Functional genomics of the filamen tous fungi: The path from yeast

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Abstract not available at time of print.

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FUNGAL (A)VIRULENCE GENES; EVOLUTION AT A MICRO-SCALE.

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The gene-for-gene hypothesis has been introduced by Flor in the early forties of last century and it was only in the last decade that some gene pairs have been isolated and characterized. The intrinsic functions of avirulence (*Avr*) genes have been unknown for a long time. In recent years it has become clear that most *Avr* genes are actually *Vir* genes in the absence of a corresponding resistance (*R)* gene. The properties and functions of a number of fungal *Avr* genes will be discussed in my talk that, however, focuses on the *Avr* genes of *Cladosporium fulvum.* So far, we have cloned four *Avr* and four *Ecp* genes that all encode small cysteine-rich peptides secreted by *C. fulvum* during penetration of tomato leaves. Recognition of the *Avr* and *Ecp* gene-encoded proteins is mediated by *Cf* resistance gene products and leads to a hypersensitive response (HR). *C. fulvum* can avoid recognition and subsequent induction of HR by various mechanisms: the *Avr* gene (i) is absent (*Avr9; Avr4E*), (ii) contains point mutations in the ORF leading to protease-sensitive elicitors or a frame shift mutation leading to truncated non-active elicitors (*Avr2, Avr4*), (iii) contains point mutations in the ORF leading to production of stable non-active elicitors (*Avr4E*) or (iv) contains transposon insertions leading to lack of Avr protein production (*Avr2*). We are also beginning to detect enormous variation among the *Cf* genes in wild tomato populations. The biochemical basis of the gene-for-gene system implies that an *Avr* gene product directly interacts with the *Cf* gene product, but this could not be proven so far. Direct interaction between Avr proteins and R proteins is rather an exception than the rule. *Ecp* genes are present in all strains of *C. fulvum* and encode active elicitors that can all be recognised by some accessions of wild tomato species. Although all *Avr* and *Ecp* genes are supposed to represent virulence functions, deletion of single genes did not have significant effects on fungal growth in susceptible tomato cultivars. Probably the Avrs and Ecps have only a minor contribution to virulence. Presently, we try to simultaneously knock down several of these genes by RNAi. For two Avr proteins we have indications for their biological functions. The Avr4 elicitor was proven to be a chitin-binding protein that can protect fungi against basic plant chitinases. Avr4 proteins encoded by virulent alleles in strains of *C. fulvum* are no longer recognised by *Cf-4* plants, but still bind to chitin, suggesting that chitin-binding by Avr4 could represent a defensive virulence function. Avr2 is a cysteine protease inhibitor. For recognition of the Avr2 elicitor, in addition to Cf-2, the tomato Rcr3 cysteine protease is required. Rcr3 can be inhibited by Avr2. Although we envisage that, like for Avr proteins of other pathogens, Avr and Ecp proteins most probably bind to virulence targets in tomato plants that are guarded by the Cf proteins, it is not clear whether Rcr3 represents such a target.

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Aspergillus Fumigatus - infection and allergenicity

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Abstract not available at time of print.

Functional genomics of plant infection by the rice blast fungus *Magnaporthe grisea.*

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The rice blast fungus *Magnaporthe grisea* causes one of the most serious diseases of cultivated rice, and understanding the early events of the infection is of paramount importance if durable control measures are to be developed. *M. grisea* forms a specialised infection structure called an appressorium which is used to penetrate the tough outer cuticle of rice leaves, allowing the fungus entry to the underlying tissues (Talbot, 2003). Appressoria are melanin-pigmented, dome shaped cells, which form in response to the hydrophobic leaf surface and generate massive turgor pressure. Turgor is translated into mechanical force and a narrow penetration hypha is formed at the base of the appressorium, puncturing the cuticle We are using a multidisciplinary approach, involving gene functional analysis, cell biology and analytical biochemistry, to investigate the biology of appressorium-mediated plant infection.
Appressorium turgor is generated by accumulation of a compatible solute within the appressoria to very high concentrations. *M. grisea* appressoria accumulate glycerol as a major compatible solute during appressorial turgor generation, and understanding the mechanisms by which glycerol is synthesised within appressoria and how this process is genetically regulated is one of the primary aims of our research. Appressoria of *Magnaporthe grisea* form in dew drops on the surface of rice leaves in the absence of exogenous nutrients. Therefore, glycerol is synthesised from precursors that are present within un-germinated spores of the fungus. We have been examining the role of trehalose, glycogen and lipids as sources for glycerol biosynthesis and progress in the analysis of glycerol biosynthetic pathways and their genetic regulation will be presented.

