Expression of *Trichoderma harzianum* genes in interactions with *Pythium ultimum*

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This work is carried out as a part of the EU-project "TrichoEST": Functional genomics and proteomics of *Trichoderma* antagonist strains for industry and agriculture (QLK3-2002-02032), which started under the 5th Framework Programme "Quality of Life" December 1. 2002. The overall aims are to identify genes and gene products from *Trichoderma* spp. with biotechnology value, to assess their industrial potential and, to exploit and commercialize them in concert with EU biotechnology strategy. cDNA libraries from several *Trichoderma* strains belonging to different species are used to construct macro-arrays for use in expression studies with various purposes defined by the different partners in the consortium. The program also contains generation of 2-DE maps and identification of potentially interesting proteins, integrated bioinformatics analysis, in vitro and in vivo expression of selected cDNA clones and functional screening of in vitro expressed cDNA clones.

Our role in TrichoEST involve construction of expressed cDNA libraries for two *Trichoderma* strains, the mycoparasite *T. harzianum* T3 and the mushroom pathogen *T. aggressivum* KNTP, grown in different simulated mycoparasitic and antagonistic situations. 1920 clones (ESTs) were sequenced from each library. Based on redundancy analysis, 767 unique ESTs were identified for T3 and used for construction of a macroarray. Using this macroarray, expression of genes in mycoparasitic interactions of T3 and the pathogen *Pythium ultimum* was studied in a time-course experiment. Interesting T3 ESTs up-regulated in the presence of *P. ultimum* have been identified and the genes have been partly identified using bioinformatics. Validation of the expression profiles of differentially expressed ESTs was performed by real-time RT-PCR analysis of selected ESTs. Future work includes exploitation of the industrial potential of selected ESTs through the TrichoEST program and studies of the role of these genes for the biocontrol activities of T3.

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Metabolic engineering of glycosylation pathway affects biocontrol properties of *Trichoderma atroviride*

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Plant diseases play a crucial role in the destruction of natural resources in agriculture. The ability of some *Trichoderma* species to antagonise and parasitise other fungi has made them effective biocontrol agents against plant pathogens. *Trichoderma* secretes a wide range of hydrolytic enzymes such as chitinases, and cellulases, having a potential role for the antifungal properties of the respective strain. Many, if not all of these extracellular proteins are glycosylated. Moreover, activity of O-glycosylation process coincides with up-regulated secretion of cellulytic enzymes. Genetic engineering of the O-glycosylation pathway by overexpression of some genes coding for enzymes connected with this process gave elevated production and secretion of cellulases or overglycosylation of cellulases resulting in higher cellulytic activity. These effects could improve biocontrol properties of *Trichoderma* strains especially when directed against plant pathogens having cellulose in their cell wall such as the root rot causing Oomycete *Pythium* spp.

In particular the overexpression of the yeast *DPM1* gene coding for dolichyphosphate mannose synthase (DPMS) in *T.atroviride* P1 led to the strains exhibiting a significantly better growth on cellulose, carboxymtheyl cellulose and xylan as a carbon sources. In consequence their anti fungal abilities against *Pythium* species were increased.

Resistance to Qo inhibitors in *Podosphaera fusca* does not correlate with the typical substitutions F129L or G143A in cytochrome B

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Powdery mildew elicited by *Podosphaera fusca* is a devastating disease of cucurbits worldwide and one of the most important diseases affecting these crops in Spain. Application of fungicides is currently the main control practice in most cucurbit crops for managing powdery mildew. Qo inhibitors (QoI) represent a relatively new and important class of agricultural fungicides, which are widely used in Spain against cucurbit powdery mildew. QoI have a single-site mode of action; they inhibit mitochondrial respiration by binding to the Qo site of the cytochrome bc1 enzyme complex, thus blocking electron transfer in the respiration pathway and leading to an energy deficiency due a lack of ATP. Shortly after the commercial introduction of QoI in 1996, resistant isolates were detected in field populations of several economically important phytopathogenic fungi. In most cases, QoI resistance was conferred by point mutations in the mitochondrial cytochrome b gene (*cyt b*). The main mutations leading to QoI resistance were the amino acid substitutions F129L or G143A.

As part of a research program on cucurbit powdery mildew management in Spain, we are carrying out a detailed study on fungicide resistance in populations of *P. fusca* from different cucurbit production areas. Regarding QoI, we have detected a resistance frequency of 32% (n=250 isolates) to azoxystrobin, kresoxim-methyl and trifloxystrobin, resistant isolates showing MIC values >500 mg/ml to the three QoI fungicides tested (cross-resistance). In addition, we have partially characterized *cyt b* alleles from isolates of *P. fusca* sensitive and resistant to QoI. Sequence analysis did not reveal any of the main point mutations, neither F129L nor G143A, previously described in several fungal plant pathogens. However, three new amino acid changes, S108A, V204A and I276Y, were observed in all QoI resistant isolates examined. The role of these amino acid substitutions in resistance to QoI fungicides in *P. fusca* will be discussed.

