (37) for an inflated production of aneuploid colonies (1). Strains of *A. nidulans*

mutant at one of several *hfa* (high frequency of aneuploidy) loci were found to be defective in the maintenance of ploidy during cell division, as shown by the generation of a high frequency of progeny exhibiting a spectrum of aneuploid phenotypes. A second group of mutations, designated *sod* (= stabilisation of disomy), produced progeny disomic for a specific chromosome (see accompanying poster, Whittaker et al.).
The *hfa* mutants have been characterised cytologically by fluorescence microscopy of DAPI- and anti-tubulin stained nuclei. For *hfaB3 and hfaL1,* aneuploid production can be attributed to defects in cell cycle control. The other mutants undergo apparently normal nuclear division at restrictive temperature and may represent defects in genes involved in spindle function or in monitoring cell ploidy as components of mitotic checkpoints. Data on the sensitivity of the mutants to DNA-damaging agents and spindle poisons will be presented and progress on cloning the genes will be discussed.
(I)Upshall & Mortimore (1984):Genetics 108, 107-21.

Genetics of *Rhizctonia solani (Thanatephorus cucumeris*)
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Rhizoctonia solani Kühn is the asexual form of the fungal species *Thanatephorus cucumeris* (Frank) Donk. *R. solani* is cosmopolitan in soils and is a destructive plant pathogen with a wide host range. The large variation between isolates of *R. solani* with respect to pathogenicity and growth characteristics and the concurrent lack of knowledge of the genetic basis of this variation make it very difficult to understand the population structure of this fungus. Isolates of *R. solani* are assigned to twelve anastomosis groups (AGs) based on the occurrence of hyphal fusion (anastomosis); isolates from the same AG anastomose, while in general isolates from different AGs are not able to anastomose.

Studies on the genetics of *T. cucumeris* have, been carried out in many of the AGs by studying the formation of heterokaryotic tufts arising in the area of contact between single-spore homokaryotic isolates. Until now only the genetics of AGs I and 4 is understood to a certain extent. It has been suggested that the genetics of AGs 2 and 3 may differ from that of AGs I and 4. We have studied the conditions for inducing the sexual stage *in vitro* of 76 isolates belonging to AGs 1, 2, 3 and 4. Homokaryons of AGs 1, 2 and 3 were obtained by growing sexual basidlospores into mycelium. To study the genetics of incompatibility, isolates were paired on cellophane placed over water agar and the area of contact between isolates was studied macro- and microscopically. Our first results reveal that:
- homokaryons from one AG anasiomose readily, but when homokaryons from different AGs are paired, no hyplial fusion takes place.
- heterokaryotic tufts form in the contact area when AG 1 homokaryons are paired in certain combinations, allowing to group the homokaryons in two mating types, but tufts are never observed when AG-2 or AG-3 homokaryons are paired.
- sexual and vegetative incompatibility are two different mechanisms that operate simultaneously in *T. cucumeris AG*- 1. Vegetative incompatibility does not prevent tuft formation.

Regulation of Asexual Spore Formation in the Homobasidiomycete *Coprinus cinereus*

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The basidomycetous fungus *Coprinus cinereus* undergoes a life cycle with transitions between two types of mycelia. The asexual homokaryon has simple septa and forms vegetative spores (oidia) on aerial byphae. The sexual dikaryotic mycelium, by contrast, has

clamp cells at each septum. It arises after fusion of two compatible monokaryons or a monokaryon with a compatible germinating oidium. The dikaryon differentiates under appropriate environmental conditions (light, temperature and nutrition) into a fruitbody in which the meiotic basidiospores are produced.

Differentiation processes in C. *cinereus* are controlled by two distinct mating type loci, *A* and *B*. To form a sexual dikaryon two nuclei with different *A* and different *B* specificity have to be present in the same cell. Specific activating mutations in both mating type loci lead from an asexual monokaryon to a homokaryon that shows growth characteristics of a dikaryon (formation of clamp cells) and produces fruitbodies in which caryogamy and meiosis occurs. These *AmutBmut* strains, however, still produce oidia typical of monokaryons. In dikaryons, oidia production is repressed by the *A* mating type locus.

In the work presented we have studied the effect of different environimental conditions on oidia formation in monokaryons, dikaryons and *AmutBmut* homokaryons. All investigated monokaryons produced oidia in the dark. The level of oldia produced was not significantly influenced by light. In contrast, spore production in *AmutBmut* strains was repressed in the dark, but oidia production could be induced by light at various temperatures. At 37 C, induction was at the same level as found in monokaryons. Light induction was seen already after 1 ininute of illumination. However, several hours of light treatment were required to reach the maximum of oidiation. A certain amount of light induction was also observed in all *Amut B* strain. This suggests that light induction must override the effect of *A* repression on oidiation over a range of temperatures, as well as that it induces fruitbody formation in dikaryoiis and *AmutBmut* strains at a temperature of 25 C.

The fact that oidiation is induced by light in *AmutBmut* homokaryons is not only interesting for developmental Studies but is also of practical value in preparing protoplasts for DNA transformations.

The A Mating Type Factor of *Coprinus bilanatus* Consists of Two Subloci Encoding Homeodomain Proteins

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Coprinus bilanatus is a two-spored secondarily homothallic basidiomycete with a multiallelic bifactorial breeding system. For dikaryon formation nuclei with different *A* and *B* mating type factor specificities have to come together either by mating or by inclusion of two distinct nuclei in a single basidiospore. *A and B* regulate different steps in the development of a dikaryon. *A* is responsible for synchronised nuclear division and clamp cell formation, *B* for nuclear migration and clamp cell fusion. To date seven distinct *A* and seven distinct *B* mating type specificities have been identified in four different collections of the species.
The *A1* factor of *C. bilanatus* was identified by homology to the *mep* gene of *C. cinereus* which flanks its well characterised *A* locus. Molecular analysis of the *C. bilanatus A1* factor revealed structural similarities to the *A* factors of *C cinereus and S. commune.* There are two closely linked subloci encoding two types of proteins with homeodomain motifs (HDI and HD2). Homology to other *A* factors of the same species is low. Localisation of the *mep* gene defines the - sublocus. The *pab1* gene which was found by chromosome walking is about 40 kb apart from *mep* and the sublocus.
Reciprocal heterologous expression of the *C bilanatus A1* factor and the *C cinereus A42* factor have been demonstrated using transformation. However, not all individual genes elicit sexual development in their respective heterologous host. Active interactions of *A* factor products are formed between compatible HDI and HD2 proteins. Heterologous expression of individual mating type genes provide evidence for interactions between HDI products of *C bilanatus* and HD2 products of *C. cinereus.* Attempts to heterologously express the *C. bilanatus* HD2 and C *cinereus* HDI genes have not proved successful; this may be due to inactivity of

individual genes in the respective heterologous host or, alternatively, there miglit be no recognition between products of HD2 genes of *C.bilanatus and* HD I genes of *C cinereus.*

Hydrophobin rodlet layers in *Agaricus bisporus* *and* *Schizophyllum commune* fruit bodies

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The hydrophobins ABHI and SC4 typically occur in the fruit bodies of *Agaricus bisporus* and *Schizophyllum commune* respectively. Both hydrophobins assemble into an SDS insoluble amphipathic membrane at hydrophilic-hydrophobic interfaces. When this occurs at a water-air interface the hydrophobic side of the membrane exhibits the typical ultrastructure of rodlet fascicles.
In *S. commune* SC4 is immunologically detected inside the fruit bodies, lining air channels present in the plectenchyma. Rodlets can be seen on freeze fractured surfaces from the plectenchyma. In *A. bisporus, ABHI* was immunologically localized primarily at the outer surface of the fruit body, conferring a high hydrophobicity to this surface (contact angles up to 120 deg) but was present also throughout the fruit-body plectenchyma. Since rodlets were seen both at the outer surface and at the surface of freeze-fractured fruit bodies, we surmise that in *A. bisporus* too, air channels are lined with a hydrophobin membrane exposing its hydrophobic side towards the air. This would prevent these air cavities and channels to collapse and become flooded with water. In addition, we propose that these hydrophobins may play a role in early aggregation of hyphae during fructification.

The Putative RNA-helicase Encoded by the *Pah-4* Gene Is Essential for Viability of the Ascomycete *Podospora anserina*

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RNA-helicases are known to be involved in various cellular processes such as translation, ribosomal biogenesis, cell growth and development. They belong to the so-called DEAD-box family of proteins, which are widely distributed among a variety of organisms ranging from bacteria to human (Linder et al., 1989).
We have identified four genes from the ascomycete *Podospora anserina* encoding typical DEAD box proteins by PCR screening using degenerated primers derived from two highly conserved regions of the protein family. One of these genes, *pah-4,* was characterized in detail. The putative PAH-4 protein shows high homology to the RNA-helicases DED1 from *Saccharomyces cerevisiae* and PL10 from mouse. The yeast gene was originally isolated as a nuclear suppressor of a nuclear pre-mRNA splicing defect (Jamieson et al., 1991) and seems to play a role in initiation of translation (Chang et al.,1995). The PL10 protein of mouse was found to be involved in spermatogenesis (Leroy et al., 1989).
In order to examine the biological role of the putative *Podospora* RNA-helicase, we have disrupted the gene by homologous recombination. However, viable homokaryotic spores isolated from back-crosses of pah-4 mutants to the wildtype strain were exclusively found to carry the wildtype gene. Moreover, under conditions selecting for the inactivated gene, heterokaryotic mycelia show an altered phenotype and sexual development is impaired. These results indicate that the PAH-4 protein is essential for the life cycle of the fungus.
We are now planning to restore the phenotype of the heterozygote by complementation with a helicase gene displaying a high homology to the *Podospora* gene.

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Chang, T. H., Chuang, R. Y. & Weaver, P., Poster presented at the RNA Processing Meeting, CSH, May 17- 21, 1995

Disruption of *Rco-3,* a Gene Involved in Glucose Sensing in Neurospora Abolished the Glucose Repression Control and Altered Conidia Development

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Conidiophore development in *Neurospora* is regulated *by rco-3,* a gene that shares homology with hexose transporters. Disruption of *rco-3* gene in the wildtype strain by gene replacement, resulted in phenotype similar to the *rco-3* strain. Growth in media containing non-glucose carbon sources did not suppress conidiation in submerged culture. However, a rich medium containing peptone did prevent development. Glucose transport studies suggested alterations in the kinetics of glucose uptake of the repressed rco-3 cells. Moreover, qa-2, a glucose repressible gene is consititively expressed in *rco-3.* The properties of the mutant suggest that *rco-3 is* primarily involved in glucose sensing. Mutation in *rco-3* resulted in signaling for starvation in nonlimited glucose levels, loss of glucose repression and altered conidia development. Our results indicating that conidiation in *Neurospora* is under glucose repression.

Cloning, Characterization and *in Vivo* Inactivation of the Phenoloxidase Genes of *Podospora anserina*. What Role Do They Play in Morphogenesis, Pigmentation and Alternative

Respiration?

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Podospora anserina is a filamentous fungus that produces four different phenol oxidase enzymes: laccases 1, II and III and one tyrosinase (MOLITORIS & ESSER, 1971). There are several developmental mutants of *P. anserina* which show abnormalities in their phenol oxidase expression, raising the question whether these enzymes play a role in morphogenesis. Additionally, most of these mutants are incapable of using the alternative respiratory pathway, which suggests a link between alternative respiration and phenol oxidases (FRESE & STAHL, 1992). Indeed, most of these nuclear mutants were originally isolated as phenol oxidase-negative mutants (PRILLINGER, 1976). Although these mutations cause similar defects, they are located on different chromosomes, indicating that the primary defect is not in the phenol oxidase genes but is caused by more general defects. For example, ihe mutant *grisea* has a defective copper metabolism, resulting in reduced female fertility and no detectable phenol oxidase activity, phenol oxidases being copper dependent enzymes (MARBACH & STAHL, 1994). With these regulatory mutants it is difficult to clarify the biological function of phenol oxidases in *P. anserina.*

We used a "reverse genetic approach" to obtain some insight into the function of the above laccases and tyrosinase in *P. anserina.* The laccase II gene and the tyrosinase gene have both been cloned and characterized on the molecular level (FERNANDEZ-LARREA, 1993). These genes were then inactivated *in vivo* and the transformants analysed with regard to differentiation, alternative respiration and pigmentation. The biological function of laccase II and tyrosinase will be discussed in respect to these mutants.

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Transformation and Karyotype Analysis of a Developmental Mutant from *Sordaria macrospora*

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Sordaria macrospora is a filamentous ascomycete, which is closely related to *Neurospora crassa* and *Podospora anserina.* In contrast to the latter species *S. macrospora* is homothallic and shows no incompatibility. After apandrous fruit body development, asci with eight linearly arranged ascospores are formed, which can be used as ordered tetrads for a genetic analysis (1). Several developmental mutants impaired in perithecial or ascospore formation were isolated using UV mutagenesis. In this contribution genetic analysis of a mutant, showing a defect in fruit body development, is provided. This mutant was named *prol* (protoperithecia) and was further characterized by electrophoretic karyotyping. Using CHEF gel electrophoresis we succeeded in separation of seven chromosomal bands, with a total size of 38 Mb. In addition, nuclear genes *(pro1-, rDNA-, tubA-, ura3-, ura5-* and mating-type-genes) were mapped on chromosomal bands using homologous gene probes. Using a recently established transfomation system (2) molecular tagging was carried out for ectopic integration of vector pRP81-1 (3) into genomic DNA. Chromosomal mapping of the integrated vector molecules was performed by Southern hybridization of chromosomal DNA separated by pulsed-field gel electrophoresis. Genetic analysis shows close linkage of a chromosomal marker with mutation *prol.* In a further approach mutation *pro1* was complemented to fertility by transformation with a wild-type cosmid library. We defined the complementing region of the cosmid clone to a 2,55 kb DNA-fragment.The molecular characterization of mutant *prol* allows identification of gene products involved in fruit body morphogenesis

in ascomycetes.
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Differential Expression of the Vegetative and Spore-bound Hydrophobins of *Trichoderma reesei*

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We have characterized hydrophobins from the cellulolytic filamentous fungus *T. reesei* and previously reported the isolation of the *hfb*1 gene. The gene is expressed in vegetative glucose-containing cultures and the protein is secreted into the culture medium. It is also found in aggregated form in fungal cell wall wherefrom it can be extracted with trifluoroacetic acid- acetonitrile solution. As a typical hydrophobin, HFBI also aggregates on air-liquid interfaces and by freezing of HFBI containing solutions. A second hydrophobin gene, *hfb*2, was isolated by heterologous hybridization using the *hfb1* gene as a probe. The HFBII protein encodes a protein of 71 amino acids (mature protein) that has a high amino acid similarity towards HFBI. The HFBII proteins was isolated from the fungal spores and it was also present in the culture medium of vegetative lactosegrown cultures. Expression of the *hfb*1 and *hfb*2 genes is divergent. *hfb*1 expression was only observed in vegetative cultures on glucose- and sorbitol-containing media. It was not found on media containing complex plant polysaccharides, cellulose, xylan, cellobiose or lactose, whereas *hfb*2 was highly expressed in vegetative cultures on these media. Expression of *hfb2*

was also strongly induced by N- and C-starvation, by light and in conidiating cultures.

Mating-type Genes in the Homothallic Ascomycete *Sordaria Macrospora*

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In the homothallic ascomycete *Sordaria macrospora* (Pyrenomycetidae, Sordariaceae) a single ascospore gives rise to hyphae that are able to enter the sexual reproductive pathway and to produce fruiting bodies (perithecia) that enclose the meiotic ascospore progeny. Sexual reproduction in heterothallic ascomycetes is similar to that of *S. macrospora* but it differs in that a haploid ascospore is not capable of completing the sexual cycle. Heterothallic species of the Sordariaceae like *Neurospora crassa and Podospora anserina* are composed of two mating type populations A and a or mat- and mat+, respectively and mating occurs only between sexual structures of opposite mating-type. The sequences conferring the mating-type behaviour in *N. crassa* and *P. anserina have* been cloned and characterized. They consist of dissimilar DNA sequences (idiomorphs), which are present at a single locus in haploid strains (1,2,3). Total DNA of *S. macrospora* was probed with A and a mating-type sequences of *N. crassa* and as was already shown (4), *S. macrospora* contains single copy sequences from both A and a idiomorphs.
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.* For further investigation of the functional conservation of the *Sordaria* mating-type genes we transformed the

mating-type DNA into mat- and mat+ strains of the closeley related fungus *P. anserina.* The mating-type genes of *Sordaria* were partially able to complement fruiting body formation in both mat- and mat+ strains of *P. anserina.*

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Gene Isolation from *Sordaria macrospora* Using an Indexed Genomic Cosmid Library

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Numerous mutants with defects in sexual morphogenesis have been isolated from the homothallic ascomycete *Sordaria macrospora* (Pyrenomycetidae, Sordariaceae) (1).
So far, genes responsible for these mutations have not been cloned and sequenced. To isolate these fungal genes, either by complementation or by chromosome walking we constructed a cosmid library of the *S. macrospora* genomic DNA using the double-cos-site cosmid vector pANsCosl (2). The average insert size of recombinant cosmid clones carrying fungal genomic DNA is about 38 kb. To improve the efficiency with which genes complementing a particular mutation can be isolated, we have established an indexed cosmid library of 4224 individual clones contained in the seperate wells of 44 microliter plates. Rapid screening methods with cosmid DNAs pooled from individual microliter dishes have been applied successfully to isolate the *S. macrospora ura5* gene encoding the orotate phosphoribosyl transferase and the *tubA* gene encoding -

tubulin. For further analysis, the *ura5* gene was used for genomic complementation of a *S. macrospora* mutant bearing an inactive *ura5* gene.

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Reverse Transcriptase Activity of Group II Introns in Mitochondria of Senescing Mycelia of the Ascomycete *Podospora anserina*

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The mitochondrial genome of the filamentous fungus *Podospora anserina* race sl contains two group IIA introns: intron *cox1-I1,* which is the first intron of the *cytochrome-c-oxidase* subunit I gene *(coxl),* and intron ND5-14, which is the fourth intron of the *NADH-dehydrogenase* subunit 5 gene. The *cox1* gene of race A contains an additional group IIA intron *(coxl-14).* In senescing cultures of *P. anserina* the complete *coxl*-I1 accumulates as a circular plasmid (p1DNA or -senDNA). The introns complete *cox1*-I1 and *coxl*-14 but not *ND5*-I4 integrate at their homologous position of the corresponding intron-containing gene leading to tandem repeats of the introns (Sainsard-Clianet et al. 1994, R. Sdgebarth, unpublished results).

As discussed for the integration of group II introns into intronless alleles during crosses (intron homing) in yeast (Kennell et al., 1993), this mobility very likely depends on the reverse trancriptase activitiy of the intron encoded protein.

Since p1DNA and the spliced intron lariat *coxl*-Il accumulates during the course of aging, we are planning to investigate, whether this accumulation also results in an increase of RT activity.

Therefore, mitochondrial ribonucleoprotein (RNP) particles from young, middle-aged and old mycelia of race A and s were prepared

and the RT activity was assayed by digesting the endogenous RNA template with RNase A and using the artificial template-primer substrate poly(A)-dT18.
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Hyphae of the thn Mutant of *Schizophyllum commune* Have a Different Cell-wall Biosynthesis than Wild-type Hyphae

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Development of aerial structures in the basidiomycete *Schizophyllum commune* is regulated by the THN I gene.
A frequently occurring spontaneous mutation in this gene prevents formation of aerial mycelium in a monokaryon and, if present in both nuclei, formation of aerial hyphae and fruit-bodies in a dikaryon, Genes specifically expressed during formation of aerial structures (e.g. hydrophobin genes) are not expressed in mutant colonies. These colonies are further characterized by very characteristic wavy (sometimes corkscrew-like) hyphae having a larger diameter than normal hyphae, by a faster radial growth rate than wild-type colonies (although the biomass of a *thn* colony is only about half of that of a wild-type colony) and by producing a pungent smell. It was observed that the tips of *thn* hyphae are easily damaged by mechanical stress not harmful to wild-type hyphae. In agar-grown cultures thn hyphae quite frequently showed balloon-like vesicles out of which sometimes hyphae can start growing again. These balloons were not observed in liquid cultures and only very rarely in wild-type colonies. Addition of Congo red (binding to non-cross-linked B-1,4linked sugars) to the medium increased the number of *thn* hyphae ending in these balloons dramatically whereas this was not seen in wildtype hyphae. Calcofluor white (binding non-cross-linked B-1,4-linked sugars) and aniline blue (reacting with B-1,3 glucan) stained a far

larger part of the apical region of thn hyphae than of wild-type hypliae and also showed dispersed staining in more subapical parts of *thn* hyphae. All these observations indicate a difference in the cell-wall biosyntliesis of thn hyphae as compared to wild-type hyphae. Further studies have to show whether this is caused e.g. by a problem with cross-linking of secreted non-polymerized cell-wall components in cell-walls of *thn* hypliae or by altered secretion of certain cell-wall components at the hyphal tips of *thn* hyphae.

apsA and apsB, **Two Genes for Nuclear Positioning in** ***Aspergillus nidulans***

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Nuclear migration is very important in many eukaryotic cells and absolutely crucial for apical extension of fungal hyphae. Microtubuies and the microtubule dependent motor protein dynein are essential components for the translocation process. Nothing is yet known about the regulation of the basic machinery and its coordination to other cellular functions, e.g. cell cycle, cell differentiation and morphogenesis. However, in *Aspergillus nidulans* two mutants have been isolated in which nuclear positioning is affected, suggesting an effect on the regulation of the process. In these mutants nuclei are clustered in hyphae in contrast to evenly distributed nuclei in the wild type. The nuclear positioning defect has a dramatic effect on asexual development. Metulaeremain anucleate and thus do not proliferate. Occasionally, nuclei enter metulae, development proceeds and thus single chains of conidia are generated.

The two corresponding genes, *apsA* and *apsB* (anucleate primary sterigmata) were cloned and sequenced. *apsA* encodes a 180 kD coiled coil protein with similarity 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 epitope tagged version of the protein were detected in Aspergillus protein extracts with polyclonal or monoclonal antibodies respectively. *apsB* will be further analyzed at a molecular level. Expression studies of *apsA* and *apsB* on the transcript and the protein level are under way.

PCR Cloning of Chitin Synthase Genes from the Cultivated Mushroom *Agaricus bisporus*

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Mushroom fruit bodies exhibit considerable expansion during morphogenesis. Cell wall biosynthesis is linked to chitin synthase activity. PCR of *Agaricus bisporus* genomic DNA with degenerate primers based on fungal chitin synthase gene sequences ainplified two fragments of 960 and 680bp. Southern hybridisation analysis of these putative chitin synthase gene fragments with a chitin synthase 2 clone from *Candida albicans* yielded positive signals. The chitin synthase gene fragments from *A. bisporus* were individually gel purified and cloned into vector pCRII using the TA cloning kit. Nucleotide sequences of these fragments were determined and analysis of the sequence data indicated homology between the *A. bisporus* 680bp fragment and the chitin synthase 2 gene from other fungi. Preliminary screening of a genomic library yielded positive signals; results of further screening and expression analysis will be presented.

Meiosis in *Aspergillus nidulans,* Cytology of Wild Type and Mutants

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The cytology of meiosis in *Aspergillus nidulans* was studied by haematoxylin staining and by acriflavin fluorescence. This study was undertaken to describe normal meiosis in this fungus and to characterise mutants blocked in meiosis. Such mutants are of' interest to identify and to clone genes that have a particular role in the progress of meiosis, either as differentiation genes or as genes involved in the process of recombination.
The meiotic cells of *A.nidulans* are small compared to other well studied fungi like e.g. *Neurospora crassa* and *Sordaria macrospora.* The sequence of events in meiosis could clearly be established and appeared not to differ significantly from that described for other fungi.
A *uvs*C mutant, defective in mitotic and meiotic recombination, was studied and was shown to be blocked at meiotic prophase 1. (Characterization of the *uvsC* gene revealed the homology to the yeast Rad5l gene, see poster by D. van Heemst et al). Specific meiotic mutants were isolated on basis of absence of mature ascospores in well developed cleistothecia (fruiting bodies). A *mei*A1 Mutant appeared to be blocked at anaphase 1. The cytology of wild type and mutant meiosis will be shown and discussed.

Isolation of meiosis specific genes from *Aspergillus nidulans.*

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A first step towards a molecular analysis of meiosis in any organism, is the cloning of meiosis specific genes from that organism. In order to clone genes involved in meiosis in the filamentous fungus *A. nidulans* several different strategies can be followed. The strategy

that will be discussed here, is transformation complementation of mutants defective in meiosis. In *A. nidulans* two classes of such mutants exist.
T'he first class consists of mutants that are both defective in the repair of DNA damage as well as in meiosis. Mutants in the A. *nidulans uvsC* gene have such a phenotype. We will describe the cloning of the *uvsC* gene of *A. nidulans* by transformation complementation of an *A. nidulans uvsCII4* mutant. Sequence analysis of the smallest fragment still giving full complementation, revealed strong homology of the predicted protein sequence with all known RAD51 homologs.
T'he second class are meiosis specific mutants. Since these were not already available in *A. nidulans,* we have set up a screening to isolate such *mei* mutants. One of them, *meiA1,* has been cytologically characterized and seems to be blocked at one of the later stages of the first meiotic division. We will report the cloning and characterization of the corresponding wild type *meiA* gene, which we are currently undertaking.

Targeted Mutation of the *Sc3* Gene of *Schizophyllum commune* Suppresses Formation of Aerial Hyphae

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Correlative evidence suggests that in *Schizophyllum commune* activation of the *SC3* hydrophobin gene is necessary for formation of aerial mycelium. Here we report disruption of the *SC3* gene by homologous integration of an *SC3* genomic fragment interrupted by a phleomycin resistance cassette. Two strains with a targeted mutation in the *SC3* gene were obtained in a sample of 163 transformants by screening for the absence of SC3 secretion followed by Southern analysis. The phenotype of the mutants is particularly clear in sealed plates in which no aerial hyphae are formed at all. In open plates the

mutants do form aerial hyphae which are however hydrophilic and not hydrophobic as in wild-type strains. In a dikaryon which is homozygous for the *SC3* mutation normal fniit bodies are produced but aerial hyphae formed by this dikaryon are hydrophilic. Dikaryon-specific hydrophobins (SC1, SC4, SC6) are apparently unable to substitute for the SC3 hydrophobin. Complementation of a disniptant strain with an *SC3* genomic clone restores formation of hydrophobic aerial hyphae in sealed plates. Currently we are working on the disruption of the dikaryon-expressed hydrophobin genes *SCI, SC4 and SC6* by using a double selection system as is also used in mammalian systems and has been described for *Neurospora crassa.*

Reversible Dimorphism and Growth Behaviour of the thermoresistant yeast *Arxula adeninivorans* Ls3

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Arxula adeninivorans Ls3 is an anamorphic, ascomycetous, arthroconidial and xerotolerant yeast, ,which was selected from wood hydrolysates in Siberia. This yeast is able to grow at temperatures as high as 48 C in minimal salts medium or yeast-extract-peptone-medium with glucose or maltose as carbon source. A morphological change from yeast to mycelia can be induced by temperature shifts and is accompanied by an altered gene expression programme This dimorphism is reversible and the mycelia can be induced at a cultivation temperature of 43 C or higher. Depending on the morphology of the strain Ls3 (yeast phase or mycelia) the secretion behaviour as well as the spectrum of polypeptides accumulated in the culture medium is altered. Besides higher concentrations of secretary proteins, the activities of the accumulated extracellular enzymes glucoamylase and invertase, were 2 to 3 times higher in cultures grown as yeast cells than in those grown as mycelia.

Role of the *sodVIC* Gene in *Aspergillus nidulans*

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Strains of *Aspergillus nidulans* carrying the *sodVIC1* (=stabilisation of disomy) mutation are unable to grow at the restrictive temperature of 42 (1). However, the mutation is only conditionally lethal, allowing growth at the sub-restrictive temperature of 37 . Upon transfer from permissive to sub-restrictive temperature, *sodVIC1* strains produce stable disomic sectors carrying an extra copy of chromosome VI. Subsequent downshift to permissive temperature causes immediate reversion to the haploid state. Study of the *sodVIC1* mutation, and others affecting the maintenance of ploidy such as *sodVIAl (1), sodVI BI* (1) and the *hfa* family ((2), see accompanying poster, Hughes & Assinder), will allow further insight into cell cycle controls ensuring high fidelity of chromosome segregation during mitosis.

The *sodVIC1* mutation has been characterised cytologically by fluorescence microscopy of DAPI-stained nuclei. Asexual conidia arrest at restrictive temperature with 1-2 nuclei and fail to produce a germ tube. Temperature shift experiments indicate that the germination block is reversible up to 4-5 hours but that more prolonged incubation at restrictive temperature is lethal.

The *sodVIC1* gene has been cloned by complementation using a chromosome VI-specific cosmid library (3). DNA sequence data for genomic and cDNA clones will be presented.

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Membranous Vesicles (Chitosomes) Are Involved in Chitin Synthase Compartmentalization and Trafficking in *Neurospora crassa*

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Polyclonal anti-chitin synthase antibodies raised against the *Saccharomyces cerevisiae CHS2* gene product were used to identify and localize chitin synthase in the filamentous ascomycete *Neurospora crassa.* A single band of approximately I 10 kDa was observed in Western blots of total protein extracts of *N. crassa,* probed with these antibodies. However, several additional bands were labeled when membrane fraction proteins (microsomes) were probed. Histo-immunochemical localization of chitin synthase confirmed that the polypeptide is compartmentalized in membranous vesicles (chitosomes), which are highly abundant in the vicinity of the hyphal tip. TEM analysis did not reveal chitin synthase in the plasma membrane. However, dense labeling of membrane-associated chitin synthase was observed by light-microscopic analysis of *N. crassa* protoplasts and at young hyphal tips.

Poster Abstracts, Biotechnology

Endoproteolytic Processing of Fusion Proteins by *Aspergillus niger*

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Optimised secretion of heterologous proteins from filamentous fungi increasingly resorts to the use of translational fusion constructs whereby the gene encoding the target protein is fused downstream of the gene encoding a naturally well-secreted carrier protein such as glucoamylase. The increased yields that result are thought to be due to a combination of factors relating to the carrier protein, e.g. improved mRNA stability and eased passage of the protein through the secretary pathway. A successful strategy for separation of the target protein from its carrier protein has been the incorporation of a DNA sequence encoding a dibasic amino acid recognition site for a kexin-like endoprotease at the fusionjunction. Cleavage occurs intracellularly and the two proteins are secreted separately. We have examined the specificity of this cleavage in *Aspergillus niger* in order to be able to predict the chance of processing at the correct site when a new target gene is expressed in a fusion construct. We used a truncated glucoamylase (sequence to amino acid 498) as the carrier protein, with a dibasic cleavage site Lys Arg at the junction with three different target proteins: hen egg white lysozyme (HEWL), the glucoamylase starch-binding domain (SBD) and bovine pancreatic trypsin inhibitor (BPTI). Cleavage sites were determined by a combination of Nterminal sequencing and electrospray mass spectrometry. The SBD was cleaved from the fusion entirely at the correct site. BPTI cleavage occurred at three sites in addition to the target processing site. The proportion of correct cleavage was increased by expressing mutant BPTI genes designed to make the BPTI resemble anti-elastase, suggesting that BPTI binds to the (major) kexin protease and interferes with its activity. Wild-type HEWL was cleaved entirely at the correct site. However, shortening the region of HEWL immediately Nterminal of the first et-helix by two amino acids caused the site of cleavage to be moved two (56%) and three (44%) residues away from the HEWL Nterminus. This suggests that correct processing requires a minimal distance away from a region of ordered structure such as an ot-helix, in addition to a preferred dibasic amino acid sequence. Cleavage of other mutant

HEWLs is being studied to test this model.

Approaches Towards Cloning of Genes from *Claviceps purpurea* Expressed during Alkaloid Metabolism

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During the infection process *C. purpurea* forms sclerotia on cereals, e.g. rye. Sclerotia are dormant structures which contain pharmacologically active ergot alkaloids. These compounds are used in the treatment of a variety of clinical conditions as for example migraine and Parkinsons disease. The genetics of the biosynthesis is not known in detail. In order to isolate genes involved in ergot alkaloid biosynthesis a differential screening of a cDNA library was performed with cDNA from alkaloid producing and non-producing mycelia (cDNA+/cDNA-) as probes, resp..Several genes could be isolated by this method: e.g. the Dimethylallyltryptophan Synthase which catalyses the first pathway specific step of ergot alkaloid biosynthesis (1); a gene showing homology to a circadian clock regulated, light and stress induced gene (*ccg-1*) of *Neurospora crassa* (2); a gene (isolated several times) showing significant homology to hydrophobins or hydrophobic proteins, resp., like cryparin, an abundant cell-surface protein from *Cryphonectria parasitica* (3) and QID 3, a cell-wall surface protein of *Trichoderma harzianum* (4). These results show that this method yields not only genes of the alkaloid pathway, but obviously also genes which might be involved in the differentiation process accompanying induction of alkaloid synthesis, i.e. formation of "sclerotial" hyphae.

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Probes for Fungal Polyketide Synthase Genes

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Polyketides are natural products derived from the successive condensation of small carboxyhc acids; assembly of the initial carbon skeleton is catalysed by an enzyme known as a polyketide synthase (PKS). Many of the polyketide metabolites so far identified are products of the fungi, particularly of the Deuteromycetes. Several of these compounds are of pharmacological interest or mycotoxins and some fungal melanins have a polyketide origin. All fungal PKS enzymes characterised have been large multi-functional proteins (MV I PKS).
We observed that the available fungal PKS gene sequences could be arranged into two subgroups on the basis of amino acid sequence conservation in functional domains. Two pairs of PCR primers (LCl/2c and LC3/5c) were designed to bind to regions where amino acid sequence was conserved within these subgroups but not between them. These primers amplified corresponding fragments containing the condensing domains from a range of fungal PKS genes in Deuteromycete genomes. The products from one primer pair hybridise strongly to each other, but not to products from the other primer pair. Each type of PCR product shows a different pattern of homologous hybridisation to the fungal genome from which it was

derived. This pattern of hybridisation may indicate two subclasses of PKS genes, both widely distributed among the filamentous fungi. The PCR products obtained using the LC primers described above should provide useful homologous probes for cloning novel fungal PKS genes.

Development of Heterologous Protein Secretion Systems in Filamentous Fungi

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This work aims to develop efficient heterologous protein secretion systems in filamentous fungus *Trichoderma reesei* . This can only be achieved by use of high performance strains and expression vectors. Suitable host strains have been obtained after several rounds of chemical mutagenesis and selection for high level secretion of various heterologous proteins. Vector construction strategy is based on the use of fusion proteins which is the most efficient system to date for secretion of heterologous proteins. The model systems in this study use *A. niger* glucoamylase (GlaA), *T. reesei* cellobiohydrolase I (CBHI) or the prokaryotic phleomycin resistance protein (Sh-ble) fused in 5' to human lysozyme (HLys). These carrier proteins have already been shown to be efficiently secreted by fungi. *GlaA::HLys, CBHI::HLys and Sh - ble::HLys* fusion vectors have been constnicted with or without introduction of modifications to the carrier protein. HLys secretion efficiency has been assessed in *T. reesei* strains endowed with different properties with respect to secretion. Findings could be applied to the production of other human proteins, commercially valuable, in *T. reesei* as well as in other fungi.

Expression of the Extracellular Aspartic Proteinase in *Penicilljum roqueforti*

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Filamentous fungi can grow under very varying environmental conditions and utilize a wide variety of compounds as nutrients. The acquisition and metabolism of the large number of different carbon and nitrogen sources are under sophisticated regulatory controls governed by several regulatory circuits, involving both wide-domain and pathway-specific regulatory genes. *Penicillium roqueforti,* an economically-important fungus secretes a number of enzymes including endo and exopeptidases. The aspartic proteinase is the major extracellular proteolytic activity in *Penicillium roqueforti.* We have cloned the gene *aspA* encoding the aspartic proteinase using a PCR probe. Nucleotide sequence data revealed that *aspA* is composed of three exons of 325, 526 and 340 bp. Two introns which interrupt the coding sequence are 57 and 67 bp in length. The deduced amino acid sequence of *aspA* presents a high degree of homology to other fungal aspartic proteinase and indicates that this aspartic proteinase is synthesized as a zymogen containing an Nterminal prepro-region of 71 amino acids followed by a mature protein of 326 amino acids. Southern blot experiments reveal that the *aspA* gene is present as a single copy in the *Penicillium roqueforti* genome.

Regulation of the aspartic proteinase production was investigated using biochemical and Northern analyses. Our results suggest that *aspA* expression is submitted to transcfiptionnal regulations. ASPA is not controlled by glucose and not produced in presence of ammoniac in the culture medium. The pH of the culture medium plays also a major role in the regulation of -this gene since it was completely turned off under alkaline conditions.

Analysis of the promoter sequence revealed the presence of putative AREA and PACC binding sites. Electrophoretic mobility shift assay experiments indicate that different transcription factors are involved according to the culture conditions. The hierarchy existing among the different regulatory systems will be discussed.

Isolation and Characterisation of the *Aspergillus nidulans* Sulphate Reduction Pathway Gene Adenosyl Phosphosulphate Kinase

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Many microorganisms, including the filamentous fungi *Aspergillus nidulans* and *Penicillium chrysogenum,* are able to assimilate sulphur by the uptake and reduction of inorganic sulphate, the suiphide thus produced being incorpotated into methionine and cystine. The sulphate reduction pathway required sulphate permease (sB), ATP sulphurylase (sC) APS kinase (sD), PAPS reductase (sA) and sulfite reductase. The sC and sA genes of *A. nidulans* have already been characterised as detailed by M.I. Borges-Walmsley et al, Mol Gen Genet (1995) 247:423-429.

It is proposed to isolate the remaining genes of the pathway in order to gain a more complete understanding of its control mechanisms. Such dats would not only be of academic interest but may also have inplications in the commercial procuction of -lactam antibiotics in which industrial production strains place a heavy burden on their cysteine pools.

By a process of heterologous colony hybridisation we have isolated the APS kinase gene from an *A.nidulans* cosmid library. A subclone of the cosmid isolated was shown, by transformation, to complement an *A.nidulans sD* mutant strain. The identity of the gene has been further verified by DNA sequence analysis displaying 59% identity with the *MET14* gene of *Saccharomyces cerevisiae.*

Work is proceeding to complete the sequence analysis of the *A. nidulans sD* gene and to iso!ate the remaining pathway genes from both *A. nidulans* and *P.chrsyogenum.*

Approach to Structural Determination of Cyp P450 and Cyp P450 Reductase in Filamentous *Fungus Cochliobolus lunatus*

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Steroid hydroxylations by filamentous fungi such as *Cochliobolus lunatus,* including 11 -hydroxylation, are economically important for the production of corticosteroids. According to CO and substrate binding spectra, the enzymes responsible for these reactions have been proposed to a family of cytochrome P450 monooxygenases as found in higer eucaryotes. The general unit of every microsomal cytochrome P450containing monooxygenase system is composed of two functional components- cyp P450 and cyp P450 reductase as electron-donor. Despite their importance little is known of their genes structure or genome organisation in fungi. We describe here the use of two DNA fragments which served as DNA probes for screening the genomic library in *E coli,* prepared in ZAPII vector. The first fragment was obtained by using a pair of oligonucleotide primers based on the conserved domain of the NADPH-cytochrome reductase *(cprA)* from *Aspergillus niger.* After PCR from *C. lunatus* genomic DNA, we amplified a 429 bp fragment which showed 74% amino acid homology with the corresponding *cprA* gene. The same approach was used in experiment looking for strain-specific cytochrome(s) P450. PCR was done with a pair of primers designed according to two structurally conserved domains as found in different *cyp* P450 from lower eucaryotes. We isolated a 273 bp fragment which showed 72% homology with the benzoate-para-hydroxylase

gene from *Aspergillus niger* belonging to the *cyp* 53 family. DNA sequencing of the positive clones is in progress and the latest results will be presented.

Expression of a Synthetic Gene Encoding the Sweet-tasting Protein *Thaumatin* in Filamentous Fungi

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The thaumatins are proteins with a very sweet taste and the ability to increase the palatability (upgrading or improving other flavours) of food; in industry they are currently extracted from the arils of the fruit of the plant *Thaumatoccocus daniellii* Benth. Thaumatins I and II, which predominate in the arils and have very similar sequences of amino acids, are much sweeter than saccharose (100,000 times sweeter according to one estimate). Besides being natural products, thaumatins I and II are non-toxic, making them a good substitute for common sweeteners in the animal and human food industries.
In this study we prepared a synthetic gene encoding thaumatin II, and used it to transform filamentous fungi. The recombinant strains obtained secrete thaumatin II into the culture medium. The recombinant protein is sweet.