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SIGNALLING AND REGULATION OF FUNGAL SECONDARY METABOLISM WITH RESPECT TO FUNGAL ß-LACTAM ANTIBIOTICS

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Fungi produce numerous of secondary metabolites. Some of these compounds are used as antibiotics such as the ß-lactam antibiotics penicillin and cephalosporin. Recent research has aimed at elucidating the molecular regulation of the biosynthesis of secondary metabolites. The identification of regulatory genes will help to elucidate both the physiological meaning of these compounds for the producing fungus and the extra- and intracellular signals controlling the biosyntheses of secondary metabolites. For the ß-lactam antibiotics it turned out that transcription of biosynthesis genes is subject to sophisticated control by nutritional factors (e. g. glucose, nitrogen), amino acids such as lysine and methionine, and ambient pH. Some regulators have been identified such as the pH regulatory protein PACC, the transcriptional complex AnCF, the bHLH transcription factor AnBH1 and the ring finger protein CPCR1. The availability of regulators facilitates to elucidate to which regulatory circuits ß-lactam biosynthesis genes belong and in particular the signals leading to their expression.

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Protein Kinase A Regulation of Secondary Metabolism is Mediated by the Transcriptional Regulators LaeA and AflR.

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Sterigmatocystin (ST) is a carcinogenic polyketide produced by several Aspergilli including *Aspergillus nidulans*. Expression of ST biosynthetic genes (*stc* genes) requires activity of a Zn(II)2Cys6 transcription factor, AflR. *aflR* is transcriptionally and post-transcriptionally regulated by a protein kinase A (PkaA) signaling pathway. Mutation of three PkaA phosphorylation sites in AflR allows resumption of *stc* expression in an overexpression *pkaA* background and demonstrates negative regulation of AflR activity by phosphorylation. PkaA transcriptional control of *aflR* is mediated via LaeA, a conserved nuclear methyltransferase found in all Aspergilli examined to date. *laeA* expression is negatively regulated by AflR in a unique feedback loop as well as by PkaA and RasA. Deletion of *laeA (ΔlaeA*) blocks expression of metabolic gene clusters including ST, penicillin (antibiotic) and lovastatin (antihypercholesterolemic agent) gene clusters in *A. nidulans* and blocks gliotoxin production in *A. fumigatus.* Both location and copy number of *aflR* is important in LaeA regulation of the ST cluster. LaeA mutants may be potent tools in identifying secondary metabolite gene clusters.

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THE ER STRESS RESPONSE IN A. NIGER; DIFFERENCES AND SIMILARITIES WITH YEAST AND MAMALIANS CELLS.

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The endoplasmic reticulum (ER) serves as a folding compartment for membrane and secretory proteins. In the oxidizing environment of the ER, protein folding is assisted by numerous protein chaperones and foldases, and a variety of quality control mechanisms ensures that only correctly folded proteins are released. A variety of physiological or environmental stress conditions can disturb protein folding and lead to the accumulation of unfolded proteins in the ER. Cells respond to this accumulation of unfolded proteins in the ER by increasing the transcription of genes encoding ER-resident chaperones and foldases, thereby increasing the folding capacity of the compartment. The responsible signalling pathway is known as the unfolded protein response (UPR). Genome-wide analysis in yeast, using DNA micro arrays, linked a large number of genes to the UPR, with functions far beyond ER chaperones (Travers et al. 2000). By affecting virtually every stage of the secretory pathway, the UPR appears to be a versatile regulatory system aiming at maintaining the homeostasis of ER functions under stress conditions.
Two key players in the UPR are HACA, a basic leucine zipper (bZIP)-type transcription factor, and IREA, a kinase/RNAse which resides in the ER membrane and senses the accumulation of unfolded proteins. Analysis of the UPR in A. niger revealed similarities with the yeast and mammalian UPR, but interestingly also some striking differences.