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Over expression of a 3-ketoacyl-CoA thiolase in the blackleg fungus *Leptosphaeria maculans* causes reduced pathogenicity on *Brassica napus*

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Leptosphaeria maculans causes blackleg (phoma stem canker), the major disease of *Brassica napus* worldwide. Random T-DNA insertional mutants of this fungus have been generated, and one (A3) produced lesions of reduced size on *B.napus* cotyledons. Genes flanking the T-DNA insertion had best matches to an alcohol dehydrogenase class 4-like gene (Adh4L) and a 3-ketoacyl-CoA thiolase gene (Thiol). Expression of *Adh4L* and *Thiol* was examined in vitro by quantitative RT-PCR and in infected cotyledons by both quantitative RT-PCR, and fluorescence microscopy using isolates with the individual promoters fused to reporter genes (GFP and mRFP, respectively). These genes were expressed in mutant A3 *in vitro* and *in planta* at significantly higher levels than in the wild type. This is the first report of a T-DNA insertion in fungi causing increased gene expression.

Transformants of the wild type isolate expressing both *Adh4L* and *Thiol* under the control of a heterologous promoter had similar pathogenicity to that of mutant A3. Ectopic expression of thiolase resulted in loss of pathogenicity, suggesting that thiolase over-expression was primarily responsible for the reduced pathogenicity of the A3 isolate. The thiolase gene encodes a functional protein and translational fusion with mRFP showed that it is located in peroxisomes. These findings suggest a link between pathogenicity and fatty acid utilisation by the fungus. Interestingly an isolate over-expressing both *Adh4L* and *Thiol* was more pathogenic than the isolate that only over-expressed thiolase, which suggests that Adh4L expression enables the fungus to obtain more nutrients through the extra alcohol dehydrogenase activity. The phenotype of the A3 mutant isolate may be due to altered nutrient balance resulting from over-expression of both alcohol dehydrogenase 4 and thiolase. Experiments are underway to elucidate the role of these genes in nutrient balance in *L. maculans*.

Identification of *Fusarium oxysporum* f. sp. *lycopersici* pathogenicity genes through *Agrobacterium*-mediated insertional mutagenesis

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Fusarium oxysporum f. sp. *lycopersici* is a soil-born fungus that causes vascular wilt disease in tomato by penetrating the plant roots and colonizing the plant xylem vessels. In order to identify genes involved in pathogenicity an insertional mutagenesis project is underway using T-DNA of *Agrobacterium tumefaciens* as an insertional mutagen. The aim is to generate 10,000 mutants, screen for those that have lost their pathogenicity and subsequently identify the tagged genes. Currently, over 5000 mutants have been generated and assessed in bioassays. So far, 12 non-pathogenic mutants and 31 mutants with reduced pathogenicity have been identified. Using TAIL-PCR the genomic regions flanking the T-DNA were isolated and sequenced. Several known pathogenicity genes were identified, such as class V chitin synthase and phosphomannose isomerase. Work is in progress to identify, verify and characterize the remaining pathogenicity genes.

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Two *Fusarium oxysporum* proteins, Six1 and Six2, are unique to isolates causing wilt disease on tomato and are secreted during infection

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Fusarium oxysporum, an asexual fungus encompassing pathogenic as well as non-pathogenic strains is a common inhabitant of soils and rizospheres worldwide. As a species, *F. oxysporum* has a very broad host range, while single isolates usually can infect only a single plant species. Isolates have therefore been grouped into formae speciales based on host specificity. Isolates belonging to the same forma specialis do not always share a common ancestor. So what determines host specificity? We identified two genes in *F. oxysporum* f.sp. *lycopersici* (Fol), which causes wilt disease in tomato, that are unique to this forma specialis. PCR amplification using specific primers as well as DNA hybridization revealed that these genes were present in all tested Fol isolates but not in closely related formae speciales. Both genes code for small, cysteine rich proteins, which are secreted during colonization of xylem vessels. The genes, *SIX1* and *SIX2*, are located close to each other on the same chromosome. The SIX1 gene was proven to be required for resistance of tomato plants carrying the *I-3* resistance gene against Fol, but also for full pathogenicity of Fol especially on older (3-4 week old) tomato plants. *SIX1* expression is highly induced during infection compared to axenic cultures. Expression starts immediately after entry of the roots and diminishes at later stages of infection, when the plants show severe disease symptoms.

Tissue cultures can be used to study transcript profiles of both partners during the interaction of Norway spruce and the pathogenic fungus *Heterobasidion parviporum*.

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Norway spruce (*Picea abies* (L.) Karst.) has a natural distribution in the northern parts of Europe and Asia and is economically the most important tree species grown in the Nordic countries. A common threat to Norway spruce is the basidiomyceteous fungus*Heterobasidion parviporum* Niemelä and Korhonen. *H. parviporum* mainly attacks Norway spruce, although Siberian fir (*Abies sibirica* Ledeb.) and Scots pine (*Pinus sylvestris* L.) occasionally get infected. One obstacle to studying host/pathogen interaction in conifers has been the limited availability of mature clones for controlled inoculations, as genetic variation within the host material and the lack of replicates complicate interpretation of the results. Somatic embryogenesis, rooted cuttings, and tissue cultures may provide solutions for this problem. Tissue cultures from mature Norway spruce trees have been proposed as a possible model system for assessing resistance toward fungal pathogens.Recent data on chitinase isoform activity in the Norway spruce/*H. parviporum* pathosystem are encouraging; clonal variation was observed in the isoforms affected by inoculation, and the isoforms showing increased band intensity following bark inoculation by *H. parviporum* were also induced in the inoculated tissue cultures of the corresponding clones.