Analysis of Heterologous Protein Production in Defined Recombinant *Aspergillus awamori* Strains

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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 objective 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 *Cyamopsis tetragonoloba* -galactosidase, no full length mRNA could be detected. With RT-PR 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 agalactosidase and human IL6, gene fusions with the *A. niger* glucoamylase gene (glaA) were constructed. Data on improved mRNA and protein levels will be presented.

Studies on The Production of Single-chain Antibody Fragments *by Aspergillus* **Species**

Hanny (J.)G..M. Hessing1, Leon G.J. Frenken2 Marian van

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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-B-endoxylanase A gene (3). Transformants comprising multiple copies of the expression cassette produced up to 10 mgA scFvLYS 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 scF-v-1,YS 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

Isolation and Characterisation of PDI-family Genes from *Aspergllus niger*

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Filamentous fungi, such as *Aspergillus niger,* secrete high levels of native protein making them attractive hosts for the production of recombinant proteins. Secreted yields of heterologous protein are often significantly lower however. Data suggest that a major problem precluding high yields occurs during transit of heterologous proteins from the endoplasmic reticulum (ER) to the Golgi apparatus, a step intimately associated with folding. The *in vivo* role of ER-specific chaperones and folding catalysts, such as protein disulfide isomerase (PDI), is poorly understood although their manipulation has increased yields of heterologous secreted proteins in *Saccharomyces cerevisiae. In Vitro* experiments show that foldases can often act synergistically to increases both the rate and the yield of folded end product. A family of ER-specific proteins which share active site homology with PDI has been identified from a variety of other systems. Several of these proteins are stress inducible, specifically by agents which cause malfolding of proteins in the ER. Here we report the isolation of two PDI-family genes from *A.niger.* Using a PCR based approach, redundant primer mixes designed against known ER retention signals and the PDI active site were used to isolate genomic and partial cDNA clones of a gene encoding a 359 aa protein. This ORF comprises a 19aa ER-targetting secretion signal and a 340 aa mature protein which includes a KDEL-type ER retention signal. It also contains two putative thiol oxidoreductase active sites with a CGHC motif, the most distal of which is created by excision of a 66bp intron. Both CT- and TATA-boxes can be distinguished in the putative promoter region and cDNA data from 2 independent clones confirm intron splice signals and define the site for polya tailing. Southern and dot blot analysis suggest the gene is present in single copy. Transcript sizing by northern analysis supports the postulated start codon. Analysis of the ORF reveals significant differences from PDI itself, the active sites are too closely spaced and there is no peptide binding site. Database searches have identified a stress-

inducible homologue, GI protein, from alfalfa. Preliminary data suggest that overexpression of heterologous proteins do not induce this gene in *A.niger* however. Data on the effects of agents known to cause protein misfolding and a preliminary functional analysis will be reported. A second gene has been isolated using the *S. cerevisiae* PDI gene as probe. Further characterisation of this gene will also be presented.

Vacuolar Targeting in *Aspergillus niger*

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The filamentous fungus *Aspergillus niger,* is able to secrete large amounts of proteins into the culture medium. When expressing heterologous genes, however, the production level is often much lower than for homologous proteins, even when the same expression signals are used. Incorrect folding of the proteins and intracellular degradation of these are likely to be one of the explanations for this. The fungal vacuole, which contains the protease machinery for such degradation, may well be involved in this proces. In the yeast *Saccharomyces cerevisiae* the targeting and function of the molecular machinery of the vacuole has been studied in detail, but hardly any information is available on this subject in filamentous fungi. We have now started research on vacuolar targeting in *Aspergillus niger.*
We are currently studying putative vacuolar targeting signals *from A.niger* genes encoding vacuolar proteases homologous to *S.cerevisiae* CarboxyPeptidase Y and Proteinase A. The functionality of these targeting signals are analysed by fusion to reporter proteins. In first instance we have chosen two homologous reporter genes encoding secreted proteins, -galactosidase A and Glucoamylase A. Biochemical data on the location of these reporter proteins in *A.niger*

strains expressing the fusions will be presented.

Ultrastructural Studies on the Localization of the Secretory Pathway of Glucoamylase in *Aspergillus niger*

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We study the secretary pathway in hyphae of *A. niger* at the subcellular level using glucoamylase (GLA) as a model protein. In order to facilitate directed sectioning of the hyphae the cells were grown between two apposed dialysis membranes, layered on solid agar plates. After two days of growth under GLAinducing conditions the colonies were flat embedded and sections could be prepared from defined parts of the colony. Specific morphological differences were observed between cells from leading hyphae and sub-apical branching cells. Leading hyphae were highly elongated *(5-10 um* thick and over 200 *um* long). The apical zone (1-3 *um*) was crowded with various types of vesicles including 'Spitzenkorper"; the sub-apical part contained numerous mitochondria, microbodies, vesicles, ER and 'tubular vesicular complexes" (TVC), which are probably derived from the ER. In branching cells the apical zone was defined by the presence of vesicles. However, accumulation of vesicles and other subcellular organelles, as in apical cells, was not observed. GLA protein was localized in these cells immunocytochemically, using monoclonal anti-GLA and secondary goat-anti-mouse antibodies coupled with 15 nm gold particles. In top cells of the leading hyphae, including the apical vesicles, labeling was never observed. Instead specific labeling was confined to the sub-apical cells and branches, including the top cells of the branches. The labeling was localized on endo-membrane structures, resembling ER and TVC, and also on the nuclear envelope. Plasma membranes, nuclei and miitochondria were not labeled. Control cells, grown at GLA-repressing conditions and cells of the glaa-deletion mutant

lacked labeling, indicating that the labeling on the induced cells was specific.
The localization of the gold particles indicated that GLA is transported via an endo-membranous system, most probably derived/related to the ER. The secretion pathway of GLA will be further studied in depth using genetically constructed mutants which are blocked at different stages of GLA secretion.

Characterisation of 5' and 3' UTR Sequence of an Alpha-amylase Hyper-expressing Gene from *Aspergillus oryzae*

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The filamentous fungus, *Aspergillus oryzae* has received increased attention as a favorable host for the production of heterologous proteins because of its ability to secrete large amounts of proteins. We have an alpha-amylase hyper -expressing *Aspergillus oryzae* strain which has been obtained by routine strain improvement methods. In order to characterise the alpha-amylase gene of this strain, the 5'UTR (618bp) and 3'UTR (709bp) regions were PCR ampfified, cloned and sequenced, The 5'UTR sequence does not show any significant changes from the reported sequence. However, the 3'UTR sequence shows more than forty alterations, some of which could play a significant role in the stability of the mRNA. The significance of these changes is yet to be established. Currently, efforts are under way to develop an expression vector in *Aspergillus oryzae* utilizing these elements.

Mutagenesis and Transformation of *Aspergillus terreus* Based on the Nitrate Reductase Pathway

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Aspergillus terreus is an efficient producer of different extracellular enzymes which are used in food industries. Thus, we are interested in developping a genetic transformation system based on the nitrate structural gene *nia*D. In order to develop this system the nitrate assimilation pathway in A. *terreus* was studied and spontaneous mutants defective in the genes required for nitrate assimilation were obtained on the basis of chlorate resistance. Of particular interest were *nia*D mutants which failed to grow on nitrate but grew as wild type on other sole nitrogen sources.
Afterwards the transformation system was developed for mutants defective in nitrate reductase of *A. terreus* using pSTA10 vector containing *nia*D gene from *A. niger.* The transformation frequency obtained was *c.a.* 0.5 per ug DNA. The vector appeared to be mitotically stable and Southern hybridization analysis of transformants showed that transformation events occurred by integration into to the recipient genome. To assess the amount of product made by the transformants, nitrate reductase assays were carried out under inducing conditions.
Experiments to improved transformation frequency and to characterize the integration are now in progress.

Regulation of Recombinant Protein Production in *Saccharomyces cerevisiae* by the Adhl Promoter

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We have cloned alliin lyase from garlic *(Allium sativum)* and expressed it in *Escherichia coli, Saccharomyces cerevisiae* and *Pichia pastoris.* This enzyme catalyzes the degradation of sulphurous amino acids, mainly alliin, resulting in the characteristic odour of garlic and other *Allium* species. Several physiological effects are linked to the product allicin, among them antimicrobial action, lowering of cholesterol levels and increase of fibrinolytic activity. Significant levels of expression could be detected in all three systems. In *S. cerevisiae,* the gene for alliin lyase was set under control of the alcohol dehydrogenase 1 (ADH 1) promoter. Transcription from this promoter has been described to be enhanced by glucose and decreased by ethanol. Therefore the regulation of product formation will be closely interrelated with the cultivation strategy, namely carbon source supply.
Results will be presented concerning the optimization of the feeding strategy to harmonize supply with carbon source, aerobic ethanol formation and regulation of product formation.

Expression of Human Insulin Gene in *Aspergillus*

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The latest data on hcterologous gene expression in fungi indicate that fungi express and sccrete significant amounts of heterologous protein under appropriate culture conditions, e.g. (1). This encouraged us to start our research on human insulin production in *Aspergillus.* The first aim of our work was to synthesize the human proinsulin gene and to clone it in fungal expression vector. Various mutations were introduced in the sequence of human proinsulin gene to study the (hopefully) positive effects of these mutations on protein secretion. The human proinsulin gene was synthesized by PCR based on

overlapping, complementary oligonucleotides used as templates and primers at the same time. The synthesis was performed in fragments of approximately 100-200 bp, which were joined together in pUC19 resulting in the following proinsulin genes: 1) wt proinsulin, using codons favored for efficient expression in *A. niger;* 2) proinsulin with a C-chain carrving a N-glycosylation consensus; 3) proinsulin from which most of the C-chain is deleted and 4) proinsulin from which the complete C-chain is deleted. Different genes were cloned into fungal expression vectors based on either the *glaA* promoter and signal sequence or the *glaA* promoter and the entire glucoamylase coding region (as secretion carrier). The various insulin expression vectors were introduced in *A. niger* and insulin activity was determined in culture supernatants of obtained transformants.
(1)M. P. Brockhuijsen *et al* (1993) J. Blotechnol. 31: 135-145.

Molecular Approaches Against Mushroom Browning

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Browning is an important aspect of common mushroom *(Agaricus bisporus)* quality loss, which is caused by mechanical injury, *Pseudomonas tolaasii* infection and by senescense. Brown discolouration has been associated with the transition of latent to active tyrosinase, possibly mediated by serine proteinase. Tyrosinase catalyses the oxidation of phenolic substrates mainly L-tyrosinase, p-amino-phenol (pAP) and -glutamylhydroxy benzene (GHB) to melanins.
Mushroom tyrosinase has been isolated before and was characterized as a monomeric protein of 43 kDa under denaturing conditions and 47 kDa under native conditions.
Work was carried out to clarify which one of the following parameters: tyrosinase activity, phenolic substrate content or protease

activity has the highest correlation with the colour and discolouration of mushrooms. Preliminary statistical analyses show that tyrosinase activity significantly contributes to the browning reaction.
Two partial tyrosinase cDNA sequences (600 hp) from *A. bisporus* encompassing the CuA and CuB binding domains of the enzyme have been isolated by PCR. The fragments (*Abtyr1* and *Abtyr2)* have been included in transformation constructs to attempt gene disruption or antisense inhibition. Transformation is based on hygromycin B resistance *(E.coli hpt* gene) driven by the A. *bisporus gpd2*-promoter (van de Rhee *et al.* in press).
Over a hundred putative co-transfonnants have been produced and are being analysed at the level of the homokaryotic transgenic mycelium, heterokaryotic mycelium (after mating) and fruitbodies. Results will be presented on the analysis of the transformants and on the possible effect on browning phenomena.

Kinetics of Protein Secretion in *Trichoderma reesei*

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In order to obtain knowledge of the protein secretion process in filamentous fungi, and to study the rate-limiting steps in protein production, we have developed methodology for measuring the kinetics of protein production and secretion by metabolic labelling in *Trichoderma.* The synthesis of the 35S- labelled proteins and their transport into the growth medium was monitored by 2D gel electrophoresis and/or immunoprecipitation. The major cellulose of *T. reesei,* cellobiohydrolase I (CBHI), was used as a model protein in the studies. To study the protein production kinetics in a controlled physiological state, a chemostat cultivation system was set up for the fungus. In the chemostat cultivations, the intracellular production rate of labelled CBHI and the accumulation rate of the protein into the culture medium was measured at different dilution rates, and the

corresponding specific steady-state mRNA levels were determined. With this methodology we have been able to follow the time course of protein synthesis, glycosylation and transport into the medium, and obtain information on the synthesis vs. secretion capacity of the fungus in relation to growth rate.

Investigation of the Genetic System of the Xylose Fermenting Yeast *Pichia stipitis*

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There is only poor knowledge on genetics of the xylose fermenting yeast *P stipitis.* We investigated the life cycle and some aspects of extrachromosomal inheritance of this yeast. Mutant frequencies indicated that wild type strains of *P stipitis* are haploid. Stable diploids could be obtained by protoplast fusion or sexual hybridization. Sexual mating was induced by a shift from complete medium to nutritionally poorer conditions. Conjugation was followed immediately by sporulation. Mitotically stable diploids could be isolated by transferring the nascent zygotes to complete medium before meiosis had started. Segregation patterns of auxotrophic markers indicated an ordered meiosis although asci contained in most cases only dyads. Further, we describe isolation and characterization of presumptive mitochondrial mutants in this petite negative yeast.

Characterization of a Protease Deficient Mutant of *Penicillium roqueforti* Generated by Heterologous Transformation

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Filamentous fungi are identified as potentially excellent hosts for the expression of recombinant proteins of interest To date, an increasing range of proteins from a variety of fungal and non fungal origins have been produced in fungi. However, the product yields are low in comparison with the yields of the native proteins. Among different factors affecting heterologous protein production i.e. vector construction, transformation, gene expression, glycosylalion and secretion, degradation of recombinant proteins by native proteases can be overcomed. We have characterized a *Penicillium* protease deficient strain following insertionnal mutagenesis . A strain of *Penicillium roqueforti* was transformed with a plasmid which confers resistance to phleomycin. Stable transformants exhibiting high resistance to the antibiotic were selected. One transformant was isolated as a proteolytic deficient mutant unable to degrade casein. The extracellular proteins profile of the strain reveals the absence of a 43 kDa polypeptide corresponding probably to the aspartyl protease of *Penicillium roqueforti.* The mutant is characterized by a tandem integration of the transformant vector in one site of the genome. The *asp* A gene which encodes aspartyl protease of *Penicillium roqueforti* is not expressed in the transformant and Southern analyses show that the *asp* A gene is not disrupted by the transformation vector.
Heterologous protein degradation was tested and demonstrated that proteins from, different origins were not degraded by the culture filtrate of the protease deficient mutant contrarly to the wild type. Result anticipate that this strain will be adequate for heterologous expression of most proteins.

Towards an Analysis System of the Secretion Pathway of *Aspergillus niger*

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Filamentous fungi, such as *Aspergillus niger,* are able to secrete large amounts of proteins into the culture medium (1). However, hardly any information is available on the molecular mechanisms of the process of protein secretion. Therefore, two lines of research have been initiated towards a systematic analysis of the mechanism of protein targeting and secretion by *A.niger (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-specific proteins have been isolated. The cloning and characterisation of the *A. niger bipA* gene will be presented together with initial results aimed at the analysis of the BipA protein and its targeting using specific antibodies.
II Based on research carried out in *Saccharomyces cerevisae,* defined (conditional) mutations in the secretion pathway will provide important information about the Organisation of the pathway. Therefore, the cloning of several secretion genes has been started. We have focused on the isolation of various genes encoding GTP-binding proteins involved in the vesicle transport between the different compartments of' the secretion pathway, using heterologous hybridisation with cloned *S. pombe* genes as probes. The successful cloning of the SAR1-homologous *A. niger sarA* gene and initial resulted on *A. niger sarA* mutants will be presented.
1. Verdoes J C et al (1995) Appl Microbiol Biotechnol 43:195-205
2. Punt PJ et al (1994) Anthony van Leeuwenhoek 65:211-216

Heterologous Gene Expression and Secretion of a Eukaryotic Protein by *Acremonium chrysogenum*

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Filamentous fungi are frequently used as host organisms for the

expression of heterologous genes or for the overproduction of homologous proteins. One major advantage is their ability to secrete large quantities of protein, which facilitates the purification procedure of the protein of interest (for a review, see [1]).
Acremonium chrysogenum is an industrially important producer of the -lactam antibiotic cephalosporin C, for which fermentation technology has already been established. Therefore it is a favourable host for the production of heterologous proteins.
We have developed a system allowing the production and secretion of heterologous proteins by A. *chrysogenum.* The expression is controlled by the promoter of the homologous *pcbC* gene that encodes isopenicillin N synthetase, a key enzyme of the cephalosporin biosynthesis. This promoter, which has previously been shown to mediate a relatively high level of expression [2], was fused to a gene encoding a small eukaryotic coagulation inhibitor. In order to achieve the secretion of this heterologous protein, we used two different signal sequences from *Fusarium* and from *A. chrysogenum,* respectively. Transformation of these expression vectors into *A. chrysogenum* resulted in the efficient secretion of the heterologous protein.
[1] van den Hondel CAMJJ, Punt PJ, van Gorcom RFM (1991) in: Bennett JW, Lasure LL (eds), More gene manipulations in fungi, Academic Press, San Diego CA, pp 396 - 428
[2] Menne S, Waiz, M, Kilck U (1994) Appi Microbiol Biotechnol 42: 57-66

Characterization of the *SAR1* Gene from the Filamentous Fungus *Trichoderma reesei*

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Filamentous fungi have a long history in production of extracellular enzymes and are well known for their high capacity in protein

secretion. The largest reported amount of secreted protein from the cellulolytic fungus *Trichoderina reesei* is about 40 g/l. Despite the above, the protein secretion machineries of filamentous fungi are poorly known. As one of the first studies in this field, we report here the characterization of the *sarl* gene from *Trichoderma reesei.*
The *Saccharomyces cerevisiae SAR1* gene product is a small GTP-binding protein involved in the ER to Golgi step of protein secretion. The Sar 1p is thought to be regulating the budding of vesicles from the endoplasmic reticulum. The corresponding gene, *sarA,* has been isolated from the fungus *Aspergillus niger (1).* Using this gene as a probe, we isolated the cDNA and chromosomal copies of *sarl* gene of *Trichoderma reesei. The Trichoderma* sequence shows 72 % conservation with yeast SAR1 and 86 % conservation with *Aspergillus sarA* at the amino acid level. The regions responsible for GTP binding are well conserved. The exon-intron structure of *Trichoderma sarl* shows some divergence when compared to that of *Aspergillus sarA.* Complementation experiments with yeast *sarl* mutants will be
discussed.
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Cloning of the Isocitrate Lyase Gene of *Ashbya gossypii* by *Saccharomyces cerevisiae* Complementation

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Ashbya gossypii is a filamentous hemiascomycete used for industrial riboflavin production. Growth experiments on glucose or soybean oil as carbon source revealed a correlation of isocitrate lyase (ICL) specific activity and riboflavin formation. Mutants resistant to a specific ICL inhibitor showed an increased ICL specific activity and an enhanced riboflavin production1. To investigate the role of ICL in riboflavin production on molecular level the respective gene was

cloned. For this purpose a plasmid library of genomic *A. gossypii DNA* was constructed with YEp 352, a yeast/E. coli shuttle vector, and used to transform *S. cerevisiae icl1 d ura3 (fs)2* which is not able to grow on ethanol. Colonies obtained by selection for *ura3* complementation were replica-plated on minimal medium with ethanol as sole carbon source. Two out of 3000 clones showed the expected phenotype i.e. grew on ethanol. Curing from the plasmid, retransformation, and detection of ICL activity indicated that the ICL-gene is located on a 2.9 kb *Sphl* fragment. Sequencing revealed an open reading frame of 1680 bp showing 65 % identity to the ICL1-gene of *S. cerevisiae* and 75 % identity to the predicted amino acid sequence. The conserved hexapeptide KKCGHM, possibly confined to the catalytic domain, as well as the C-terminal tripeptide AKL, described as targeting signal for peroxisomes, were found.

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Cloning of Specific Genes of the Gibberellin Pathway from *Gibberella fujikuroi*

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The gibberellins are a group of natural plant hormones with various effects on growth and differentiation of plants. The rice pathogen *Gibberella fujikuroi* accumulates large amounts of Giberellic acid (GA3) 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 the relationship to the biosynthetic pathway in higher plants are not well understood until now. Therefore, we have started to isolate and characterize 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, farnesylpyrophosphate synthetase and geranylgeranylpyrophosphate synthetase were isolated by screening genomic and expression libraries of this fungus with heterologous probes or with PCR-fragments synthesized on the basis of sequence homologies to other fungal prenyltransferases. The sequence comparison with analogous genes of other fungi and plants showed that the pathway must be highly conserved.All the three genes isolated from *Gibberella fujikuroi* were found as single copy genes which are not linked to each other.
Beside those genes from the central terpenoid pathway, we are trying to isolate the kaurene synthetase gene and the C20-oxidase gene from the gibberellin-specific part of the pathway using the recently isolated corresponding plant genes (Sun and Kamiya,1994; Lange et al.,1994) as heterologous probes and designed specific PCR-primers on the basis of sequence comparison between these plant genes in order to amplify parts of the corresponding *Gibberelia* genes.Additionally, REMI mutagenesis was used to isolate tagged gibberellin-defective mutants.
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Heterologous Expression ofthe *Fusarium solani pisi* cutinase gene in *Aspergillus awamori*

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Filamentous fungi are used as hosts for the commercial production of heterologous proteins because they have an enormous capacity for secretion. The production of many hetcrologous proteins are, however, rather low compared with the high levels which are obtained with homologous proteins such as glucoamylase. This study hits been started to sort out the bottlenecks in the production of heterolagous proteins, and subsequently to identify the factors, involved in these ratelimiting pathways. Therefore, the effect of different pro- and presequences and the presence of multiple copies of the gene on the production of heterologous proteins is studied. The cutinase protein from *Fusarium solani pisi* is used as a model for the production of heterologous proteins.
A synthetic copy of the cutinase cDNA was constructed and expressed under the control of the endoxylanase II expression signals from *A. awamori.* Four different constructs were used to test the effect of different pre- and pro-sequences. A single copy of these expression cassettes was integrated at the *pyrG* locus of *A. awamori.* Shake flask induction experiments revealed that the pre-sequences used were equally efficient in the production of extracellular cutinase. The absence of a pro-sequence, howcver, resulted in a two-fold increase in extracellular cutinase. To study the effect of multi copy gene expression, one of the constructs was integrated in multiple copies into the genome of *A.awamori.* There was no linear correlation between copy number and extracellular cutinase production, but the amount of active enzyme produced correlated with the level of cutinase specific mRNA. These data and the fact that a relatively small amount of cutinase was found inside The cell suggest that there is no limitation in the secretion of this protein by *A.awamori*.

Improvement of Cytochrome P450 Activities in *Aspergillus niger*

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Recently the gene encoding cytochrome P450 reductase *(cprA)* was cloned from the filamentous fungus *A. niger.* CPR functions as an electron donor for many different cytochrome P450 enzymes and is thus essential for P450 activity.
We used the *cprA* gene to generate *A. niger* strains with increased cytochrome P450 activities. For this purpose, strains were constructed which contain multiple copies of the *cprA* gene, multiple copies of a cytochrome P450 encoding gene or multiple copies of both genes. T\vo fungal cytochrome P450 encoding genes were used in this study: the *A. niger bpha (cyp53)* gene, encoding benzoate para-hydroxylase, and the *P. italicum cyp5l* gene, encoding eburicol 14 -demethylase.
For both sets of transformants it was found that the highest cytochrome P450 activity was detected in strains which contained multiple copies of both genes. Increasing the copy number of only one of the components of the enzyme system (the P450 encoding gene or the *cprA* gene) resulted in a significant, but much smaller increase in cytochrome P450 activities as compared to wildtype. These results show that for an optimal increase of cytochrome P450 activity in filamentous fungi simultaneous overexpression of CPR and of the cytochrome P450 is needed.

Transport Steps Involved in Penicillin Biosynthesis *by Penicillium chrysogenum*

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The filamentous fungus *Penicillium chrysogenum* is well known for its ability to produce penicillin. The biosynthesis of penicillin involves three steps which are catalyzed by three different enzymes, -aminoadipyl-cysteinylvaline synthetase (ACVS), isopenicillin N synthetase (IPNS), and isopenicillin N acyltransferase (AT) (1). Evidence exists that the activity of the different enzymes is confined to specific intracellular compartments (2-5). This sublocalisation, together with the import of precursor molecules and the secretion of the produced penicillin, imposes the need for several active and/or passive transport steps.
Recently, we reported the isolation and characterisation of plasma membranes obtained from low- and high-producing strains of *P. chrysogenum* (6). Subsequent studies showed that the uptake of the penicillin G side-chain precursor phenylacetic acid across the plasma membrane occurs by passive diffusion (7). Further studies are being directed at transport steps across the vacuolar membrane. The vacuole has been implicated to play various roles, e.g. as a storage compartment for (basic) amino acids and/or as the compartment where the first reaction of the penicillin biosynthesis, i.e. the formation of the tripeptide a-aminoadipyl-cysteinyl-valine by the enzyme ACVS, takes place (5). First results from experiments that are eventually aimed at elucidating the role of the vacuole in penicillin biosynthesis will be presented.
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Development of a Virus-resistant Strain of *Agaricus bisporus*

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Fruit bodies of *Agaricus bisporus* affected by La France disease contain a set of nine unique dsRNA molecules (Harmsen *et al.,* 1989). In addition to the nucleic acid sequences of the L3, M1 and M2 dsRNAs determined by Harmsen *et al.* (1991), the sequences of the L1 and L5 dsRNAs have now been detemined (Van der Lende *et al.,* 1996; in press). These dsRNAs are contained in 34 nm virus particles that also contain at least three immunologically unrelated proteins of 120, 115, and 90 kDa (Van der Lende *et al.,* 1994). The virion-associated protein of 115 kDa proved to be the RNA-dependent RNA polymerase encoded by the L1 dsRNA, whereas the virion-associated protein of 90 kDa is encoded by the L3 dsRNA. *In vitro* translation experiments of the individual dsRNA segments indicate that L2 dsRNA encodes the protein of 120 kDa. Antibodies raised against a protein encoded by M2 dsRNA, reacted with a protein present in diseased fruit bodies but not with any of the virion-associated proteins (Van der Lende *et al.,* 1996; in press). A number of essential virus genes have now been characterised. Current research is directed towards the development a virus resistant strain of *Agaricus bisporus* using pathogen-derived-resistance (PDR). For this purpose the development of an efficient transformation protocol for commercial strains is in progress. In addition, different expression vectors are being developed in order to express viral sense RNAs and antisense RNAs and/or viral proteins in *Agaricus* which may inhibit viral replication as has been shown for virus-resistant transgenic plants.

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Secretion of Mammalian Glycosyltransferases by *Aspergillus*

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We have investigated the production and secretion of mammalian glycosyltransferases by *Aspergillus niger.* These enzymes are involved in the glycosylation of proteins and are normally located within the secretary apparatus of the cell. They typically possess a transmembrane anchor sequence which serves to localize them on the membrane. Recently, there has been interest in the use of these enzymes for the enzymatic synthesis of therapeutic complex carbohydrates. However, production of these enzymes at commercially viable levels has been difficult. We tested secretion of rat alpha 2,3 sialyltransferase (ST) in *A. niger* by production of a fusion protein between native glucoamylase and a truncated form of ST (without the transmembrane domain). A KEX2 cleavage site was optionally incorporated between glucoamylase and ST. Either full-length or truncated glucoamylase (lacking the starch binding domain) was employed. Initial yields were low and inconsistent and ST purification was not possible due to interaction with media components. However, media optimization gave increased and consistent yields and allowed ST purification. Yields of 1700 U/L (85 mg/L) were obtained with *A. niger* compared to 7 and 0.2 U/L obtained from Baculovirus and mammalian cell expression systems respectively. A similar strategy also allowed a much higher level of expression of human alpha 1,3 fucosyltransferase (FIF) in *A. niger*

compared to the Baculovirus and mammalian cell systems. The yield and quality of both ST and FT produced in A. *niger* are sufficient to allow their commercial use in synthesis of complex carbohydrates.

Trehalose-6-phosphate Synthetase Activity of *Aspergillus niger* Is Encoded by Two Differentially Exxpressed Genes

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Aspergillus niger is of major industrial importance because of its production of several hydrolytic enzymes as well as organic acids. All these processes are strongly influenced by the carbon source supplied. In order to investigate a possible glucose signalling mechanism in *A. niger* we have cloned the homologue of the yeast *GGS1/TPS1* gene, which encodes a trehalose-6-phospate synthase. The *GGS1* gene product has been reported to be involved in the transduction of several glucose dependent regulatory phenomena in *Saccharomyces cerevisiae* and *Kluyveromyces lactis.*
The *A. niger ggsA* gene contains four introns and its deduced aa-sequence shows 65.2 % and 64.5 % identity to the corresponding gene products of *S cerevisiae* and *K. lactis,* respectively. Northern analysis demonstrated that its expression is growth correlated. Neither gene disruption nor gene amplification had a significant effect on the glycolytic flow in *A. niger.* Disruption of *ggsA* gene reduced most of the trehalose-6-phosphate synthase (TPS)activity of growing mycelia, whereas mycelia exposed to heat shock still exhibited high TPS-activity, suggesting the existence of at least one further isoenzyme. Using PCR, we amplified a genomic fragment, whose nt-sequence showed areas of high similarity to *ggsA,* yet is clearly distinct and therefore encodes *ggsb.* Its nt-sequence and expression pattern are currently investigated.

Poster Abstracts, Fungal-host- interaction and signal transduction

Molecular Analysis of Cytokinesis Mutants in *Ustilago maydis* Michael Bolker and Regine Kahmann. Institut fur Genetik und Mikrobiologie, Universitat Munchen Maria- Ward-Str. la, 80638 Munchen, Germany

The phytopathogenic fungus *Ustilago maydis* exhibits a dimorphic life style. Haploid sporidia grow yeast-like by budding and are nonpathogenic. 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 *don1 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. Molecular analysis of these genes revealed that the final step of cytokinesis seems to be regulated by a rho/rac GTPase. The *don1* gene shows high similarity to the family of rho/rac guanine exchange factors, *the don3* gene codes for a kinase that is homologous to the yeast STE20 and the mammalian pak/p65 kinase. Since these kinases are known to be activated by the active form of the rho/rac GTPase we propose that a similar signaling pathway is involved in the regulation of cytokinesis.

Differentiation-specific Expression of Chitin Deacetylase in Infection Structures of *Uromyces viciae-fabae* H. Deising1 and J. Siegrist2 1 Universitat Konstanz, Fakultat fur Biologie, Phytopathologic, D-78434 Konstanz, and 2Universitat Hohenheim, Institut fur Phytomedizin, D-70593 Stuttgart, Germany

Uredosporelings of rust fungi differentiate complex infection structures in order to penetrate the host leaf through the stomatal openings. Probing with FITC-conjugated wheat germ agglutinin

revealed that chitin is a major cell wall component only of infection structures formed on the plant surface, but not of those differentiated in the intercellular space of the plant.
Radiometric enzyme assays and analyses of extracellular proteins of different developmental stages by substrate inclusion-SDS PAGE showed that at least five chitin deacetylases are secreted, beginning with the penetration process. These enzymes remove the acetyl groups from the chitin molecule to give rise to chitosan. Since chitosan is resistant to degradation by plant chitinases, secretion of chitin deacetylases and enzyme-mediated cell wall modifications may represent a mechanism to avoid hyphal lysis after penetration into the plant.
A fragment of the cda (chitin deacetylase) cDNA of *Mucor rouxii* was used to screen a gt 1 0 cDNA library of *Uromyces viciae-fabae.* Two gt 1 0 clones hybridizing to the heterologous probe were purified. The cDNA inserts were amplified by PCR and subcloned into plasmid vectors. Exonuclease deletions were made for sequence analyses, and sequencing of the rust cDNAs is currently in progress.

Characterization of P-glycoprotein Encoding Genes in *Aspergillus nidulans*

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P-glycoproteins are members of the ATP-Binding Cassette (ABC) superfamily of transporters. They consist of two transmembrane domains (each with six membrane spanning -helices) and two ATP-binding domains that couple ATP hydrolysis to transport of substrates. These substrates can be either non-toxic (e.g. the a-factor pheromone of *Saccharomyces cerevisae)* or toxic (e.g. antibiotics). P-glycoprotein transporters became especially known for their role in multidrug resistance (MDR) of mammalian tumor cells. MDR is

defined as the simultaneous resistance against a wide variety of both natural toxic compounds and synthetic drugs. MDR can be ascribed to increased efflux of the drugs caused by increased expression of P-glycoprotein encoding genes and resulting in decreased cellular accumulation. Phenotypic, genetic and biochemical evidence indicates that MDR also plays a role in resistance of *Aspergillus nidulans* to fungicides which inhibit sterol demethylation (DMIS) and non-related compounds such as cycloheximide.
The research presented in our poster gives a characterization of P-glycoprotein encoding genes of *A. nidulans* and a functional analysis of these genes in fungal physiology and MDR.
Heterologous hybridization of a genomic library of *A. nidulans* resulted in cloning of two P-glycoprotein encoding genes, coded *Anpgpl and Anpgp2.* Both genes were fully sequenced and their deduced gene products show a high degree of homology with other members of the ABC superfamily. Northern analysis experiments demonstrated that the basic level of expression *of Anpgpl* is higher in DMI-resistant mutants than in the wild-type isolate. Expression could strongly be induced by treatment. of mycelium with DMIs and cycloheximide, especially in some of the DMI-resistant mutants. The basic level of expression of *Anpgp2* was the same for wild-type and DMI-resistant mutants, but again transcription could be induced by a several nonrelated toxicants among which DMIs. Time-course experiments demonstrated that transcription of both genes was already induced within 5-15 min of treatment of mycelium with the toxicants. These results strongly suggest that the cloned *Anpgp* genes indeed play a role in MDR of *A. nidulans. The* natural physiological role of the encoded gene products may be to protect this saprophytic fungus against naturally toxic compounds which do occur in the environment.

The Putative Role of P-glycoproteins in Pathogenesis of *Botrytis cinerea*

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P-glycoproteins are members of the ATP-Binding Cassette (ABC) superfamily of transporters. They consist of two transmembrane domains (each with six membrane spanning -helices) and two ATP-binding domains that couple ATP hydrolysis to transport of substrates. The enzymes have a low substrate specificity and can simultaneously secrete a variety of toxic, hydrophobic compounds. Therefore, it is believed that P-glycoproteins play a major role in protection of both pro- and eukaryotic organisms against naturally toxic products. The transporters can be regarded as "membrane or cytoplasmic vacuum cleaners" which expel toxicants as soon as they are detected in the plasma membrane or cytoplasm.
In view of these considerations we propose that P-glycoproteins of the filamentous plant pathogen *Botrytis cinerea* can play a role in pathogenesis. This fungus has a wide host range which may contain a wide variety of phytoncides and phytoalexins. The pathogen is obviously able to cope with these toxins and it is proposed that the mechanisms involved is based on secretion by P-glycoproteins as soon as they accumulate in the pathogen during plant penetration and further colonization. In addition, it may also be possible that P-glycoproteins are involved in secretion of fungal toxins involved in pathogenesis. In order to test these hypotheses we started to characterize P-glycoprotein encoding genes in *B. cinerea.*
Heterologous hybridization of a genomic library resulted in the isolation of three independent clones. One of them has been fully sequenced and contained a P-glycoprotein encoding gene of which the deduced amino acid sequence shows a high degree of homology with other members of the ABC superfamily. Northern analysis experiments indicated that transcription of the gene can be induced by various toxic compounds. Time-course experiments showed that induction takes place within 15 min after mycelial treatment, thus suggesting that P-glycoproteins of *B. cinerea* may be involved in secretion of plant toxins preventing their accumulation in fungal cells.

This would implicate that P-glycoproteins can be regarded as a new pathogenicity factor. The validity of this hypothesis will be further tested by studying the pathogenicity of isolates with disrupted P-glycoprotein encoding gene(s).

Variation in Conidial Size of Some *Pyricularia spp.*

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The size of the conidia has been used as an indicator of ploidy levels in fungi. Conidial size and volume have been used to detect diploids since diploid spores are larger than haploid spores. The size of conidia of six forms of *Pyricularia* (from *Oryza sativa, Eleusine coracana, Setaria italica, Pennisetum typhoideum, Panicum repens* and *Leersia hexandra*) produced in culture and on their respective host lesions were compared. The study revealed certain interesting features about conidial widths in *Pyricularia* that have not been so far recognized. Regardless of the isolate, populations of conidia could be grouped into three classes based on their width (6, 9 and 12 um) and their proportion varied with the isolate and source of conidia. In general, 9 um conidia predominated in populations derived from culture or host lesions. Among the six isolates studied here, the 12 um wide conidia were present to a greater extent with *Leersia* isolate than others both in culture and on lesions. The presumptive mean volumes of the three classes of conidia revealed that the volume of the widest conidia (12 um) was 1.5-2.0 times the volume of the less wide conidia (9 um) in five of the six isolates studied. In culture, the *S. italica* isolate showed the greatest variation in length (15-45 um) and the *E. coracana* isolate, the least (18-30 um). On host lesions, however, the *S. italica* isolate showed the least variability (21-30 um) and *P. typhoideum* isolate, the greatest variability (21-45 um). It seems possible to differentiate different forms and isolates of *Pyricularia* on the basis of frequency distribution of their conidial lengths as demonstrated here for the first time. A bimodal

distribution of conidial lengths was evident with certain monoconidial isolates of *Pyricularia* which was considered to indicate the heterokaryotic nature of the isolates. Although conidia of *Pyricularia spp*. could not be distinguished on the basis of their widths, a study of the frequency distribution of lengths of 9 um wide conidia showed differences in the frequency patterns which appeared to be adequate to differentiate conidia of *Pyricularia spp.*

Purification and Cloning of Protein Phosphatases from *Neurospora crassa*

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Protein phosphorylation is a universal regulatory mechanism in eukaryotic cells. The phosphorylated state of a protein is affected by the conflicting activities of protein kinases and phosphatases. Nearly all Ser/Thr specific dephosphorylation reactions can be attributed to four classes of protein phosphatases (PP's): PPI, PP2A, PP2B, PP2C, which are differentiated on the basis of inhibitor sensitivity and metal ion dependence. We found that all the four main classes of Ser/Thr protein phosphatases are present in *N. crassa.* The catalytic subunits of PP2A and PPI (PP2AC and PPIC) were successfully purified to near homogeneity by using ammonium sulfate-ethanol precipitation, DEAE Sephacel, Heparin-Sepharose and MonoQ FPLC chromatography. The molecular mass of PP2AC proved to be 33 kD by SDS PAGE. PP2AC,. was completely inhibited by 1 nM okadaic acid, was insensitive to rabbit muscle inhibitor 2, and was specific for the -subunit of rabbit muscle phosphorylase kinase as substrate. Antipeptide antibodies raised against the N-terminal and C-terminal ends of human PP2A. did not cross-react with *N. crassa PP2AC* .The

catalytic subunit of PPI was found to be 33 kD in size, was inhibited by okadaic acid and inhibitor-2. It could be established that PPI and PP2A of *N. crassa* showed striking similarity to their mammalian counterparts with respect to biochemical characteristics. The pSV50 cosmid library of *N. crassa* was screened using a *Drosophila* PPI CDNA probe. Four recombinant cosmids of different restriction patterns were isolated. The heterologous CDNA probe hybridized to 4 - 5 BamHl and Hindlll fragments in a Southern hybridization experiment. Subclones from two recombinant cosmids were sequenced with primers homologous to the conserved regions of PPI. One of the sequences proved to be a truncated version of the other. The obtained sequence showed high degree of homology to *Saccharomyces cerevisae* PPZI and PPZ2 enzymes which are considerably larger (75 and 78 kD) than PPI and may play a role in osmotic stability and the PKC-mediated signal transduction pathway. The new *N. crassa* protein phosphatase gene was mapped by RFLP mapping to the left arm of chromosome 1.
This work was supported by OTKA grant #T6005 and FEFA grant # 1559.