PROTEIN PRODUCTION IN FILAMENTOUS FUNGI: CELLULAR RESPONSES AND STRAIN IMPROVEMENT

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In recent years our understanding of cellular responses in *Trichoderma* and *Aspergillus* towards secretion load has increased significantly. Production of large amounts of native proteins or production of difficult foreign proteins provokes secretion stress that is believed in particular to be caused by folding limitations within the ER. Impairnment of folding causes the so called unfolded protein response (UPR), which is mediated by the regulatory protein HAC that causes modification of expression of a number of genes involved in various cellular processes related to protein secretion. In addition to the classical UPR, also other cell responses have been discovered using genome-wide approaches. Furthermore, a novel mechanism (RESS) has been identified that down-regulates expression of genes encoding native secreted proteins such as cellulases and glycoamylase under secretion stress conditions. These results show that the function of the secretory pathway is interlinked with other cellular processes, and that several feed-back mechanisms may exist that serve to balance between expression, folding, glycosylation and secretion of proteins. It is possible to alter these cellular mechanisms and create improved production strains. For instance, overexpression of HACA increases production of calf chymosin and laccase in *A.niger.*
The EC-funded Eurofung consortium has studied protein secretion in filamentous fungi and some of the results obtained will also be presented.

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Aspergilli – The ultimate cell factory for production of chemicals

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Aspergilli are widely used as cell factory in many different industries, i.e. in the food industry (citric acid, food grade enzymes), the chemical industry (detergent enzymes) and the pharamaceutical industry (statins). Many species of *Aspergilli* fulfill the requirements for being efficient cell factories, namely the possibility to produce metabolites at high rates, the ability to efficiently secrete many different enzymes, the ability to sustain low pH, and the ability to utilize complex substrates. Particularly the last two abilities make many *Aspergilli*, e.g. *Aspergillus niger*, particularly suited for industrial production of bulk chemicals, where it is important to be able to use cheap raw materials based on biomass. The natural chemical diversity expressed by many *Aspergilli* do, however, also make them well suited as hosts for industrial production of complex natural products. At Center for Microbial Biotechnology we are developing a chemical production platform based on *A. niger*. The platform will be used to produce both low value added products like succinate and high value added products like polyketides. In order to develop an efficient cell factory platform it is necessary to have a number of tools implemented, and this involves efficient gene targeting techniques, tools for genome wide transcription analysis and tools for quantitative analysis of the cellular metabolism. As a genomic sequence is not public available for *A. niger*, many of the tools are developed using *A. nidulans* as a model organism. In the presentation the different tools will be presented together with the vision for the use of *A. niger* as the ultimate cell factory for chemical production.

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THE GENETIC BASIS OF ADAPTATION: NEUROSPORA DISCRETA

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Adaptation to novel environments is easily inferred by comparisons between species, for example, Darwin's finches. Characterizing adaptation within a species and its underlying genetic basis, perhaps as a prelude to or simultaneous with speciation, is much more difficult. The keys will be to study a confirmed single species that ranges across different environments and to concentrate on traits that contribute to fitness and for which genetic control can be assigned. Neurospora discreta will be our model species because it thrives in different environments over a tremendous latitudinal gradient in western North America (Jacobson et al. 2004 Mycologia 96:66-74), a range that offers variation in abiotic factors (e.g., day length and temperature) and in biotic factors (e.g., host tree and coexisting microbes). To ensure conspecificity, we are applying phylogenetic species recognition to the diverse N. discreta clade as we have done for the other outbreeding Neurospora species (Dettman et al. Evolution 57:2703-2741). We will start with comparing a simple trait, mitospore germination, between N. discreta individuals in a common garden experiment, using strains collected at the extreme ends of the range and using temperature as the environmental variable. In time, we will expand to measure other fitness components, such as growth, mitotic sporulation and meiotic sporulation. To assay genome activity, we are profiling transcription using microarrays designed from the N. crassa genomic sequence. Funded genome sequencing projects for Podospora and Chaetomium should improve N. crassa genome annotation and microarray design, as would the proposed sequencing of N. discreta itself. Hypotheses about the adaptive role of differentially expressed genes identified by transcriptional profiling (e.g., as compared to drift) combined with existing knowledge of Neurospora biology can be challenged by genetic investigation; the latter might range from QTL analysis of adaptive traits to molecular manipulation.