To investigate the biological relevance of tissue cultures in host-pathogen interaction studies, transcript levels of selected host and pathogen genes in tissue cultures of Norway spruce were compared to those in bark of 33-year-old ramets of the same clones upon challenge by the pathogenic fungus *H. parviporum*. Similar transcript profiles of the pathogen and host genes were observed in both tissues, this supporting the use of tissue cultures as experimental material for the pathosystem. Higher transcript levels of the host genes phenylalanine ammonia lyase, peroxidase, and glutathione-S-transferase were observed in the more resistant clone #589 than in the less resistant clone #409 during the early stages of colonization. The most striking difference between the spruce clones was related to gene transcript levels of a class IV chitinase, which showed a continuous increase in clone #409 over the experimental period, with a possible association of this gene product to programmed cell death. Several of the fungal genes assayed were differentially expressed during colonization, including putative glutathione-S-transferases, laccase, cellulase, cytochrome P450 and superoxide dismutase genes. The transcriptional responses suggest an important role for the antioxidant systems of both organisms.

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Towards defining determinants of symptom development in smut fungi

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The two related smut fungi *Sporisorium reilianum* and *Ustilago maydis* both parasitize the same host plant, *Zea mays*. While the initial phase of infection is similar in both pathosystems, progression of the respective diseases is different. Both fungi have to undergo mating to form an infectious dikaryon, which then enters the plant via specialized infection structures. Mating and pathogenic development are controlled by the *b* mating type genes, encoding two subunits of a transcription factor, that is only active if the two subunits are derived from different alleles. After penetration, *U. maydis* ramifies locally within the plant tissue and leads to the induction of tumors in which spore development takes place. *S. reilianum*, however, grows towards the meristem of the plant and produces spores only in the inflorescence without prior production of tumors. To define the molecular basis of the difference in symptom development of these two related smut fungi, we employed two independent approaches.

In the first approach, we isolated RNA of maize seedlings after infection with either *S. reilianum* or *U. maydis*, at a time point where differences in proliferation between the two fungi can be microscopically detected. These samples were compared by cDNA-AFLP to mock-infected plants and axenically grown fungal material. This led to the identification of 330 differential fragments, of which 90% corresponded to plant genes. Differential genes of fungal origin are now under investigation.

In the second approach, we generated forced interspecies diploids by protoplast fusion of an *U. maydis* strain deleted for the *b* genes and a recombinant solopathogenic *S. reilianum* strain, in which the *bE2* gene was replaced by the *bE1* gene. The interspecies hybrids were weakly pathogenic and produced small amounts of spores. These spores were germinated and isolated haploid progeny were analysed for symptom development. Different isolates showed distinct phenotypes. One produced tumors on leaves, however, compared to the tumor morphology observed after *U. maydis* infection these were altered in shape. Another isolate produced tumors in the flowers only and thus combines traits of *U. maydis* and *S. reilianum*. The genomic content of these putative hybrids is currently being analysed by Affymetrix arrays representing about 6300 of the *U. maydis* genes. This should allow to map the altered traits to specific regions of the genome.

The *yap1*-like gene of *Ustilago maydis* is a virulence factor

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One of the most effective plant defense responses to pathogen attack is the production of reactive oxygen species (ROS). However, it is presently unknown how plant pathogenic fungi cope with these responses during a successful infection. In *Saccharomyces cerevisiae* this signalling pathway has been extensively studied and it has been shown that YAP1 serves as the central regulator for ROS signalling. The biotrophic fungus *U. maydis* contains one gene related to *YAP1*. To analyse the role of *U. maydis yap1* during pathogenic development, knockout mutants were generated in such way that the *yap1* ORF was substituted by eGFP. In addition, *yap1:eGFP* fusions were constructed to localize the Yap1 protein during *U. maydis* development. The *yap1* deletion mutants were significantly more sensitive to H_2O_2 than respective wild type strains and produced a dark pigment whose nature is currently unknown. This could indicate that under conditions where Yap1-regulated genes are no longer expressed, *U. maydis* is able to switch on the synthesis of other protectants. Compatible *yap1* knockout strains were able to mate, produced dikaryotic hyphae, invaded plants and induced disease symptoms. However, compared to the respective wild type strains, of the *yap1* mutant strains were significantly less virulent. This was reflected by reduced number of tumors as well as reduced tumor size. Using a *yap1:eGFP* fusion, we have observed that Yap1 is only activated during the early stages of the biotrophic growth, becoming inactive later. Staining with diaminobenzidine (DAB), a compound that forms a dark precipitate in the presence of ROS, showed the production of ROS in the vicinity of hyphal tips of *yap1* deletion strains only in these early stages when the Yap1 protein was activated. No DAB staining was observed after infection with the wild type strains. These results indicate that Yap1 controls genes which allow *U. maydis* to detoxify ROS during early infection. Transcriptome analysis showed, that Yap1 from *U. maydis* regulates genes involved in the detoxification of ROS as well as other with a putative function in virulence.