Molecular Characterisation of Plant-induced Genes in the Take-all Fungus *Gaeumannomyces graminis*
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Our work focuses on the use of different approaches for the isolation of genes controlling pathogenicity in *Gaeumannomyces graminis*. One approach involves the identification of fungal genes that are induced *in planta* by differential screening of a library of fungal genomic DNA. This approach has allowed the isolation of nine *in planta*- induced genes from the potato blight fungus *Phytophthora infestans* (Pieterse et al.,1993). In the use of this approach it is very important to solve some problems that arise in the production of the infected material:

1) To imitate the natural conditions of infection
2) To use inoculation procedures which give uniform infection
3) To find a time point at which to harvest infected material for RNA extraction
4) To isolate a constitutively expressed fungal gene to use as a standard in Northern blot analysis.

The first two points have been solved with the use of different inoculation procedures in parallel, and the third point has been partially solved with microscopic observations of the infected roots harvested at four different time points.

To solve the last point it is necessary to characterize a constitutively expressed gene of the fungus to use as an internal standard in Northern blots, since the signal obtained with this probe should give an indication of the proportion of fungal RNA in the mixture of RNA of plant and fungal origin.

Genes encoding actin and the elongation factor EF- I a have been shown to be very useful markers. (Mahe et al.,1992; Pieterse et al,, 1993).

Since it has been demonstrated that many plant-induced genes can also be induced *in vitro* by starvation conditions (Oliver et al., 1994), we want also to screen a genomic library of *Gaeumannomyces graminis* with cDNA from the fungus grown under condition of nitrogen and/or carbon starvation.

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Role of in Planta-expressed Genes in Biotrophic Nutrient Uptake of the Rust Fungus *Uromyces viciae-fabae*

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Haustoria are specialized hyphae of biotrophic fungi which play a crucial role in parasitic nutrition. To identify proteins involved in nutrient uptake, we have isolated genes which are specifically expressed in rust haustoria. Differential screening of a haustorial cDNA library revealed a high proportion (approx. 20%) of haustoria-specific cDNAs. Some of these cDNAs (PIGs = *in planta* induced genes) were strongly expressed in haustoria (each 1% of the total haustorial mRNA). By sequencing, one PIG was identified which encodes a membrane protein with high similarity to yeast amino acid permeases. With antipeptide antibodies, the protein was localized to haustorial membranes. Heterologous expression of the protein in yeast was achieved, but no complementation of amino acid transport mutants was observed. We are currently using *Xenopus* oocytes to determine the substrate specificity of the putative amino acid transporter.

In rust haustoria, the activity of the plasma membrane H+-ATPase is strongly increased compared to rust spores and germlings, indicating that this enzyme generates an electrochemical potential across the haustorial plasma membrane. We have cloned and sequenced a full-length cDNA of the H+-ATPase, and have studied its expression using Northern analysis and RT-PCR (see poster by Wernitz et al.). Taken together, our data suggest an active, carrier-mediated uptake of nutrients by rust haustoria.

The Pheromone Response Factor Coordinates Filamentous Growth and Pathogenicity in *Ustilago maydis*

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Dimorphic growth and pathogenic development of the corn smut fungus *Ustilago maydis* is regulated by the two unlinked mating type loci a and b. Haploid cells grow by budding and are non pathogenic. Cells which differ in both mating type loci can fuse and form the filamentous growing dikaryon, which is able to infect corn plants. Cell fusion is controlled by the biallelic a locus, which encodes a pheromone based cell recognition system. After fusion, pathogenicity is triggered by heterodimers of homeodomain proteins encoded by the multiallelic b locus. Filamentous growth of the dikaryon requires an activated pheromone pathway in addition to the active heterodimer.
Transcription of all genes in the a and b mating type loci is induced in response to pheromone. We have isolated an HMG box protein (Prfl) which binds to short pheromone response elements present in both loci. *prf1* mutants do not express the pheromone and receptor genes even after pheromone stimulation and thus are sterile. Disruption of *prf1* in pathogenic haploid strains results in loss of pathogenicity, which can be restored by constitutive expression of the b genes. In addition these strains grow filamentous in the absence of pheromone stimulation. We present a model in which Prfl coordinates cell fusion, pathogenicity and filamentous growth by regulating the expression of the a and b genes in response to different stimuli.

Assimilatory Nitrate Reductase in the Arbuscular Fungus *Glomus*

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There is ample but circumstantial evidence that nitrogen is among the nutrients supplied by arbuscular mycorrhizal (AM) fungi to the host. Nitrogen is mainly taken up as nitrate and then reduced by assimilatory nitrate reductase. By PCR, a part of the gene coding for the apoprotein of nitrate reductase could be amplified from the fungi *Aspergillus nidulans, Pythium intermedium, Phytophtora infestans, Phytophtora megasperma, Glomus D13, Glomus fasciculatum, Glomus intraradices* and *Glomus mosseae.* Sequencing of four of the amplificates as well as DNA hybridizations revealed strong homologies with the nitrate reductase gene in all cases. The digoxigenin labeled amplificate from *Glomus* D 13 hybridized with DNA isolated from *Glomus* spores (Kaldorf et al., Mycorrhiza 5, 23-28, 1994).

Expression of the nitrate reductase genes of maize and *Glomus* has been investigated by Northern hybridization experiments with gene probes from maize and *Glomus.* Expression of maize nitrate reductase is about tenfold lower in the roots of plants infected with *Glomus intraradices* compared to noninfected roots. The difference in the level of maize nitrate reductase mRNA is smaller but also detectable in the leaves of infected and noninfected maize plants. Hybridization with the gene probe for the nitrate reductase from *Glomus intraradices* gave a clear signal with RNA from infected maize roots. RNA from roots and leaves of control plants also gave a hybridization signal which was, however, distinctly weaker than the signal in infected plants and might be the result of cross-hybridization.

In situ hybridization experiments with the gene probe from *Glomus D13* have been performed with infected maize roots. A significantly enhanced labeling was obtained in arbuscules but not in vesicles of *Glomus D13* indicating that the arbuscules are the active sites of nitrate reduction. Experiments with the gene probe for maize nitrate reductase are currently under way.

A Gene Encoding a Protein Elicitor of *Phytophthora infestans* is

Down-regulated During Infection of Potato

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Most species of the genus *Phytophthora* produce 10 kDa extracellular protein elicitors, collectively termed eliciting. Elicitins induce hypersensitive response specifically in the Solananceae family. The role of eliciting in the interaction between *Phytophthora infestans* and potato was investigated. A cDNA encoding INFI, the major secreted elicitin of *P. infestans,* was isolated and characterized. The expression of the *inf1* gene in relation to the disease cycle of *P. infestans* was analyzed. *inf1* was shown to be expressed in mycelium grown in various conditions, whereas it was not expressed in sporangiospores, zoospores, cysts, and germinating cysts. During the early stages of infection of potato, *inf1* expression was downregulated compared to *in-vitro* and late (sporulating) stages of infection. The low level of expression of *inf1* during infection could be an adaptation of *P. infestans* to evade effective defense responses, and could be typical for elicitor or avirulence genes encoded by multiple copies. In order to test this hypothesis, transformation experiments using constitutive promoters fused to *inf1* are being conducted.

Nip1, a Bifunctional Signal Molecule from the Barley Pathogen, *Rhynchosporium secalis*

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The imperfect fungus *Rhynchosporium secalis* is the causal agent of barley leaf scald. The interaction of this pathogen with its host complies with the gene-for-gene hypothesis. *R. secalis* secretes a

family of small toxic proteins (necrosis inducing proteins, NIPS) which exert unspecific activity towards mono- and dicot plants. Toxicity is at least in part mediated through a stimulation of the plant plasmalemma H+-ATPase. In addition, one of these proteins, NIP1, was found to be a race-specific elicitor of defense reactions in *Rrs1*-barley at concentrations 2-3 orders of magnitude lower than those needed for toxic activity.

Genetic complementation and gene disruption demonstrated that NIP1 is encoded by the fungal avirulence gene, *AvrRrs1,* that is complementary to the barley resistance gene, *Rrs1*. Fungal races virulent on *Rrs1*-barley either lack the *nip1* gene or secrete a gene product that lacks elicitor activity due to a single amino acid alteration. However, while NIP1 producing races are highly virulent on *rrs1*-barley, *nip1*-deficient races show a lower degree of virulence on both *rrsl- and* Rrsi-barley. A similar phenotype was found with transformants in which the functional *nip1* gene was replaced by a non-functional gene through homologous recombination. This indicates that in addition to its avirulence function the *nip1* gene also plays a significant role in the development of virulence.

We have synthesized 3 oligopeptides spanning the amino acid primary sequence of NIP1. These peptides, alone or in all possible combinations, were elicitor-inactive. However, combinations including the central and the C-terminal peptide were toxic on *Rrs1*- as well as on *rrsl*-barley indicating that the structural constraints for toxicity are higher than those for elicitor activity. In addition, one of the amino acids that is essential for elicitor activity of the mature protein is located in the non-toxic N-terminal peptide indicating that different parts of the molecule appear to be involved in its two functions. Furthermore, the receptor mediating the toxic activity may be different from the elicitor receptor. Models including 1 or 2 NIPI receptors will be discussed.

Cutinase: a Virulence Factor in the Interaction Chickpea / *Ascochyta rabiei*?

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Ascochyta rabiei, the main pathogen of chickpea *(Cicer arietinum L.),* penetrates its host directly through the cuticle (Hohl, 1992). Therefore, the cuticle is the first barrier that must be overcome by *A. rabiei.* Our aim is to construct a cutinase-deficient *A. rabiei* strain via transformation mediated gene replacement. The cutinase gene from *A. rabiei* had been cloned (Tenhaken, 1992) and a gene replacement vector had been constructed. Several transformants were analyzed with respect to the integration pattern of the vector and the cutinase enzyme activity. Infection studies of such transformants in comparison to the wildtype with resistant and susceptible chickpea cultivars will provide information on the importance of the cutinase in this interaction.
About 70% of the transformants carried the replacement vector in the "downstream" homologous integration site; but the gene was not replaced, which was supported by Southern- and PCR-analyses. Nevertheless, this integration of the vector into the left border fragment of the gene led to a substantial loss of exogenous esterase activity in comparison with activities of wildtype and ectopic transformants. These transformants are not impaired in virulence (infection of the susceptible cultivar ILC 1929 with transformants carrying homologous integrated vector or ectopic integration). Disease symptoms developed as quickly and as intensively as in the control infection with the wildtype. This was also observed, when the spore inoculum was decreased from 1.0*105/ml to 1.0*104/ml. Interaction with the resistant cultivar ILC 3279 was also not affected.

Mycoparasitic Interaction Relieves Binding of the Cre1 Carbon Catabolite Repressor Protein to Promoter Sequences of the *Ech42* (Endochitinase-encoding) Gene in Trichoderma harzianum

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The fungus *Trichoderma harzianum* is a potent mycoparasite against various plant pathogenic fungi. In order to study the molecular regulation of mycoparasitism. we have used *Botrytis cinerea* as a model plant pathogen, confronted it on agar plates with *T harzianum* PI, isolated cell-free extracts from various stages of mycoparasitism, and used them in electrophoretic mobility shift assays (EMSAs) with two promoter fragments of the ech-42 (42 kDa endochitinase encoding)-gene of *T harzianum.* This gene was chosen since ist expression has been demonstrated to be triggered during mycoparasitic interaction (Carsolio et al.. 1995-, Proc. Natl. Acad. Sci. USA 91, 10903 ff.). Cell-free extracts from *T harzianum* mycelia, harvested before contact with *B. cinerea.* and those harvested upon mycoparasitism on *B. cinerea* both produced high-molecular weight protein-DNA complexes. The complex obtained from mycoparasitic mycelia exhibited a smaller size. Competition experiments, using oligonucleotides containing functional and non-functional consensus sites for binding of the carbon catabolite repressor Crel provided evidence that the complex from nonmycoparasitic mycelia involves binding of Crel to both *ech-42* promoter fragments. These findings are consistent with the presence of two consensus sites for binding of Crel in the *ech-42* promoter. In contrast, the protein-DNA complex from mycoparasitic mycelia does not involve Crel as its formation is unaffected by the addition of the competing oligonucleotides. The mycoparasitic complex can. however. be changed into the non-mycoparasitic complex by the addition of equal amounts of protein of cell-free extracts from non-mycoparasitic mycelia, or by the addition of the purified Crel-.:glutathione-S-transferase protein. These findings suggest that a model for regulation of *ech-42* expression in *T. harzianum* which involves at least (a) binding of Crel to two single sites in the *ech-42*

promoter-, (b) binding of a “mycoparasitic" protein/protein complex to the *ech-42* promoter in close vicinity of the Crel binding sites- and (c) functional inactivation of Crel upon mycoparasitic interaction to enable the formation of the “mycoparasitic" protein-DNA complex.

Phytophthora infestans Population Divergence on Potato and Tomato in Moscow Region

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Earlier parasitic specialization of T0 and T1 races to potato and tomato and a difference for phenotypes rates among *P. infestans* isolates collected from the host plants were detected. In 1993 130 isolates were collected from adjacent fields and shown to be of A1 mating type. In contrast to potato, 7% of isolates from tomato were metalaxyl-resistant , 90% were of T1 race and 50% carried “old” Ib type of mtDNA. Potato isolates carried “new” Ia and IIa mtDNA and had higher mean number of virulent genes than the tomato isolates. According to frequencies of different pathotypes the isolates from potato and tomato could be grouped separately. Difference in frequencies of mtDNA types and pathotypes were found in the isolates from tomato fruits and leaves. Population divergence induced by specialization of *P. infestans* was genetically fixed by a larger fertility of T1 race hybrids in comparison with T0 and T0x T1 races

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Mitotic Stability of Transforming DNA in Antagonistic Fusarium *oxysporum in Vitro* and After Release in Soil Microcosms

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Before planning the large scale release of both genetically manipulated and wild type organisms, their behavior in natural and agricultural environments should be carefully evaluated for risk assessment. One of the main factors for consideration to predict the environmental risk of transformed biocontrol fungi is their genetic stability: any evidence for genetic instability in contained preliminary experiments should prompt a re-evaluation of the antagonist and its deliberate release, as unpredictable changes in the transforming sequences may lead to erroneous risk evaluation. A benomyl resistant, dark red-pigmented *Fusarium oxysporum* mutant antagonistic against phytopathogenic *formae speciales* of *F oxysporum* was transformed with the *Escherichia coli* hygromycin B phosphotransferase gene *(hph),* conferring hygromycin B resistance. Hybridization with the complete plasmid suggested that the integration had generally occurred in a multiple-tandem array at multiple sites. Both hygromycin B resistance and mitotic stability of nine transformants were evaluated after *in vitro* growth and after release of the transformants in soil microcosms. Three of the transformants were mitotically stable after four rounds of vegetative growth with no selective pressure, while six showed various changes in the integration pattern, mainly consisting in excision and rearrangement of plasmid copies. One transformant had lost the ability to grow in the presence of hygromycin B. Four weeks after release in soil microcosms all the transformants maintained the hygromycin B resistant phenotype, but six of them showed rearrangement of transforming DNA. Only one strain underwent no obvious rearrangement of the transforming DNA both after *in vitro* growth and after recovery from the soil microcosm. The construction of genetically engineered strains of antagonistic *F. oxysporum* marked with altered pigmentation and with double resistance to

benomyl and hygromycin B will be very helpful for further studies on biological activity, population dynamics and behavior of this biocontrol agent under various conditions. This will provide additional knowledge to the build-up of a risk assessment protocol to predict the fate and effects of genetically altered microbial antagonists in agricultural environments.

Isolation of the *Ashbya gossypii Genes AgLEU2* and *AgSTE7* by Heterologous Complementation and Characterization of Non-reverting Mutants

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The filamentous ascomycete *Ashbya gossypii* was isolated as a phytopathogenic fungus of cotton. *A. gossypii* can naturally excrete riboflavin (vitamin B2) and improved strains are used for the commercial production of this vitamin. Systematic molecular genetic investigations with this fungus started only a few years ago. A transformation system based on the resistance to Geneticin by expression of the bacterial Kanamycin-resistance gene was established. Surprisingly free replication of *Saccharomyces cerevisiae ARS* plasmids and efficient homologous recombination takes place in this fungus.
Two A. *gossypii* genes were isolated by heterologous complementation of *S. cerevisiae* mutations with a genomic A. *gossypii* library. The cloning of the *AgLEU2* gene was an essential prerequisite for the construction of *A. gossypii* strains with a non-reverting leu2 deletion. This was achieved by one step gene replacement and direct repeat induced homologous excision. The *AgLEU2* gene was successfully used as a selection marker on freely replicating plasmids and as a selection marker for one-step gene disruption. We also have started to study the biological function of an A. *gossypii gene* which is a structural homolog of the *S. cerevisiae*

STE7 gene. The *STE7* gene of *S. cerevisiae* codes for a protein kinase and is involved in mating of haploids and in control of pseudohyphal growth in certain diploids under nitrogen limitation. The *A. gossypii homolog (AgSTE7)* could be cloned by heterologous complementation and a knock-out mutation *(Agste7)* was constructed using the *AgLEU2* marker gene. The *AgSTE7* protein shows significant homology to serine/threonine protein kinases. The *AgSTE7* gene is non-essential and does not influence sporulation, germination of the spores and growth on full medium. However, on minimal medium with asparagine as N-source the *Agste7* mutant strain grows significantly slower than the wild type strain.

Mating Populations of *Gibberella fujikuroi (Fusarium* Section *Liseola)* from European and Their Maize and Their Toxigenic Profile

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Gibberella fujikuroi (Saw.) Ito *(Fusarium* section *Liseola),* a fungus distributed worldwide occurring on a variety of host, is one of the most important pathogens of maize. On the basis of the fertility, *G. fujikuroi* has been subdivided into at least six different, genetically distinct mating populations, termed "A"-"F". We tested members of the *Liseola* section isolated from maize from some European countries (Austria, Hungary, Italy, Poland) for their capability both to form sexual stage and to produce toxins fumonisin BI, beauvericin and fusaproliferin. Strains were identified as belonging to the "A","D", and "E" mating populations. Fumonisin B, was produced by isolates belonging to "A" and "D" mating populations. Beauvericin was produced at high level by isolates belonging to "D", "E", , whereas isolates of the "A" mating populations produced by isolates

little, if any, of this toxin. Fusaproliferin was produced by isolates from the "D" and "E" mating populations, but not by isolates from the"A". These results revealed the occurrence of three different mating populations of *G. fujikuroi* from European, maize and support the fact that each mating population has a characteristic toxicological profile.

Isolation and Replacement of a Cellulase Gene from the Fungal Pathogen *Claviceps purpurea*

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Cytological analyses indicate that the degradation of cellulose might be an important factor during the infection of rye by *Claviceps purpurea.*

Using cbhI from *Trichoderma reesei* as a probe, a putative cellulose gene (CEL 1) was isolated from a genomic library of *C. purpurea* strain T5. The derived amino acid sequence shows significant homology to other fungal cellobiohydrolases, but it obviously lacks the substrate binding domain, which is also regarded for the cellulose encoding genes cbh1-1 from *Phanerochaete chrysosporium* (Covert et al. 1992) and CEL 1 from *Cochliobolus carbonum* (Sposato et.al 1995).

Transcription analyses by northern indicate that CEL 1 is induced by crystalline cellulose on solid medium.

CEL 1 was inactivated by targeted gene replacement. Transformants with homologous integration of a deleted CEL 1 gene still show cellulolytic activity in axenic culture, but no transcription of CEL1 could be detected after induction with crystalline cellulose.

To reveal the role of the CEL 1 gene in pathogenicity of *C purpurea infection* studies with the isolated transformants on rye were performed. Evaluation of the data will be presented.

A CEL 1 complementation vector was constructed by cloning the

CEL1 gene with adjacent promotor and terminator regions in the hygromycin resistance vector pAN7- 1. Complementation of the CEL 1 mutants with this vector will show, if the reduced pathogenicity of the transformants is due to the absence of the CEL 1 gene product. The complementation vector could also be useful to obtain multi copy CEL1 transformants for further analysis of the regulation of the CEL 1 gene and the corresponding protein. References: Covert et al. (1992), Appl.Env.Microbiol. 58:2168-2175, Sposato et al. (1995), MPMI 8(4):602-609.

Clustering of Trichothecene Producing *Fusarium* Strain Determined from Partial rDNA Sequences

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Fusarium genus includes several species which produce trichothecenes, toxigenic secondary metabolites involved in plant and animal disease. The genetic relationship among trichothecene producing *Fusarium* species was investigated by PCR amplification and by analysis of partial sequences of a variable region at the 5' end of the larger nuclear rDNA. All trichothecene producing strains clustered together and two principal monophyletic groups were resolved. The first clade includes strains of *F acumitialtim*, *F. sambucinum, F. tumidum, F conipactitm, F. camptoceras* (red pigmented), *F sporotrichioides* and *F venenottini* which produced type A trichothecenes (T-2 toxin, HT-2 toxin, neosolaniol and diacetotoxyscirpenol). A second clade consist of *F. crookwellense, F. culmorum* and *F graminearum* producing type B trichothecenes (fusarenone-X nivalenol and deoxynivalenol). Such study shows a good relationship between genetic affinity and mycotoxin profile in *Fusarium* genus. Moreover, this region appears quite reliable for a

rapid determination and phylogenetic arrangement of uncertain and atypical toxigenic *Fusarium* strains.

Ectomycorrhizal Fungus Talks to Plant - Up-regulation of the par Gene Expression of Eucalyptus globtilus by Pisolithus hypaphorine During the Symbiosis Development

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Several soil fungi are able to colonize roots of woody plants to develop a symibiotic association, the ectomycorrhiza. In this symbiosis both, root and fungus function no longer independently, but form a novel organ with adapted metabolic pathways controling the exclhange of metabolites between both partners. The most pronounced morphological changes of the plant occurring during ectomycorrhisa development are an enhanced formation of secondary roote, the loss of root hairs and swelling of the apex of infected roots. Comparable morphological changes of the root architecture are obtained by, the application of exogenous auxins to plant.
To investigate the molecular mechanisms which take place during the ectomycorrhiza development, we have isolated a full length cDNA clone from Eucalyptus globulus which shares homology with an auxin-induced gene, the parC from tobacco and Arabidopsis. In these plants. the expression of' the parC gene coincides with cell divisions accompanying the initiation of root primordia. The eucalypt parC gene was expressed at a constitutive level in roots and other tissues of the eucalypt seedlings. However, the steady state of the parC gene was several fold up-regulated in eucalypt roots during the early steps of the interaction with the ectomycorrhizal Gasteromycete Pisolithus tinctorius.
An enhanced level of the parC transcripts was also obtained in roots

of seedlings incubated in growth medium containing micromolar concentrations of IAA or its analogs. A dramatic up-regulation of the level of parC transcripts was also obscrved in roots incubated in the presence of either Pisolillitis extracts or the indolic compound hypaphorine, a tryptophane betaine, abundantly excreted by this fungus. The latter data indicated that the fungal hypaphorine is able to triger 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 difusible signal from an ectomycorrhizal fungus.

Isolation and Characterization of Phase Specific Clones of the Grass Pathogen *Claviceps purpurea*

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The ergot fungus *Claviceps purpurea* infects the florets of rye and other grasses . The infected ovary is fully replaced by fungal tissue. During this process a balanced biotrophic interaction and a distinct border between fungus and plant is established; the fungus switches from a conidium producing form (sphacelium) to an alkaloid producing resting structure (sclerotium). We are characterizing the interaction between *C.purpurea* and its host *Secale cereale.* We have isolated two clones out of a genomic gene bank of *C. purpurea* which seem to carry regions which are expressed in the honeydew(plant colonization, conidia producing) phase, but not in axenic culture. Both clones were sequenced, and the transcribed regions were localized. The identification of phase specific ORFs turned out to be difficult. As an additional approach we are now trying to construct at least a partial cDNA bank from infected plant florets.

Expression of *Septoria lycopersici* Tomatinase in Heterologous Fungi

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The steroidal glycoalkaloid saponin -tomatine has been implicated in the resistance of tomato to attack by saponin-sensitive fungi (Schonbeck and Schlosser, 1976). In general, fungal pathogens of tomato are more resistant to this compound than fungi which do not infect tomato (Arneson and Durbin, 1968). A number of tomato-infecting fungi produce extracellular enzymes known as tomatinases, which detoxify -tomatine by the removal of sugars (for review see Osbourn, 1995). The tomatinase enzyme produced by the tomato leaf spot fungus *Septoria lycopersici* has recently been purified and the cognate gene cloned (Osbourn *et al.,* 1995; Sandrock *et al.,* 1995). Experiments are currently in progress to test the role of this enzyme in pathogenicity of *S. lycopersici* to tomato, using the techniques of targeted gene disruption. This poster will describe the expression of *S. lycopersici* tomatinase in the heterologous fungi *Neurospora crassa* and *Cladosporium fulvum*, neither of which produce -tomatine-degrading enzymes. The effects of tomatinase expression on the ability of *N. crassa* to colonize green and ripe tomato fruits, and on the compatible and incompatible interactions of races of *C. fulvum* with tomato cultivars, will be presented.

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Characterization of a Repetitive DNA Sequence from *Erysiphe graminis* fsp. *tritici*

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Erysiphe graminis is an obligate pathogen causing powdery mildew of cereals. It adapts rapidly to new environmental factors and is able to overcome both host-plant resistance and fungicide control measures. Increased understanding of genome organization of *E. graminis* should aid both the development of techniques leading to the cloning and analysis of pathogenicity and fungicide resistance genes and improved disease management. Repeated DNA sequences have already been described in filamentous fungi including *E. graminis fsp. hordei* (Rasmussen *et al.* Mol. Gen. Genet., 1993, 239: 298-303). The ability of these repetitive elements to move throughout the genome may be involved in the adaptive potential of powdery mildew.

We have previously described a genomic clone from *E. graminis fsp. tritici* (pAT5B, Grosjean-Cournoyer *et al.* ECFG2, 1994, D4) which contained a repetitive element which showed some homology to pBTEG20, the clone containing the B-tubulin gene of *E. graminis fsp. hordei* (Sherwood and Somerville, Nucleic Acid Res., 1990, 18: 1052). We have further characterized this repetitive sequence and determined by Southern analysis, that it is at least 700 bp in size. Slot blot analysis estimated the repetitive element to be present in several hundred copies per genome. The nucleotide sequence of the cloned repetitive sequence has been determined and this showed no structural similarity with repetitive elements described in other organisms. Restriction analysis and Southern hybridization of several different genomic clones from a *E. graminis fsp. tritici* genomic library indicated that this repetitive element is distributed throughout the genome and has identified sequence differences within a

population of repetitive elements.
We are currently working to define the exact size of the repetitive element. Sequence variation within this repetitive element family in *E. graminis fsp. tritici* will be investigated further by Southern analysis of restricted genomic clones. To complement these approaches a second repetitive element from the *E. graminis fsp. tritici* genomic library will be isolated and sequenced.

Cloning the Tomatinase Gene from *Fusarium oxysporum* f.sp. *lycopercisi.*

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A possible determinant of resistance of tomato to fungi has been attributed to the presence in the plant of a preformed inhibitor of fungal growth: the -tomatine. Previous studies have shown that tomato pathogens are less sensitive to -tomatine that are most non-pathogenic fungi. In this way some fungi are resistant to tomatine because of their membrane composition, while others produce specific tomatine-detoxifying enzymes known as tomatinases. *F oxysporum* f. sp. *lycopersici* produce an inducible tomatinase (1). The significance of -tomatine detoxification by this fungus in the pathogenic process has not been determined. In order to clone the tomatinase coding gone different approaches were used. One of them was to utilize as a probe a fragment of cDNA of the avenacinase gene from the oat pathogen *Gaeumannomyces graminis var. avenae (2).* Avenacinase degrade the oat saponine avenacine, a compound related to -tomatine. A genomic library from F *oxysporum. f.sp. lycopersici* constructed in the vector -EMBL3 was screened against the heterologous probe. Several clones were isolated that hybridised specifically with the cDNA fragment from the avenacinase gene. The pattern of hybridization indicated that the positive clones corresponded at least to four different DNA genomic fragments.

Hybridising segments have been subcloned characterized and sequenced. We found that three of them were represented as unique copies in the genome. The second approach was to construct a cDNA library of the fungus using mRNA obtained from mycelia induced with -tomatine. A degenerate oligonucleotide deduced from the aminoend of the purified tomatinase was used as a probe to screen the library. The nucleotide sequence of the fragments containing the putative genes are being determined and their expression under different induction conditions are being studied.
(1) Ford J E, D J McCance, R B Drysdale (1977) Phytochemistry 16:545-546
(2)Bowyer P, B R Clarke, P Lunnes, M J Daniels, A E Osbourn (1995) Science 267:371-374

Differential Screening of a Genomic Library to Isolate *Botrytis cinerea* Genes Induced During Infection of Tomato.
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The Ascomycete *Botrytis cinerea,* a.k.a. Grey Mould, causes severe pre and post-harvest crop loss to a wide range of ornamentals and other plants, but there is a lack of fundamental insight into molecular aspects of the infection process.
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.
In the *B. cinerea*-tomato interaction, the isolation of genes expressed during infection is carried out by means of a non-biassed approach. This is performed by differential screening of a genomic library of *B. cinerea.* For the probes, poly(A)' RNA was isolated from *B. cinerea grown* in *vitro* and the tomato-Botrytis interaction, and reverse

transcribed into cDNA in the presence of 32P- dATP. Since the fungal poly(A)+ RNA in the interaction sample only represents 3%, the *in vitro* probe was supplemented with a tenfold excess of tomato poly(A)+ RNA to establish a better comparison and to exclude fungal clones hybridizing to tomato cDNAs.
Phages which hybridized differentially after the first and second screening were used to isolate DNA. Hybridizing bands, selected on Southern blots, were cloned and checked on genomic blots containing *B. cinerea DNA* and tomato DNA to confirm the origin. Finally, the expression *inplanta* and *in vitro* is checked on northern blots containing RNA from *B. cinerea* grown *in vitro, Botrytis-tomato* interaction RNA and appropriate controls. First results of this screening procedure will be presented.

Detoxification of -tomatine by the Phytopathogenic Fungus *Botrytis cinerea* - Molecular and Biochemical Studies

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Botrytis cinerea is the causal agent of "grey mould", a severe disease of many economically important fruits, vegetables and flowers. Our main interest is in the mechanisms of virulence and pathogenesis during the interaction process between *B. cinerea* and its hosts. Since the detoxification of saponins - preformed fungitoxic compounds involved in plant defense against pathogens - has been shown to determine host-specificity in the *Gaeumannomyces-Avena* pathosystem (Osbourn et al., 1994a, 1994b), 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 (Verhoeff and Liem, 1975). In contrast to literature data, we identified the detoxification product as 1-tomatine, suggesting a xylose removing activity of the *Botrytis* tomatinase. Analyses of field isolates from different hosts indicate that not all of

them are able to detoxify -tomatine. A tomatinase deficient isolate showed enhanced sensitivity towards -tomatine in in-vitro assays compared to tomatinase producing isolates. Infection tests on detached tomato leaves with both tomatinase-producing and non-producing strains showed significantly reduced virulence in the nonproducing strain.
Using the tomatinase cDNA of the tomato pathogen *Septoria lycopersici* (Osbourn, pers. comm.) as a heterologous probe we isolated a corresponding gene *(toml)* from a genomic library of *B. cinerea SAS* 56. The putative tomatinase gene showed significant homology with the probe and with the avenacinase gene of *Gaeumannomyces graminis* on both nucleotide and amino acid level. Analyses *of B. cinerea* field isolates derived from different hosts showed that the cloned gene of the reference strain is present in all isolates.
The importance of -tomatine detoxification for phytopathogenicity is under investigation using complementation of a tomatinase deficient wild type strain with the *toml*-locus of strain SAS 56, together with gene replacement in the tomatinase-positive strain B. 05. 1 0.

The G Protein Alpha Subunit Gpa3 Is Involved in the Pheromone Response of *Ustilago maydis*

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In the maize pathogenic fungus *Ustilago maydis* mating and sexual development are regulated by the a and b mating type loci. The multiallelic b locus governs pathogenicity while the a locus, which occurs in the alleles *a1* and *a2*, is responsible for cell-recognition and fusion. The cloning and molecular analysis of the a locus revealed the existence of genes for pheromone precursors (*mfal* and *mfa2)* and their respective receptors (*pral* and *pra2).* These pheromone receptors show significant homologies to serpentine receptors,

structurally characterized by seven transmembrane spanning domains and functionally coupled to heterotrimeric G proteins. Thus, the signal transduction of the pheromone stimulus in *Ustilago maydis* is expected to occur via a G protein mediated mechanism. Using cross hybridization studies and PCR of genomic DNA with degenerated primers three genes coding for alpha subunits of G proteins could be identified. These genes have been designated *gpal, gpa2* and *gpa3*. Null mutants were generated for all three genes and tested for mating competence. Only the *gpa3* null mutants showed an alteration in morphology and were sterile. In haploid *gpa3* strains the basal expression level of the pheromone inducible *mfal* gene was unchanged. However, upon pheromone induction no increase of the mfal expression could be detected in *gpa3* strains. This result indicates that the loss of gpa3 abolishes pheromone stimulation of *mfal* gene transcription. By side directed mutagenesis we have created a constitutively active gpa3 allele. This mutation causes a significant increase in *mfal* gene expression in haploid strains. We take this to indicate that Gpa3 plays an active role during the pheromone-signaling in *Ustilago maydis.*

Stress-inducible Cell Wall Proteins from the Mycoparasitic Fungus *Trichoderma harzianum*.

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cDNA clones encoding cell wall proteins (QID3 and QID74) were isolated from a library prepared from chitin-induced mRNA in cultures of *Trichoderma harzianum.* Whereas QID3 gene was induced by , at least, chitin, fungal cell walls and nutrient-stress. QID74 was also induced by abiotic stress conditions such as heavy metals (Cu and Zn), saline solutions and high temperatures, even in the presence of glucose.

When QID3 protein was introduced in yeast, the growth rate was similar to that of the control but the protein gave rise to cell division arrested in cytokinesis and cell separation. Qid74 protein does not alter the phenotype of the yeast cell. They divided normally but those cells which expressed the protein seemed to be more resistant to mechanical forces such as vacuum: under the scanning microscope, the cells were more turgent and well preserved in comparison with the control. In addition, both conjugation and sporulation were slowed down and the yield was also lower. Western analysis has shown QID74 protein to be located in the cell wall.
The sequence of the QID3 protein shows similarities both with cell wall plant proteins and fungal hydrophobins: small cell wall proteins highly hydrophobic which contribute to the fungal morphogenesis as well as cell-cell attachment and/or pathogen recognition and appresories formation. The sequence of QID74 has homologies with cell wall proteins involved in resistance mechanisms such as extensines or the Balbiani Ring Proteins.
When the expression pattern of QID74 gene was compared in two *T. harzinaum* mycoparasit-ic strains, one of them (CECT 2413) which produces high amounts of hydrolytic enzymes and the other one (IMI 206040) whose levels of enzyme production are very low QID74 was strongly induced only in the former strain.
Results points to QID74 and QID3 proteins being cell wall components of *T. harzinaum,* expressible under stress conditions and probably with different function: QID74 as a defense mechanism against biotic (lytic enzymes) and abiotic stress (i.e. heavy metals) and QID3 in cell-cell recognition by increasing cell hydrophobicity.

The Reproductive Biology of *Cryphonectria parasitica* at the Edge of the Chestnut Blight Epidemic in Europe.

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Chestnut blight caused by the introduced ascomycete *Cryphonectria parasitica* is still spreading in Europe. We have used vegetative compatibility (VC) and mating type to characterize three populations that have recently infested chestnut stands in northern Switzerland. The dsRNA virus that causes hypovirulence was not found in these populations. VC and mating type markers indicated that each population was founded by only one or two genotypes. Many isolates of two populations produced perithecia in the presence of both mating type testers and were also able to self-fertilize. Single ascospore and conidiospore isolates derived from two selfing strains showed segregation for mating type. Only few cultures of both spore types again self-fertilized. We conclude that selfing strains are heterokaryons for mating type probably as a result of mating type switching. The biological significance of these phenomenon may lay in 1) the production of ascospores important for long distance dissemination, 2)the possibility to outcross in the second generation of otherwise sexually incompatible strains and thus increasing genetic diversity, and 3) the defense against viruses which are not transmitted into ascospores.

ITS-sequence Analysis Shows That Flower Bulb Attacking *Rhizoctonia solani* Strains Are a Distinct Subgroup in Anastomosis Group 2- 1.

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Rhizoctonia solani Kuhn (*Thanatephorus cucumeris* (Frank) Donk) occurs word-wide and is a soilborne plant pathogen with almost unlimited host range. Many isolates exist that are saprotrophs or

mycorrhizal on orchids or other plants. The *Rhizoctonia solani* species complex is currently divided in 12 Anastomosis Groups (AG, designated AG 1 to 11 and AG BI), and several of these AGs can be divided in subgroups. For insight in pathogenic behavior and for the design of management control strategies it Is necessary to understand the population structure and the relationship between different isolates. Today molecular identification methods can be used to resolve questions on relationship.
AG2 can be divided into three subgroups. AG 2-1, AG 2-2 and AG 2-3, based on hyphal fusion frequency. AG2-2 can be subdivided in AG2-2-HGIII and AG-2-2-HGrV based on DNA base sequence homology. In addition, isolates belonging to AG-2 can anastomose in low frequency with isolates belonging to AG-8 and AG BI (bridging isolates). AG-2-1 is a typical pathogen of crucifers, but some strains defined as AG21 are also able to attack flower bulb.
34 strains belonging to AG 2 have been analyzed at sequence level, by asymmetrically PCR amplified ITS regions. The analyzed strains have been isolated from different diseased plants in The Netherlands, Japan and Italy. Phylogenetic trees were constructed according to ITS sequence variations (with the 5.8s rDNA being identical in all the isolates). All strains attacking flower bulb cluster together as a separate group within AG-2- 1. These results confirm that AG-2-t (the strains attacking flower bulb) is a specific subgroup within AG-2.

Inducible Tomatinase Activities in Different Formae Specialis of *Fusarium oxysporum* Not Pathogenic to Tomato Plant

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Fusarium oxysporum is a very common and worldwide existent soilborne plant pathogen that causes severe losses in a broad range of agricultural crops. Within the species there is a considerable host

specificity with over 120 described formae speciales and races capable of causing vascular wilt diseases on particular hosts. Plants have evolved different defense mechanisms to protect themselves against a great variety of invasive pathogens. The antifungal compound -tomatine, present in tomato plants, has been reported providing a preformed chemical barrier against phytopathogenic fungi. F *oxysporum f. sp. lycopersici,* a tomato pathogen, produces an inducible extracellular enzyme able to detoxify -tomatine. We only detected a unique inducible protein with tomatinase activity in extracellular filtrates. This protein was purified and characterized and was found to be a monomer of 50 kDa. The native tomatinase showed at least five isoforms with pi's ranging from 4.8 to 5.8. Treatment with Nglycosidase F gave a single protein band of 45 kDa, indicating that the 50 kDa protein was N-glycosylated. This result suggests that there is only one gene coding tomatinase. Tomatinase degrading activity was also inducible in other formae speciales not pathogenic on tomato such as *melonis, niveum* and *tuberose.* The host plant of these formae speciales (muskmelon, watermelon, potato) do not produce -tomatine. On the other hand tomatinase was absent in other formae speciales (e.g. *lini and conglutinans).* These enzymes have molecular weights similar to the enzyme from F *oxysporum* f. sp. *lycopersici.* The mechanism of action of these enzymes was identical; all of them detoxify -tomatine by cleaving the glycoalkaloid into the tetrasaccharide lycotetraoside and tomatidine. This finding suggests a possible evolutive parentage between different formae speciales of *F oxysporum.*

Rearrangements at a DNA Fingerprint Locus in the Rice Blast Fungus

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A family of repetitive elements, called MGR586, are dispersed throughout the genomes of rice pathogens of *Magnaporthe grisea,* and are associated with a high incidence of restriction fragment length polymorphisms (RFLPs). The analysis of MGR586 RFLPs resolves collections of field isolates into a small number of clonal lineages. The asexual mechanisms that produce variation among members of a lineage, as well as different lineages, are not known. In the process of analyzing the segregation of certain MGR586 RFLPs we identified a novel polymorphism, called MGR586-P2, in one member (designated 4395-12-3) of a sister spore pair from a complete, tetrad. Molecular cloning suggests that MGR586-P2 was generated by a novel, long terminal repeat (LTR)-containing retrotransposon called *fosbury.* Genetic analysis shows that MGR586-P2 and its progenitor polymorphism (MGR586-PI) are alleles of a single genetic locus termed the MGR586 polymorphic locus or MGR586-PL. Surprisingly, we also found that strain 439512-3 and its clonal descendants produced strains containing as many as three allelic forms of MGR586-PL (i. e. these strains are heteronuclear). These results suggest a pathway that produces clonal variation in *M. grisea* in which recurrent nonrandom rearrangements at a single genetic locus lead to the formation of a heteronuclear mycelium. Sporulation of this heteronuclear strain may then produce an array of clonal variants.