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Targeting mitochondrial respiratory and oxidative stress response systems for control of fungi

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Signal transduction and stress-response genes of fungal pathogens play important roles for exerting pathogenesis and, in some cases, biosynthesis of mycotoxins. As such, they should serve as potentially viable targets for antifungal compounds. Results of our research show that targeting the mitochondrial respiratory chain, MAPK or vacuolar H(+)-ATPase (V-ATPase) system using safe, natural compounds can significantly elevate the sensitivity of fungi to commercial fungicides or antifungal drugs. The use of such compounds can result in lowering effective dosages, costs of treatment and potential for development of resistance. Our rationale is based on the fact that cellular targets of several conventional antifungal compounds are already known. Examples of these targets include macromolecular synthesis (e.g., nucleic acids, amino acids, cell wall, etc.), cell division, signal transduction and respiration. We theorize that disruption of cellular redox homeostasis using phenolics may inhibit fungal development and invasiveness. Targeting these systems with drugs and additional safe, natural compounds leads to cellular oxidative stress responses, with a resultant decrease in cell viability. We illustrate the use of this target-based strategy to significantly improve control of fungi such as aspergilli. The molecular target for strobilurin-related fungicides, such as azoxystrobin or kresoxim-methyl, is the mitochondrial respiratory bc1 complex. Inhibition of this complex eventually leads to cellular oxidative stress caused by abnormal release of electrons from the respiratory chain. Using deletion mutants, we found at least five phenolic compounds that disrupt the normal function of mitochondrial respiration. Combined treatments of these phenolic agents and conventional fungicides that are inhibitors of the mitochondrial respiratory chain have a 100 to 1000-fold synergistic fungicidal effect due to disruption of respiration and inhibiting the oxidative stress-response of the fungus. In addition, we found that the alkaloid berberine targets the activity of oxidative stress genes, and combined treatment of this alkaloid and certain phenolics resulted in > 10, 000 times greater fungicidal activity than either compound alone. In addition to directly targeting mitochondrial respiration we also found effective synergistic control targeting the vacuolar H(+)-ATPase (V-ATPase) system by using phenolics as synergists for the V-ATPase inhibitor concanamycin A. We conclude that natural compounds (i.e. phenolics or alkaloids) can be developed as useful antifungal agents when the molecular target is identified. The potential use of this approach to effectively control a broad spectrum of fungal pathogens is discussed.

Characterization of *Fusarium oxysporum* f.sp. *phaseoli* isolates from scarlet runner bean (*Phaseolus coccineus* L.) in Spain

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The *Fusarium oxysporum* f.sp. *phaseoli* isolates used in this study were recovered from diseased plants of scarlet runner bean (*Phaseolus coccineus* L.) in the area of El Barco de Avila in West-Central Spain (where have been exclusively cultivated for decades). The isolates were characterized by means of pathogenicity tests, vegetative compatibility groups, polymorphisms of the intergenic-spacer (IGS) region of ribosomal DNA and distribution of mating-type (MAT) idiomorphs. Results were compared to those previously obtained for *F. oxysporum* f.sp. *phaseoli* strains isolated from common bean (*Phaseolus vulgaris* L.). In those previous works we had defined two virulent groups, highly virulent and weakly virulent, among the pathogenic strains isolated in Spain. The pathogenicity assays developed on *P. coccineus* allow the distribution of the new isolates into three groups: weakly and highly virulent, as happens in the strains isolated from *P. vulgaris*, and a new group of super virulent strains. All the new characterized strains show MAT1-2 genotype and most of them belong to the IGS A group (the IGS group of the pathogenic strains isolated from *P. vulgaris*). On the contrary, the strains here analyzed belong to new VCGs, namely VCG 169, 1610, 1611 and 1612.

The results here presented indicate that there is no clear correlation between virulence and genetic characteristics, suggesting that changes in virulence profiles may happen in any clonal lineage, as determined by VCG o IGS analyses. Also, the finding of only the MAT1-2 idiomorph among the Spanish *F. oxysporum* f.sp. *phaseoli* strains is indicative of the absence of sexual reproduction in the field.

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Elucidating the role of the F-box protein Frp-1 in pathogenesis of *Fusarium oxysporum*

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During evolution, plants have developed effective ways to defend themselves against microbial invasion. A microbial pathogen has to break through these defences in order to colonize the plant. To investigate the genetic basis of this ability of pathogens, we use the interaction between the soil-borne fungus *Fusarium oxysporum* f.sp. *lycopersici* (Fol) and its host tomato as a model system. From an insertional mutagenesis screen, a gene for an F-box protein called Frp1 was found to be required for pathogenicity. It was shown that Frp1, like other F-box proteins, binds to Skp1, a subunit of E3 complexes. These complexes are involved in the ubiquitination of proteins recruited by F-box proteins. One approach towards determination of the function of Frp1 is to find interacting proteins. To do so, a yeast two-hybrid screen using of a genomic library of Fol is being carried out with Frp1 as bait. Another way to find interactors is to use tagged Frp1 for isolation of protein complexes. GST-tagged Frp1 was produced by *E. coli* to fish interactors from Fol cell lysates. In addition, TAP-tagged Frp1 will be expressed in Fol and purified from cell lysates together with its interactors. The second approach to determine the function of Frp1 is to study the phenotype of the *frp1* knock out mutant. Microscopic studies of GFP-labelled wild type Fol and *frp1* knock out mutant showed that the mutant has lost its ability to colonise the roots. The mutant also showed reduced growth on agar plates with alcohol, organic acids or plant cell wall components as the sole carbon source. Probably, the mutant has a defect in assimilation of certain carbon sources which might be related to the loss of pathogenicity.

Separated and simultaneous inactivation of two Exo-β-1,3-glucanase genes has a significant effect on the biocontrol efficiency of *Pichia anomala* (strain K) against *Botrytis cinerea* on apples

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Pichia anomala (strain K), antagonistic yeast against *Botrytis cinerea* and *Penicillium expansum* on apples, may constitute an effective solution to be included in an integrated pest management programme aiming at reducing the environmental depredation caused by synthetic fungicides. Previous studies aiming at understanding the mode of action of *P. anomala* (strain K) led to opposite conclusions about the implication of *PAEXG1* and *PAEXG2,* two genes coding for Exo-β-1,3-glucanase. To further study any contribution of *PAEXG1* and *PAEXG2* in the biocontrol, both genes had to be simultaneously inactivated in a single strain. This result was achieved thanks to the *URA3*-blaster technique, previously validated on *P. anomala*. The biocontrol efficiency, against *B. cinerea,* of the resulting strains was significantly affected after application on wounded apples. For the first time, glucanase-mutated strains of yeast offered a lower level of protection as compared to the level of parental strains. Furthermore, the experiments underlined the complexity of the antagonistic relationship established within the host-antagonist-pathogen system, since the difference of efficacy between mutated and wild strains was modulated by yeast concentration and apple maturity.

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Proteomics of the biological control strain *Trichoderma atroviride*

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Trichoderma atroviride has a natural ability to parasitise phytopathogenic fungi such as *Rhizoctonia solani* and *Botrytis cinerea* therefore providing an environmentally sound alternative to chemical fungicides in the management of these pathogens. Two-dimensional electrophoresis was used to display cellular protein patterns of *T. atroviride* (*T. harzianum* P1) grown on media containing either glucose or *R. solani* cell walls. Protein profiles were compared to identify *T. atroviride* proteins up-regulated in the presence of the*R. solani* cell walls. Twenty four protein spots were identified using matrix assisted laser desorption ionisation mass spectrometry, liquid chromatography mass spectrometry and N-terminal sequencing. Three novel proteases to *T. atroviride* were up-regulated and identified as vacuolar serine protease, vacuolar protease A and trypsin-like protease. Two of these proteases, vacuolar protease A and vacuolar serine protease have been sequenced and cloned using chromosome walking PCR. Vacuolar protease A has two predicted introns, a predicted signal peptide and contains an aspartic proteinase conserved domain. Vacuolar serine protease has one predicted intron, a predicted signal peptide and contains a peptidase S8 conserved domain.

Sm1, a Proteinaceous Elicitor Secreted by *Trichoderma virens* Induces Plant Defense Responses and Systemic Resistance

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The soil-borne filamentous fungus *Trichoderma virens* is a biocontrol agent with a well known ability to produce antibiotics, parasitize pathogenic fungi and induce systemic resistance in plants. Even though a plant-mediated response has been confirmed as a component of bioprotection by *Trichoderma* spp., the molecular mechanisms involved have not been as well defined, compared to responses by other beneficial organisms such as rhizobacteria. Here we report the identification, purification and characterization of an elicitor secreted by *T. virens,* a small protein designated Sm1 (small protein 1). Sm1 lacks toxic activity against plants and microbes. Instead, native, purified Sm1 triggers production of reactive oxygen species in monocot and dicot seedlings, rice and cotton, and induces the expression of defense related genes both locally and systemically in cotton. Gene expression analysis revealed that *SM1* is expressed throughout fungal development, under different nutrient conditions and in the presence of a host plant. Using an axenic hydroponic system we show that *SM1* expression and secretion of the protein is significantly higher in the presence of the plant. Pretreatment of cotton cotyledons with Sm1 provided high levels of protection to the foliar pathogen *Colletotrichum* sp. These results indicate that Sm1 is involved in the induction of resistance by *Trichoderma* through the activation of plant defense mechanisms. This is, to our knowledge, the first report of the cloning, purification and characterization of a proteinaceous elicitor from *T. virens*.