Functional Analysis of the *Cladosporium fulvum Avr9 Promoter*

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Physiological and biochemical aspects of the interaction between the fungal pathogen *Cladosporium fulvum* and tomato have been studied

quite intensively. From *C fulvum* several *in planta* induced genes have been cloned: the avirulence genes *Avr9* and *Avr4* and the putative pathogenicity genes *ecp1* and *ecp2.* The avirulence gene products e.g. the race-specific elicitor proteins AVR9 and AVR4, are thought to interact with the gene products of the complementary resistance gene Cf9 and Cf4, resp.) in tomato, thus giving rise to a hypersensitive-response (HR) which is a characteristic resistance reaction of the plant against pathogens. The *Avr9* gene is proven to be responsible for race-specific resistance by transformation and replacement studies (Van den Ackerveken et al. 1992) Expression of the *Avr9* gene is induced upon growth *in plaina.* When grown on liquid medium, expression of the *Avr9* gene could be induced by limitation of amount of nitrogen (present as nitrate, ammonium, glutamate or glutamine Van den Ackerveken et al. 1994)
Detailed sequence analysis of the *Avr9* promoter showed the presence of several putative regulatory elements with the consensus sequence (GATA) of tile binding site for major wide domain nitrogen regulator proteins like NIT2 of *Neurospora crassa* and AREA *of Aspergillus nidulans.* Using the *Avr9* promoter-GUS fusion in combination with the *argB* site directed integration system for *A. nidulans,* data have been obtained which suggest that functional sites for the binding of a regulatory protein homologous to Nin and AREA are present, and that the initial analysis of the *in vitro* induced mutations in the potential regulatory elements in the *Avr9* promoter is feasible in *A. nidulans.*

Van den Ackerveken GFJM, Van Kan JAL, De Wit PJGM (1992) Molecular analysis of the avirulence gene avr9 of the fungal tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. Plant J 2:359-366

Van den Ackerveken GFJM, Dunn RM, Cozijnsen TJ, Vossen P, Van den Broek HWJ, De Wit PJGM (1994) Nitrogen limitation induces expression of the avirulence gene avr9 in the tomato pathogen *Cladosporium fulvum* . Mol Gen Genet 243:277-285

Molecular Genetics of Infection-Related Development by the Rice Blast Fungus *Magnaporthe grisea*

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The blast fungus *Magnaporthe grisea* infects rice by elaborating infection structures known as appressoria. We are investigating appressorial development and the mechanism of appressorial infection by using a combination of biochemical and genetic approaches.
First we are studying the pathogenicity gene *MPG1* which is highly expressed during appressorial development and is required for efficient appressorial formation. *MPG1* encodes a class I hydrophobin and we have evidence that it encodes a novel rodlet protein composed of 5nm interwoven rodlets. This protein appears to interact with the rice surface during the prepenetration phase. Attachment to the rice surface is a multi-component process but chemical extractions clearly indicate an MPGlp hydrophobin mediated interaction occurs. This suggests the presence of MPGlp at the fungal-rice interface is a pre-requisite for efficient appressorial development and may transfer the correct inductive signal(s) to facilitate morphogenesis. Functional relatedness of MPGlp to other hydrophobins is also being studied by cross-species complementation to determine the conserved features required for surface interaction, appressorial development and pathogenicity restoration.
Secondly, we are studying the mechanism of appressorial infection. Appressoria generate up to 8MPa of pressure during the infection process which is transferred into mechanical force to breach the host surface. We are studying the physiology and molecular genetics of turgor generation. We have determined that in order to generate such high pressure a solute accumulates within appressoria to concentrations in excess of 3M. Appressorial extractions have identified the solute and we are carrying out genetic studies to determine the pathway by which it is synthesized and the regulation

of its accumulation.
Lastly we are carrying out a number of differential cDNA screens to identify genes expressed under nutrient limiting conditions which are also highly expressed during pathogenesis of *M. grisea.* We have identified and sequenced a number of cDNAs and mapped the corresponding loci and progress in this are will be reported.

Infection by the Ergot Fungus *Claviceps purpurea*: Structure and Function of Two Polygalacturonase Genes in Relation to Host Pectin Alteration

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C. purpurea is a biotrophic ascomycete infecting only florets of grasses. The fungus mainly colonizes the ovary by an intercellular mode of growthl. Therefore, pectinolytic enzymes have been thought to be of special importance2. Such activities had been detected in axenic and parasitic culture. We combined molecular genetics with ultrastructural studies in order to prove the fungal origin of such enzymes in infected ovaries and their operation on host pectin components.
Two putative polygalacturonase genes (pgl *and pg2)* were isolated from *C. purpurea,* using the *pgall* gene of *A. niger* as a probe. The two genes are closely linked head-to-tail and are highly homologous. They probably code for mature proteins of 343 and 344 amino acids, showing significant homology to endopolygalactuonases of filamentous fungi. We study the expression of *pgl and pg2* in axenic and parasitic culture using RT PCR.
Their corresponding substrate, polygalacturonic acid, was demonstrated to be a component of the host cell walls in rye ovaries, using immunogold TEM with a monoclonal antibody (JIM5) specific for homo-galacturonic acid regions of pectin. JIM5 epitopes were localized along the usual infection path in healthy carpels. At the

interface of penetrating and of intercellular hyphae, JIM5 label was heavily increased. The observed host wall alterations provide evidence for the secretion and activity of fungal extracellular enzymes *in planta.*

1 Tenberge, K.B., Tudzynski, P. (1994) BioEngineering 10 (S3/94), 22.
2 Tudzynski, P., Tenberge, K.B., Oeser, B. (1995) In: Pathogenesis and host specificity in plant diseases: histopathological, biochemical, genetic and molecular basis, Bd. 11, Eukaryotes, pp. 161-187, Kohmoto, K., Singh, U.S., Singh, R.P., eds., Oxford, Elsevier Science, Pergamon Press.

Functional Analysis of the *in planta* Induced Gene *ipiO of Phytophthora infestans*

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An *in planta* induced gene, *ipiO,* of the potato late blight pathogen *Phytophthora infestans,* was isolated from a genomic library by differential hybridization. *P. infestans* has two *ipiO genes, ipiO1* and ipiO2, which are very similar and closely linked. The encoded proteins, IPI-01 and IPI-02, have no homology with known protein sequences (Pieterse *et al.,* Gene 138: 67-77). The *ipiO* genes are expressed at high levels in the early stages of the pathogenic interaction of *P. infestans* with its host plants potato and tomato suggesting that the IPI-0 proteins have a function in pathogenicity (Pieterse *et al.,* MGG 244: 269-277). ipiO mRNA is also detectable prior to infection i.e. in zoospores, in cysts and in germinating cysts. In sporangiospores no *ipiO* mRNA is found, whereas germinating sporangiospores contain only little *ipiO* mRNA. In order to assay the role of IPI-0 in pathogenicity, we transformed *P. infestans with* constructs carrying a strong oomycete promoter fused to the *ipiO*

coding sequence in anti-sense and sense orientations. In the anti-sense and sense transformants the endogenous ipiO mRNA level was determined and in addition, the transformants were tested for their ability to cause disease on potato leaves. Characterization of the transformants will be presented and the possible role of IPI-0 during pathogenesis of *P. infestans* on its hosts will be discussed.

The Differentiation of *Leptosphaeria maculans* Strains from Poland

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The ascomycete *Leptosphaeria maculans* (Demn.) Ces. et de Not., together with its conidial stage *Phoma lingam* (Tode ex Fr.) Desm., is one of the most common and devastating fungi to oilseed winter rape *(Brassica napus* L.) in Poland. In the last decade the pathogen has spread from the northwestern part of Poland to all intensive rapeseed cultivation areas of the country.
Multidisciplinary studies to assess the distribution of aggressive pathotypes have been undertaken. Fifty strains collected from distant geographical areas were described in terms of colony morphology, sirodesmin production, RFLP and RAPD-PCR patterns. The results indicate the prevalence of non-aggressive isolates, with few aggressives - coming mainly from the northwestern part of Poland. However, the studies of potential isolates pathogenicity to rapeseed (1) suggest the high heterogeneity within “non-aggressive" group, varying from weakly to fairly harmful for rapeseed plants.
(1)Joqczka M., Lewartowska E., Frencel I. (1994). Phytopath.Polonica 7 XDC): 71-79

Molecular Analysis of Mixed Fungal Infections on Oilseed Rape *(Brassica napus)*

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Cultivation of oilseed rape has increased in importance during the past two decades especially in the European Community. This rise in popularity was accompanied by a succession of serious fungal epidemics caused by a variety of pathogens, which often occur in mixed infections. In Germany, probably the most important pathogen is *Phoma lingam* (sex. form: *Leptosphaeria maculans),* the causative agent of blackleg disease, leaf spot and stem canker. Other fungi commonly found in the blackleg complex are *Cylindrosporium concentricium* (light leaf spot), *Sclerotinia sclerotiorum* (stem rot), *Fusarium moniliforme, A1ternaria brassicae and A. brassicicola* (dark leaf and pod spot), *Verticillium dahliae, Pythium* sp. and *Rhizoctonia solani.* There is considerable interest in a fast and reliable procedure for analyzing these fungi within the blackleg complex.
We have identified RAPD-PCR amplificates which represent DNA sequences for most of these fungi. The species specificity was tested by eluting PCR bands from agarose gels and using them as probes in Southern blot experiments. For *Phoma lingam,* bands which turned out to be specific for the species and even for the major pathotype groups,”"aggressive” and "nonaggressive", have been characterized by sequencing. On the basis of these sequences, pathotype specific PCR primers have been constructed (1). This method allows the fast and reliable *Phoma* diagnosis without the known drawbacks of the RAPD-technique for routine analysis.
(1) Voigt, K., Wostmeyer, J. (1995). Microbiol. Res. 150, in press

Genetics and Toxicity of the Phytopathogenic Ascomycete *Calonectria morganii* (Imperfect State *Cylindrocladium*

scoparium)

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To study genetic diversity of the phytopathogenic *Calonectria morganii* (imperfect state *Cylindrocladium scoparium),* thirty two strains isolated from ericaceous hosts and two specimens from ATCC were examined by Random Amplification of Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP). Five oligonucleotides were chosen as primers to differentiate the isolates. RAPD patterns of the ATCC strains differ significantly from those of the field isolates. Diversity among field isolates is low. Results obtained in RFLP analysis using telomere repeats of *Neurospora crassa* as a probe were highly consistent with the RAPD data. To distinguish different mating populations isolates were paired in all possible combinations. Fertile perithecia occurred only in one combination, from which ascospores were analyzed by formal genetics and RAPD. A bipolar mechanism of homogenic incompatibility was found. Ascospore derived strains were much more variable than field isolates. Phylogenetic trees suggested a correlation to host plants from which strains were isolated.
Toxic compounds were detected in culture fluids as well as in mycelia. Among the strains tested toxin producers and non producers could be detected. The toxin was active in bacteria and also in eukaryotic organisms including yeasts, filamentous fungi, animals and plants. Preliminary results suggested the toxin to be a cyclic tetrapeptide carrying an epoxy decanoyl acid.

Molecular Genetic Mapping of Virulence in the Barley Net Blotch Pathogen, *Pyrenophora teres*

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Two strains of the fungal ascomycete *Pyrenophora teres,* one avirulent (strain 15A) and the other virulent (strain 0-1) on the barley cultivar 'Harbin', were mated and the progeny from the cross were isolated. Avirulence in the 76 progeny tested was observed to segregate at a near 1:1 ratio. Genomic DNA was prepared from fungal cultures of 15A, 0-1 and the cross progeny. Progeny DNA was combined into virulent and avirulent pools for bulked segregation analysis (BSA) using random amplified polymorphic DNA markers. Five markers were obtained that were amplified from DNA of avirulent progeny pools and the avirulent parent (1 5A), but not from the virulent progeny pools and parent (0-1). Estimates of marker order and marker genetic distance from the avirulence locus will be presented. The use of these markers in the potential cloning of the *P. teres* avirulence gene that is active on the barley cultivar 'Harbin' will be discussed.

Characterization of Two Genes of the Phytopathogenic Fungus *Gibberella pulicaris,* which are Induced by the Phytoalexin Rishitin

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Gibberella pulicaris is one of the causal agents of potato dry rot. The fungus enters the tubers via wounds where it is confronted with different pre- and postinfectional inhibitors. These compounds like the phytoalexins rishitin and lubimin are produced by the tubers as part of a general defense reaction induced by wounding and invasion of microorganisms. In order to identify attributes of phytopathogenic fungi like *G. pulicaris* affecting their virulence and pathogenicity towards their host plants we have started to isolate genes induced by the above mentioned defense compounds. The goal is to study the regulation of these genes and their importance for the virulence of

the fungus. By differential cDNA screening two clones of a genomic library of *G. pulicaris* have been isolated each carrying a gene induced by the phytoalexin rishitin. The first gene called *rinl* codes for a 400 bp transcript, which is also induced by the phytoalexin lubimin within 15 min. The second gene called *rin-2* coding for a 500 bp transcript is induced 3-4 hours later and only by rishitin. Comparison of the sequences of both gene so far did not reveal any homology to know sequences. The construction of knock out transformants is under way to investigate the importance of these genes for tolerance of *G. pulicaris* of phytoalexins and their possible impact on the virulence of the fungus on different host plants.

Metabolism of the Saponins -tomatine, -solanine and -chaconine by *Gibberella pulicaris*

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Gibberella pulicaris is a phytopathogenic ascomycete with a very broad host range. Among others the fungus infects potatoes and tomatoes. These plants are reported to accumulate saponins like -solanine, -chaconine, and -tomatine as preinfectional inhibitors in the vacuoles of their outer cell layers, from which they are liberated upon microbial attack or wounding. Therefore an invading fungus like *G. pulicaris* has to overcome this chemical barrier either by tolerating or by detoxifying these compounds.
G. pulicaris is able to metabolize -solanine and -chaconine by cleaving off part of the sugar moiety. In contrast, -tomatine is metabolized by removing the complete sugar residue resulting in the transient accumulation of the aglycon tomatidine. This compound is further hydroxylated to a product, which is not degraded any further. A 2 kb *BamHI* fragment was isolated from a genomic cosmid clone of *G. pulicaris* by hybridization with the tomatinase gene of *Septoria lycopersici.* Partial sequence analysis revealed 62 % and 66 % homology to the tomatinase gene of *S. lycopersici* and the

avenacinase gene of *Gaeumannomyces graminis,* respectively. Further sequences analysis and the production of knock out transformants are in progress to find out the function of the encoded enzyme.

The Plasma Membrane H+-ATPase of the Rust Fungus *Uromyces viceae-fabae*: Genetic and Functional Analysis

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Rust fungi are biotrophic parasites which differentiate haustoria within plant cells. We are interested in the mechanism of nutrient uptake by haustoria and the role played by the plasma membrane ATPase.
The activity of the vanadate-sensitive plasma membrane H+-ATPase was found to be severalfold higher in haustoria than in rust spores and germlings. This indicates that the H+-ATPase generates an electrochemical potential across the haustorial plasma membrane which drives the secondary active transport of nutrients. Using plasma membrane-enriched fractions, the biochemical properties of the enzyme were determined and found to be similar to those of other fungal and plant H+-ATPases. For purification and a full enzymatic characterization, we are planning to express the rust H+-ATPase gene in yeast. A full-length cDNA of the rust H+-ATPase was cloned and sequenced. Surprisingly, the deduced amino acid sequence showed significantly higher homology to the H+-ATPases of plants than to those of ascomycetous fungi. Northern analysis revealed in haustoria a transcript of different size as compared to the transcripts of rust hyphae growing *in vitro*. Nevertheless, no differences in the cDNAs sequences from *in vitro* grown hyphae and from haustoria were detected so far. We are now testing which kind of changes in H+-ATPase transcript processing are taking place during rust development.

Poster Abstracts, Fungal Transposons

Recent Advances in the Molecular Biology of *Phycomyces*

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Protocols for transformation of *Phycomyces* protoplasts have been improved: the production and regeneration of protoplasts has been optimized and new vectors have been developed with a bacterial geneticin resistance gene under the control of regulatory *Phycomyces* sequences. A plasmid has been recovered from a -carotene superproducing strain obtained after microinjecting DNA from a plasmid genomic library into *Phycomyces* sporangiophores. The plasmid, containing a 3.1 Kb insert of *Phycomyces* DNA, confers upon microinjection the superproducing phenotype. No similarity was found by comparing the 3.1 Kb sequence with gene databases.
The gene *hmgA,* encoding the key enzyme in terpenoid biosynthesis HMG-COA reductase, has been cloned from a lambda genomic library. A 3 Kb segment containing the entire gene is now being sequenced.
Southern analysis indicates the presence of one copy of the *hmgA* gene in *Phycomyces.*
Highly repetitive DNA sequences of *Phycomyces* have been characterized. About 5% of the total genome is a repetition of any of two consensus sequences of 31 bp, named *PrAl and PrA2.* Higher order repetitions of groups of *PrA* units have been identified, surrounded by more complex repetitive sequences.
We have isolated two sequences *of Phycomyces* with similarities to ORFs encoded in two different transposable elements: Tcl from *Caenorhabditis elegans* and *Txl* from *Xenopus laevis.* Preliminary southern analysis suggest the presence of 10-20 copies of the Tcl-like transposable element in the *Phycomyces* genome.

Flipper, a Bacterial-like Transposable Element in *Botrytis cinerea*

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Botrytis cinerea (Botrylinia fuckeliana) is a phytopathogenic fungus having a broad host spectrum whinc includes grapevine, tomato, flower bolbs and otnamentals. There is no apparent specialization in the fungus for this wide host range. From a wild strain isolated from sweet pepper, a mutant deficient int he nitrate reductase gene nia) was selected through its spontanewus resistance to chlorate. A Southern Blot hybridization with the *nia* gene of *Bortytis cinerea* showed that a size polymorphism appeared between the mutant strain and the wild strain. By PCR we have shown that the mutation correspond to an insertion of 1.7 Kb in the coding region. The sequence of this insertion is characterized by two ITS and one ORF which has strong similarity with bacterial-like transposable elements, Pol2 of *Magnaorthe grisea* and Fot1 of *Fusarium oxysporum.* This new transposable element hot yet reported in discomycetes is named Flipper. Analysis by Southern blot hybridization have shown that the number of Flipper copies varied from zero to about 20 copies depending on the strains.

Evolutionary Origin of the *Impala* Element Within the *Fusarium oxysporum* Species

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The *impala* element was first identified as an insertion within the *niaD* gene. *Impala* is a DNA transposon (1,280 hp in length, 27 hp ITRS, TA insertion specificity) belonging to the *Tcl-mariner* superfamily (Langin et al. MGG, 1995). This inserted copy is

transposase defective due to the presence of several stop codons but it can be mobilized by a source of transposase. In order to identify an autonomous element, the 8 *impala* copies present in the genome of the F24 strain have been cloned and sequenced. Two copies carrying an ORF able to encode an active transposase have been identified. In addition, this analysis revealed the existence of a high level of nucleotide polymorphism leading to the identification of four subfamilies. Within a subfamily, the polymorphism is low (0.20 to 1.4%) whereas between subfamilies, it ranges between 9 to 30%. To gain insights on the evolutionary origin of the *impala* family, an analysis of the distribution of the different subfamilies has been carried out within the *F. oxysporum* species using Southern blots and PCR amplification. *Impala* elements belonging to the different subfamilies have been detected in most of the *formae speciales* analyzed. These results indicate that the *impala* element is an ancient component of the *F. oxysporum* genome with a presence of the different subfamilies before host specialization.

Restless, an Active Transposon from the Filamentous Fungus *Tolypocladium inflatum*

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Transposons in eukaryotes can be divided in two major groups (McDonald 1993; Flavell et al. 1994). Class I transposable elements are able to transpose via an RNA intermediate, thereby employing a reverse transcriptase. Class II elements transpose via DNA transposition intermediates, demonstrating their activity by direct excision and subsequent integration into target sequences. Transposons of both classes have previously been identified in filamentous fungi (reviewed in Daboussi and Langin 1994). We previously described the isolation and characterization of a new type of fungal class II transposons from *Tolypocladium inflatum (synonym: Beauveria nivea)* which so far has not been found in any

other fungus. 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. Its transcription and splicing characteristics were analyzed in detail. Based on cDNA sequencing, alternate RNA splicingmay lead to two different proteins, both encoded by the transposon. The function of these proteins is currently under investigation. In addition we will present data concerning the activity of the transposable element.
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Evolution of the *Fot1* Element in the Fusarium Genus: Horizontal Versus Vertical Transmission?
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Fot1 is a DNA transposon identified in filamentous fungi *Fusarium oxysporum* and recognized recently in other species. To understand the evolutionary origin *of Fot1,* we have undertaken an extensive survey of the *F. oxysporum* species. The presence of *Fot1* was detected in strains belonging to 15 *formae speciales* with a copy number ranging from I to more than 100. PCR amplications using the ITRs demonstrated that *Fot1* exists in this species essentially as full-length copies. Partial sequencing of *Fot1* copies in different strains showed that the nucleotidic polymorphism is very low (<1%). All these results suggest that *Fot1* was present in this species before the

host specialization and has been vertically transmitted. The existence of a few number of strains devoid of *Fot1* copies can be interpretated as the result of stochastic loss. The presence of *Fot1* in different *Fusarium* species was analysed by Southern blot analysis at high stringency conditions. Although this element appeared to be absent in most of the *Fusarium* species examined, *Fot1* -related elements were detected in species genetically distant from *F. oxysporum.* This discontinuous distribution can be explained by two non-exclusive hypotheses: (1)- *Fot1* is an ancient element present in the common ancestor of *Fusarium* species and the discontinuity is the result of a complete loss in some species; (2)- the presence of *Fot1*-related elements in distant species would correspond to a recent acquisition resulting from horizontal transfer. To gain insights on the mechanisms of spreading of *Fot1* within the *Fusarium* genus, the nucleotide divergence of *Fot1* elements from different species and compared to those observed for non-transposable chromosomal sequences.

Identification of Autonomous Copies of the *Fusarium oxysporum Fot1* Transposable Element

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Insertional mutagenesis is a powerful method for gene isolation that does not require prior knowledge on gene product. One of the tags efficiently used is represented by transposons. The discovery of two families of' DNA transposons (*Fot1 and Impala)* in the genome of the fungal plant pathogen *F. oxysporum,* offers the opportunity to develop a transposon gene tagging system, a strategy not yet available in fungi. These elements transpose at high rate by excision and reinsertion, which are features pertinent when designing gene tagging experiments. To gain insights on the autonomy of the *Fot1* copies we cloned them as insertions within the nitrate reductase gene

(niad) by developing a phenotypic assay based on the restoration of the *nia* function af'ter excision of the *Fot1* elements. Plasmids carrying the *niad* gene disrupted by two different copies of' *Fot1* were introduced by co-transformation with the pAN7-1 plasmid into stable nia- mutants deriving from two strains free of active *Fot1* copies. NiaD+ colonies have been recovered, thus indicating that the gene function was restored after excision of the *Fot1* copy. Molecular analysis of these *niad*+ revertants indicates that in more than 50% of them the excised copy has reinserted in a new genomic position, indicating that both *Fot1* copies encode all functions necessary for transposition. These results constitute the first identification of autonomous copies of transposons in filamentous fungi.

Molecular Epidemiology of *Aspergillus fumigatus* with AfuT1, a Retrotransposon-like Element

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Epidemiological studies of the opportunistic fungus *Aspergillus fumigatus* has required the development of molecular methods for fingerprinting strains isolated from patients and clinical environment. Repeated DNA sequences specific *of A. fumigatus* have been cloned and used as probes for Southern blot hybridization. This method allowed to show that immunocompromised patients with invasive aspergillosis are mostly contaminated by a single strain, whereas several strains are isolated from cystic fibrosis patients. However, when the latter patients develop an aspergillosis, the infection is due to a single strain. Moreover, the nosocomial origin of invasive aspergillosis was suggested in some cases.
The repetitive sequence has been characterized. It is a retrotransposon of 6914 bp, bounded by Long Terminal Repeats

(LTR) of 282 bp, with sequence and feature characteristic of retroviruses and retrotransposons. The organization of the domains homologous to the reverse transcriptase, RNase H and endonuclease domains of retroviruses revealed that A*fut*1 is a member of the gypsy group. However, *Afut*l is a defective element because of an accumulation of transitions from C:G to T:A generating multiple stop codons in the putative coding domains.

Tan and Vader - Transposable Elements Found from *A. niger* var. *awamori*

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A.niger var. awamori has transposable elements which we refer to as Vader (Amutan *et al,* 1996) and Tan (Nyyssonen *et al,* 1996). Vader was isolated using a transposon entrapment strategy, in which unstable nidD (nitrate reductase) mutants were screened for transposons. Four of the isolated *nidD* mutants were shown to contain a small insertion element. This 441 bp, ATrich insertion element, Vader, is flanked by 44 bp inverted repeats and is present in approximately 15 copies in the genomes of the two *A. niger* var. *awamori* strains examined (Amutan *et al.,* 1996). Vader contained no ORF and hence it was deduced that the mobility of Vader was dependent upon a transposase activity present elsewhere in the genome. A synthetic oligomer corresponding to the inverted repeat was used to clone a 2.3 kb element, Tan, which is present as a single copy in the *A. niger var. awamori* genome. Tan has a single ORF (1668 bp) encoding a putative transposase, which is bounded by IRs and Vader contiguous with it (i.e. IR-ORF-IR-IR-Vader-IR). Protein alignment of this 555 aa Tan encoded protein with other proteins in the EMBL and Genbank databases revealed 31.2% and 27.9% identities throughout the entire length of the protein to the *Fusarium oxysporum Fot*1 and *Magnaporthe grisea* Pot2 ORFS, respectively.

This result suggests that Tan was the element which had provided the activity for the Vader element to transpose. We hypothesize that at some stage the independent Vader element, although inactive by itself, has arisen from Tan resulting in current strains with only one copy of Tan providing transposase activity and numerous mobile copies of Vader dispersed in the genome.

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Mobile Genetic Elements Involved in the Control of Senescence of *Podospora anserina*

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In the ascomycete *Podospora anserina* life-span is controlled by nuclear and mitochondrial (mt) genetic traits. In particular, a mitochondrial plasmid of circular structure (p1DNA or senDNA) was demonstrated to be crucially involved in the control of senescence (1,2). The plasmid is derived from the first intron *(mobile intron)* of the mitochondrial cytochrome oxidase subunit I gene (3). Intron mobility was demonstrated by molecular approaches (4) and appears to be involved in the destabilization of the juvenile mtDNA leading to gross DNA rearrangements and to impaired cellular functions.

The analyses of an extrachromosomal long-lived mutant of wild-type strain A led to the identification of an extrachromosomal DNA

species (pAL2-1) with the typical characteristics of linear plasmids (5,6). This element was shown to interfere vhth the age-related liberation and/or amplification of p1DNA. In addition, pAL2-1 is able to integrate into the mtDNA leading to integrated plasmid copies with long terminal repeated DNA sequences at the integration site. Finally, the ability of pAL2-1 to modulate the life-span of *P. anserina* was demonstrated by plasmid transfer experiments. These experiments revealed that the *loss-of-plasmid* results in a decreased life-span, whereas *gain-of-plasmid* strains displayed a long-lived phenotype. Interestingly, all strains to which the plasmid was transferred were found to contain pAL2-1 in both, the autonomous and the integrated form.

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Rearrangements of Fusarium Retrotransposon Sequences Induced under Stressing Conditions

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Retrotransposons are mobile elements which replicate via an RNA intermediate and have been described in different fungal species (Mc Hale et al., 1989, 1992; Dobinson et al., 1993; Julien et al., 1992).

Recently a retrotransposon has been identified in *F*. oxysporum f. sp. *lycopersici*. (Anaya and Roncero 1995). This element designated *skippy* is 7,846 bp in length, it contains all conserved features of *gypsy* class retrotransposons and all the characteristic sequences presumably required for integration and functional transposition. The mobility of this retroelement as for other retroelements from filamentous fungi remained still unproven. Rearrangement of the retroelement *skippv (skp)* has been induced under stressing growth conditions in the presence of potassium chlorate. Three fungal strains, one showing wild type phenotype *str* and two of them resistant to chlorate and deficient for nitrate reductase, were studied by Southern analysis of their genomic DNA. Polymorphism in their hybridization banding pattern was detected in comparison with the wild type (wt) grown in the absence of chlorate. Results are consistent, with three different events occurring in the *str* strain: genomic amplification of *skp* in tandem copies, integration of new *skp* elements, and deletion of resident *skp* elements. The analysis of mutants *nidD*65 and *nidD*94 showed that only integration and deletion of copies of the elements had accursed. Amplification of genomic DNA from *str* using divergent primers belonging to the retroelement originated a new band of 589 bp corresponding to one LTR that was not present in wt strain.

Isolation and Characterization of a Transposable Element from *Fusarium* oxysporum fsp. *lycopersici*

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Fusarium oxysporum, like many other plant pathogens displays considerable phenotypic variability, a characteristic of organisms harboring transposable genetic elements. Bacterial-like transposable elements have been previously isolated from races of *F. oxysporum* f. sp. *melonis* (reviewed by Daboussi and Langin, 1994). We have used

the strategy of transposon tagging for isolating mobile elements *in F. oxysporum f.sp. lycopersici.* A total number of ninety nitrate deficient spontaneous mutants from strain 42-87 (race 2), positively selected for their chlorate resistant phenotype, were characterised by Southern analysis using the cloned nitrate reductase homologous gene as a probe. Among them two mutants (#66, #108) showed to harbor an insert of about 2,5 kilobases as deduced from their hybridization pattern compared with that of the parental strain. Total genomic DNA from mutants #66 and #1 08 was subjected to amplification by PCR using two specific primers from the 5' and 3' ends of the nitrate reductase gene. An unique band, with the expected size of 8kb, was amplified from each mutant and they were cloned in PGEMT vector for subsequent subcloning. The physical maps of the DNA inserts interrupting the nitrate reductase gene showed an identical restriction pattern. Their nucleotide sequences are being determined for identification of ITRs and transposase coding gene, The presence of homologous elements in other formae speciales is been studied in order to identify elementfree strains and to establish the autonomy of this *F. oxysporum* f. sp. *lycopersici* transposable element.

Yeast Transposable Elements

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Ty elements are a family of LTR retrotranposons found in the yeast *Saccharomyces cerevisiae.* To date five distinct families designated Tyl-Ty5 have been described. They can be divided into two classes related to the *copia or gypsy* class of plant and animal elements. The yeast elements are structurally and functionally similar to animal retroviruses. Like retroviruses they contain large internal coding regions flanked by long terminal direct repeats (LTR). They transpose through an RNA intermediate and use the same replication and integration strategy as metazoan retroviruses. The structure and

life cycle of the Ty elements will be briefly described. Transposable elements contribute to the fluidity and shaping of the eukaryotic genome. Chromosomal rearrangements which result in duplication or deletion are induced by recombination between regions of homology. Insertion of a retroelement DNA in or near a cellular gene can affect its regulation or inactivate it. Although Ty element insertion can occasionally be beneficial, the average fitness of host yeast,cells declines with increasing Ty copy number. The balance must be maintained between the level of transposition and the viability of the host cell. The recent studies showing that Ty retrotransposons integrate selectively into certain regions of the genome which can readily tolerate insertions will be discussed.

Poster Abstracts, Molecular Karyotyping and Gene Mapping

Single-read Sequencing Reveals Synteny Between *Ashbya gossypii* and *Saccharomyces cerevisiae* at the *Thr4* Locus

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Eight clones of a genomic *Ashbya gossypii* library were identified by Southern analysis to contain inserts derived from the smallest of the seven chromosomes. The termini of these clones were single-read sequenced and compared to data bases. This revealed the following homologies to *Saccharomyces cerevisiae* genes*: THR4* (threonine synthase), *PWP2* (periodic tryptophane protein), *CYRI* (adenylate cyclase), *ERGIA* (squalene epoxidase), *SSK2 (MAPKKK), CPR3* (cyclophilin-3).
For future use as an auxotrophic marker the entire *AgTHR4* gene was cloned and sequenced. This represents the first reported sequence for a threonine synthase from a filamentous fungus. The overall identity to the *S. cerevisiae* protein is 67.4 % on amino acid level. The disruption of the *AgTHR4* gene lead to threonine auxotrophy, which could be complemented by transformation with replicating plasmids

carrying the *AgTHR4* gene.
The homologies for *THR4* and for *PWP2* were found at the two termini of one plasmid, In *S. cerevisiae* these two genes map on chromosome III and are only separated by one other ORF. By sequencing the entire *AgTHR4* and the adjacent regions one further ORF and a partial ORF were identified. Surprisingly, these four adjacent ORF's are arranged in *A. gossypii and S. cerevisiae* in the same order and orientation. We have started sequencing the termini of many more A. *gossypii* clones to search for homologies and additional cases of synteny between this filamentous fungus and *S. cerevisiae.*

Vegetative Compatibility Groups and Electrophoretic Karyotype Variation among Isolates of *Fusarium oxysporum* from Common Bean Fields in Spain

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We have undertaken a comparative study of the genetic structure and variability of pathogenic and non pathogenic isolates of *F. oxysporum* from common bean fields in Spain. As a first step here we describe the vegetative compatibility grouping of *F. oxysporum* isolates and the correlation of these VCGs with electrophoretic karyotype (EK) variation.
F. oxysporum isolates were sampled from rizosphere or colonized tissues of common bean plants *(Phaseolus vulgaris L.)* from several plots in the zone of Barco de Avila in the province of Avila (Spain). Monoconidial cultures were derived from microconidia from the original isolate and assayed for production of Fusarium wilt in pathogenicity tests carried out on the bean variety "blanca redonda". Isolates which produced no external symptoms of wilt were classified as non pathogenic and used to generate nit mutants on PDC plates, following the procedure described by Correll (1987). *nit1,*

nit3 and *nitM* mutants from each isolate were tested for vegetative compatibility with complementary nit mutants from other isolates. Up to now we have found 15 VCGs with at least one *nitM* tester per VCG. No heterokaryon self-incompatible strains have been found among the isolates analyzed so far.
Chromosomal DNAs were prepared from all the isolates belonging to the 15 VCGs and subjected to CHEF electrophoresis. Different conditions were tried in order to resolve the maximum number of chromosomal bands. Most commonly the different EKs were composed of 9 to 11 chromosomes ranging in size from 0.7 Mb to around 10 Mb. EK variation was greater between members of different VCGS. Some VCGs had remarkably uniform EKs. For instance the four members of VCGI showed identical EKs even though these isolates were collected from different plots.
The results obtained show a high number of genetically distinct isolates, as determined by VCGs and EKs, among *F. oxysporum* colonizing common bean rizosphere. The next step will be to determine whether pathogenic isolates are also highly diverse or they belong to the same clonal lineage.

Genetic Evidence for the Occurrence of Dispensable 'B' Chromosomes in the Phytopathogenic Ascomycete *Leptosphaeria maculans* (Phoma lingam)

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Leptosphaeria maculans electrokaryotypes are easily established using CHEF (Contour-clamped Homogeneous Field) electrophoresis and reveal major chromosome length polymorphism between field isolates or following meiosis. All field isolates displayed a small-sized chromosome clearly separated from the overall electrokaryotype using appropriate electrophoretic conditions. Minichromosome ('MC') size polymorphism ranged from 650 to 950

Kb. Tetrad analyses of crosses between isolates displaying size polymorphism for the MC revealed either parental ditype segregation, monoparental segregation, or generation of MC of intermediate size. MC with higher or lower size than that of the parents were also generated. Genetic analyses thus demonstrated that MC from different field isolates were homologues. Eighteen percent of the tetrads analysed had lost the MC band for 1 or 2 of the 4 genotypes of the tetrads. Crosses between isolates differing in the presence:absence of the MC revealed non-mendelien segregations and demonstrated that some isolates could display at least two copies of the MC. Finally, saprophitic or parasitic fitness was not modified when isolates lacked the MC. All these data strongly suggested that the small-sized chromosome of *L. maculans* behaves like a dispensable 'B' chromosome.

Chromosomal Rearrangements and Despersed Repetitive Sequences in *Fusarium oxysporum*

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The discovery of at least seven families of transposable elements in the genome of the phytopathogenic fungus *Fusarium oxysporum* raises a number of questions on their role in the evolution and adaptation of natural populations. Indeed these elements could represent the origin of multiple chromosomal rearrangements which are responsible for the extraordinary karyotype variation detected among strains of *F. oxysporum*. These rearrangements (deletion, inversion, translocation) would be the consequence of homologous recombination events among copies of dispersed repetitive sequences. In order to better understand the role of transposable elements on the genome structure of *F. oxysporum* we developed an experimental system which is based on the introduction, within the same strain, of truncated copies of the *niaD* gene with a common sequence of about

2 kb, representing the substrate for recombination. The frequent observation of colonies able to utilise nitrate as a consequence of gene runction restoration by mitotic crossing over indicates that this process is highly efficient in *F. oxysporum*. We therefore analysed the karyotypic variation in order to identify the nature of observed rearrrangements. Pulsed-field gel electrophoresis conditions allowing the separation of chromosomes within 1 and 7-8 Mb range were defined. A polymorphism in the number (11-14) and in size of some chromosomes was observed among subcultures for the same strain and among strains in which a transposition event was selected. The precise nature of events is now under evaluation by probing the electrophoretic karyotypes with different single copy genes or transpoaable elements.

Mucor circinelloides Strains Differing in Mating Type Show Electrophoretic Karyotype Heterogeneity

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The electrophoretic karyotypes of strains NRRL 3631 *(M. circinelloides* f. *lusitanicus)* and NRRL A-7420 *(M. circinelloides* f. *gryseo-cyanus)* have been reported recently. They show a considerable amount of variation, both in chromosomal number and size. It would be desirable to assess whether these polymorphisms extend to other *M. circinelloides* strains and if they may be correlated with mating type.
Chromosomal DNAs were prepared from strains CBS 276.49 (incorrectly named CBS 277.49 in previous works), ATCC 1216a (-) and ATCC 1216b (+) and subjected to contour clamped homogeneous electric field gel electrophoresis (CHEF) under different conditions. 276.49 and 1216a, both strains of mating type (-), share the highest chromosomal homology, all the bands resolved being the same size except for the smallest chromosomes. 1216b,

mating type (+), has an electrophoretic karyotype that shows greater differences, both in chromosome sizes and number of bands. Disimilarities are more evident among the largest chromosomes. Genome sizes estimated by adding the sizes of individual chromosomes are around 31-33 Mb for the mating type (-) strains and around 46 Mb for the mating type (+) strain.
The degree of homology between chromosomes of similar size was further investigated: several single copy probes were used in Southern hybridizations to detect their chromosomal location. These probes are genes previously described *(pyrG and leuA)* or recently characterized by our group *(chs1* and *chs2,* genes involved in the chitin biosynthetic pathway). Hybridization data indicate that *pyrG, leuA and chs2* are each located in equivalent chromosomes of the three strains analysed, while *chs1* shows hybridization against the 5.7 Mb chromosome of (-) strains and the 2.7 Mb chromosome of the (+) strain.
These results suggest that polymorphisms at the chromosomal level may constitute a source of genetic variation among *M. circinelloides* strains differing in mating type. Whether this variation may be of some importance in hampering the outcome of genetic crosses needs further verification. A survey of *M. circinelloides* strains showing reasonably homologous chromosomes would be of help to determine the most adequate fertile pairs to perform genetic crosses in this fungus.