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Transcriptomic response of *Alternaria brassicicola* exposed to host plant defense compounds

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The Ascomycete *Alternaria brassicicola* is the causative agent of the black spot disease of crucifers belonging to the *Brassica* and *Raphanus* genus. During colonization of host tissues, *A. brassicicola* is exposed to several antimicrobial plant defense products such as phytoanticipins and phytoalexins. Significant residual growth of this necrotrophic fungus has been observed even in the presence of high concentrations of these toxic compounds suggesting that it must be able to overcome these chemical barriers. In order to better understand the detoxification mechanisms responsible for such relative tolerance to host plant metabolites, we have used Differential Display RT-PCR (DD) and Suppression Subtractive Hybridization (SSH) to identify genes that are up-regulated in the presence of either glucosinolates-derived isothiocyanates (Allyl and benzyl ITCs) or phytoalexins (camalexin and brassinin). DD was first used to analyze subsets of the *A. brassicicola* transcriptome in the presence of benzyl ITC. Apart the identification of a sequence encoding an ITC-inducible Glutathione S-transferase, DD was poorly informative and generated a lot of aberrant and non informative sequences. By contrast, the SSH methodology was successfully applied to generate two cDNA libraries enriched for genes expressed in response to allyl ITC and camalexin. Allyl ITC treatment induced the expression of several genes involved in cell detoxification, especially drug efflux and response against oxidative stress. Following exposure to the phytoalexin camalexin, expression of genes involved in drug efflux and melanin, sterol and sphingolipid biosynthesis was induced. The results of *in planta* gene expression studies will be also presented for some of the identified genes.

Normalized cDNA libraries and some preliminary chemical analysis of *Ophiostoma clavigerum* growth on lodgepole pine metabolites associated with defense

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Ophiostoma clavigerum (Robinson-Jeffrey & Davidson) Harrington, is a pathogenic associate of the bark beetle, *Dendroctonus ponderosae* (Mountain Pine Beetle, MPB). When MPB attacks its primary target, healthy lodgepole pine, it introduces *O. clavigerum* and other associated microorganisms, which grow rapidly in the sapwood. The staining fungi produce melanin and within a few weeks fungal growth can occupy the entire sapwood region. Mutualistic fungi are thought to contribute to MPB outbreaks by increasing the survival of beetle offspring. This might be accomplished by modification of oleoresin metabolites or phloem/sapwood moisture dynamics. Improved brood vigor could also result from nutrient mining and sequestering during *O. clavigerum* sapwood invasion. To examine the processes of fungal growth, development and fungal tolerance towards lodgepole pine oleoresin it is necessary to develop molecular tools for characterizing *O. clavigerum* gene expression. We have created an EST resource of ~2700 unique genes and are currently using this resource for the development of a cDNA microarray. On this poster we describe our normalized EST annotations and our current work characterizing the growth of *O. clavigerum* on lodgepole pine oleoresin (the main component of tree defense) and biotransformation of select oleoresin compounds.

IXp-18

Isolation of genes involved in appressorium formation from *Colletotrichum gloesporioides* via differential gene expression

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Species of *Colletotrichum* are considered as major plant pathogens as these fungi cause economically significant damages to crops worldwide. These fungi penetrate the host via appressoria formation and therefore, identifying the genes that are induced or highly expressed in the pathogen during this morphological stage could provide potential novel pathogen-specific targets for plant disease control. In this research, we demonstrate the isolation and identification of genes differentially or uniquely expressed in the appressoria of a *C. gloeosporioides* strain. Treatment of conidia with wax extracted from papaya fruits and rubber leaves induced large-scale synchronous appressoria formation. Total RNA samples of appressorial and mycelial cells were isolated and used in the screening for differential expressed genes. Ten transcripts that were differentially or uniquely expressed in appressoria were isolated and cloned. These genes include genes involved in pathogenicity such as the isocitrate lyase gene reported to be involved in the establishment of appressoria turgor pressure and a gene, designated as *CAS*1, that encodes an appressoria specific protein (62% identity to a *Magnaporthe grisea* appressoria specific protein (MAS3)). Other transcripts isolated included genes that have not been described to date. These transcripts have been identified as encoding hypothetical proteins as they showed high identities to ESTs of other fungi. Results of the *in silico* analyses to predict the function of these hypothetical proteins indicated that 2 proteins have transmembrane functions while 6 may be proteins involved in signal transduction. Northern analysis of selected genes confirmed the differential gene expression results. The full length sequence of *CAS*1 has been cloned and characterised. Preliminary gene disruption results indicate that *cas1* gene disrupted mutants have been generated and that *CAS*1 may be required for appressoria formation.

Small GTPases in *Botrytis cinerea* – their role in signaling networks and impact on pathogenicity

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The necrotrophic ascomycete *Botrytis cinerea* causes gray mold diseases on various different hosts, among them many agriculturally important plants and ornamentals. Up to the present, several components of signaling pathways were identified that are involved in pathogenicity, e. g. the Galpha-subunit BCG1 (Schulze Gronover *et al.*, 2004). So far little is known about the impact of small GTPases belonging to the Ras superfamily on pathogenicity and possible links to other signaling pathways in *B. cinerea*.
Members of the Ras superfamily are highly conserved guanine nucleotide binding proteins and act as molecular switches, as they are active in a GTP-bound state and inactive in a GDP-bound state. They are known to be involved in proliferation, regulation of actin localization, intracellular transport, differentiation and pathogenicity.
A gene replacement approach was performed to inactivate *bcras1*, encoding one of two Ras-homologues existing in *B. cinerea*. Δ*bcras1* deletion mutants formed small, compact dark colonies and were not able to form conidia. Morphology of hyphae in deletion mutants differed from wildtype morphology, as they did not show polarized growth but misshapen blistered cells. Δ*bcras1* mutants were fully apathogenic on different host plants. A phosphorylation assay indicates that the MAP kinase BcSak1, which is responsible for osmotic stress response, is not phosphorylated in the deletion strain Δ*bcras1* under osmotic stress conditions (N. Segmüller, unpublished). Thus, BcSak1 is a possible downstream target of BcRas1. Additionally, a dominant active form of BcRas1 was generated. The DABcRas1 mutants showed a normal growth rate and hyphal morphology but increased sclerotia formation.