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. We identified *M. grisea* isolates pathogenic to rice and fertile in crosses. These isolates were used to identify fungal avirulence genes involved in interactions with race-specific rice resistance genes. Unravelling the mechanisms involved in such an interaction will help us in the identification and deployement of genes that confer durable resistance. Three genetically independent avirulence genes were identified in a cross between two isolates Guy 1 1 and 2/0/3, pathogenic to rice : Avr-MedNoi-1, Avr-Irat7-1, Avr-Ku86-1 (1, 2). These three avirulence genes are likely to interact with so far undescribed resistance genes in rice. In order to clone these avirulence genes by chromosome walking, we constructed a partial genetic map using 77 random progeny from this cross. This map includes 75 RFLP markers corresponding to either repeated, single copy or telomeric sequences and 25 RAPD markers. Two avirulence genes mapped to chromosome tips (Avr-MedNoi-I and AvrKu86-1). Using bulk segregant analysis, we found either one or two RAPD markers linked (I to 4 cM) to each avirulence gene. Most of these RAPD markers were composed of repeated and dispersed sequences alongside with single copy sequences. Single copy sub-clones were obtained from some of these RAPD markers. These single copy probes revealed polymorphisms between the two parents which could be due to chromosomal rearrangements (deletions) around avirulence loci. Linked single copy probes will be used as starting point for chromosome walks toward these genes.

1. Silue *D.et al.*. 1992. Phytopath., 82:577-580
2. Silue *D.et aL.* 1992. Phytopath., 82:1402-1407

A Genetic Linkage Map of *Phytophthora sojae*

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Phytophthora sojae belongs to the oomycetes which are characterised by gametangial meiosis, thus having a diploid life cycle. Until recently the genetics of virulence/avirulence in *P. sojae* was considered intractable due to its homothallic nature. Two crosses between genetically different isolates of *P. sojae* were established by coculturing parental isolates followed by screening the predominantly selfed progeny to identify hybrids using RAPD markers. Hybrids were recovered at a rate of approximately 2 % for both crosses. One isolate was used as a common parent in both crosses. F2 populations comprising over 200 individuals were generated for each cross. A subset of 53 F2 individuals from each cross was selected at random for analysis of segregations of avirulence genes and molecular markers, and finally the construction of a genetic linkage map. The genetic linkage map developed for *P. sojae* is based on 35 RFLP, 229 RAPD, and 7 dominant avirulence markers. The linkage map comprises ten major linkage groups and fourteen small linkage pieces covering a total of 968.4 cM. Close linkage (0.2 cM) of a RAPD marker to two avirulence genes (Avr4 and Avr6) may form the starting point for chromosome walking strategies directed towards cloning avirulence genes from *P. sojae.*

Chromosomal Mapping of an Endochitinase Gene from *Trichoderma hamatum*

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A PCR-amplified DNA fragment (1,450 bp) from the antagonistic fungus, *Trichoderma hamatum* was subjected to sequence analysis. This sequence proved to be highly homologous (93.1 %) to that of a chitinase gene from *Trichoderma harzianum,* but much less homology (66 %) was found with the chitinase gene from *Aphanocladium album.* The chitinase gene of *T hamatum* contains three introns, 57, 65 and 66 bp in sizes, as well as two highly

conserved regions located in the catalytic domain and probably code for the active site of the enzyme. When chromosome-sizd DNA-fractions of four other *Trichoderma* species (*T. atroviride, T. harzianum, T. koningli, T. viride)* were probed with the chitinase gene from *T. hamatum,* hybridisation signals developed in all cases on a chromosome aggregate of -5.5 Mb in size.

A Repetitive DNA Sequence from *Fusarium poae*

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Fusarium poae is a trichothecene producing asexual fungus and lacks a complete parasexual cycle, as well; all strains of this species harbour virus-like particles with double-stranded RNA genome. A 1209 bp clone, named ZITI isolated from *F. poae* strain 72.187 was found to be a moderately repetitive DNA sequence, which selectively hybridised to the polymorphic chromosomal regions of various *F. poae* strains. Sequence analysis of ZITI revealed retrotransposon-like structures, among them a characteristic zinc-finger motif was the most prominent. ZITI may play a role in the genome plasticity of F. *poae.*

Differentiating Interspecies Hybrids of Aspergilli by RAPD Analysis and Electrophopetic Karyotyping.

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Interspecific hybridisation between auxotrophic mutants of the filamentous fungi *Aspergillus nidulans, A. rugulosus* and A. *ochraceus* was studied using polyethylene glycol induced protoplast fusion. Viable prototrophic fusion products from crosses between A.

nidulans and *A. rugulosus* were exposed to benomyl containing medium which promotes haploidisation and resulted in the formation of haploid segregants. The aim of this investigation was to assess the suitability of Randomly Amplified Polymorphic DNA (RAPD) and electrophoretic karyotyping in distinguishing interspecific hybrid haploid progeny from their parental species.
Several 10-mer oligonucleotide primers which gave unique RAPD profiles for the parental species were used to analyse over twenty segregants. RAPD profiles different to those of either parent were generated demonstrating that recombinant hybrids had been isolated through an induced parasexual cycle.
Electrophoretic karyotypes were established by pulsed field gel electrophoresis using the contour-clamped homogenous electric field (CHEF) system. Unique karyotypes were obtained for the parental species. A. *nidulans* and A. *rugitlosits* were each separated into five chromosomal mobility groups. In both cases some groups contain more than one chromosome of similar size, which may be further identified by Southern analysis using chromosome specific probes. Nine segregants karyotyped to date indicate chromosomal reassortment has occurred.
Following protoplast fusion the established procedures for confirming and determining the extent of hybridisation between two species are based on genetic marker recombination studies. However in some circumstances it may be undesirable to introduce several genetic markers into the parental species through mutagenesis. The presented results clearly demonstrate both RAPD analysis and electrophoretic karyotyping to be valuable in the differentiation of interspecies hybrids and would provide a rigorous non-invasive analysis of hybridisation.

Characterisation of a Gene of the Blackleg Fungus, *Leptosphaeria maculans* Conferring Virulence on Indian Mustard

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Leptosphaeria maculans causes blackleg disease of oilseed *Brassicas* worldwide. The blackleg fungus is a model system for studying the genetic basis of host-pathogen interactions as it has a haploid vegetative state, is outcrossing, can be cultured on defined media, has a relatively small genome size and a high efficiency DNA transformation system. We are examining the interaction of blackleg isolates on *Brassica juncea* (Indian mustard). In a cross between a virulent blackleg isolate (attacks varieties of Indian mustard) and an aviruient isolate (cannot attack), the Fl and backcross progeny segregate in a 1:1 ratio, suggesting the presence of a single locus for virulence (Chen et al. 1995). We are determining the molecular basis of this trait.

One way that virulent *L. maculans* isolates may invade Indian mustard is by breaking down preformed host defence compounds. Seeds and leaves of this plant contain high levels of glucosinolates, sulphur-containing compounds that give mustard a pungent taste. Upon wounding of the plant, these compounds are cleaved into glucose and volatile compounds such as isothiocyanates, which are toxic to some insects and fungi. We have shown that volatiles from Indian mustard seed meal (a rich source of glucosinolates) inhibit the growth of avirulent blackleg isolates (cannot attack Indian mustard) much more strongly than they inhibit the growth of virulent isolates. We are currently testing the progeny of a cross between avirulent and virulent blackleg isolates to see if the ability to grow in the presence of the volatiles segregates with the virulence trait. We are also looking at the ability of virulent blackleg isolates to metabolise isothiocyanates.

Reference:

Chen CY, Plummer KM and Howlett BJ (1995) Ability of an *Leptosphaeria maculans* isolate to form stem cankers on Indian mustard segregates as a single locus. *European Journal of Plant Pathology, in* press.

Mapping the Genome of *Claviceps purpurea* by Means of RFLP Analysis and Electrophoretic Karyotyping

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Claviceps purpurea (Fries) Tulasne, belonging to the pyrcnomycetous ascomycetes, is a phytopathogenic fungus with a wide host range parasitizing more than 200 species of Poaceac. It is not known whether this is due to the existence of subspecies or physiological races.
Different strains of *Claviceps purpurea* vary frequently in their chromosomal equipment. It is possible to perform sexual crossings between strains with a different karyotype resulting in a fertile progeny.
In this work, the progeny of a crossing between two Claviceps-strains differing largely in their karyotypes were examined by means of Pulsed Field Gel Electrophoresis (PFGE), the analysis of Restriction Fragment Length Polymorphisms (RFLP) and Random Amplified Polymorphic DNA (RAPD). The majority of the molecular markers showed a 1: 1 -segregation. Pulsed Field Gel Electrophorcsis revealed six chromosomes for each parental strain, whereas nine linkage groups could be found by the analysis of the segregation patterns of the molecular markers (65 RFLP-markers, 32 RAPD-markers).

Genome Structure and Chromosomal Polymorphisms in Field Isolates of *Colletotrichum lindemuthianum*

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The filamentous ascomycete Collelotrichrim lindemuthiuanum causes anthracnose of the common bean *Phaseolus vulgaris,* and is responsible for significant yield losses in tropical regions. The fungus lacks a sexual stage but exhibits nevertheless a large degree of diversity in both pathotype, as determined by pathogenicity testing on a set of differential bean cultivars, and genotype, as measured by RAPD and RFLP analyses (Fabre *el al,* 1995). The genome structure was investigated at different levels. Flow cytometry was used to determine the haploid DNA content of the nucleus, as well as ploidy levels in the growing mycelium. Strains representing genotype groups I and 11 (as defined by ITS and RFLP analyses of the ribosomal DNA unit) had similar DNA contents, suggesting that the evolutionary divergence between these groups has not resulted in large-scale reductions or expansions of the genome. At a finer level, physical techniques (notably pulsed-field separation of chromosomes, separation of rare cutter digests, and hybridisation analysis) have been used to identify variable regions of the genome, with the aim of finding evidence of structural plasticity which might explain in part the high level of variability found in the fungal population.
The results of pulsed-field separations of nuclear DNA of twenty strains (representative of a worldwide collection offield isolates) reveal molecules of two remarkably disparate size classes, the first consisting of chromosomes which are larger than 10 Mb in size and not resoluble by pulsed-field electrophoresis, while the other comprises between one and four chromosomes in the range of 0,5 - 2,0 Mb. Within this latter class, there is great variation in both number and size of chromosomes indicating that at least a portion of the genome is subject to frequent rearrangements. Chromosome-specific sub-libraries are currently being prepared to obtain probes for the investigation of homology among chromosomes from different isolates.
Reference :
Fabre, J. V., Julien. J., Parisot D., & Dron. M. (I 995) Myc. Res. 99 (4), 429-43 5

AFLPc Mapping of the *Phytophthora infestans* Genome

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A new, powerful DNA fingerprinting technique called AFLP (Vos *et al.* NAR 1995 in press) has been used to generate molecular markers of the Oomycetous plant pathogen *Phytophthora infestans.* The AFLP technique is based on PCR amplification of genomic DNA restriction fragments to which linkers with known sequence are ligated. The PCR primers consist of the linker sequence but are extended with 1, 2 or 3 bases to select a subpopulation of the genomic fragments for amplification. In this way up to a hundred different amplified fragments can be obtained in a single PCR reaction. Electrophoresis of the radioactive labelled fragments on polyacrylarnide and autoradiography results in DNA fingerprinting patterns which are reproducible and easy to score. With the AFLP technique it is possible to analyse many markers in a short time on a population large enough to establish a linkage map saturated with molecular markers. Currently, we are analysing the segregation of AFLP markers in the Fl progeny of a cross between two *P. infestans* isolates (80029x88133). In this progeny there is also segregation for several avirulence genes, among which *avrl.* If there is a Mendelian segregation of AFLP markers in this cross then DNA from AVR+and AVRI- progeny will be pooled and used for Bulked Segregant Analysis (BSA). In this way AFLP markers linked to the *avri* gene can be obtained and these will be used as starting point for map based cloning of the *avrl* gene. A preliminary AFLP linkage map of *P. infestans* will be presented.

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Ashbya gossypii - a Filamentous Fungus with a Genome of less than 10 Mb

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Ashbya gossypii is a phytopathogenic ascomycete which was first isolated from cotton but which also can infect citrus fruits and tomato. Furthermore, about 30 % of the word industrial riboflavin output is produced with this organism. *A. gossypii* is an unusual filamentous fungus. According to sequence homologies and behaviour of transforming DNA it resembles more the yeast *Saccharonzyces cerevisiae* than other filamentous ascomycetes. In addition, its genome is extremely small. Electrophoretic separation of chromosome sized DNA revealed seven chromosomal bands which range between 690 kb and 2020 kb and add up to a total genome size of 9.7 Mb. This value is very low compared with the genomes of other filamentous fungi (generally between 25 Mb to 40 Mb) and even compared with the genomes of unicellular fungi like *S. cerevisiae* (13.5 Mb) and *Schizosaccharonyces ponibe* (13.8 Mb). We therefore investigated if *A. gossypii* has larger chromosomes which did not enter the gel. Furthermore, we analysed if the seven bands really represent only seven chromosomes or if they consist of equally sized chromosomes comigrating within the gels. With hybridisation analyses using 80 randomly chosen clones as probes the seven bands were shown to represent the whole nuclear genome. To investigate the existence of double bands, the 18 bp recognition site for the restriction enzyme *I-SceI* was integrated by homologous recombination into chromosomes corresponding to each of the seven bands. Cleavages at these I-SceI-sites revealed that each band represents a single chromosome. Hence, the *A. gossypii* genome really consists of only 9.7 Mb and is therefore one of the smallest eukaryotic genomes described so far.

Molecular Analysis of Chromosome Polymorphism in *Beauveria bassiana* by Using Electrophoretic Karyotype and a Telomeric Probe

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The imperfect fungus *Beauveria bassiatia* is an entomopathogenic species widely used as biological control agent against several insects. The karyotypes of isolates from different host insects have been studied using two complementary approaches :
- Pulsed-Field Gel Electrophoresis was used to separate chromosomes. The electrophoretic karyotypes present bands between 1,2 and 7,5 megabase pairs and a high chromosome length polymorphism between isolates from different host insects. On the other hand, isolates from the European Corn Borer *(Ostrinia nubilalis)* could not be distinguished.
By Southern Hybridization with homologous probes, the chromosomal location of known genes (Nitrate reductase, -tubuline, Histone 4, rDNA) and gene of the Proteinase I (which is implicated in penetration of the host cuticule) have been mapped. Results show that similar sized chromosomes do not always bear the same information.
- The second approach involved a telomeric probe from *Botrytis cinerea*. By Southern hybridization on total DNA digested by restriction enzymes, the minimum number of chromosomes of each isolate has been estimated. This probe was also used onto Southern blots of restriction digests of individual chromosomes in order to detect doublets.
Molecular knowledge of the genome organisation in *B. bassiatia* is a milestone in the genetic improvement by protoplast fusion. These studies would facilitate the identification of strains with pathogenicity-related genes -on different chromosomes which would be good candidates for somatic hybridization in order to increase

gene copy number and the diversity of pathogenicity genes.

Poster Abstracts, Beta-lactam Antibiotics

Genetic Analysis of the Putative Thioesterase Region Of ACV Synthetase

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The *acvA* gene encodes -(L-(-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) which catalyses the first step in -lactam biosynthesis pathways. The non-ribosomal synthesis of the tripeptide ACV follows the thiotemplate mechanism in which the release of the ACV from the enzyme, presumably bound as a thioester, requires the activity of a thioesterase. Inspection of the amino acid sequence revealed the motif GX-S-X-G in the C-terminal region which is found in a number of thioesterase enzymes. To determine the significance of this putative thioesterase in the C-terminal region of ACVS, deletions were made from the 3' end using the available full length cloned *P. chrysogenum acvA* gene. The first deletion removed the thioesterase motif and all sequences 3' to it. The second deletion removed the 3' end of the *acvA* but it retained the motif. Both of these deletions removed most of the ACVS activity, as determined by penicillin bioassay in a strain of *A.nidulans* in which the *acvA* was precisely deleted.

Attempts were made to complement the deleted strains with the expressed C-terminal end of ACVS. Expression under the control of the alca promoter was detected by SDS-PAGE and western blotting. This strain was crossed with the deleted strains and analysis of progeny by PCR made it possible to obtain a strain in which the separated domains were co-expressed. Penicillin bioassay showed no changes in penicillin production compared to the deleted strains. This suggests that the expression of the C-terminal end and deleted ACVS in trans cannot restore penicillin production.

Further purification of the truncated enzyme may reveal whether the

ACV is trapped on the enzyme and if so, whether the valine is in the L- or D form in order to establish when epimerization occurs.

Cloning and Analysis of an Intron Containing Peptide Synthetase Gene from the Entomopathogenic Fungus *Metarhizum anisopliae*

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The entomopathogenic fungus *Metarhizium anisopliae* produces a family of related cyclic hexapeptide toxins called destruxins. The precise role of these toxins is unclear, with reported effects including immunosuppression, causing paralysis and impairing osmotic control of the host. The significance of these effects in the disease process is not clear and in order to define the role of the toxin more fully we aim to disrupt the gene(s) responsible for destruxin synthesis.
Many peptide synthetase genes have been extensively characterised and several conserved regions identified within each of their domains. Oligonucleotides designed against two of these conserved areas have been used to screen a genomic EMBLIII library and positive clones identified. A region of more than 12kb has been completely sequenced and this spans three of the six predicted domains. These domains are typical of peptide synthetase genes, containing all of the expected core sequences. In contrast to the other peptide synthetase genes characterised to date, the open reading frame of this gene is split by at least two introns. These show typical fungal intron consensus sequences and their presence has been confirmed bv RT-PCR, also confirming that this gene is transcribed. Progress towards the complete sequencing and disruption of this gene will be presented.

Comparison of the Penicillin Gene Clusters and RFLP Patterns of Penicillin Gene Specific PCR Products of *P. nalgiovense and P. chrysogenum*

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Penicillium nalgiovense a terverticillate species of the genus *Penicillium* is a frequently used starter culture for mold ripened foods. This fungal species can preferably be isolated from fermented meat products f.e. salami. From the physiological as well as the genetical point of view P. *nalgiovense* seems to be related very closely to *P. chrysogenum* a well known producer of the a-lactam antibiotic penicillin. It was possible to prove the complete set of genes -(L-(-aminoadipyl)-L-cysteinyl-D-valine synthetase (acvA), isopenicillase N synthase *(ipna)* and acylCoA:6-aminopenicillanic acid acyltransferase (aat) necessary for biosynthesis of penicillin in *P. nalgiovense* as well by gene specific polymerase chain reactions using oliginucleotide primers derived from sequences of *P. chrysogenum,* as in Southern hybridization experiments using DIG labeled DNA probes. For further judging the degree of relation between these two species cluster analysis of penicillin biosynthetic genes in *P. nalgiovense* have been made and results of these experiments have been compared to the penicillin gene cluster in *P. chrysogenum.* These investigations showed that penicillin genes in *P. nalgiovense* are clustered in nearly the same cluster as in *P. chrysogenum.* RFLP analysis of PCR fragments of about 1 kb showed that PCR products from *P. nalgiovense* and *P. chrysogenum* yielded exactly the same restriction fragments when they have been digested by enzyme MboI, but a slightly different pattern of restriction fragments could be found when enzyme HaeIII have been used. The results of these investigations further confirm the assumption that *P. nalgiovense* and *P. chrvsogenum* are two very closely related fungal species.

The Penicillin Gene Cluster Is Amplified in Tandem Repeats Linked by Conserved Sequences

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The penicillin biosynthetic genes *(pcbAB, pcbc and penDE) of Penicillium chysogenum* AS-P-78 were located in a 106.5 kb DNA region that is amplified in tandem repeats (five or six copies) linked by conserved TTTACA sequences. The wild type strains *P. chrysogenuni* NRRL 1951 and *P. notatum* ATCC 9478 contain a single copy of the 106.5 kb region. This region was bordered bv the same TTTACA hexanucleotide found between the repeats in the strain AS-P-78. A penicillin overproducer strain*, P. chrysogenum* El, contains a large number of copies in tandem of a 57.9 kb DNA fragment that mostly overlaps the right half of the amplified region in the strain AS-P-78. The tandem repeats in strain El are linked by the sequence TGTAAA, which is the reverse complementary of that appearing in strains AS-P-78 and NRRL 1951. The occurrence of one or the other pattern of amplification seems to depend on the orientation of a 3.4 kb fragment (shift fragment: SF) located at the right border of the amplified region. The penicillin non-producer mutant *npe*10 showed a deletion of a 57.9 kb fragment that corresponds exactly to the amplified region in strain E I. The conserved hexanucleotide sequence was reconstituted at the deletion site in strain *npe*10. The amplification has occurred within a single chromosome (chromosome 1), no other copies were located in any of the remaining chromosomes in the strains studied. The tandem reiteration and deletion appear to arise by mutation-induced site-specific recombination at the conserved hexanucleotide sequences.

Isolation and Characterization of Mutants Affecting the

Expression of the ACVS Gene in *Aspergillus nidulans.*

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The expression of penicillin biosynthetic genes in *Aspergillus nidulans has* been examined by the use of the reporter genes lacZ and uidA which have been fused in frame to the promoters of the acvA and ipnA genes1 The acvA gene encodes for the enzyme -(L-(-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) - and is the first of the three key enzymes responsible for penicillin production in *Aspergillus nidulans.*

In an attempt to find regulatory genes affecting acvA expression, mutants of the strain carrying the acvA-lacZ fusion were produced by UV irradiation, and those that showed either an increased expression of *lacZ* or those showing a loss of expression were selected for further analysis. Mutants showing loss of -galactosidase activity were probed to check for the loss of the fusion gene, and those retaining the sequence were analyzed by sexual crosses. All mutations appeared to be linked to the fusion gene, and the promoter was sequenced to look for mutations.

The mutants that displayed an increase in -galactosidase activity were crossed to establish the location of the mutation. Those showing a mutation not linked to the fusion gene were used to form diploids in an attempt to map the linkage group of the mutation, whilst those that showed linkage were sequenced to detect the position of the mutation.

1 Brakhage et al, 1992

Functional Consequences of Structural Variation of the Thioesterase Domain of ACV Synthetase

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ACV synthetase forms the tripeptide -(L-(-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) from the respective amino acids and ATP. It is considered the rate-limiting enzyme in penicillin and cephalosporin fermentation, and serves as a model system for peptide biosynthetic systems. We are studying the involvement of the thioesterase-like site in the terminating tripeptide cleavage and the mechanism of epimerization of the valine residue by site directed mutagenesis.

We have constructed a strain of *A.nidulans* in which the *acvA* gene encoding ACV synthetase has been deleted. If a wild-type P. *chrysogenum* acvA gene is reintroduced by transformation, penicillin production is restored to wild type levels. Mutations can be introduced *in vitro* anywhere in the acvA gene and studied for expression and functional properties. We have altered one of the functionally important sefine residues of the thioesterase to alanine. This strain shows a strongly reduced production of antibiotic. The altered multienzyme has been isolated and characterized. Evidence is presented for the intermediate -(L-(-aminoadipyl)-L-cysteinyl-D-valine (LLL-ACV) and impaired peptide release. The data support the timing of epimerization at the tripeptide stage.

Construction of Hybrid Peptide Synthetases

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Peptide synthetases catalyse the non-ribosomal synthesis of small peptides. The peptides produced by such systems include amino acids and hydoxy acids unavailable to the ribosomal system such as the D isomers of, and methylated amino acids. They also often have important biological activities, acting as antibiotics, immunosuppressants, plant toxins, or insect toxins. The primary

structure of peptide synthetases reveals the presence of repeated similar domains, with one domain for each amino or hydroxy acid added to the chain.
ACV synthetase, which catalyses the production of the tripeptide ACV, the first step in penicillin biosynthesis, was used as a test system for the construction of hybrid peptide synthetases. Two different strategies have been employed in order to produce hybrid systems. The first of these involved the expression of ACV synthetase as two separate polypeptides. In order to mimic the systems found in gramicidin and tyrocidin biosynthesis in which two or three enzymes interact to produce the peptide product. The activity was monitored in a strain of *A. nidulans* in which the *acvA* gene had been deleted, with penicillin production indicating the presence of ACV synthetase activity. The second method involved the production of a fusion gene in which domains of the *acvA* gene from different organisms were linked. This was again analysed in the *AacvA* strain.
It was found that the expression of ACV synthetase as two separate polypeptides failed to produce any penicillin while the hybrid ACV svnthetase was found to be active. Work is now progressing towards the construction of hybrid peptide synthetase genes, mixing domains from entirely different veptide svnthetases, for the biosynthesis of novel peptide products.

Identification of Upstream Activating Regions Important in Cis-acting Regulation of Expression of pcbAB Gene in *Penicillium chrysogenum*

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Expression of the *Penicillium chrysogenum gene* pcbAB, encoding

the -aminoadipyl-cysteinyl-valine synthetase (ACVS) that catalyzes the first step of penicillin biosynthesis was analysed since the control of expression of this gene is particulary important. Expression of the pcbAB gene is believed to be limiting for penicillin biosynthesis. Accumulating evidence suggests that the 1. 1 6 -kb intergenic region between the *pcbAB and pcbC* genes regulates their expression. Functional elements in the pcbAB upstream region have been defined by assaying -galactosidase activity in extracts from recombinant strains carrying deletion derivates of the *pcbAB* promoter fused to the *Escherichia coli lacZ* as a reporter gene. Transformants with a single copy of the lacZ gene at the *pyrG* locus were selected. Strains were grown in penicillin production medium under carbon catabolite repressing or derrepressing condition. Analysis of the -galactosidase activity of several single copy transformants revealed the presence of two upstream regions that are important for expression of the pcbAB gene and both regions appear to be involved in glucose catabolite regulation of expression of the pcbAB gene. Both fragments were shifted in a gel retardation assays by DNA-binding proteins.

Regulation of the Penicillin Biosynthesis Gene *aat (PenDE) of Asperillus nidulans* Encoding Acyl Coenzyme A:6-aminopenicillanic Acid Acyltransferase

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Penicillin is produced as a classical secondary metabolite by some filamentous fungi. Because penicillin is still an important antibiotic for the therapy of infectious diseases, knowledge about the genetics of its biosynthesis will have an impact on production and modification of this -lactam antibiotic.
The last reaction of the 3-step biosynthetic pathway of penicillin is catalysed by acyl coenzyme A:6-aminopenicillanic acid acyltransferase (AAT). The enzyme is encoded by the *aat (penDE)*

gene which is located together with the two other penicillin biosynthesis genes *acva (pcbAB)* and *ipna (penDE)* in a single gene cluster. To analyse the expression of the *aat* gene, its promoter region was fused in frame with the *E. coli lacZ* reporter gene. In a fermentation run, the expression of *aat-lacZ* gene fusions was maximal during the first 24 h when the growth of mycelia was maximal as well. The use of glucose instead of lactose as the carbon source led to reduced AAT specific activity. The *aat-lacZ* expression, however, was not affected by the carbon source, indicating that the glucose effect is posttranscriptionally mediated. For the identification of cis-acting regulatory sequences in the promoter of the *aat* gene and of corresponding DNA binding proteins, band shift experiments with partially purified protein extracts of A. *nidulans* were carried out. By analysing different DNA fragments spanning the whole or parts of the aat promoter, a recognition site for a DNA binding protein was localised within a 20 bp DNA fragment. The introduction of mutations in this DNA fragment abolished the binding of the protein *in vitro. An aat-lacZ* gene fusion carrying the same mutations in the aat promoter region showed a different expression pattern compared with that of a wild-type gene fusion, providing evidence for the significance of the identified protein binding site *in vivo.* Taken together, a comprehensive picture of the molecular regulation of this secondary metabolism gene is emerging.

Transcription of the *pcbAB, pcbC* and *penDE* Genes of *Penicillium chrysogenum* is Strongly Repressed by Glucose and the Repression is Not Reversed by Alkaline pHs

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Glucose repressed transcription of the penicillin biosynthesis genes *pcbAB, pcbc* and penDE genes in *Penicillium chrysogenum when*

added at inoculation time but it had little repressive effect when added at 12 h to batch cultures and no effect when added at 24 or 36 h. The rate of penicillin biosynthesis and expression of the three genes *pcbAB, pcbc* and penDE was reduced at pH6.0 as compared to pH 7.0. A slight increase of the expression of *pcbC and penDE* (and to a smaller extent of pcbAB) was observed in glucose grown cultures at pH values 7.1, 7.8 and 8.5 but alkaline pHs did not override the strong repression exerted by glucose. Transcription of the actin gene used as control was not significantly affected by glucose or alkaline pH values. Glucose repression and the lack of reversal of repression by alkaline pHs was also observed when the promoters of the *pcbAB, pcbc* and *penDE* genes were coupled to the LacZ reporter gene and the fusions integrated as single copy transformants (by directed targeting) at the *pyrG* locus. Glucose repression of the three genes encoding enzymes of penicillin biosynthesis appears, therefore, to be exerted by a regulatory mechanism independent from pH regulation.

Structure-function Analysis of ACV Synthetase from *Penicillium chrysogenum* Substrate Specificity of the Third Domain by Fragment Expression

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Biosynthesis of the penicillin precursor -(L-(-aminoadipyl)-L-cysteinyl-D-valine (ACV) is catalyzed from the constituent L-amino acids and ATP by a multifunctional peptide synthetase (ACV

synthetase, ACVS). Its structure basically consists of three modules, each containing key motifs involved in ATP-binding, acyladenylate formation and aminoacylation. The modules have been tentatively associated with activation of the respective amino acids in order of their sequence in the peptide, and this association has been proved by limited proteolysis for the middle domain activating cysteine. Investigation on the substrate specificities of the other modules has been approached by fragment expression. We here present data on the third module thought to activate L-valine. The adenylate region has been subcloned and is expressed in *Aspergillus nidulans.* Unexpectedly also -aminoadipic acid forms an adenylate.

Use of Reporter Genes to Identify Recessive Trans-acting Mutations Specifically Involved in the Regulation of *Aspergillus nidulans* Penicillin Biosynthesis Genes

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Starting from three amino acid precursors, penicillin biosynthesis is catalysed by three enzymes which are encoded by the following three genes: *acvA (pcbAB), ipnA (pcbC) and aat (penDE).* To identify trans-acting mutations which are specifically involved in the regulation of these secondary metabolism genes, a molecular approach was employed by using an *Aspergillus nidulans* strain (AXTII9) carrying *acvA-uidA* and *ipnA-lacZ* gene fusions integrated in double copies at the chromosomal *argB* gene. On minimal agar plates supplemented with X-Gal, colonies of such a strain stained blue, indicative of *ipnA-lacZ* expression. After mutagenesis with UV-light, colonies were isolated on agar plates with lactose as carbon source, which produced only a faint blue colour or no colour at all. Such mutants (named Prg for penicillin regulation) most likely were defective in trans-acting genes. Control experiments revealed that the mutants studied still carried the correct number of gene

fusions. In a fermentation run, mutants Prg-I and Prg-6 exhibited only 20 to 50% of the *ipnA-lacZ* expression of the wild-type strain and produced only 20 to 30% of the penicillin produced by the wild-type strain. Western blot analysis showed that these mutants contained reduced amounts of *ipnA* gene product, i.e., isopenicillin N synthase. Both mutant Prg- I and mutant Prg-6 also differed in *acvA-uidA* expression levels from the wild-type. Segregation analysis indicated that for both mutants the Prg phenotype resulted from mutation of a single gene. Two different complementation groups, which were designated *prgA1* and *prgB1,* were identified.
The identification and characterisation of cosmid clones complementing the *prgA1* mutation will be presented.
Brakhage, A. A. and J. Van den Brulle (1995). *J. Bacteriol.,* 177: 27812788

The Aspergillus nidulans lysF Gene Encodes Homoaconitase an Enzyme Involved in the Fungal Specific L-lysine Biosynthesis

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In *Aspergillus nidulans,* the biosynthesis of L-lysine proceeds via the L-2aminoadipate pathway which is specific for higher fungi. L-2-Aminoadipic acid is a key intermediate of the L-lysine biosynthetic pathway and simultaneously, besides L-cysteine and L-valine, one of the precursor amino acids of the penicillin biosynthesis. Hence, L-2-aminoadipic acid is a branch point of primary and secondary metabolism. The analysis of regulation of genes / enzymes leading to formation of L-2-aminoadipic acid thus represents an excellent tool to investigate how the regulation of primary and secondary metabolism interacts, in order to coordinate the supply of L-2aminoadipic acid for both L-lysine and penicillin biosynthesis. In *Saccharomyces cerevisiae,* the expression of nearly all genes encoding enzymes of the L-lysine biosynthetic pathway is repressed

by L-lysine. Interestingly, in *A. nidulans* the expression of the penicillin biosynthesis genes *acvA (pcbAB) and ipnA (pcbC)* is regulated by L-lysine, too.
Because, so far, nothing is known about the molecular basis of the L-lysine biosynthesis in filamentous fungi, we cloned the *lysF* gene by complementation of the corresponding lysine auxotrophic mutant of *A. nidulans.* Biochemical analysis revealed that *lysF* affects a step before formation of L-2-aminoadipic acid of the L-lysine biosynthetic pathway. Genomic DNA used for complementation derived from a chromosome specific cosmid library of *A. nidulans.* Subcloning from a 19 kbp *Hind*lII fragment and DNA sequence analysis revealed an open reading fraine encoded by at least 1.8 kbp. Within the deduced amino acid sequence of *lysF, a* motif characteristic of an iron-sulfur cluster is present. Computer analysis did not reveal major homologies of the deduced amino acid sequence of *lysF* to any other known gene. Further biochemical analysis indicated that *lysF* encodes homoaconitase. We thus present the first cloning of a gene encoding homoaconitase activity. The addition of L-lysine to the fermentation medium led to drastically reduced homoaconitase specific activity implying that the level of homoaconitase is regulated by L-lysine. The physiological role of the *lysF* gene product for both L-lysine and penicillin biosynthesis will be discussed.

Poster Abstracts, Gene Expression

Aspergillus nidulans FacB Transcriptional Activator and *Neurospora crassa* Acetate-induced Protein Compete for Their Respective Cognate Promoter DNAs
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The *fac* B gene product mediates acetate-induced gene expression in

Aspergillus nidulans . Gel mobility shift scanning with the N-terminal DNA-binding domain of the FacB protein (FacB4-417) synthesised in *Escherichia coli*, demonstrates region-specific DNA-binding to the native acetate-inducible promoter *acuD* (gene coding for the isocitrate lyase) and a serie of analogous promoters from *Neurospora crassa.* Conversly a partially purified protein extract from *N. crassa* mycelium induced with acetate binds the corresponding *A. nidulans* promoter DNA fragments, Protein/DNA gel retardation assays with the respective isocitrate lyase gene promoter fragments identify the same 5' non -coding regions as those reported to be involved in acetate induction based on promoter deletion analyses. Competitive DNA-binding experiments between the FacB4-417 protein and the *N. crassa* protein extract demonstrate that each can effectively titre the other, implying they have DNA recognition motifs in common.

Cloning and Analysis of *areA and creA* -Like Genes from the Insect Pathogenic Fungus *Metarhizium anisopliae*

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The insect pathogenic fungus *Metarhizium anisopliae* invades its host insect by direct penetration of the cuticle. a complex laminate structure comprising primarily chitin and protein. Several extracellular cuticle-degrading proteases have been characterised from *Metarhizium* and the role of one of these, PRI, in infection has been established. PRI production is regulated in response to C and N-derepression (and specific induction by insect cuticular protein) and consequently the *pr1* promoter region was searched for the presence of putative binding sites for C and N response regulator proteins as defined in *Aspergillus nidulans* and *Neurospora crassa.* In *A. nidulans,* C-repression is mediated by CREA, a repressor protein which binds to the sequence 5' SYGGRG in C-repressed genes. N-

derepression in *N. crassa* is effected by an activator protein NIT2 which binds to the sequence 5' GATA. 3 putative binding sites for a CREA-like protein were identified in the *pr1* promoter. Two of these have been shown to bind CREA *n vitro.* Seven GATA sites were also identified, however, the DNA-binding requirement of NIT2 for 2 closely, spaced GATA sites would suggest that the *pr1* promoter contains only 2- functional binding sites. PCR using oligos based on the zinc finger DNA-binding domain of CREA and MIGI (from *S. cerevisiae)* have been used to amplify fragments from *Metarhizium* genomic DNA. Partial sequencing of one of these fragments confirmed strong identity to CREA. A genomic clone was subsequently isolated and sequenced. This crea-like gene is present in a single copy in the *Metarhizium* genome. A similar approach using oligos based on homology between NIT2 and AREA (of *Aspergillus)* has allowed the isolation of a 800 bp PCR fragment which shows strong sequence similarity to AREA. The full length gene has been isolated from a *Metarhizium* genomic library and sequenced.

Increasing Expression of *Neurospora crassa NADP*+ -Dependent Glutamate Dehydrogenase

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NADP+-dependent glutamate dehydrogenases (GDH) found in many bacteria, plants and fungi catalyse the reductive amination of 2-oxoglutarate to L-glutamate which is an important step in the amino acid assimilation pathways.
Work has been undertaken to increase the expression of the NADP+ dependent GDH protein, transcribed from the *N. crassa am* gene, as an aid to protein crystallisation studies of wt and mutant enzymes. The *am132* deletion strain of *N. crassa* was transformed with the *am* gene coupled to the strong promoters *grg-1*, and also *crp-2*.

Integration of the *am* gene occurred and the corresponding protein assays showed high enzyme levels, but over successive subcultures the quantity of GDH enzyme declined, possibly because the gene expression was being affected by methylation or quelling. The same result was obtained with both *N. crassa* promoters. Stabilisation only occurred at levels of expression lower than those produced by the *N crassa* wt.
Expression of the *am* gene was also attempted in *Aspergillus nidulans* using the strong *alcA* promoter, but although levels of expression were obtained these were not higher than the *N. crassa* wt equivalent.
In vitro mutagenesis is now in progress to investigate subunit interaction in this hexameric protein.

Cloning of the GCN4/cpc-1 Homologue of *Aspergillus niger*

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Imbalances in the pool of amino acids in certain fungi is known to result in a coordinated derepression of amino acid biosynthetic genes. This phenomenon, called General Control of amino acid biosynthesis in *Saccharomyces cerevisiae* (Hinnebusch, 1992) and Cross-Pathway control in *Neurospora crassa* (Carsiotis et al., 1974), was also known to exist in *Aspergillus nidulans* (Piotrowska 1980).
The genes coding for the main trans-acting factor mediating this control mechanism are identified in *S. cerevisiae* (aCN4) and N. *crassa* (cpc-1). These genes encode bZIP-type transcriptional activators which bind to palindromic general control recognition elements (GCRE's, consensus sequence 5' ATGA(C/G)TCAT 3';

Hope and Struhl, 1985, Arndt and Fink 1986; Ebbole et al., 1991) in the promoter sequence of many amino acid biosynthetic genes and stimulate their transcription.
Here we report the cloning and initial characterization of OcA, the GCN4/CPC-1 homologue of the filamentous fungus *Aspergillus niger.*
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The Phytoene Dehydrogenase Gene of *Phycomyces:* Regulation of the Expression by Blue Light and Vitamin A

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By using a PCR-based cloning strategy, we have isolated the first carotenogenic gene from the filamentous fungus *Phycomyces blakesleeanus,* encoding the enzyme phytoene dehydrogenase. The structural region of the gene includes 1935 nucleotides and consists of two exons separated by one intron, 183 bp in length, whose processing brings about a 1752-nucleotide open reading frame that encodes a 583-residue polypeptide with a predicted molecular mass

of 65.9 kDa. The deduced protein showed a high homology to carotenoid dehydrogenases from other fungi and bacteria, specially in the amino-terminal region, and the main conserved regions found in other phytoene dehydrogenases, that are thought to be essential in the enzymatic activity, are present in the sequence from *Phycomyces*. Heterologous expression of the *Phycomyces* gene in *Escherichia* coli showed that, like in other fungi and bacteria, a single phytoene dehydrogenase catalyzes the four dehydrogenations from phytoene to lycopene in the *Phycomyces* carotenoid biosynthetic pathway. RNA measurements showed that the level of expression of the phytoene dehydrogenase gene in wild-type mycelia increased in response to blue light. The kinetics of the mRNA levels of this gene after blue light induction (0.1 and 0.4 W/m2) exhibits a two-steps (biphasic) dependence on fluence rate, suggesting the possibility that there could be two separate components in the reception of the low and high blue light exposure. The presence of vitamin A in the medium stimulated the phytoene dehydrogenase transcript accumulation in wild-type and some carotenogenic mutants (genotype *car*). Diphenilamine, a phytoene dehydrogenase inhibitor, did not affect the level of transcription of this gene.

Mutational Analysis of the Proline Pathway Positive Regulator PRNA in *Aspergillus nidulans*

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The *Aspergillus nidulans* proline catabolism is subject to three types of control:a general repression by glucose and ammonium and a specific induction by proline. At least three regulatory proteins are involved in this process: CreA, AreA and the specific regulator PrnA. The positive regulator PrnA is a 818 amino acid protein with a binuclear zinc complex and other domains common for regulatory proteins like a NLS or an activation domain. We study the PrnA domains for their interactions with other cellular factors. We are also

interested in the role of the intramolecular interactions in the protein function.
This study is based on the analysis of cold- and thermosensitive mutations and their revertants. Six of these mutations are mapped and three are sequenced. Two cold-sensitive mutations appear to be localized in the bipartite nuclear entry signal and lead to an abolition of the consensus sequence. The *prnA29* mutation changes an Arg into Leu and in the *prnAI21* mutant the same Arg becomes a Pro. An aleatory mutagenesis was done on these mutant strains and gave several types of intragenic suppressors in the NLS spacer restoring the consensus sequence and the wild type phenotype of the mutant strains. We are now about to confirm the role of this sequence in the nuclear transport by using a tag construction and fusions with the Green Fluorescent Protein. We are also looking for some extragenic suppressors which could touch other proteins of the nuclear transport system.
We work also on the PrnA "central" region highly homologous to other specific regulators domains. The *prnA27* mutation is localized in this region and changes a Thr into an Ala. We have found intragenic suppressors in other domains of the protein, upstream from this mutation. These results suggest the existence of some intramolecular interactions necessary for the protein function.
We are currently sequencing other *prnA* mutations. Some of them would be localized in the C-terminus and therefore could be in the glutamine-rich putative activation region of the protein.

Position Effects in the Transcriptional control of the *Aspergillus nidulans prn* Cluster

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Proline catabolism in *Aspergillus nidulans* is subject to three levels of control: specific induction, mediated by the PrnA protein, carbon

catabolite repression, mediated by the negatively acting protein CreA, and nitrogen metabolite repression, mediated by the positively-acting protein AreA. The 1.7 kb intergenic region between genes *prnb* and D has been shown to play a crucial role in the regulation of the expression of all *prn* genes with the exception of *prnA*. Deletion analysis in this region has confirmed the existence of a previously predicted enhancer element for *prnC* more than three kb upstream of its putative start of transcription. This enhancer is necessary for maximal transcription of *prnC* at 25 C but not at 37 C.
Using the same type of analysis we have confirmed our hypothesis that integration between carbon catabolite repression, and nitrogen metabolite repression is mediated by a factor, different from AreA, whose activity is counteracted by that of CreA. The binding site for this factor is located more than 500 pb appart from the CreA binding sites responsible "in vivo" for glucose repression.
We have used DNAsel footprint analysis with crude extracts of *Aspergillus nidulans* to localize some potential targets for both the enhancer and the other new factor in the regions defined by deletion analysis.
Other interesting observation from these deletions is that the binding of CreA shows a very stringent position requirement to be effective in repression. Chromatin structure analysis by DNAse1, micrococcal nuclease and several restriction enzymes also suggests the involvement of these regions in transcriptional control of proline catabolism. We propose a model integrating all these data and accounting for the positional requirement of the CreA binding sites.

A GATA-factor Encoding Gene *(sreP) from Penicillium chrysogenum,* Homologous to the *Ustilago maydis urbs1* Gene

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GATA-binding proteins constitute a family of transcription factors

that recognize a target site conforming to the consensus motif GATA. This group includes a range of major regulatory proteins from organisms as varied as fungi, mammals, birds, insects and plants. The different members of this family are related by a high degree of amino acid sequence identity within their DNA-binding domains, which consist of either one or two zinc chelating fingers. Intriguingly, in a number of different organisms multiple functionally distinct GATA factors with similarity of binding specificity have been reported. Recently, we have isolated and characterized the *nre* gene encoding the major nitrogen regulatory gene of *Penicillium chrysogenum,* containing a single Cys2/Cys2-type zinc finger motif which recognizes the consensus sequence GATA (1). To characterize additional *Penicillium* GATA factors we have used Polymerase Chain Reaction amplifications of cDNA using degenerate oligodeoxynucleotide primers derived from the conserved DNA binding domain of the GATA protein family. Using this approach the *srep* gene encoding a protein of 533 amino acids which contains two GATA type zinc finger motifs was isolated. The deduced amino acid sequence reveals 50% identity to the URBS I protein from *Ustilago maydis* over a stretch of 150 amino acids containing the two DNA binding motifs and the intervening sequence (2). The sequence homology to URBSI, which interacts directly or indirectly to repress biosynthesis of siderophores, suggests a similar role for *sreP* in *Penicillium.* In order to investigate the promoter region and structural organization of *sreP* gene, a genomic 10-kb fragment was isolated and partially sequenced. Comparison of the nucleotide sequence of the genomic fragment and the cDNA clones revealed the presence of two introns. In Northern blot analysis of total cellular RNA two transcripts, 2 kb and 2.5 kb in length respectively, were detected due to two different major transcriptional start sites.

The cloning and characterization of *sreP* and other GATA factor encoding genes from filamentous fungi will provide a suitable model system to investigate the regulation of iron metabolism as well as to enlighten how structurally related proteins maintain distinct roles within the same organism.

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Promoter Analysis of a Heat Shock Gene ***(Hsp70) of Aspergillus nidulans.***

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A member of the *hsp7O* gene family of the filamentous fungus *Aspergillus nidulans* has been isolated and cloned (data being prepared for publication). The DNA sequence shows strong homology with *hsp7O* genes of other organisms, especially the *ssb* genes of *Saccharomyces cerevisiae.* Interestingly, this *A. nidulans hsp7O gene* contains several introns, which is an uncommon feature in highly expressed heat shock genes of higher eukaryotes. Among other upstream sequence elements common to fungal promoters, six copies of the highly conserved Heat Shock Element (HSE) can be found in the 5' flanking sequences of this heat shock gene. A PCR amplification product of the promoter sequences has been fused in frame to the *Escherichia coli lacZ* gene, in order to study the promoter's functionality and efficiency. Site directed integration of a single copy of the vector pCK70 (carrying this promoter-*lacZ* fusion) into the *Aspergillus* genome has been accomplished. Enzyme assays to determine the -galactosidase activity of these fungal strains were performed under control conditions and after different heat shock treatments. Surprisingly, no significant increase in enzyme activity was apparent after heat stress. Although several HSEs can be found in the promoter sequences, this *A. nidulans hsp7O* gene seems to be constitutively expressed. Results of a promoter deletion study to elucidate this phenomenon are shown.

High levels of -galactosidase activity can be found under many different growth conditions. Little or no effect could be found after treatment with heat and other inducers, like heavy metals, ethanol and cold shock. However, growth phase related changes in enzyme activity were apparent and have been studied. Results of these studies will be presented and discussed.

SCONB, An *Aspergillus nidulans* Sulphur Metabolism Regulatory Gene Encodes a -Transducin-like Protein

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Expression of genes coding for enzymes of sulphate assimilation and enzymes participating in the metabolism of sulphur amino acids are controlled by four regulatory *scon* (sulphur controller) genes. Mutations in these genes relieve sulphur metabolism from sulphur metabolite repression - a regulatory system which shuts off the synthesis of the enzymes of sulphate assimilation pathway when a favourable sulphur source such as methionine is available. Two of these genes *(sconB* and *sconC)* have been cloned by transformation of the *A. nidulans* with cosmid gene library.
Genomic and cDNA sequence of *sconB*+ gene indicated a 2034bp open reading frame interrupted by one intron of 47bp. It encodes a polypeptide of 678 amino acids which belongs to the expanding family of G-protein - subunit related eukaryotic regulatory proteins. Each -transducin-like protein is defined by the presence of four to eight highly conserved WD-40 repeats. The SCONB protein contains seven repeated G -homologous domains spanning the C-terminal half of the protein. It also possesses an amino-terminal domain found in several -transducin homologs including the mouse protein *MD6, Xenopus* protein BTrCP, *Neurospora crassa* protein SCON2 and the yeast proteins MET30 and CDC4. The SCONB protein shows

considerable similarity to SCON2 protein of *N. crassa (74%)* and *S. cerevisiae* MET30 protein (63%); both are involved in the regulation of sulphur metabolism.
The analysis of the *sconC* gene is in progress.

Identification of a Regulatory Gene in *Ustilago maydis* That Affects the Expression of Genes Regulated by the B Locus

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In the maize pathogen *Ustilago maydis* mating, filamentous growth and pathogenic development are controlled by the two mating type loci *a* and b. The a locus exists in two allles and controls fusion of haploid cells while the multiallelic b locus regulates pathogenicity and sexual development. Filamentous growth requires the concerted action of the *a* and *b* locus. The *b* locus encodes a pair of unrelated homeodomain proteins termed bE and bW. These proteins dimerize only when they are derived from different alleles and are then able to switch on pathogenic development.
One of the genes regulated by the b locus is *egl1* a gene encoding an endoglucanase. *egl1* is expressed only in the filamentous phase when an active bE/bW heterodimer is present in the cell. Expression *of egl1 can* easily be monitored in a plate-assay. *egl1* was used as a reporter to screen for mutants which allow *egl1* expression in haplid cells where *egl1* is normally not expressed. With this screen one mutant was found which expresses *egl1* constitutively. 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. The mutation was complemented with a cosmid library. After recovery of the cosmid a complementing subclone containing a 8.9 kb Bglll-Xbal

fragment was isolated. We will provide the sequence of the respective gene and discuss how this negative regulator fits into our current scheme on gene regulation exerted by the bE/bW heterodimer.

Cpc-3 of *Neurospora cassa* Is Homologous to Yeast *GCN:* an eIF-2 Kinase Involved in General Amino Acid Control

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In lower eukaryotes, like *Neurospora crassa and Saccharomyces cerevisiae,* starvation for any one of a number of amino acids leads to simultaneous induced transcription and derepression of enzymes in many amino acid biosynthetic pathways. This mechanism is called general amino acid control. The transcriptional activator of the regulated genes is encoded by the homologous genes *cpc-1 (N. crassa* ,l) *and GCN4 (S. cerevisiae*,2)*,* respectively. In *S. cerevisiae* expression of *GCN4* itself is under translational control mediated by the *GCN2* encoded eIF-2 kinase (3).
To find out whether substantial differences exist in the details of general control between the two ascomycetes we cloned the *GCN2* homolog of *N. crassa,* called *cpc-3.*
cpc-3 was identified using PCR and degenerate primers representing highly conserved sequences of eIF-2 kinases. The open reading frame encodes a putative protein with a two domain structure, an eIF-2 kinase and juxtaposed histidyl-tRNA synthetase-like domain, and shows 29% amino acid identity over the entire length with yeast GCN2p.
The *N. crassa* genomic sequence was used to engeneer a disruption mutation of the *cpc-3* gene. Strains carrying the recessive *cpc-3* allele were unable to induce increased transcription and derepression of amino acid biosynthetic enzymes under conditions of amino acid starvation indicating that CPC3p like yeast GCN2p is involved in the mechanism of general amino acid control. The *cpc-3* mutation does

not interfere with *cpc-1* transcriptional regulaton.
(1)Barthelmess 1982 Genet. Res 39:169-185 Paluh et al. 1988 Proc. Natl. Acad. Sci. USA 85:3728-3732
(2)review: Hinnebusch 1994 TIBS 19:409-414
(3)review: Wek 1994 TIBS 19:491-496

Control of *SC3* Expression in *Schizophyllum commune*

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The monokaryon of the basidiomycetous fungus *Schizophyllum commune* abundantly expresses the SC3 hydrophobin gene which is accompanied by intensive formation of aerial hyphae. In the *thn* mutant SC3 expression is suppressed. Also in a common-MATA heterokaryon with two different MA7B mating-type genes present, *SC3* is down-regulated. In both the *thn* mutant and the common-*MAT*A heterokaryon, few aerial hyphae are formed.
To study the regulation of *SC3,* the availability of a reporter-gene system would be convenient. However in *S. commune* foreign genes are mostly not expressed. To overcome this problem a genomic SC3 clone was introduced into a SC3 disruptant. Restoration of SC3 expression could be, detected on RNA as well on protein level. In these cotransformation experiments using the UR,41 gene as an auxotrophic marker, reconstitution of *SC3* expression was very poor. DNA analysis showed no correlation between copy number and SC3 expression. Using the phleomycin resistance gene as a selective marker on the same vector as the SC3 genomic fragment, gave much better SC3 expression after transformation to the SC3 disruptant. Not only did more transfortnants show SC3 expression, also the average level of expression was higher. Southern Blot analysis again showed no clear correlation between copy number and the level of *SC3* expression. These results taken together strongly suggest position-dependent expression of integrated DNA.

However, regulation of the introduced SC3 gene was as the original endogenous gene both with respect to temporal and mating-type gene regulation. Apparantly all cis-regulatory sequences were still present on the transformed genomic fragment. Experiments using this reporter system with promoter deletion constructs of the *SC3* gene, are currently underway.

Cloning and Heterologous Expression of the *creA* Gene of *Penicillium chrysogenum*

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As an enzyme saving response, in the presence of less favoured carbon sources microorganisms usually turn off the synthesis of a range of enzymes when more readily utilized carbon sources such as glucose are present by carbon catabolite repression. The *mig1* gene of *Saccharomyces cerevisiae* and the *creA* genes of *Aspergillus nidulans* and *A. niger* are the best studied genes mediating carbon catabolite repression in fungi.
As our intentions are directed towards *Penicillium chrysogenum,* it was our aim to identify, clone and characterize creA gene of *P. chrysogenum.*
Performing a high stringency plaque hybridization, a DIG labeled *NcoI-NcoI* fragment containig the zinc finger domain of the *creA* gene of A. *nidulans* was found to hybridize to a genomic Lambda EMBL3 DNA library of *P. chrysogenum.* From one of five selected positive plaques DNA was isolated and sequenced directly, starting with primers designed from sequences within the highly conserved regions known from the *creA* genes of A. *nidulans* and A. *niger* respectively from the *crel* genes of *T. reesei* and *T. harzianum.* The complete coding region of the *P. chrysogenum crea* gene was amplified using a PCR reaction and the product was cloned into the prokaryotic protein expression vectors pSE420 and pMS470.
At the moment overexpression using *E. coli* BL21 and TOP1OF'

hosts is under investigation.

Glucose Repression of *xyn1* (Xylanase I Encoding Gene) Expression by Crel in *Trichoderma reesei* Requires Binding of a Protein Complex to an Inverted Repeat of the Crel Consensus Sequence.

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To degrade xylan in plant hemicelluloses, the filamentous fungus *Trichoderma reesei* forms a xylanase enzyme system comprising two specific, inducible xyianases encoded bv *xyn1* and *xyn2.* Their expression is affected by glucose in different wavs: *xyn1* transcript is absent in the presence of glucose as sole carbon source, whereas *xyn2* is expressed at a low constitutive level under these conditions. *xyn1* induction by xylan is not affected by the simultaneous presence of glucose, whereas induction of *xyn2* is prevented. Repression of *xyn1* is mediated bv the carbon catabolite repressor protein Crel. Binding of Crel, either overexpressed as a truncated fusion to GST in E *coli,* or present in cell-free extracts, to the *xynl* promoter requires the consensus binding site 5'-SYGGRG-3'. In the *xynl* promoter, *in vivo* functionality of this consensus is dependent upon arrangement as inverted repeat: *T. reesei* strains, bearing a *xynl:hph* construct in which four nucleotides from the center of the repeat has been removed, expresses *hph* on glucose at a level comparable to that observed under carbon catabolite derepressing conditions (e.g. growth on lactose). Conversely, the insertion of a double consensus into the xyn2 promoter does not result in glucose repressability of constitutive *xyn2* transcript formation. We conclude that that the mere presence of an inverted repeat Crel binding site is not sufficient to mediate glucose repression but the functionality of such site is dependent on the nucleotide context and/or on the position in the

promoter. Native Crel protein, present in cell free extracts of *T. reesei* participates in formation of a Crel specific multi-protein complex, regardless the presence of a functional or non-functional (four base-pair delition) double consensus site. From these results we conclude that Crel strongly interacts with other components of the transcriptional machinery but functionality as a repressor is dependent on binding of Crel to its target.

A Ring-finger Protein Involved in the Regulation of the Biosynthesis of Carotenoids in the Fungus *Mucor circinelloides*

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Most of the mucorales species that produces carotenoids accumulate -carotene as the end product. *Mucor circinelloides* is one of the few mucorales that is suitable for genetic transformation and can therefore be used for gene cloning. *M. circinellioides* possesses a light-regulated carotenoid biosynthetic pathway like other related species, including *Phycomyces blakesleeanus.* We have obtained a collection of *M. circinelloides* mutants blocked in each of the enzymatic steps from famesyl pyrophosphate to -carotene and three different regulatory mutants affected in the total production of carotenes or/and in their regulation by light.
By complementation of this collection of mutants with a gene bank, we have already isolated a truncated sequence that produces a high increasing in the production of -carotene, both in the dark and light conditions, when introduced in the wild type strain of *M. circinelloides.* The protein deduced from this truncated sequence includes three main features: (i) a RING-finger motif at the amino end, (ii) an acidic domain and, (iii) a poly-glutamine stretch. Experiments carried out to elucidate the function of the gene which codes for this sequence will be presented.

Control of Taka-amylase A gene: CCAAT binding protein in *A. nidulans*

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Taka-amylase A, TAA, is a typical inducible enzyme induced by starch and repressed by glucose. The deletion analysis of *Taa* gene revealed that a 55bp DNA fragment containing a CCAAT sequence (312/-308) conferred starch inducibility. This prompted us to identify regulatory proteins which bind in a sequence-specific manner to the promoter region.

A.nidulans carrying *Taa* gene isolated from *A. oryzae* was grown under either inducible or repressive conditions, from which nuclear protein was prepared. Protein was further fractionated on a heparin Sepharose column and used for both gel shift and DNase I footprinting assays. A nuclear protein (AnCPI) extracted ftom starch-grown mycelia was found to bind to a DNA fragment containing CCAAT element. Its precise binding sequence was determined by DNase I footprinting analysis and found to be a 14 -9 bp sequence containing CCAAT element. A nuclear protein (AnCP2) isolated from glucose-grown mycelia protected almost the same region protected by AnCPl against DNase I digestion, suggesting that both factors might be the same protein. This further suggests that CCAAT binding protein could be constitutively synthesized. Purification of AnCPI is in progress. The other CCAAT binding protein (AnCF) has been also detected in *A. nidulans* using *amdS* gene. Although the relationship between AnCP and AnCF remains to be determined, AnCP protected a sequence containing CCAAT element on *amdS* gene as well as that on *gatA* gene. Therefore, AnCP would function as a global regulatory protein.

As to carbon catabolite repression of *Taa* gene, CREA was found to bind to three sites with high affinity and one site with low affinity, indicating *creA* gene product participates in the repression of *Taa* gene. All these sites contain sequences related to the CREA

consensus binding sequence, SYGGRG.

Regulation of the Gibberellin Biosynthesis in *Gibberella fujikuroi*

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The rice pathogen *Gibberella fujikuroi (* imperfect stage: *Fusarium moniliforme),* produces high amounts of gibberelins,a group of diterpenic plant hormones.The economic importance of these plant growth regulators has led to an extensive study of the regulation of gibberellin biosynthesis by physiological methods in the last years. It is well known that ammonium and glucose in high concentrations drastically reduce the yield of gibberellin formation. (Bruckner, 1992; Candau et al., 1992) However, the target genes of nitrogen and carbon catabolite repression are still unknown.
Recently we have isolated the *Gibberella fujikuroi* HMG-CoA-reductase- FPPS-, and GGPPS-genes. Northern analyses showed that those genes are expressed constitutively as expected for genes which play a role in primary and secondary metabolism as well.Therefore, 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. In order to study the nitrogen and glucose repression of gibberellin formation in more detail in next future, we isolated and sequenced the global nitrogen regulatory gene *areA*. Furthermore, cloning of the carbon-catabolite repression gene (*creA*) is on the way.

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Candau,R.,Avalos,J. and Cerda-Olmeda,E., 1992. Regulation of gibberellin biosynthesis in *Gibberella fujikuroi,* Plant Physiol., 100, 1184-1188.

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 in both gene control regions involved in benzoate dependent induction of gene expression. In gel mobility shift assays, using specific DNA fragments obtained from both promoters, we were able to further localize these Benzoate Responsive Elements (BRE's).
Another mechanism involved in regulation of the BPH enzyme system is the use of different promoters'). 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.

Cloning of *cpcB* Encoding a WD - Protein from *Aspergillus nidulans*

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Imbalances in the pool of amino acids in fungi result in a coordinated derepression of amino acid biosynthetic genes (general control) and has been described in *Neurospora crassa, Asperaillus nidulans,* and *Saccharomyces cerevisiae.* Recently, a gene *(cpc-2)* from *Neurospora crassa* has been cloned (MGG, 248, 162-173) which influences general control as well as formation of female sexual organs (protoperithecia).
Here, we report the cloning of *cpcB*, the *Neurospora crassa cpc-2* homologue from *Aspergillus nidulans*. *cpcB* encodes a protein entirely composed of WD-repeats and is highly homologous to similar proteins from eukaryotes including fungi, plants, and animals, indicating a conserved function during evolution.

The Elongation Factor 1 (EF-1) of *Schizophyllum commune*

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The translation elongation factor lot (EF-l) plays an essential role during protein synthesis by transferring aminoacyl-tRNA into the acceptor site of the ribosome in a process requiring GTP. In evolutionary terms the amino acid sequence of EF-l has been highly conserved. PCR using degenerate oligonucleotide primers allowed the amplification of a 750 bp fragment from genomic DNA. This PCR fragment was then used to obtain genomic clones containing the entire tef-locus. By sequence analysis seven introns interrupting the

tefl open reading frame were identified. Southern blot analysis revealed only one copy of the tefl-gene in genomic DNA. The level of tefl mRNA is very high in growing cells allowing for the use of the tefl promoter for gene expression in *S. commune.* Amino acid sequence alignments of Tefl of *S. commune* showed a high degree of similarity to other fungal EF1- sequences.

Poster Abstracts, Novel Molecular Tools

Identification of Basidiomycetes by PCR Assay Using Primers Targeted on Specific Region of Nuclear 18S rDNA

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The objective of the research was to develop PCR-based method suitable for reliable identification of ectomycorrhizal fungi to study species composition of fungal symbionts of forest-forming trees. The idea was to elaborate a method allowing the identification of ectomycorrhizal basidiomycetes using DNA extraction from rootlets and PCR with primers targeted on basidiomycete specfic sites of 18S rDNA. Two primers of our design were found to be able to specifically amplify ca 260 bp long fragment in basidiomycete 18S rDNA. Using this fragment digested with Sau3A and Taql enzymes it was possible to divide basidioma taxa and mycorrhizae samples into two groups. One large group included majority (12 of 16) of mycorrhizae as well as Russula taxa, *Lactarius rufus*, and *Amanita citrina*, while another one was consisted of three mycorrhizae samples as well as *Leccinum vulpinum,* two Amanita taxa, Cordnarius, Laccaria, Tricholoma and Hygrophorus taxa. However, the restriction analysis of the fragment chosen did not allow the reliable identification of all fungal spruce symbionts studied. For this purpose we used dd fingerprinting technique, which allowed us to reliable distinguish near all mycorrhizal samples and to find their counterparts.

Plasmid Partitioning *in Aspergillus nidulans.* Now in Full Colour

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A system has been devised which allows easy monitoring of behaviour and maintenance of autonomously replicating plasmids in a fungal colony. It consists of the *yA* gene from *A*. *nidulans* cloned on an AMA l -containing plasmid along with the selective marker gene *argB*. Distribution of vector molecules in the growing fungal colony is easily observed as green-coloured areas on the backgound of yellow-conidial recipient phenotype. The system has made it possible to demonstrate that, although typical phenotypic stability of replicative transformants on selective media does not exceed 60%, this figure does not reflect the frequency of mitotic loss. Mitotic loss of plasmid seems to be an infrequent event, but portions of mycelium lacking the plasmid remain viable due to cross-feeding with plasmidbearing portions.

The system was used to create a genomic library for screening sequences which affect behaviour of transforming DNAs in the fungus. It was demonstrated that, contrary to a common belief, unstable transformants are generated by a genomic library at a relatively high frequency. Experiments aimed at isolating sequences from the *A*. *nidulans* genome which affect plasmid replication and partitioning are currently under way.

Insertion of a Human Telomere at the End of a Chromosome of the Filamentous Fungus *Podospora anserina* Leads to the Formation of a Functional Telomere

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The repeat T2AG3 has been recently identified at telomeric locations on the chromosomes of the fungus *Podospora anserina.* About 200 bp of uniform repetitions of T2AG3 are present only at the ends of the chromosomes. The same telomeric motif is observed in other filamentous fungi *N. crassa, F. oxysporum, C. fulvum* as well as in most eucaryotes including humans.
A human telomeric sequence of 600 bp (HTEL) containing mainly T2AG3 repeats has been shown to be able to function as telomeres when inserted into chromosomes of human cell leading to the breakage of the chromosomes. When inserted at both ends of a linear vector, this sequence makes the plasmid behaving as an unstable minichromosome in the fungus *P. anserina* suggesting that the telomeric function is conserved.
In order to demonstrate that the human sequence can fullfil all the telomeric function in the fungus *P. anserina,* the HTEL sequence has been introduced by transformation at the end of a chromosome. The results presented show that in the transfon-ned strains, the HTEL sequence became functional leading to the breakage of the chromosome.
In such strains, the selectable *ura5* marker used for transformation is now located very close to the telomere. In yeasts, it has been shown that the expression of genes placed in the direct viccinity of a telomere is strongly repressed. This is known as the telomere position effect (TPE) and attributed to the invadment of the gene by proteins of the telosome. By studying the expression of the *ura5* gene, it will be investigated if such a telomeric position effect is observed in the fungus *P. anserina.*

UP-PCR (RAPD Like) and Virulence Analysis of Host Specificity in Phytopathogenic Fungus *Cochliobolus sativus*

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The objective was to evaluate at the genome and the virulence levels whether *C. sativus* is specialized with respect to its hosts. The 8 fungal populations were sampled from barley, wheat and wild barley and analyzed by the UP-PCR technique. Each population was found to be characterized by one major type of UP-PCR patterns. Of 7 types observed in total, two polymorphisms proved to be characteristic of wheat, one type was specific for wild barley and four others were barley specific. The phylogenetic analysis of polymorphisms showed the close relatedness of polymorphisms specific for wheat but not for barley.
The virulence analysis revealed that the isolates of two main UP-PCR polymorphisms from wheat population are more virulent to wheat than barley, while the isolates from barley population were of equivalent virulence regarding these two hosts.
Thus., *C. sativus* possesses the wheat specificity which is based on genome differences as well as pathogenicity variation.

Multiplex PCR Reaction for the Detection of Potential Aflatoxin and Sterigmatocystin Producing Fungi

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A multiplex PCR reaction was developed to amplify the aflatoxin biosynthetic genes: norsolorinic acid reductase (*nor-1*), versicolorin A dehydrogenase (*ver-1*) and sterigmatocystin O-methytransferase *(omt-A).* The reaction gives a triplet banding pattern with aflatoxin producing strains of *Aspergillus flavus, A. parasiticus* and also with sterigmatocystin p -producing strains of *A. versicolor.* The pattern of aflatoxin negative *A. flavus* strains varied. One strain showed no signal. In one strain the band for the gene of the *omt-A* gene is missing but in a third strain the triplicate pattern is complete. *A.*

oryzae and *A. sojae,* which do not produce aflatoxin, but are closely related to *A. flavus* and *A. parasiticus* apparently carries sequences homologous to the *ver-I* and the *omt-A* gene, but the primer set specific for the *nor-1* gene gave no signal indicating sequence variation or deletion of that gene. Most other food related strains tested showed negative results for all genes. The exception was *Penicillium roqueforti* which showed two bands. One with an identical fragment length as that of the *nor-1* PCR product of *A. parasiticus* and one with an increased length compared to the *ver-I* PCR signal. The multiplex PCR reaction therefore seems to be specific for potential aflatoxin and sterigmatocystin producer.

Use of Internal Transcribed Spacers (ITS) for Detection of Penicillium by the Polymerase Chain Reaction

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Rapid identification of filamenentous fungi is becoming increasingly important in food myclogy, both for monitoring a production process and for the identification of food spoilers.
A 610 bp region spanning the ITS regions and 5.8 S gene of 72 strains belonging to 28 Penicillium subgenus Penicillium species was sequenced and the data used for phylogenitic analysis and identification of specific PCR primers. The region is very conserved with only 29 individual positions differring in one or more species. The largest number of differences was found for *P. rogueforti* and *P. carneum,* as 13 nucleotide differences separated these two species from the rest of the penicillia. Bootstrapped parsimony analysis showed that the penicillia investigated could be allocated to three main groups.
The primer sets were tested by performing PCR on DNA extracted from a range of Penicillum subgenus Penicillium, other Penicillium and non Penicillum species. A 336 bp fragment was specifically

amplified from Penicillium subgenus Penicillium DNA by the primers ITS212d/ITS549 and a 300 bp fragment was specifically amplified from *P. rogueforti* and *P. carneum* DNA by the primers ITS183/ITS401. In addition we tested the primer sets and PCR conditions on cheese extracts. Fungal hyphae or spores were partially purified from blue veined or camenbert cheese by aqueous two-phase polymer partitioning and DNA was extracted by CTAB after proteinase K digestion. The Penicillium subgenus specific fragment was amplified from both DNA extracts and the *P. roqueforti* / *P. carneum* specific fragment was amplified from DNA extracted from the blue veined cheese only.

Mutational and Differential Display Analyses of the Yeast to Hypha Transition in *Mucor circinelloides*

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Two different approaches are being employed to analyze the dimorphic transitions in *Mucor circinelloides.*
A program was designed to isolate mutants affected in the yeast-to hypha transition. Nitrosoguanidine mutagenesis of *M. circinelloides* spores led to the isolation of several mutants which can grow only as yeasts under aeorobic conditions. Characterization of the mutants and complementation analyses are now in progress. We have designed also a procedure to isolate mutants affected in the hypha-to-yeast transition. The differential display technique is a powerful tool to detect and isolate genes which are differentially expressed in two cell types, two stages of development, or, as it is the case two alternative growth models (Liang, P. and Pardee, A.B, (1992). Science 257:967). We have displayed mRNAs from yeasts, hyphae, and the yeast-to-hypha transition. Total RNA was obtained from yeasts grown for 16 hours under a nitrogen atmosphere (yeasts), germlings grown for two days (mycelium), and yeasts grown for 16 hours under a nitrogen

atmosphere and then allowed to Initiate the hyphal growth for 4 hours aerobically (transition). A number of cDNA fragments have been isolated which are differentially expressed during the transition as compared to yeast or mycelial growth. One of these cDNA clones shows high sequence similarity (about 70% at the amino acid level) with ornithine decarboxylase genes from several organisms. ODC activity was previously shown to increase 40-fold during the yeast-to hypha transition in *Mucor*- spp. (Calvo-Mendez C., Martinez-Pacheco, M. and Ruiz Herrera, J. (1987). Exp Mycol 11:270-277). Other cDNA clones specfically expressed during the phase shift show no relevant homology to any DNA or protein sequences in the databases and their function remains unknown.
These preliminary data suggest that analysis of *M. circinelloide* dimorphic transition can effectively be carried out by means of the display technique. Mutants altered in the phase shift can also contribute to elucidate the genes involved in the dimorphic response.

Restriction Enzyme-mediated DNA Integration in *Coprinus cinereus*

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Tagging genes by insertion of foreign DNA is an elegant way to create mutations for genetical studies. In filamentous fungi, the use of transposons is up till now not possible but recently an alternative technique (restriction enzyme-mediated DNA integration, REMI) has been introduced. By action of restriction enzymes added to the transformation mix, linear DNA is inserted into the host genome. Here we describe effects of restriction enzymes on transformation in the basidiomycete fungus *Coprinus cinereus*. It is our aim to establish an efficient system for tagging developmental genes in this fungus.
First we constructed pPAB2, a vector for REMI which has a number

of unique restriction sites in poly-linker regions and which is able to complement pab1 mutations in *C. cinereus.* Two different strains and three different enzymes *(Bam*Hl*, Eco*Rl*, Pst*l*,* 20-180 units per transformation sample) were used. We observed that transformants appear earlier when restriction enzymes were present and that transformation rates are strongly influenced by the amounts of enzyme added. Each enzyme has a specific concentration where the highest number of transformants is obtained. Transformation rates rapidly decrease with further increase of enzyme.
We isolated about 7500 transformants of *C. cinereus* and determined mutation rates in fungal development and mode of plasmid integration in relation to enzymes and enzyme units used. In line with the effects on transformation, mutation rates are lowest with optimal transformation rates and increase with higher enzyme doses. Plasmid integration varies in type and number. True REMI events and single integrations are preferentially found with lower enzyme concentrations. High enzyme concentrations can cause multiple insertion events and may accumulate mutations created by incorrect repair of DNA damage induced by added restriction enzyme.
Based on our experiences we therefore propose to use those enzyme concentrations for REMI mutagenesis which generate the highest amount of transformants and show the lowest rates of mutation. Under such conditions, the probability seems to be highest for a specific mutation induced by a single, restriction enzyme mediated integration of the selection marker.

Specific Detection of *Phytophthora nicotianae* Using the Polymerase Chain Reaction and Primers Based on the Sequence of its Elicitin Gene ParA1

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Primers based on the sequence of the elicitin gene ParAl of *Phytophthora nicotianae* were used to detect specifically the fungus

by the polymerase chain reaction (PCR). Six primers from flanking and coding regions of the ParAl gene, were tested in various combinations. One combination, EL7/IL8, with EL7 in a flanking region and IL8 in a coding region of the gene, gave an intense 378 bp signal with a diverse collection of isolates of *P. nicotianae,* that included some from black shank disease of tobacco and others from a variety of hosts. The sequence of the primer product obtained with an isolate that produces elicitin and one that does not, was homologous with the known sequence of the ParAl gene. The same primer combination gave no signal with sixteen other *Phytophthora* species tested except for two isolates *P. palmivora* with which it gave a weak 800 bp signal. It gave no signal with DNA from healthy tobacco and tomato plants but *P. nicotianae* was detected in inoculated tobacco and tomato plants. Small numbers of zoospores (>100) trapped onto a nitrocellulose membrane after filtration from suspension were also detected after two successive rounds of PCR.

Gene Targeting in *Schizophyllum commune*

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To aid site-directed integration of transforming DNA two gene targeting vectors have been constructed for *S. commune.* The vectors, pUT1 and pUT2, allow the investigation of gene expression excluding the positional effects of integration sites observed with ectopically integrating constructs.
Both vectors, pUT1 and pUT2, contain 31-truncated *ural* genes missing the last 162 bp or 312 bp, respectively. These constructs are not sufficient to complement the mutant *ural*- phenotype upon ectopic integration. only homologous recombination within the *ural* locus yields active *ural*+ genes and therefore can be detected by screening for uracil prototrophy.
Transformation efficiency of both pUT1 and pUT2 is approximately

500-fold reduced in comparison to transformation with a wild-type allele.
Molecular analysis of the transformants obtained with pUT1 confirmed the homologous integration event expected for gene targeting. The pUT2 transformants revealed properties of an replacement event instead.

Molecular Data Suggest Further Re-classification of the Genus *Trichoderma*

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The genus *Trichoderma* introduced by PERSOON (1794) and later revised by RIFAI (1969) and BISSETT (1984, 1991) consists of deuteromycetous *Trichoderma* species as well as anamorphs of the ascomycetous genus *Hypocrea*. From comprehensive morphological studies on this genus two taxonomical keys are provided to differentiate *Trichoderma* species into 9 aggregates (RIFAI 1969) or 5 sections *(Trichoderma, Longibrachiatum, Saturnisporum, Pachybasium, Hvpocreanun;* BISSETT 1984). A taxonomy in terms of phylogenetic relatedness of the genus is still unresolved.
We have used a series of molecular methods (RFLP and sequence analysis of the internal transcribed spacers ITS-1 and ITS-2 of the rRNA gene complex, RAPD assay) differing in their sensitivity with respect to the characterization of relationships within the genus *Trichoderma* at different taxonomic levels (inter/intrageneric, inter/intrasectio, inter/intraspecies). We analyzed 117 strains belonging to the different species of section *Longibrachiatum.* All species within this section were found to be closely related. In order to evaluate these data with respect to "taxon" definition and taxon borders we compared ITS sequence data from the type species and related strains of the remaining sections of *Trichoderma.* Special emphasis was put on the inclusion of a broad spectrum of.strains

representing anamorphs of *Hypocrea spec.* The molecular data strongly suggest a re-classification of the *Trichoderma* taxonomy according to BISSETT (1984, *1991):* I. The type species of section *Saturnisporun* clearly falls into section *Longibrachiatum.* II. There is no clear. separation between sections *Trichoderma* and *Pachybasium.* III. Part of the *Hypocrea* species is clearly related to distinct *Trichoderma* species, i.e. we found new anamorph-teleomorph connections.

Heterologous and Homologous Transformation of *Acremonium crysogenum*

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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 compound. The *pcbAB/pcbC* and *cefEF/cefG* genes, both of which are involved in this process, have been cloned and characterized. It has been shown, that *pcbAB and pcbC* are closely linked and transcribed in opposite directions, as are the *cefEF and* cefG genes (for review, see [1]). To study potential controlling sequences, which are expected to be located in the intergenic regions, reporter gene constructs have been made. The non translated sequences of both pairs of genes have been translationally fused to the lacZ reporter gene derived from plasmid pSI8.8 [2]. Using both constructs, a set of promoter deletions was transformed into *A. chrysogenum* for measuring the coresponding -galactosidase activities.
In previous work, the *pcbC* promotor turned out to mediate relatively strong activity [2]. To identify additional sequences with similar activity but different expression pattern, we used the reporter vector SI8.8 to screen a library of *A. chrysogenum* DNA fragments. Such

sequences are suitable to express heterologous genes in the eukaryotic host *A. chrysogenum.*
A system which would allow the targeted integration of DNA into the genome of *A. chrysogenum* would be desirable. For this reason the tubulin gene of the fungus has been cloned. By introducing a point mutation into the gene it has been altered in such a way as to confer resistance to the fungicide benomyl [3]. This is the first step in developing a vector system, that allows targeted integration and positive selection of transformants in this organism.
[1] Martin JF, Gutierrez S, Fernandez FJ, Velasco J, Fierro F, Marcos AT & Kosalkova K (I 994) Antonie van Leeuwenhoek 65: 227-243
[2] Menne S, Walz M & Kuck, U (1994) Appl Microbiol Biotechnol 42: 57-66
[3] Nowak C & Kilck U (1994) Curr Genet 25: 3440

The Phylogeny of the Genus Claviceps
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Claviceps species colonize wide variety of grasses and sedges. *C. purpurea* producing peptide ergot alkaloids exhibits the broadest host spectrum. Other *Claviceps* species are restricted to smaller host taxa: *C. fusiformis* (Panicoidea) producing clavine alkaloids, *C. paspali (Paspalum)* producing clavines and simple lysergic acid amides and *C. gigantea* (varieties of *Zea* mays), where we found not only clavines but also simple lysergic acid amides. Other strains used in this study were *C. grohii (sedge), C microcephala* (pearl millet), *C. viridis* (Oplismenus) *and C.phalaridis (Phalaris).* In the past, two major reclassifications were suggested: the separation of *C. purpurea* isolates to different taxa (cf Loveless 197 1) and placement of C *paspaii, C gigantea* and C *grohii* into a new genus *Mothesia* (Oddo

and Tonolo 1967). The above species were used for phylogenetical study based on RFLP and RAPD DNA fingerprinting and the analysis of ITSI and ITS2 DNA sequences.
Both genome fingerprinting methods confirmed the higher intraspecific variability among *C. purpurea* isolates when compared with strains of *C. paspali and C. fusiformis.* However, their variability did not correlate with the host species or the geographic origin and therefore did not substantiate the sub-division of *C purpurea* species. The RFLP fingerprints of C *purpurea and C. fusiformis* were related as well as those of *C. paspali and C. gigantea.* The phylogeny tree obtained by the neighbor joining method from the comparison of ITSI and ITS2 confirned the close relatedness of *C. purpurea* and *C. fusiformis* as well as *C. grohii.* On the other side, *C. paspaii and C. gigantea* were closer to endophytic Balansiae. *C.Paspali* as well as Balansiae produce alkaloids on high phosphate media whereas *C. purpurea* and *C. fusiformis* are subjected to strong phosphate inhibition (Pazoutovai et al. 1983).The genetical and physiological distance of *C. paspaii* and *C. gigantea* from the "purpurea" group could substantiate their placement into a new genus .
Loveless AR (197 1) Trms. Br. Mycol. Soc. 56, 419434
Oddo N and Tonolo A (1967) Ann. Ist. Super. Sanita 3, 16-25
Pazoutovai S et al, (1983) Etir. J. Appl. Microbiol. Biotechnol. 16: 208-21 1,

Application of DDRT- PCR to the Analysis of Differential Gene Expression in a Plant-fungal Pathogen Interaction

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Botrytis cinerea is pathogen to a wide range of host plants. A non-biassed approach is being carried out in order to detect those fungal genes which are differentially expressed during the interaction with

the host plant. Among those genes specifically expressed *in planta,* one would expect to find fungal genes with a role in pathogenicity. To this end the expression pattern of *B. cinerea* during the pathogenesis process on tomato is being compared with its expression pattern during saprophytic growth by "differential display of mRNA" (DDRT-PCR). As a first step a standard inoculation procedure of *B. cinerea* on detached tomato leaves has been developed. Inoculation conditions have been optimized and high infection efficiencies have been achieved by inducing high germination rates on the leaves as well as synchronization of the infection process in different lesions. The infection process has been followed visually and microscopically. At the molecular level the progress of infection has been estimated by determining the proportion of fungal RNA in the total interaction RNA population in a time course experiment. Since in the interaction two organisms are present, by "differential display" cDNAs from both, the fungus and the plant, are detected. Most of these cDNAs represent fungal or plant genes constitutively expressed. Their origin can be discriminated by comparison of the expression pattern displayed in the interaction with the expression pattern of the fungus grown *in vitro* or the expression pattern of a non infected tomato plant. Interaction specific cDNAs are also detected which will represent either fungal *in planta* induced genes or plant defense genes induced in response to a pathogen. In order to discriminate these last two categories of cDNAs, samples from control infections of tomato leaves with two other pathogens *(Phytophthora infestans* and Tobacco Necrosis Virus) are being used.