Furthermore, deletion of *bcrac* and *bccdc42* – members of the Rho subfamily of Ras proteins – is under way to analyze their role in fungal growth, morphology, pathogenicity and their cross-talk to other signaling pathways.

Schulze Gronover C., Schorn C., Tudzynski B.: Identification of *Botrytis cinerea* genes up-regulated during infection and controlled by the Galpha subunit BCG1 using suppression subtractive hybridization (SSH). Mol Plant Microbe Interact. 2004 May;17(5):537-46

IXp-20

Evolutionary relationships of genes encoding candidate pathogenicity factors from different intersterility groups of the root-rot fungus *Heterobasidion annosum*

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Heterobasidion annosum sensu lato (s.l.) is the economically most important pathogen in the northern hemisphere where it causes root and butt rot in conifers. The basidiomycete *H. annosum* (s.l.) species complex consists of several biological species and intersterility groups with different host preferences and overlapping geographic distribution. The intersterility groups are called S, F and P based on their main host specificity. In Europe, the P group grows on *Pinus sylvestris*, other conifers and broad leaved trees, the S group is found on *Picea abies* and *Abies sibirica*, and the host of the F group is *Abies alba*. In North America, the P group is found on *Pinus* species and the S/F group is found on *Abies*,*Tsuga*,*Picea*,*Pseudotsuga* and *Sequoiadendron*.

Adaptation to different hosts of the distinct intersterility groups will be tested by analysing selection forces on different genes. Genes encoding putative pathogenicity factors such as transcription factors, ras-protein, G-protein, glutathione-S-transferase, ATPase, hydrophobin and superoxide dismutase, identified from a North American P isolate have been selected for the study. We found examples of genes with substitutions only in exons, substitutions in exons and introns as well as genes with no substitutions between North American S and P isolates. Substitution frequencies vary between 1,6 % and 12 % in introns and between 0,6 % and 8,2 % in exons. The number of synonymous (K_s) and nonsynonymous (K_a) substitutions has been calculated to identify positive selection ($K_a/K_s>1$). Preliminary results indicate that the genes glutathione-S-transferase 335 and a gene with unknown function that is upregulated during the interaction between *H. annosum* and Scots pine seedlings might be under positive selection in the North American P and S groups.

The *Mycosphaerella graminicola* ABC transporter *MgAtr8* is part of a dicistronic messenger involved in iron homeostasis

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The ABC transporter *MgAtr8* from the wheat pathogen *Mycosphaerella graminicola* has a high homology to fungal ABC transporters implicated in azole-fungicide sensitivity. As *MgAtr8* disruptants did not show any alterations with respect to fungicide sensitivity, the genomic organization of this gene was examined in further detail. Upstream of *MgAtr8* an ORF was identified containing a motif characteristic for a pyoverdine / dityrosine biosynthesis protein (DIT1_PvcA, Pfam; PF05141). Interestingly, analysis of the *Fusarium graminearum* genome indicated the presence of an orthologous ABC transporter with the same association to a DIT1_PvcA motif. Such an association was not found for any other fungal DIT1_PvcA motif identified sofar. The *F. graminearum* genome annotation predicts that the DIT1_PvcA motif is fused to the ABC transporter part, suggesting that this association could also be present in *M. graminicola*. Since this would reveal a new exceptional class of ABC transporters, the transcription of the MgAtr8 / DIT1_PvcA locus was analysed to assess whether this locus encodes one or two genes. RT-PCR indicated that indeed both parts (the DIT1_PvcA motif and the ABC transporter) were present in the same mRNA transcript. However, sequencing of the derived cDNA fragments showed that translation of this mRNA would result in two independent proteins and not one fusion-protein. This indicates that the *MgAtr8* and *DIT1_PvcA* motif are expressed as part of a dicistronic messenger, a rare phenomenon in eukaryotes in general and filamentous fungi in particular. cDNA analysis also revealed a remarkable conservation of intron positions between the *MgAtr8* / *DIT1_PvcA* dicistron from *M. graminicola* and the predicted *F. graminearum* ortholog.

Dicistronic expression suggests that both components are needed in the same physiological proces and that coordinated expression is favourable in that respect. The PvcA motif is associated with the biosynthesis of the bacterial siderophore pyoverdine. This may imply that this dicistron is involved in iron metabolism. Growth assays under varying iron conditions using *MgAtr8* knock-out mutants indeed point to a function in iron homeostasis, particulary under high iron conditions.