The DDRT-PCR procedure has been adapted to the analysis of the interaction *B. cinerea-tomato* and the controls indicated above have been included. Several fungal interaction specific cDNAs have been isolated. A higher level of expression or a differential expression *in planta* for some of the corresponding genes has been demonstrated by Northern blot analysis.

RAPD Analysis of *Trichophyton verrucosum* Strains

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20 random primers (dekamers) were tested for their ability to amplify genomic DNA of *Trichophyton verrucosum* using random amplified polymorphic DNA (RAPD) analysis. Six of these primers were selected for further study aimed at discrimination of wild and vaccination strains of *Trichophyton verrucosum.* The estimated proportions of false positives and false negatives in the RAPD data were calculated from repetitive experiments to prevent PCR artifacts. Corrected values of Nei and Li's coefficient of similarity were then used for the comparison of typed strains.
The results indicate the ability of RAPD to distinguish strains *of Trichophyton verrucosum.* The method is even able to discriminate between the avirulent vaccination strains *(Trichophyton verrucosum TV*M-9 *and Trichophyton verrucosum TV-M-310)* prepared by UVmutagenesis originating in the standard wild strain *Trichophyton verrucosum Straznice* and the original wild strain *Trichophyton verrucosum Straznice.* These outcomes suggest new possibilities for epidemiological analysis, for discrimination between different vaccination strains and for studies of fungal population in infected host.

Typing of *Aspergillus fumigagus* Isolates by the Random Amplified Polymorphic DNA Technique

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Aspergillus fumigatus Fresenius is an opportunistic human pathogenic fungus, which may cause several diseases, such as

allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis. Typing of these isolates is at the center of interest of epidemiological studies. We have tested the applicability of a PCR-based method for this purpose. Sixty-one isolates and collection strains of *Aspergillus fumigatus* were compared by the random amplified polymorphic DNA (RAPD) technique. Although the patterns of the strains were very similar for most of the primers, the application of three primers (OPC-06, OPC07, and OPC-10) made it possible to cluster the 61 *A. fumigatus* isolates into 47 groups. The results allowed the RAPD technique to be used more efficiently for typing *A. fumigatus* isolates than isoenzyme analysis, or restriction enzyme analysis of the mitochondrial DNA and the ribosomal gene cluster of these strains (1). The application of another primer (OPC-08) permitted the distinction of *A. fumigatus* from other potentially pathogenic aspergilli *(A. niger, A. flavus and A. terreus)* and also from closely related species (species of section *Fumigati, e.g. Aspergillus fennelliae and A. thermomutatus).* The only cross-reacting species was *A. fischerianus,* which is the species most closely related to *A fumigatus.*

(1) E. Rinyu, J. Varga and L. Ferenczy (1995) Phenotypic and genotypic analysis of variability in *Aspergillus fumigatus.* J. Clin. Microbiol. 33: 2567-2575

Comparison the Isolates of the Postharvest Pathogen *Mucor piriformis* Using Molecular Markers

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The soilbome fungus *M. piriformis* Fischer is an important

postharvest pathogen of different fruits and vegetables. This can cause the substantial decay of such agricultural products if they are stored or transported at low temperature for a prolonged time.
The genetic diversity of *M. piriformis* was studied by isozyme polymorphism and random amplified polymorphic DNA (RAPD) analyses. Six enzyme activities, catalase (CAT), -esterase (EST), glucosc-6-phosphate dchydrogenase (G6P), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and superoxide dismutase (SOD) have been tested for 10 (+), 9 (-) and 10 neutral isolates. Some isozyme markers (EST, G6DH, and MDH) were useful to differentiate the strains with mating abilities from neutral ones. The six different 10-bp primers used for RAPD analyses revealed different levels of diversity, three to nine different amplification patterns were detected. The results indicated a higher degree of variability than that found in isoenzyme studies. The dendrogram produced bv the unweighted pair group method from the unified data sets revealed a correlation between the two clusters obtained and the mating potency of the isolates. These results demonstrate that both isoenzyme and RAPD analysis provides an efficient approach for genetic studies of *M. piriformis.*
This work was supported in part by the Hungarian Scientific Research Fund (OTKA) # F/4 017677.

Highly Efficient Homologous Integration via Tandem Exo-6-1,3-glucanase Genes in Common Mushroom, *Agaricus bisporus*
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Recently, a transformation system was developed for common mushroom, *Agaricus bisporus.* Plasmids carrying the hygromycin resistance gene controlled by regulatory sequences from either *Aspergillus nidulans* (pAN7-1) or A. *bisporus* were introduced into *Agaricus* protoplasts by electroporation. Approximately 1-100

transformants arose from 0.2-1 x 108 protoplasts using 10 ug linearized DNA. Linearization of plasmid DNA increased the number of transformants 2-5 fold. A random 3 kb genomic fragment from A. *bisporus* was cloned into plasmid pAN7-1, downstream of the A. *nidulans* terminator. The resulting plasmid pHAG3-1 was linearized within this homologous region (called AbGH3) before transformation. Transformation frequency was not enhanced by this plasmid, although it was found that homologous integration occurred in about 50% of the transformants. Homologous integration was found with pHAG3-1 linearized at three different positions within the AbGH3 sequence generating either blunt, 5'- or 3'-overhanging ends. Tandem integrations were also observed at the homologous position with restoration of the restriction sites used for linearization.
The AbGH3 fragment was found to contain two open reading frames in tandem, which showed 60% similarity to exo-B-1,3-glucanases from *Saccharomyces cerevisiae* and *Candida albicans.* The function of these enzymes is unknown, but they are thought to be involved in cell wall metabolism. The upstream gene (AbEXGI) encodes a polypeptide of 419 amino acids, whereas only the start of the second gene (AbEXG2) is present on the genomic fragment, encoding the N-terminal 159 amino acids. Both polypeptides contain a predicted signal peptide region. The genes are interrupted by numerous short introns at conserved positions. Expression at the mRNA level is low in vegetative mycelium and relatively high in fruitbodies. Exoglucanase mRNA was increased in vegetative mycelium of a transformant with tandemly integrated pHAG3-1 plasmids at the homologous position. Fruitbodies are currently grown of this transformant to study the effect of exoglucanase overexpression.

Isolation of Meiosis Specific Genes from *Aspergillus nidulans*

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A first step towards a molecular analysis of meiosis in any organism, is the cloning of meiosis specific genes from that organism. In order to clone genes involved in meiosis in the filamentous fungus *A. nidulans* several different strategies can be followed. The strategy that will be discussed here, is transformation complementation of mutants defective in meiosis. In *A. nidulans* two classes of such mutants exist.

The first class consists of mutants that are both defective in the repair of DNA damage as well as in meiosis. Mutants in the *A. nidulans uvsC* gene have such a phenotype. We will describe the cloning of the *uvsC* gene of *A. nidulans* by transformation complementation of an *A. nidulans uvs*CII4 mutant. Sequence analysis of the smallest fragment still giving full complementation, revealed strong homology of the predicted protein sequence with all known RAD51 homologs.

The second class are meiosis specific mutants. Since these were not already available in *A. nidulans,* we have set up a screening to isolate such *mei* mutants. One of them, *mei*Al, has been cytologically characterized and seems to be blocked at one of the later stages of the first meiotic division. We will report the cloning and characterization of the corresponding wild type *meiA* gene, which we are currently undertaking.

Characterization of *Phytophthora infestans* Ham34::GUS-Transformants

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A few years ago Judelson *et al* (1991, MPMI 4: 602-607) have established a DNA-transformation procedure for the oomycetous plant pathogen *Phytophthora infestans.* In this method, CaCl2 and

polyethylene glycol are used to introduce DNA complexed with cationic liposomes into protoplasts which are made from germinating sporangia. After DNA uptake the protoplasts are allowed to regenerate on selective medium. With this procedure we obtained a variety of stable *P. infestans* transformants. Among those are transformants containing a gene construct in which the *Ham34* gene promoter of *Bremia lactucae* is fused to the coding region of the GUS-reporter gene. The activity of the *Ham34* promoter was analyzed by GUS staining of *in vitro and in plants* grown mycelium, sporangia and germinating cysts. Although the *Ham34* gene promoter is thought to be constitutive in *P. infestans,* it appeared that mycelium of a number of Ham34::GUS-transformants contains blue and white sectors, and in some transformants not all sporangia or germinating cysts stain blue. In other transformants blue staining was found in all hyphae, germinating cysts and sporangia. These observations raised questions about the transformation procedure and urged us to investigate the transformants in more detail. Results of the characterization will be presented.

REMI-Mutagenesis in *Claviceps purpurea*

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The ascomycete *Claviceps purpurea* attacks young flowers of many grass- and cereal species, especially *Secale cereale*1. To interrupt and to tag genes, which are involved in pathogenesis, we generated mutants of a possibly haploid benomyl-derivate from *Claviceps purpurea* with the REMI-technique (restriction enzyme mediated integration). The transformation-rate was lower with REMI than with the established transformation-system in *Claviceps purpurea,* in contrast to the results in many other systems. We isolated 1031 REMI-clones, which were generated with different enzymes. Most of these clones have only one copy of the vector. In 12,3 % of the tested strains it was possible to recover the vector with the REMI-enzyme.

So far 64 transformants were tested on rye-flowers with regard to their virulence. Six of these clones not substantially differing in growth and conidia-germination-rate to the wildtype, showed a clearly reduced pathogenity on plants, representing the first pathogenity mutants of *Claviceps purpurea.* From two of these strains vector-flanking sequences were isolated and sequenced.
1Tudzynski, P., Tenberge, K. B., Oeser, B. (1995) *Claviceps purpurea*, in: Pathogenesis and host specificity in plant diseases. (U. Singh, ed.), Vol. IV Eukaryotes. Pergamon Press, New York, pp 161-187.

Molecular Analysis for Virulence in *Fusarium oxysporum* F.sp. *dianthi*

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Isolates of *Fusarium oxysporum f.sp dianthi* show a large variation in virulence for different cultivars of carnation. Previously we have identified more than 10 races, which all appear to belong to different VCGS. However, within VCG 0022, two races (1 and 8) occur that evoke similar reactions on most differentials, with the exception of cv. Pallas. This cultivar is highly susceptible to race 8 and completely resistant to race 1. Both races can be considered as near-isogenic as they belong to the same VCG and on the basis of electro-karyotyping and isozyme, RFLP and RAPD analyses.
Since both races only reveal differences in pathogenicity assays, we are currently investigating whether the gene-for-gene theory is applicable in this plant-pathogen interaction. In this patho-system the avirulent race 1 would differ from the virulent race 8 by one or a few genes involved in specific recognition by cv. Pallas. Two approaches have been chosen to test this hypothesis.
Firstly, mRNAs from the infected xylem of stem inoculated cv. Early Sam, that is susceptible to both races are analyzed by differential

display. In this cultivar both races form sufficient biomass whereas the avirulence factor(s) from race 1 are assumed to be expressed. Secondly, genomic subtraction is being performed using the DNA from the avirulent race 1 as tester-DNA and the DNA from the virulent race 8 as subtractor-DNA. Genomic fragments supposed to be involved in the race specificity of race 1 isolates are currently being analyzed.

Successful Crosses and RAPD Analysis of Progenies Indicate a Heterothallic Bipolar Mating System in *Mycosphaerella graminicola*

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Monospore isolates of *Mycosphaerella graminicola* derived from ascospores that were considered to originate from a single ascus were studied by the polymerase chain reaction (PCR) with 33 RAPD primers. The results of these analyses indicated that 7 of these monospores were indeed derived from the same ascus. One monospore was crossed with the remaining six and three of these crosses were successful, directing to a bipolar mating system in *M. graminicola.* Three RAPD primers were used to study segregation of markers in random ascospore progenies of 162 isolates from these crosses. Mendelian segregation of RAPD markers was observed, and in one progeny individual genotypes were confirmed by hybridization analyses. These results prove the existence of a bipolar heterothallic mating system in *M. graminicola,* which has important epidemiological consequences, and provides an explanation for the vast genetic variation in this pathogen.

Phylogenetic Relationships of *Fusarium oxysporum* Isolates as Revealed by RAPD-PCR, Isozyme and rDNA RFLP Analyses

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Differences in RAPD-PCR and isozyme polymorphisms between 26 isolates of *Fusarium oxysporum* were compared using native polyacrylamide gel electrophoresis and agarose gel electrophoresis. 13 of the isolates were collected from barley, while the rest of the isolates were collected from various cultivated dicotyledonous plants in Finland. The species designation of all isolates was verified by morphology.

Clear isozyme polymorphims was detected in five enzymes by which the isolates could be divided into six main groups by UPGMA analysis at the similarity level of 55 % . The phenotypes from RAPD-PCR analysis formed 7 main groups in UPGMA analysis at the similarity level of 60 % and the composition of these groups was somewhat different from that of isozyme analysis. The phylogenetic relationships between *F oxysporum* isolates were studied bv the heuristic search mode of PAUP 3.1.1. and by the Neighbor Joining and branch and bound analyses of PHYLIP 3.5. When the data matrices from isozyme and RAPD-PCR analyses were combined, the F *oxysporum* strains could be divided into six main clades in phylogenetic analyses. In the Neighbor Joining tree each of these six clades was supported by bootstrap values higher than 50 %. A correlation was found between the geographical origin and the phylogenetic relationship of isolates collected from barley.

Five of the *F.**oxysporum*** isolates representing five of the six main clades of phylogenetic trees were furthermore studied by rDNA RFLP analysis on polvacrylamide gel together with 6 isolates of *F. avenaceum* and one isolate of *F. redolens, F. graminearum, and E equiseti.* The isolates of all species formed their own groups in UPGMA and branch and bound analyses of PHYLIP, except for one isolate of *F oxysporum,* which was grouped to the same group with the only isolate of *F. redolens.* The bootstrap values of all six groups

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of branch and bound analysis were higher than 70 %. Thus it is possible that at least one of the isolates that was morphologically identified as *F. oxysporum* actually belongs to the species *F.redolens,* which is known to be closely related to *F. oxysporum.*

Poster Abstracts, Extracellular Enzymes

A Reporter Gene System for Analysis of Promoter Functions in the Basidiomycete Fungus *Phanerochaete chrysosporium*

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Lignocellulose degradation by *Phanerochaete chrysosporium* involves families of genes with differential expression. To establish a system for functional analysis of gene promoters involved in the process, we made a DNA construct comprising the minimal promoter region of the *cbhI.1* gene of *P. chrysosporium* fused to the *phlR* gene of *Streptoallotiechus hindustanus.* This was used to transform P. *chrysosporium* to phleomycin resitance.
Southern blot analysis revealed that, in phleomycin-resistant transformants, the transforming DNA was maintained extrachromosomally. The incoming donor DNA was also methylated by the fungus. Inhibition of such methylation elevated expression of the *phlR* gene. RT-PCR analysis of gene expression revealed that the *cbhl.1* promoter/*phlR* gene construct was transcribed, demonstrating the successful use of this vector for a reporter system. However, an intron within the *cbhl.1* gene promoter, which was excised from transcripts of the *cbhI.1* gene, was not excised from transcripts of the transforming DNA construct.

Purification and Partial Characterisation of an Glucosidase (GLUA) from the Entomopathogenic Fungus *Metarhizium anisopliae*

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Efficient mechanisms for carbon uptake by entomopathogenic fungi are likely to be important factors in disease development. The predominant carbon source in the hemocoel of many insects is the glucose disaccharide trehalose. We describe the purification and characterisation of an extracellular -glucosidase (GLUA) produced by *M. anisopliae* which shares a number of characteristics with -glucosidases from other sources.
Purification was achieved using ammonium sulphate precipitation, preparative isoelectric focusing and fast-flow anion exchange chromatography. This resulted in a 109-fold purification of the enzyme. SDS-PAGE resolved a major protein of 37kDa. *GLU*A hydrolysed a range of -linked disaccharides but showed no detectable activity toward any -linked disaccharides tested. *GLU*A was inhibited by EDTA however no metal ion requirement was determined. *GLU*A displayed transglycosylation activity which resulted in non-linear MchaelisMenton kinetics. Transglycosylation products were resolved using thinlayer chromatography. Progress toward the characterisation and cloning of this gene will be presented.

Expression of the Starch-binding Domain of Glucoamylase from *Aspergillus niger and* Determination of its Structure

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The starch-binding domain (SBD) of glucoamylase 1 (GI) of

Aspergillus niger is a separate domain required for the binding of glucoamylase to granular starch. SBD has been produced by proteolysis of GI but the yields were poor and the method was time and labour consuming. An expression vector, using the glucoamylase sequence (residues 1-498) as carrier in a fusion construct has been used to produce from *Aspergillus niger* the starchbinding domain on its own. The endoproteolytic cleavage recognition site, Lys-Arg, introduced between G498 and the SBD sequences, was correctly processed *in vivo.* Yields of secreted SBD in different culture media have been studied and up to 300mg/l achieved in shake flasks. Biochemical and physical analysis showed that the engineered SBD is functionally active and 2 mol cyclodextrin/mol protein are bound with an affinity very similar to the proteolytically-derived SBD. The threonine residues 510, 511 and 513 are glycosylated with an average of three mannose residue per SBD molecule. 15N labelled SBD has been successfully produced at 20-40 mg/l with a specific activity of >99% for high resolution NMR analysis. The labelled protein was used to determine NMR ambiguities. The secondary structure has been resolved and it consists of one parallel and five antiparallel pairs of B-strands forming two B-sheets. The three dimensional structure of the SBD has been also completed. In order to study the molecular basis of starch binding, mutagenesis of specific amino acid residues of the SBD, which are thought to be involved in the binding of polysaccharides, has been undertaken. Binding affinity studies with the mutated SBDs are underway.

Sequence Analysis of Esterases from the Rumen Anaerobic Fungus *Neocallimastix patriciarum:* Members of a New Family of Hydrolases

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A Neocallimastix patriciarum cDNA library was screened for esterase activity and four different esterase-encoding genes *(bnaI, bnaII, bnaIII* and *bnaIV*) were isolated. Two of the esterases *bnaI* and *bnaIII* were shown to possess acetyl xylan esterase activity. The complete nucleotide sequences of *bnaI, and bnaIII* and a partial sequenceof *bnaII* have been determined and encode proteins with several distinct domains. BnaI and BnaIII each contain a putative signal sequence followed by the catalytic domain, a threonine-rich linker and two copies of a cysteine-rich repeat at the carboxy-terminus. The carboxy terminal repeats of BnaI and III are homologous to the carboxy terminal domains of the *CelB* and the *XynA* proteins from *N. patriciarum.* These domains may constitute a novel cellulose binding domain or alternatively be involved in cellulosome assembly. From the *bnaII* cDNA clone (only a partial clone) the complete nucleotide sequence of the catalytic domain only has been determined. The catalytic domains of BnaI,II and III are members of a new family of serine active site hydrolases. Candidate active site aspartic acid and histidine residues have been identified as part of a consensus sequence for the new family of enzymes. The amino acid sequence of BnaII was found to exhibit homology with a domain of the *Clostridium thermocellum* CelE enzyme, while the amino acid sequence of BnaIII exhibits extensive amino acid sequence identity with a domain from *XynB* from *Ruminococcus flavefaciens* and a lower level of identity with a domain of the *C. thermocellum* CelX enzyme. *BnaI*, possibly the major extracehular esterase of *N. patriciarum* is much less similar to any of the members of the family than BnaII or BnalIl and appears to represent the first member of a new branch of the family. The partial sequence of BnaIV is related to a group of esterases that includes lipases, carboxyl esterases and a para-nitrobenzyl esterase from *Bacillus subtilis.*

Isolation and Characterization of Pectinolytic Enzymes and Encoding Genes from *Fusarium oxysporum*

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Pectinolytic enzymes, produced by the vascular tomato wilt pathogen *Fusarium oxysporum f.sp. lycopersici* have been proposed to be important pathogenicity factors. We have purified and characterized an endopolygalacturonase (PG1), an exopolygalacturonase (PG2), and an endopectate lyase (PL1) produced by this pathogen on tomato vascular tissue. The three enzymes were N-glycosylated and their secretion was inhibited by tunicamycin, indicating a role of glycosylation in the secretary pathway. Sequencing of N-termini showed high homology of PGl and PL1 with a *F. moniliforme* endopolygalacturonase (Caprari et al., Mycol. Res. 97:497-505) and a *F. solani* pectate lyase (Gonzalez-Candelas and Kolattukudy, J. Bacteriol. 174:6343-6349), respectively. The N-terminal sequence of PG2 showed no homology with other proteins in the databases. cDNAs of the *F moniliforme* and the *F solani* genes were used as heterologous probes to screen a -EMBL3 genomic library of *F. oxysporum* f.sp. *lycopersici,* race 2. Hybridization patterns of four phage clones hybridizing to the *F. moniliforme* gene indicated that they all encompassed the same genomic region. A 2.2 *kb Hind III* fragment containing the putative PGl gene was subcloned into pUC118. On the other hand, a 2.5 kb Sal I fragment from a phage clone hybridizing to the *F solani* gene was subcloned and is being characterized.

Molecular Characterization of the Endoxylanolytic System of the Rumen Anaerobic Fungus *Neocallimasix frontalis*

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Anaerobic fungi belonging to the class Chytridiomycetes are potent producers of enzymes degrading the various components of plant cell wall material in the rumen of herbivorous animals. The secreted xylanolytic system of *Neocallimastix frontalis* is characterized by a multiplicity of activities as revealed by zymogram analysis. In order to know if these polypeptides are the products of different xylanase genes or the result of post-traductional modifications of the products of a limited number of genes, we started upon the molecular characterization of the xylanolytic system. Different cDNAs designated xyn3 and xyn4 were isolated from an expression library of the anaerobic rumen fungus *N. frontalis*. Xyn3 was further characterized and was shown to contain a single open reading frame of 1821 bp coding for a protein XYN3 of Mr 66000. The predicted primary structure of XYN3 consisted of two large N-terminal reiterated regions of 223 amino acids with high homology (88.3%). Each domain of XYN3, XYN3A and XYN3B showed significant homology with fungal and bacterial xylanases belonging to endoxylanase family G. Xyn3 and xyn3A were cloned in a bacterial expression plasmid harbouring a 6His-C terminal tag and the recombinant proteins XYN3 and XYN3A were purified from *Escherichia coli.* The recombinant proteins had a Mr of 66800 and 34000 respectively and hydrolysed xylan to xylo-oligosaccharides. Analysis of truncated forms of XYN3 confirmed that the full length protein contained two catalytic domains which displayed similar substrate specificity. Western blot analysis using antiserum raised against XYN3 showed that the *N. frontalis* xylanase was not extensively glycosylated. XYN3 antiserum recognized similar polypeptides in the culture medium of two other rumen fungi *Piromyces rhizinflata* and *Caecomyces communism* Xylanases have potential industrial applications in the feed industry, food processing and paper industry. The availability of recombinant fungal xylanases offers a potentially attractive source of xylanase for industrial applications. Experiments are in progress to obtain expression of the *N. frontalis* xylanases in species of aerobic fungi that are more amenable to genetic manipulations.

Molecular Characterization of the Xylanolytic System from the Fungal Pathogen *Claviceps purpurea*

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Calviceps purpurea is a phytopathogenic fungus which is a parasite on grasses and cereals. Cell wall degrading enzymes probably play a role during colonization of *Secale cereals* ovary by *C pupurea.* As the primary cell walls of monocotyledonous plants consist of approximately 40% arabinoxylan xylan-degrading enzymes might be an important factor in pathogenicity of the fungus. In axenic culture C *purpurea* secretes at least two different xylanases of which one has been purified by ion exchange chromatography and isoelectric focusing.

Using the XYL I -gene from *Cochlioboliis carbonum* as a probe a xylanase encoding gene (XYL 1) was isolated from a genomic library of *C. purpurea* and subsequently sequenced. The XYL I -gene consists of 83 1 bp, the open reading frame is interrupted bv an 182 bp intron. The sequence exihibits great homology on both nucleic acid (up to 66%) and amino acid (up to 67%) level to other xylanases and belongs to family G xylanases according to the classification of Gilkes et al. (1991). Southern hybridizations indicate the presence of at least one more gene with homology to XYL I from *C. purpurea.* A further genomic region shows homology to *Magnaporthe grisea* xylanase gene Xyn33 belonging to family F xylanases.

The expression and regulation of XYL I in axenic culture and in planta is currently being examined by Northern and Western hybridization and RTPCR, respectively.

A disrupted copy of Xyl I was used to create a xylanase lacking mutant by transformation mediated gene replacement. Integration of the vector into the homologous genomic Xyl I region in the

chromosome was confirmed bv Southern analyses. Disruption of Xyl I results in reduced total xylanase activity. Western analyses using monoclonal antibodies raised against *Trichodernia reesei* Xylanase Xyn I as a probe show the disappearance of one out of three crossreacting bands wrih a MG of 30.000 Da strongly indicating that this is the XYL I gene product.
In order to elucidate the role of Xyl I for pathogenicity the Xyl I - Mutant is currently being examined in pathogenicity tests.
References: Gilkes, N.R., Henrissat, B., Kilbourn,D.G., Miller R. C. and Waffen R.A.J. (1991), Microbiol. Rev. 55, 303-315

Kinetics of Endo- and Exocellular Production of Glucose Oxidase by Recombinant *A. niger*

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The kinetics of endo- and exocellular production of glucose oxidase (GOD) by recombinant *Aspergillus niger* NRRL-3 (GOD3-18) were investigated using enzymatic activity measurements as well as gel electrophoresis techniques. Cells were grown in batch culture on glucose minimal medium supplemented with yeast extract. An improved method for cell disruption was developed that subsequently allowed to monitor quantitatively the time dependence of endocellular GOD activity and concentration during bioreactor cultivations. During fast growth in the first part of the cultivation, rapid endocellular accumulation of GOD up to 40% of soluble cell protein was observed followed by a slow release of the enzyme into the culture fluid. A second, weaker phase of GOD synthesis that did not cause a significant increase in endogenous enzyme activity occurred concomitantly with a second phase of slower growth. At the end of the cultivation, about 97% of total GOD recovered were detected in the culture supernatant. Two dimensional gel

electrophoresis analysis provided evidence that endo- and exocellular GOD are indistinguishable clearly demonstrating identical posttranslational modifications (e.g. signal sequence cleavage, glycosylation pattern). These results also show that the initial steps of the secretary pathway are fast and that the release of the enzyme into the culture fluid is most likely delayed due to cell-wall association.

Molecular Genetics of Xylanase Production in *Aspergillus nidulans*

Perez-Gonzalez, JA., MacCabe, AP., De Graaff, LH., Visser, J., Fernandes-Espinar, M.T. and Ramon, D.

Aspergillus nidulans produces at least three xylanases of molecular masses 22, 24 and 34 kDa (X22, X24 and X34). These enzymes have been purified and biochemically characterized. The genes *xlnA, xlnB* and *xlnC* encoding the three enzymes have been isolated and sequenced. *xlnA* and *xlnB* were cloned by cross-hybridisation with the *xlnB* gene of *A. tubingensis and* share a high degree of homology. Each ORF is interrupted by a single intron. Both genes have been overexpressed in *A. nidulans* multicopy transformants and their cDNAs expressed in yeast. *xlnC* which encodes X34 was cloned by heterologous hybridisation with an *A. kawachii xynA* gene probe,*xlnC* contains nine introns, one of which has atypical donor/acceptor sites. Overexpression *of xlnC* in *A.nidulans* multicopy transformants resulted in elevated levels Of X34 but no apparent overexpression of *xlnA* and *xlnB.*
Synthesis of the xylanases is induced by xylan and xylose and, with the exception Of X24, is subjected to carbon catabolite repression by glucose. Regulation of xylanase gene expression is currently being studied at the transcriptional level. Expression of *xlnA* and *xlnC* in wild type is i) repressed by glucose (2%), ii) commences approximately nine hours after
transfer to xylan medium and iii) is temporally advanced in the carbon catabolite repression mutant *creAd30. xlnA* and *xlnC*'

transcription remains repressed in the presence of 2% glucose in the *creAd30* strain.
Analysis of the sequences upstream of the xylanase genes has revealed the presence of both putative CREA and PACC binding sites. Nucleic acid protein binding assays and promoter deletion experiments are being performed in order to determine the functional significance of these motifs.
This work was supported by grants from DG XII of the European Commission (BIOTECH BIO2-CT93-0174) and the Spanish Government (CICYT AL193-0809).

A CCAAT-box Mediates Sophorose-induction of CBH2 (Cellobiohydrolase II- Encoding)-gene Expression in the Filamentous Fungus *Trichoderma reesei*

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The cellulose system of the filamentous fungus *Trichoderma reesei* consists of several cellobiohydrolases, endoglucanases and -glucosidases, encoded by separate genes, which are coordinately expressed in the presence of cellulose or the disacharide sophorose. In order to identify nucleotide-motifs involved in the induction of cellulases, we prepared protein extracts from induced and noninduced mycelia of *T. reesei,* and used them for electrophoretic mobility shift assavs (EMSA) with a 615-bp fragment of the *cbh2* promoter. This assay detected a specific protein-DNA complex present only in sophorose-induced mycelia. Using various overlapping fragments and competitive oligonucleotides, the DNA target motif for this protein complex was shown to be a CCAAT-box. CCAAT-motifs were also shown to be present in the 5'-noncoding region of other sophorose-inducible cellulase*(cbh1, egl1, egl2)* and xylanase *(xyn2)* genes. To study the *in vivo* role of the CCAAT-box

in cellulose induction, *T. reesei* strains were constructed, which carried a copy of the *cbh2* gene in which this CCAAT-motif was mutated to CCTTT. Sophorose did not induce *cbh2* transcription in these strains. Protein extracts were also prepared from a mutant strain of *T. reesei,* in which cellulose formation could not be induced by cellulose or sophorose, but which formed a complete cellulose system upon cultivation under carbon catabolite derepression. These protein extracts did not form the sophorose induced CCAAT binding protein complex. It is proposed that binding of a protein complex, of which at least one component is induced by sophorose, to a CCAAT-box mediates the triggering of expression of *cbh2* (and probably also that of the other cellulose genes) by sophorose in *T. reesei.*

Isolation and Characterization of a Novel Arabinofuranosidase from *Aspergillus niger*

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Arabinofuranosidases are enzymes that split off arabinose substituents from arabinose-containing hemicelluloses such as arabinoxylans, arabinans and arabinogalactans. We have purified and partially sequenced an arabinofuranosidase from the culture medium of *Aspergillus niger* grown on sugar beet pulp/wheat bran. The purified arabinofuranosidase has a molecular mass of 33,270 D $\pm$ 50 D, determined by laser desorption mass spectrometry and a pI of 3.7. The purified protein has been shown to specifically cleave arabinose side chains from arabinoxylans, branched arabinan and sugar beet pectin. This protein in combination with Xylanase A, synergistically breaks down the xylan backbone.

Degenerate oligonucleotides designed from the partial amino acid sequence were used to amplify a 90 bp genomic DNA fragment. The PCR product obtained was used to screen an *Aspergillus niger*

genomic library. A genomic clone was isolated, and the structural part of the gene encoded a 26 amino acids long hydrophobic signal sequence and a mature protein of 270 amino acids. The deduced polypeptide has 35% amino acid sequence identity to the catalytic domain of a 59 kDa Xylanase C from *Pseudomonas fluorescens subsp. cellulose.*

Purification and Characterization of a -galactosidase from *Schizophyllum commune*

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Schizophyllum commune secretes extracellular -glycosidases, which possess activity for the substrate o-nitrophenyl-galactoside. The enzyme with the highest activity was purified and characterized. Purification to apparent homogenety was achieved by affinity chromatogaphy, gel filtration and ion exchange chromatography.
A highly active -galactosidase was induced in the presence of lactose in the medium, lowest activity was observed when a medium with glucose as carbon source was used.
Denaturing gelelectrophoresis of this preparation showed a single band of an apparent molecular mass of about 66 kDa.
We intend to use the N-terminal sequence of the purified enzyme to identify the corresponding gene. This may be used as a homologous reporter gene to study gene expression in *Schizophyllum commune.*

Acetyl Xylan Esterase from *Trichoderma reesei* Contains an Active Site Serine and a Cellulose Binding Domain

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The hemicellulose gene, *axe*l, encoding acetyl xylan esterase, was isolated from an expression library of the filamentous fungus *Trichoderma reesei* using antibodies raised against the purified enzyme. Apparently *axe*I codes for the two forms, pI 7 and pI 6.8, of acetyl xylan esterase previously characterized. The *axeI* encodes 302 amino acids including a signal sequence and a putative propeptide. The catalytic domain has no amino acid similarity with the reported acetyl xylan esterases, but has a clear similarity especially in the active site with fungal cutinases, which are serine esterases. Similarly to serine esterases AXEI was inactivated with PMSF. At its C-terminus AXEI carries a cellulose binding domain (CBD) of fungal type, which is separated from the catalytic domain by a serine, glycine, threonine and proline-rich region. 'ne CBD can be separated from the catalytic domain by limited proteolysis without affecting the activity of the enzyme towards acetylated xylan, but abolishing its capability to bind cellulose.

Cloning of Hemicellulase Genes Encoding Alpha-galactosidase and Alpha-arabinofuranosidase Activities from *Trichoderma reesei* by Expression in Yeast

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In order to clone novel genes encoding hydrolytic enzymes without no need of pre-existing data, a cDNA expression library of *T. reesei* RutC-30 has been constructed in the yeast *S. cerevisiae.* We have previously reported isolation of new -glucanase genes with this strategy. Here we report the cloning of three different -galactosidase genes and two -L-arabinofuranosidase genes by screening the expression bank in the presence of the substrates pNP- -galactopyranoside and pNP- -arabinofuranoside, respectively. The -galactosidase genes, *agl1, agl2* and *agl3*, encode for 444, 746 and 624 amino acids respectively. AGLI and AGLIII show similarity

between them and with the -galactosidases of the family 27 of glycosyl hydrolases. AGLII shows high similarity with the -galactosidases of family 36. The -L-arabinofuranosidase genes, *abf*1 and *abf*2, encode for proteins of 500 and 758 amino acids, respectively. ABFI displays high similarity with the aarabinofuranosidase B (ABF B) of *A. niger.* ABFII shows similarity with the -glucosidases of family 3. The yeast produced enzymes were tested for enzymatic activity against different substrates. The three -galactosidases showed different specificities. AGLI was able to liberate galactose from small galactose containing saccharides and polymeric galacto(gluco)mannan. AGLII was almost inactive towards polymeric substrates. AGLIII was active against all substrates tested but at a very low extend compared with the other enzymes. AGLI acted synergistically with the -mannanase, and AGLII and AGLIII showed synergism with - mannanase and -mannosidase. The -arabinofuranosidase ABFI released .arabinose from pNP- -arabinofuranoside and arabinoxylan. ABFII did not acted on arabinoxylan and has both -arabinofuranosidase and -xylosidase activity, the second activity being higher.

Improved Production of *Trichoderma harzianum* Endochitinase in *T harzianum* and *T reesei.*

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The mycoparasite fungus *T harzianum* P1 has been used as a biocontrol agent against plant pathogenic fungi. The endochitinase secreted by *T. harzianum* has been suggested to play an important role in the biocontrol capacity, alone or acting in synergy with hydrolases produced by the fungus. In order to obtain improved biocontrol strains or enzyme preparates increased in endochitinase,

the chromosomal endochitinase gene *(ThEn-42)* of *T. harzianum* P1 was isolated and overexpressed in *T. harzianum* P1 and *T. reesei* RutC-30, respectively, under the control of the promoter of the major cellulose gene *cbh I* of *T. reesei.*
T. reesei RutC-30 did not produce any endogenous endochitinase activity. The prepro region of the *T. harzianum* endochitinase was correctly processed in *T. reesei.* On the other hand, no differences in expression were observed when the prepro region was replaced with the CBHI signal sequence. Shake flask cultivation yielded 130 mg/l of active enzyme which is about 20 fold increase to the endochitinase activity produced by the non transformed *T. harzianum.* The presence of multiple copies of the expression cassette in the transformant lead to limitation in transcription and/or regulation factors needed for full activity of the cbhl promoter, although this was not the major limiting factor for even higher production of endochitinase. The *T. reesei* strain RutC-30 appeared to be tolerant to the endochitinase and can be used as a production host for this enzyme.
The transformed strain of *T. harzianum* P1 cultivated in shake flask with cellulose inducing medium increased the production of extracellular endochitinase in 4 to 5 fold compared with the non-transformed *T. harziatium.* However, the extracellular activity towards colloidal chitin increased approximately 10 fold, most likely because of the synergistic action of the endochitinase with other chitinases produced by the fungus. The expression of the *T. reesei cbhl* promoter was regulated in *T. harzianum* in a similar manner than in *T. reesei.* The endogenous endochitinase gene of *T. harzianum* was expressed at a low basal level on glucose or sucrose containing media. No specific induction of the endogenous gene by chitin could be observed.

Regulation of Cellulase and Hemicellulase Genes *of Trichoderma reesei*

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The filamentous fungus *Trichoderma reesei* uses plant polysaccharides to survive in nature producing a great array of cellulases and hemicellulases that act synergistically to degrade complex substrates. A large number of the *T reesei* genes encoding cellulases and hemicellulases have been isolated recently. This allows expression of several genes to be compared simultaneously in the same conditions and possible coregulation mechanisms to be studied. Here we present a study on the expression patterns of three cellulose and ten hemicellulase genes of *T reesei.* The analysis was carried out at the level of mRNA produced by the fungus cultivated in media containing different complex plant polysaccharides, oligosaccharides or monosaccharides.

Phytases from Thermotolerant Fungi

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We screened a number of thermotolerant fungi for phytase production and cloned the complete phytase genes from 2 candidates *(Aspergillus fumigatus, Talaromyces thermophilus),* using degenerate oligonucleotides based on known phytase and histidine acid phosphatase sequences. Sequencing reveals that the presumed translation products are 465 and 466 aa, respectively, both containing a single short intron. Comparison of the sequences to each other and to published phytases show amino acid identity levels between 47-88%. The identity with known acid phosphatases are between 21-29%. Overexpression of these phytases in *Aspergilli* are under way and will facilitate the analysis of the enzymatic activities of both enzymes (e.g. pH activity profile with phytic acid and 4-nitophenylphosphate, temperature stability) as well as the testing of

their performance in "in vivo" feed trials.

Cloning and Characterisation of an -amylase gene from the thermophilic fungus *Thermomyces lanuginosus*

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Fungal -amylases have been used for many years in food industry, especially in starch and bakery industry. Fungal amylases have traditionally been fermented from *Aspergillus oryzae*. However this amylase is not very heat stable and have little if any activity during starch gelatinasation. Several thermophilic fungi, among these *T lanuginosus,* produces -amylases (1) with significant higher heat stability, unfortunately in rather low amounts.
In order to study the -amylase from a thermophilic fungus we have cloned and characterised the gene encoding the -amylase from *T lanuginosus* . A PCR fragment of the gene was isolated by designing primers specific to conserved regions of fungal -amylases. The cloned fragment was used to screen a genomic library of *T lanuginosus* and 5 independent clones were found. The clones contained an ORF encoding a 493 residues long protein with a putative signal sequence of 18 residues. The ORF was interrupted by eight introns. Two peptides of purified -amylase was sequenced which confirmed the isolated gene encodes the correct enzyme.
This ORF was expressed in *Aspergillus niger* resulting in high expression of -amylase in the transformants. The heterologous protein was purified and characterised biochemically, which showed that it was substantially identical to the native protein. In contrast to the heterologous protein the native protein was N-terminally blocked. The N-terminal sequence of the mature protein confirmed the putative identified signal sequence cleavage site.
The cloning and expression of this gene in *A. niger* have given a sufficient and reliable source of T *lanuginosus* -mylase for characterisation and application tests.

(1) Jensen, B. and Olsen J. (1992) Enzyme Microb. Technol. **14:** 112-116

Hyperhydrolytic Mutant of *Trichoderma harzianum* with Improved Antifungal Activity

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Strains of the filamentous fungus *Trichodermia harzianum have* been described as biological control agents against plant fungal pathogens. The cell wall degradation and further assimilation of phytopathogenic fungi have been proposed as a major mechanisms accounting for the antagonistic activity of the *Trichoderma* strains. It is therefore expected that strains with increased lytic activity will be more efficient biological control agents.
After nitrosoguanidine treatment of a mycoparasitic strain of *T. harzianum* mutant strains were isolated which produced halli of lysis in media with pustulan, a -1,6-glucan polymer, longer than that of the wild type. One of the mutant characterized, PFI, had higher levels of - 1,3-glucanase and chitinase, in addition to -1,6-glucanase, under both basal and induction conditions. Extracellular protein separation and deten-nination indicated each individual protein to be more abundant in PF1 than in the wild type, what seems to indicate that the mutant is hypersecretor. Preliminary results in experiments carried out *in vitro* in dual cultures with *Rhizoctonia solani* indicated that PFI possessed higher antifungal activity than the wild type. Finally, the mutant grows less than the wild type and sporulates sooner. Experiments in progress will allow us to establish whether this behavior reflects a higher sensitivity of the mutant to the lytic enzymes and/or a pleiotropic effect of the altered permeability, i. e. a higher energy consumption.

Isolation and Characterisation of Genes Encoding Cell Wall Degrading Enzymes of the Pathogenic Fungus *Botrytis cinerea*

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The pathogenic fungus *B. cinerea* causes major problems in diverse agricultural crops. At this moment control of *B. cinerea* infection is hampered for several reasons including high genetic variation and the various infection strategies employed by this organism. Fungal extracellular hydrolases presumably play an important role during infection by degrading the plant cell wall.
In order to develop an effective control strategy, the ftmgus is extensively studied, however *B. cinerea* is still poorly characterised at the molecular level. Therefore, we recently started a molecular genetic study in order to identify and characterise extracellular hydrolases of the fungus and to elucidate the roles the various enzymes play in the process of pathogenicity.
A genomic DNA library was constructed from *B. cinerea* strain SAS56. Via heterologous hybridisation, using DNA-probes derived from several *Aspergilli* species, genes coding for pectinolytic enzymes were isolated. Amino acid sequence alignments showed that the Bcpgal gene encodes a polygalacturonase, which is highly homologous to the PG genes of *Sclerotinia sclerotiorum.* Interestingly, as in *S. sclerotiorum* the ORF is not interrupted by introns. The Bcpel1 gene encodes a pectin lyase and shows significant homology with *pelA of A. niger.* Compared to the *A. niger pelA* gene, only one intron is conserved both in respect to position and consensus intron sequences, whereas the other three introns are absent in Bcpel1.
To manipulate the fungus genetically several methods have been modified and developed. Disruption of chromosomal copies of the Bcpga1 and Bcpel1 genes, using a haploid strain, derived from

SAS56, is currently in progress.
The effect of gene disruption on the level of pathogenicity will be tested in a well defined bioassay using tomato leaves. Gene expression, enzyme activities and the effect on the process of maceration will be determined. Obtained results and prospects will be discussed.

Organisation and Expression of Two Laccase Genes in the Cultivated Mushroom *Agaricus bisporus*

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Laccase encoding sequences from the cultivated mushroom Agaricits *bisporus* were first isolated as cDNA. Two non-allelic sequences were found, one of which was also sequenced from genomic DNA (Perry et al., 1993. J. *Gen. Microbiol.* **139,** pp 12091218). Cosmid clones containing laccase GDNA sequence all gave a comparable southern blot hybridisation pattern to total *A. bisporus* genomic DNA suggesting that the two genes were not segregating to independent clones. Restriction fragment mapping of cosmid clone LA16E2 was not completely unambiguous, but was consistent with both laccase genes residing within a few thousand base pairs of each other. PCR reactions gave a product of ca. 1.5 kb when specific primers were used directing synthesis (5' - 3) from the 3'-end of *lcc 1* and (3' - 5') from the 5'-end of *lcc2*. The 1.3 kb sequence between the 3'-end of *lcc1* and the 5'-end of *lcc2* has been analysed and will be presented, together with studies of laccase gene expression by northern blot and competitive RT-PCR assays.

Fermentation Studies on the Overproduction of Glucoamylase in *Aspergillus niger*

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Batch fermentation studies with a wild type *A. niger* and strain B36, derived from the wild type, containing multiple copies of the A. *niger* glucoamylase gene (*glaA*) were carried out in pH controlled fermentors with a working volume of 1.5 liters. In both strains the expression of the *glaA* genes is driven by the wild type glucoamylase promoter and glucoamylase production was induced with maltodextrin. For both strains, the production of glucoamylase was 2-3 times higher in the fermentor than the production in erlenmeyers. In addition, production in fermentors was much faster than in erlenmeyers. Strain B36 produced 5-10 times more enzyme than the wild type strain and there was low production of aspergillopepsin in the fermentor. The glucoamylase production in strain B36 was pH dependent. At pH 4 the glucoamylase production was higher than at pH 4.5 and pH 5.5 The addition of extra carbon source to the medium resulted in a higher biomass and a higher glucoamylase production. In this way, we were able to produce almost 4 g/l glucoamylase within 70 hours. Further studies will focus on fed-batch cultures with strain B36.

Cutinase of *Botrytis cinerea* Is Not Essential for Infection of Gerbera Flowers and Tomato Fruits

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Cutinase was proposed to play an early role in the infection of host tissues *by Botrytis cinerea.* To investigate this role, the enzyme was purified, characterized and amino acid sequences of the purified cutinase were used to design primers for PCR based gene cloning from strain SAS56; the protein sequence shows significant homology to known fungal cutinases.
Expression of the cutinase gene during the infection of gerbera flowers and tomato fruits was studied, using a cutinase promoter-GUS construct, transformed into *B. cinerea.* During germination and penetration of host tissue, fungal structures demonstrated high GUS activity, indicating that the cutinase 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 for penetration of host tissue by *B. cinerea,* cutinase deficient mutants were obtained by means of gene disruption. These mutants were tested on gerbera flowers and tomato fruits for their pathogenicity. The ability of these mutants to penetrate and cause infection was not altered. Microscopically, infection structures produced by the mutant did not differ morphologically from the wild type. Therefore we conclude that cutinase in *B. cinerea* is not an essential pathogenicity factor.

Novel Fungal Phytase Genes and Enzyme Activities
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We isolated and characterised the complete genes encoding novel

fungal phytases from *Aspergillus terreus and Myceliophthora thermophila* and PCR fragments of the phytase genes from 4 other fungi. The encoded proteins show 48-60% identity compared to the phytase of *Aspergillus niger* and have 21-29% identity when compared with known acid phosphatases. The phytases form a separate subclass of the histidine acid phosphatase family. Phytase genes of additional fungal species were identified by cross-hybridization experiments. Interestingly, no cross-hybridization with the *Aspergillus niger* phytase gene was seen. The phytases of *Aspergillus terreus and Myceliophthora thermophila* showed novel enzyme activity profiles and have a higher preference for phytic acid as substrate compared to 4-nitrophenyl phosphate, than the *A. niger* phytase. Further characterization of the genes and enzymes is underway.

The SC3 Hydrophobin Is among the Most Surface Active Molecules

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Hydrophobins are small secreted proteins involved in emergent growth of fungi. These proteins are characterized by the conserved presence of 8 cysteine residues and similar hydropathy plots. Although more than 20 hydrophobin genes have been cloned, SC3 of *Schizophyllum commune is* the only hydrophobin that has been partially characterized. SC3 self assembles at a hydrophilic/hydrophobic interface into an SDS-insoluble amphipathic protein membrane. Self-assembly of this hydrophobin is solely determined by the degree of hydrophobicity and not by other chemical or physical properties at the interface. When an aqueous

solution of SC3 is dried on a hydrophilic solid surface SC3 assembles at the water/air interface exposing its hydrophobic side (water contact angles 110) that is characterized by a mosaic of parallel rodlets. On the other hand, when a hydrophobic surface, e.g. Teflon, is incubated in an aqueous solution of SC3, the hydrophobin assembles at the water/Teflon interface making the Teflon wettable. The hydrophilic side of the SC3 membrane (water contact angles 48) appears smooth. The amphipathicity of assembled SC3 is accompanied by a different atomic composition at different sides of the protein membrane. Self-assembly of SC3 was also studied *in situ* by Axisymmetric Drop Shape Analysis by Profile (ADSAP). ADSAP allows simultaneous analysis of self-assembly of SC3 at the water/air and water/solid interface in a water droplet placed on a Teflon surface. The interfacial tension at the water/air interface decreased sharply from 70 to 28 mJ.m-1 at 35 ug.ml-1, which shows that SC3 is among the most surface-active molecules identified. This decrease was mainly caused by a conformational change and was not limited by difftision of SC3 monomer to the interface. At the water/Teflon interface, however, the contact angle did not decrease but increased from 110 (bare Teflon) to 122 (coated Teflon) after 30 min. of incubation. This contrasts to the low water contact angle measured after dipping Teflon in an aqueous solution of SC3. A model explaining this paradox will be presented.

Poster Abstracts, Free Topics

Hypervariability in Copy Number of rDNA in the White Button Mushroom *Agaricus bisporus*

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Pulsed field electrophoresis (CHEF) of intact chromosomes of the white button mushroom *Agaricus bisporus* revealed that chromosome IX can vary considerably in length. Length variations were observed during vegetative propagation in both parental nuclei of strain Horstreg. Ul. Hybridisation of CHEF-blots with a genomic

clone containing all the rRNA genes showed that these are located on chromosome IX. *Sal*l restriction, which leaves the rDNA unit intact, and a subsequent digestion of the *Sal*l fragment with *Bam*Hl, which cuts once in the rDNA unit, revealed that the rDNA units are organized as a head-to-tall cluster on chromosome IX. The length variation of chromosome IX was exclusively due to variation of the number of rDNA repeats in the cluster, i.e. when chromosome IX specific probes were used located outside the rDNA cluster, no variations in length were found.
The length of the rDNA cluster in one parental line varied between 220 to 360 kb and in the other between 430 and 860 kb. Assuming the length of the rDNA unit 9.2 kb, the copy number of rDNA repeats in the parental lines varied between 24 and 34 and between 47 and 93, respectively.
Often, more than two chromosomal bands were observed in fruit bodies and in vegetative clones of heterokaryons. Occasionally, a ladder of bands was found when *Sal* I digested chromosomal DNA was separated by CHEF and hybridized with the rDNA probe indicating the hypervariability of the number rDNA repeats in *A. bisporus.* Currently, influences of culture conditions are investigated on the number of rDNA repeats and the effect of this variation on strain behaviour.

Inheritance of Resistance to Streptomycin, Blasticidin and Dimethomorph in *Phytophthora infestans*

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Induced streptomycin- (Str,900 mkg/ml) and blasticidin- (Bl, 120 mkg/ml) resistant mutants were crossed on oatmeal agar. Fl progeny of this pair was found to be resistant to Str and Bl in concentration 900 mkg/ml and 60 mkg/ml, respectively. Dominant to Str and semi-dominant to Bl inheritance agree with Poedinok and Dyakov (1981) reported resistance at somatic recombination of drug-mutants.

Induced dimethomorph-resistant (Dm, 8 mkg/ml) mutant accumulated two step-mutations was crossed with compatible susceptible to Dm strain (lethal dosage 2 mkg/ml). Both of mutations occurring in diploid fungus are suggested to be dominant. 80 isolates of progeny were established with susceptibility and 2 with resistance to 8 and 6 mkg/ml. Segregation appears to be different from Mendelian inheritance pattern. Lethality of Dm-resistant zygotes and epistatic effect were assumed. Negative effect of Dm-resistant mutations on fitness of isolates was found.

The Relationship Between Conversion at the *AM* Locus in *Neurospora crassa* and Crossing over

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The incidence of crossing over between flanking markers during meiosis is enhanced when gene conversion is observed at an intervening locus. This is taken as evidence that gene conversion and crossing over are intimately associated. Estimates of the level of association between conversion and crossing over in Neurospora based on closely flanking genetic markers are similar to that for yeast (r ~ 0.33) (Perkins *et al* 1993). The Neurospora *am* locus is not extraordinary in this respect (r = 0.26).

We have used restriction site and sequence polymorphisms at the *am* locus to re-examine recombination events in Neurospora at a resolution higher than is possible using conventional genetic flanking markers. We find a much lower association of conversion and crossing over (r = 0.07). Only 7% of *am* conversions enjoy a crossover nearby.

The modal minimum conversion tract length is about 741 base pairs, similar to that observed in yeast. In 8 of 14 strains which had a crossover at *am,* this was remote from the conversion event. In the remaining 6 strains, no evidence of gene conversion was detected. The spectrum of recombination events at *am is* similar to that seen at

a number of loci in yeast, suggesting that *am* is not atypical, adding weight to the argument that the level of association between conversion and crossing over may have been generally overestimated. This suggests either that the two modes of recombination result from distinct mechanisms or that there is a mechanism that strongly biases resolution of a common intermediate, such as a Holiday junction, in favour of preserving the parental combination of flanking markers.
Perkins, D. D. *et al,* 1993 Genetics, **133:** 690-691.

Molecular Analysis of Meiotic Reconmination in the *Histidine-3* Region of Neurospora

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Meiotic recombination in *Neurospora crassa* is differentially regulated by at least three *trans* acting genes: *rec-1, rec-2* and *rec-3* each polymorphic in the wild collections used to establish the commonly used laboratory strains (reviewed by Catcheside 1986). In each case dominant alleles *(rec)* are known to reduce recombination in specific target regions on at least two chromosomes. Collectively, the three *rec* loci influence recombination in approximately one third of the genome sampled and cause about ten fold variation in recombination at their target sites. Recombination in the *his-3 region* is influenced both by *rec-2,* which affects crossing over between *his-3* and *ad-3* and recombination across the *his-3* gene, and by the *cis* acting element *cog*, which is also polymorphic amongst laboratory strains. The dominant allele *cog*+ permits higher recombination but events initiated by either allele are blocked by *rec-2*+. It is presumed that *cog* like recombinators with the relevant *rec* gene sensitivity exist within all DNA segments under *rec* control.
We have found that *cog* is within highly polymorphic DNA and have used the polytnorphisms to map *cog* to a region 2.3 - 3.2 kb 3' of *his-3* (Yeadon and Catcheside 1995). We have also found substantial polymorphism within the *his-3* coding region, providing markers for

determining the position of recombination events. We report here that although events stimulated by *cog* which lead to recombination between *his-3* alleles only rarely have termini within *cog*, there are regons where termination is common; that conversion tracts are discontinuous in some recombinants; and that the chromosome *cog*+ is preferentially the recipient of information unless *rec-2* + is present. Catcheside DEA (1 986) Genet Res Camb 47:157-165 Yeadon PJ & Catcheside DEA (1995) Current Genetics **28:** 155-163.

Isolation and characterisation of the gene encoding isocitrate lyase in *Coprinus cinereus*

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Like many other microorganisms, the basidiomycete fungus *Coprinus cinereus* can utilize acetate as sole carbon source for growth. Acetate is utilized via acetyl CoA through the action of the mobilising enzyme acetyl CoA synthetase. Acetyl CoA is metabolised via the anaplerotic glyoxylate pathway which replenishes the TCA cycle in the absence of glycolysis. The two key reactions of this cycle are catalysed by the enzymes isocitrate lyase and malate synthase which bypass the two decarboxylation steps of the TCA cycle.

The structural gene for isocitrate lyase, *acu-7,* was isolated from a genomic library by heterologous hybridization using a probe derived from the *Aspergillus nidulans* gene*, acuD* (Mellon *et al.* 1987). This gene, together with corresponding cDNA clones has been sequenced and the gene sequence and predicted protein sequence will be presented. The coding sequence is interrupted by three introns, two of which occupy conserved positions with respect to introns in the corresponding genes of *A*. *nidulans* and *Neurospora crassa.* The C. *cinereus* protein, unlike the protein from these other fungi, has a C-

terminal SKL tail which may play a role in import into the glyoxysome.
Progress with promoter analysis will be presented.
Mellon, F.M., Little, PFR. and Casselton, L.A. (1987) Mol. Gen. Genet. 210:352-357.

The Autonomously Replicating Plasmid pAB4-ARpl Transfers Between Nuclei in a Heterokaryon of *Aspergillus.*

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Genetic analysis of transformants carrying the autonomously replicating (AR) plasmid pAB4-ARpl were performed in *Aspergillus niger and A.nidulans.* The following results were obtained. Like in *A.nidulans,* diploid analyses showed that the AR plasmid segregates independently from chromosomal markers in *A.niger*. Heterokaryon analyses confirmed that the plasmid is nuclear rather than cytoplasmic in Aspergillus, however upon testing large numbers of conidia, transfer of the AR plasmid from one nuclear genotype to the other in the heterokaryon could be observed. The frequency of this transfer from plasmid bearing to naive nuclei in such heterokaryons is considerably higher for *A.niger* than for *A.nidulans.* Parasexual behaviour of these heterokaryons was normal. Upon transfer the plasmid seems to remain unchanged and can be further transferred to other nuclei in heterokaryons. Possible modes of transfer will be considered.

Effect of Medium Composition on Cell Growth and Extracellular Production of Glucose Oxidase by Recombinant *Aspergillus niger*

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The effect of medium composition on cell growth and extracellular production of glucose oxidase (GOD) by recombinant *A. niger* NRRL-3 (GOD3-18) was investigated. The recombinant strain carries multiple copies of the *god* gene with the -amylase-signal peptide from *A. oryzae* under the control of the *gpd*-promoter of *A. nidulans*.
The addition of yeast extract to a mineral salt medium containing glucose as carbon source enhanced cell growth and production of extracellular GOD. However, an increase of the yeast extract concentration above a critical value caused declining extracellular concentrations of GOD.
Studies on the influence of different carbon and nitrogen sources on cell growth and extracellular production of GOD revealed highest productivities using glucose, xylose or mannose as carbon and nitrate as nitrogen source. Again, an increase of the nitrate concentration above a critical value caused a decline in the extracellular production of GOD.

Breeding Systems in the Genus *Agaricus*

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The breeding systems of homobasidiomycetes are typically homothallic or heterothallic. In the genus *Agaricus,* which contains the principal cultivated species *A. bisporus,* clamp-connections are absent, mating reactions may not be evident and fruiting tests are not easily performed. Determining the breeding systems present in *Agaricus* spp. is therefore not straightforward. This study aims to use RAPD markers to clarify what breeding systems are present in a range of wild *Agaricus* species. In a heterothallic species, diversity would be expected amongst single spore progeny: in a truly

homothallic species uniformity would be expected.
To date five species have been studied, *Agaricus nivescens* and A. *bitorquis* which are demonstrably heterothallic, *A. subfloccusus* which is believed to be homothallic on the basis of fertility of single spore progeny and two other species whose breeding systems have not been classified. In *A. subfloccosus* the variation between parental strains and their single spore progeny was 2% based on 55 scorable markers with a size range from 340 to 3170 bp. In *A. nivescens* and *A. bitorquis* the percentage score fell between 18.2% (32 markers: 660 - 2230 bp) and *11.1%* (15 markers: 990-2210 bp) respectively. Of the two undefined species *A. silvicola* showed 4.3% variation (32 markers: 440-3620 bp) suggesting it is homothallic whilst *A. campestris* showed 18.9% variation (22 markers: 440 to 3120 bp). The study is being extended to other species and defined markers are being used to test for heterokaryon formation between single spore progeny. The use of RAPDs is clearly informative in determining the breeding system present in an unstudied species.

Population Genetical Aspects of the Incompatibility System of Higher Basidiomycetes

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In population genetics the kind of sexual reproduction is an important parameter. For instance, to the population of a bisexual species the Hardy-Weinberg law applies if panmixia exists. The situation is more complex in populations of higher basidiomycetes since many different incompatibility factors (IF) may be present and because some species are tetrapolar. For bipolar as well as tetrapolar species, we devised mathematical models which make possible to calculate the intrinsic changes of the IF frequencies in consecutive generations. Regardless of the original composition, all populations approach an equilibrium in which each IF has the frequency 1/N**,** where N is the number of rare IFs. Some instructive examples will be

presented. During the process of convergence, frequency dependent selection takes place in favor of the rare IFs. If in a bipolar species (N > 2), a deleterious gene (relative fitness < 1) is closely linked to an IF, the extinction of this gene is prevented if > (N - 2) / (N - 1).

Presence of Introns in Nuclear 18s rRNA-genes of Ascomycetal Black Yeasts

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Fungi belonging to genera of so called “black yeasts” with ascomyccetal affiliation are of medical interest because of their association to variable severe phaeomyphomycoses in human and animals. The pleomorphism exhibited bv these fungi impedes the identification of such clinical isolates. In order to establish a reliable method for differentiation utilizing a molecular approach we sequenced the nuclear 18S rRNA-genes of 36 well characterized strains of black yeasts so far.

Within the 18S rRNA-genes of the following fifteen species we found introns ranging in size from 377 to 475 bp: *Exophiala “bergeri"* CBS 353.52, *Exophiala “gougerotii"* CBS 526.76, *Exophiala jeanselmei v. heteromorpha* CBS 232.33(3) , *Exophiala “mesophila ", Exophiala moniliae* CBS 520.76, *Nadsoniella nigra* CBS 535.94,*Nadsoniella nigra hesuelica* CBS 546.82, *Phaeoannellomyces elegans* UTMB 1286, *Pullularia prototropha* CBS 534.94 (3), *Rhinocladiella aquaspersa* CBS 313.73 (3), *Exophiala dermatitidis* CBS 207.34 (2), and four allied synanamorphs: *Exophiala “ schawii*” CBS 292.49 (2), *Wangiella dermatitidis* ATCC 34 100 (2), *“Phaeotheka" dermatitidis* KU A-0052 (2). *Sarcinomyces phaeomuriformis* CBS 131.88 (2). Some 18S rRNA-genes were shown to habor two or three different introns (see the numbers in brackets) simultaneously.

A structural analysis of three of these introns *(E. dermatitidis* and

Phaeoannellomyces elegans) indicated that they could be classified as group I introns. A distinctive feature of group I introns is their ability for autocatalytic splicing. For the two introns of *Exophiala dermatitidis* this autocatalytic splicing could be experimentally demonstrated using a novel non-radioactive assay (E-PCR). Phylogenetic analysis using 18s rRNA-gene sequences revealed that all these intron-haboring dematious hyphomycetes could be placed among species of the Herpotrichiellaceae.
Our-findings parallel observations of other groups that introns can occur in some nuclear 18S rRNA-genes of higher fungi as a consequence of their genetic mobility. The potential presence of such introns in fungal nuclear 18S rRNA-genes should be observed when applying techniques like ribotyping for (epidemic) identification of fungal pathogens.

Detailed Map of Mitochondrial Genom of *Aspergillus carbonaris*

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Aspergillus carbonarius (Thom) is possibly the most distinct member of the black *Aspergilli* exhibiting some unique characters. Studying the molecular polymorphism of this species among thirteen examined strains two main mitochondrial DNA (mtDNA) groups could be distinguished (designated type 1 and type 2 respectively). These groups correspond to their nuclear rDNA types. The mtDNA type 1 could be divided into two subgroups (labelled type la and type 1b respectively) based on slight alterations of their RFLP patterns. Despite of the significant similarities of their restriction patterns considerable differences were estimated between the genome sizes of two subtypes (51-52 kb, 60-61 kb respectively). For interpretation of these differences between two subgroups an attempt was made to construct their more detailed restriction maps. Isolated mtDNA was digested by *Eco*RI and the fragments were separated by

electrophoresis and cloned into pbluescript KS plasmid. The cloned fragments were digested by several restriction enzymes, such as *Eco*RV; *Pst*I; *Hind*III; *Hinc*II; *Xho*I; *Kpn*I; *Xba*I; *Bam*HI;*Hae*III, *Pvu*II. On the basis of these data linearized physical maps of both subtypes were developed. Digoxigenin-labelled hybridisation probes were applied for identifying the possible positions of certain regions.

Phylogenetic Analysis of the Genus *Aspergillus* Based on RFLP of Repetitive and Single Copy Sequences.

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Species of Aspergillus belong to the first fungal organisms that were cultivated on artificial media and studied for biochemical properties, they are also among the most common fungi in man's environment. Classical methods for systematic studies of this genus have been very successful and have provided a relatively good classification. However, morphological characters used for these studies probably do not reflect the real phylogenetic and evolutionary relationships between the taxa.
In our work to determine the relationship among several species of the genus (39 strains representing 5 subspecies and 12 sections) we used restriction fragment length polymorphism (RFLP) of the unit coding three classes of rRNA - 5.8S, 18S) 26S.
Using a plasmid containing the cloned rRNA unit from *A.nidulans* as a probe we found this method to be useful on the level of section. For more detailed analysis of strains representing section *Nigri* (22 strains) we additionally used RFLP analysis for a single copy gene - the gene coding glucoamylase from *A.awamori.*

Recombination of Mitochondrial DNA Following Directed Mitochondrial Transmission among Black *Aspergilli*

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Strains of the *Aspergillus niger* aggregate can be divided to three main groups based on their *Hae*llI-*Bgl*ll digested mtDNA patterns. The first and second groups correspond to *A. niger* and *A. tubingensis species* respectively, while the third type is represented only by some wild-type isolates derived from Brazil (mtDNA types 1, 2 and 3 correspond to rDNA groups I, II, and III, respectively). The mtDNA types I and 2 consist of several subgroups (labelled la - le, and 2a - 2f, respectively) [1]. Successful mitochondrial transfers were performed by protoplast fusion. Recombination of mtDNA occurred among strains representing different mtDNA groups or subgroups. All these strains showed full incompatibility in respect of nuclear complementation. Transfer experiments were carried out under selection pressure using a mitochondrial oligomycin resistant (oliR) mutant as a donor, which exhibits type la mtDNA and type I rDNA. Mitochondrial oliR progenies could be recovered following protoplast fusion in the presence of oligomycin by selecting for the nuclear phenotypes of the oliS recipient strains. The transfers were successful in all possible combinations. Within the group of strains belonging to mtDNA type I the transfers resulted in a single type of recombinant RFLP profile in each subgroup. The recombination events were more complex when the transfer of oliR occurred between strains representing different species. A great variety of recombined RFLP profiles of the partners appeared.
In mtDNA transfers la 2b and la 2d, resistant clones harbouring possibly unchanged donor mitochondria with the nuclear background of the recipient strain were also recovered in addition to the

recombinant types.
1. Varga, J. et al. (1994) Can. J. Mcrobiol. 40: 612-621

Selection of ***mutS*** **Homologs of** ***Aspergillus nidulans***

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Recent findings linking the *mutS* homolog *(MSH2)* to lymphoid and colon cancer in mice and humans respectively (Reitmair *et al.,* 1995, and Fishel, *et al.,* 1994), has renewed interest in the effects of mismatch repair genes in eukaryotic cells.
The bacterial *mutS* gene functions in the recognition and binding of a mismatched base pair (New *et al.,* 1993). *Saccharomyces cerevisiae* has been found to encode 4 proteins (MSH 1 -4) which show strong amino acid similarity to *mutS (Alani, et al.,* 1995).
The aim of this work is to isolate *mutS* homologs of *Aspergillus nidulans* by homology to genes from related species including *Neurospora crassa* and *S. cerevisiae* and to investigate the effect of knockout mutations on the polarity of gene conversion found to exist between the *niiA* and *niaD* genes of *Aspergillus nidulans* (Thijs*, et al.,* 1995).
Considerable sequence homology (up to 78%) exists in the 3' regions of the *mutS* genes of various bacterial and eukaryotic species. Selection of *mutS* homologs of *A. nidulans* was based on the construction of degenerate primers from these regions of high homology. Primers were used with both *A. nidulans* genomic DNA and a cDNA library. Selected PCR products were partially sequenced and used as probes against a cDNA library for selection of a complete copy of the *mutS* homologs.

Genomic Comparison among Wild-type and Mutant Strains of *Phaffia rhodozyma*

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P. rhodozyma is the onlv known yeast that produces the pigment astaxanthin (3,3'-dihydroxy- , -carotene-4,4'-dione) as its principal carotenoid. Though this can be manufactured svnthetically, the demand for the available natural sources has substantially increased. Eighty mutants obtained from two *P. rhodozyma* strains (ATCC 24203 and ATCC 24229) by a recently described method (1) were investigated. Protoplast fusion experiments were carried out between mutants with auxotrophic and morphologic (colour) markers and the resulting fusion products were isolated. CHEF separation of the chromosomal size DNAs (2) revealed several new chromosomal patterns after mutagen treatment. Analysis of chromosomal rearrangements proved useful for a more exact estimation of chromosome number and genome size. No correlation was found between a given type of chromosomal aberration and a phenotypic character. When hybrid strains resulting from protoplast fusion were analysed- both the disappearance of certain bands and the appearance of new bands were detected.

(1)Zs.Palagyi, A. Nagy, Cs. Vagvolgyi and L. Ferenczy (1995) Biotechnol. Tech. 9, 401-402.
(2)A. Nagy, N. Garamszegi, Cs. Vagvolgyi and L. Ferenczy (1994) FEMS Microbiol. Lett. 123, 315-318.

Genetic Inactivation of Mitochondrial Acyl Carrier Protein in *N. crassa* and *S. cerevisiae*

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The nuclear genes encoding the mitochondrial acyl carrier protein *(acp-1, ACPI)* were disrupted in *Neurospora crassa* and *Saccharomyces cerevisiae.* In *N. crassa,* the acp-I is a peripheral subunit of the respiratory NADH:ubiquinone oxidoreductase (complex 1). *S. cerevisiae* lacks complex I and its ACPI appears to be located in the mitochondrial matrix. The loss of the acp- I in *N. crassa* causes two biochemical lesions. First, the peripheral part of complex I is not assembled, and the assembly of the membrane part is disturbed. The respiratory ubiquinol:cytochrome c oxidoreductase (complex III) and cytochrome c oxidase (complex IV), however, are made in normal amounts. Second, the lysophospholipid content of mitochondrial membranes is increased fourfold. In *S. cerevisiae,* the loss of the ACP I leads to a pleiotropic respiratory-deficient phenotype
The phenotype of the *acp-1* deficient mutant of *N. crassa* was restored by complementation with the *acp-1* gene of *Aspergillus niger.* This was accomplished by the use of the heterologous selection marker *bar* providing resistance against the herbicide BASTAreg. (phosphinotricin).

Studies on the Function and the Regulation of the *AOX* Gene from *Penicillium chrysogenum*

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The specifically regulated *aox* gene, dependent on the physiological state and on external pH conditions shows strong homology to alcohol oxidases of methylotrophic yeasts.
In order to investigate the functionality and the regulation of the *aox* promoter we fused a 0.8kb promoter fragment to the *E.coli*

glucuronidase gene *(uidA)* as the reporter gene. A transformant containing one single copy integrated at the homologous marker gene was used for further studies. -glucuronidase expression was examined on plates under conditions of different carbon sources adjusted at different pH values.
To analyse the function of the *aox* gene we overexpressed this gene in *E.coli* as well as in *P. chrysogenum.* On the other hand we constructed plasmids to inactivate the *aox* gene by targeted gene disruption. We are currently analysing the respective transformants.

Using Monospore Pellets in Genetic and Population Analysis of *Pleurotus ostratus*

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We report here the novel method of producing monospore mycelial pellets from the spore prints of *Pleurotus ostreatus* fruit bodies which is less time consuming and useful for genetic analysis. The culturing conditions under which each basidiospore gives rise to an individual haploid pellet are described. A 20-30 offspring pellets can be obtained from one basidiome (dikaryotic parent) in a flask. The resultant monospore progeny can then be tested for genetic polymorphism(s) using isozyme protein electrophoresis and RAPD technique, a mycelium quantity of each pellet (a basidiospore derived monokaryon) being enough for the analysis.
P. ostreatus presents an exellent model organism because of well-established haplo-diploid life cycle and the following advantages:
- dikaryotic and homokaryotic clones can be reproduced on artificial media perrmtting analysis of their mating relationships; - mating criteria are easily testable in *Pleurotus;*
- along with multiallelic mating compatibility system the fungus has genetically controled somatic incompatibility system which delimits individual clones.

Spore prints of different origins are presently being screened. Data on clonal and population structure, equation between outbreeding and inbreeding processes in *P. ostreatus* is expected to be obtained. The results will be discussed in relation to the population ecology and reproductive strategies in *Pleurotus.*

Mitochondrial DNA Recombination among Compatible Strains of the *Aspergillus niger* Aggregate Without Using Selection Pressure

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Heterokaryon formation is a rare event between individual isolates of asexual black *Aspergillus* species *(*section *Nigri),* even if they derived from the same geographical area. The reason for this extreme heterokaryon incompatibility is still not understood. Heterokaryosis might occur very rarely only between those strains which harbour the same molecular markers, but never occur between isolates bearing polymorphic molecular characters, even if they represent the same species. The lack of genetic or molecular markers also hampered the examination of mtDNA inheritance even between compatible strains. When oligomycin resistant mitochondria were transmitted into incompatible sensitive partners (la 2b and la 2d), two resistant clones were isolated bearing possibly unchanged donor mitochondria as regards their mtDNA RFLP patterns (mtDNA type la) with the nuclear background of the oliS recipient partner (rDNA type II, and other phenotypic characters). The resistant strains harbouring either recombinant or substituted mtDNAs were crossed with a compatible sensitive parent of isogenic origin by anastomosis. Oligomycin was not applied for selection during heterokaryon formation. Heterokaryons possessing mixed mitochondrial populations showed different growth rates. After prolonged cultivation, conidia of the heterokaryons were harvested. Both parental nucleotypes could be

recovered from the progeny. An overwhelming majority of the progeny harbours recombined mitochondria. This result suggests that mitochondrial recombination may occur without any selection pressure. This model is a useful tool for studying mitochondrial inheritance and the mechanism of mtDNA recombination following parasexual processes. These types of recombined mtDNA RFLP profiles will be compared with those isolated following strong selection procedure.

Mitochondrial DNA Inheritance in *Aspergillus nidulans-A. tertazonus Hybrids*

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Interspecific hybrids were produced between an *Aspergillus nidulans* ‘master strain', and an A. *tertazonus* auxotrophic mutant by protoplast fusion. The haploid segregants obtained after treating the allodiploid hybrids with haploidizing agents like benomyl were examined for their niitochondrial DNA (mtDNA) restriction patterns. Without selecting for any of the parental mtDNA genomes, some of the progeny harboured recombinant mtDNAs, while some others carried the mtDNA of the *A*. *nidulans* parent. The observed recombinant mtDNAs were different from those obtained by selecting for the mitochondrial oligomycin resistance marker of the *A. tertazonus* parent. Only the mtDNA patterns of the *A*. *nidulans* parent were observed in the progeny when selection was carried out for the mitochondrial oligomycin resistance marker of *A*. *nidulans.* Simple physical maps of the two different recombinant mtDNAs were constructed and compared to the parental mitochondrial genomes. The recombinant mtDNAs differed from the parental mtDNAs in the presence of a new *Hae*III fragment, which indicates

that the recombination event took place in the *coxl* gene. The two recombinant mtDNAs possibly differ from each other in the presence or absence of an intronic sequence in the *cog* gene.

Studies on the Electron-pathway of *Neurospora crassa* Complex I Using Site-directed Mutagenesis

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The NADH:ubiquinone oxidoreductase (complex 1) is the most complicated enzyme of the respiratory chain. The eucaryotic complex I couples the transfer of two electrons from NADH to ubiquinone with the translocation of four protons across the inner mitochondrial membrane. In the ascomycete *Neurospora crassa* complex I consists of some 35 subunits with one FMN and four EPR detectable iron-sulphur clusters serving as prosthetic groups.
The 78 kDa subunit possesses two binding motifs for FeS clusters. For further understanding of the electron-pathway we inactivated the gene NUO 78 by homologous recombination with an in vitro deleted copy of the gene. We used the heterologous hph-gene for a dominant selection marker.
Moreover, we use site-directed mutagenesis for specific analysis of FeS clusters by exchanging distinct amino acids of putative binding motifs. Using the bar-gene, which confers resistance to the herbicide Basta, as another heterologous dominant marker, the deletion mutant nuo 78 can be transformed with the point-mutated copy of the gene NUO 78. FeS clusters with changed characteristics can subsequently be identified by EPR spectroscopy.

Detection of Endoparasitic Fungi in Nematodes

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Many nematode species are parasites of plants and animale, including economically important crops and livestock. Nematodes, in their turn, can be parasitised by several parasitic fungi, the so-called nematophagous fungi. These fungi are used for development of control methods for nematode pests in plants and animals. Endoparasitic, nematophagous fungi develop their hyphal system within thier host where they spend their entire vegetative lives; only spore bearing structures will be produced outside the body of the nematodes.
The purpose of this work was to investigate the possibility to use molecular techniques to detect and identify endoparasitic fungi inside single infected nematodes. PCR was used to amplify the ITS (internal transcribed spacer) region of the rDNA. The amplification product was cut with different restriction enzymes to produce RFLP patterns.
Six different species of endoparasitic fungi, that were possible to identify in axenic culture by the PCR/RFLP method were used to infect nematodes. The amplification products from the infected nematodes were represented by two bands: one band resulted from the amplification of the nematode ITS-region and the other band from the ITS of the fungi. After restriction analysis it was possible to identify three out of six fungal species, within the infected host.

Ectomycorrhizal Community Diversity in Forests with Different Nitrogen Deposition, Measured by PCR/RFLPS from Single Mycorrhizas

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Circumstantial evidence suggests that functional progerties of

ectomycorrhiazal communities can be influenced by antrhropogenic stress. To be able to study how, we need information about changes in themycorrhizal community at the level of individual mycorrhizal roots, since those are the organs for nutrient exchange. Ideally such studies should be followed by directed physiological laboratory studies on the species identified to be of interest. We have used PCR/RFLP methods to analyse the ITS-region of the ribosomal DNA of the mycobionts forming mycorrhizas on spruce at two sites with different nitrogen deposition. The data revealed a total of sixeen different ITS types in both forests, and three of thosewere found at both sites. In the high N-deposition site a total of eight ITS-types were found wheras the low N-deposition site had fifteen different ITS types. Identification of the ITS- types by comparison with those of identified fruitbodies from the same regrion is in progress. Our data show a higher ectomycorrhizal diversity in the non-polluted site. Inter and intra specific variation in the ability to utilise organic-N sources is now being examined in the mycobionts from both sites to determine whether changes in community structure can be explained in terms of differences in nitrogen deposition.

Development of an Easy and Rapid Method to Screen Transformants Directly by PCR

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A method has been developed for screening large numbers of transformants of different filamentous fungi by PCR. Colonies were grown in 96 well microtiter plates. Protoplasts were generated in these wells by NOVOZYM234 treatment and thewe were heated to 95 C. The resulting solution sould be immediately be used for PCR. Different PCR enzymes were tested for their performance on this template. PCR fragments sized up to 6 kb could be reproducibly

detected,
In an application *Beauveria bassiana* transformants were screened for a deletion in a gene docing for a cytochrome P450 enzyme, for which no phenotype was available. In a screen of 250 colonies 5 transformants with a deletion genotypoe were detected using this method.

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I apologise for the lack of formatting in this file.
If there are any confusions, please contact the FGSC
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