IXp-22

Using genome information and sequence similarity searches to speed up the identification and characterization of genes induced in planta in *Botrytis cinerea.*

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The causal agent of grey mold, *Botrytis cinerea* Pers. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) is a filamentous fungus with a broad host range and responsible of important economic losses. In order to understand the molecular mechanisms involved in the infection process of *B. cinerea*, an experimental approach based on the analysis of differential gene expression by DDRT-PCR during the plant/fungus interaction was applied which made it possible to identify several cDNA fragments derived from *B. cinerea* genes differentially expressed in planta. One of these fragments, ddB47, is a 209 nt fragment which includes a 136 nt region with a 88,5% purine nucleotides content. When this fragment was used as a probe on a time course northern blot analysis, two different mRNAs, about 1.4 and 1.0 kb in size respectively, were detected, both derived from genes differentially expressed during the interaction with tomato. The first gene, named *Bde47A*, was cloned by hybridization using high stringency conditions. It encodes a mitochondrial protein and its deletion generates mutants showing earlier germination of spores and increased aggressiveness on several host species. We are interested now in cloning the gene encoding the 1.0 kb mRNA, provisionally named *Bde47B*. Two different experimental approaches are being considered. First, hybridization under low stringency conditions using as a probe the ddB47 cDNA fragment. And second, genome analysis searching for sequences similar, but not identical, to the sequence of fragment ddB47. This analysis yielded twelve “GA” rich genomic regions containing sequences showing significant similarity with the sequence of fragment ddB47. Specific probes for each of these sequences are being generated for Northern blot analysis in order to identify the ORF coding for the 1.0 kb *Bde47B* mRNA. This has not been identified yet, but the results already obtained allowed us to detect two novel *B. cinerea* genes whose expression is enhanced *in planta*. The first one encodes a helicase. Interestingly, the second one appears to encode a specific component of the mitochondrial translation system. This is the second *B. cinerea* nuclear gene encoding mitochondrial products which is strongly induced during the establishment and progress of the infection process. Work is in progress in order to analyze all the twelve “GA” rich genomic regions and to functionally characterize the gene encoding the specific component of the mitochondrial translation system.

Genetic linkage between growth rate and intersterility genes in the basidiomycete *Heterobasidion annosum* s. l.

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Quantitative trait loci (QTL) for mycelial growth rate were identified and positioned on a genetic linkage map of *Heterobasidion annosum sensu lato* (s.l.), a devastating root rot pathogen on conifers. The mycelial growth rate was analysed among 84 progeny isolates in two different temperature regimes, 12 and 24 °C, and segregated as a continuous character. The assay identified three QTL for growth rate at low temperature positioned on linkage groups 1, 17 and 19 with peak LOD values of 3.2, 2.9 and 4.8, respectively. At high temperature corresponding QTL on the same linkage groups, with peak LOD values of 1.3, 2.8 and 2.2, were identified. The QTL for the low temperature regime explained 20.9 %, 18.1 % and 24.0 % of the variation in mycelial growth rate, respectively. The broad-sense heritability was estimated to 0.97 and 0.95 for growth rate at low and high temperature, respectively. Two of the QTL for mycelial growth rate were shown to be tightly linked to the intersterility genes S and P, which control mating within and between closely related species and intersterility groups of *H. annosum* s.l. Isolates with a plus allele at the intersterility loci had a higher growth rate than isolates that harboured minus alleles.

IXp-24

Pathogenicity factors in the conifer - *Heterobasidion* pathosystem

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Root rot caused by the basidiomycete *Heterobasidion annosum* s.l. is one of the most destructive diseases of conifers in the northern boreal and temperate regions of the world. The fungus consists of three European intersterile subspecies P (*H. annosum*), S (*H. parviporum*) and F (*H. abietinum*) with differing main host preferences; pine, spruce, and fir, respectively. In North America, two intersterile groups are present, P and S/F, but these have not yet been given scientific names.

About 4000 ESTs were collected from *H. annosum* growing in contact with its host. About 70 % of the genes identified showed high similarities to known proteins and 16 % had similarity only with proteins with unknown functions. Detailed expression studies confirm up-regulation *in planta* of putative pathogenicity factors e.g. toxin production, cell wall degrading enzymes and proteins known to be involved in oxidative stress. Recently, an AFLP-based genetic linkage map was established that allowed mapping QTLs for pathogenic growth in seedling roots and pine phloem. The next step underway is to verify the identity of candidate genes located within the QTLs. Future functional analysis of both QTL and EST-derived candidate genes will be aided by the recently established *Agrobacterium*-mediated transformation system in *Heterobasidion*.

Host responses to the pathogen have been studied using microarrays of*Pinus taeda* genes. Up-regulation of phenyl propanoid and stilbene pathways as well as antimicrobial protein production was detected within 5 and 15 days of infection. The antimicrobial protein was located in the cell wall according to immuno gold labelling. Infection experiments on seedling material have shown that resistance to *H. annosum* has a relatively high heritability. Work is in progress to identify genes involved, with the long term goal to guide resistance breeding.

Fost12, a *Ste12* homologue is involved in pathogenicity and pH regulaed in *Fusarium oxysporum*

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Fusarium oxysporum is a ubiquitous phytopathogenic fungus able to infect an extremely wide range of crops. An important change is produced in fungal pathogens when the colonization of their hosts takes place. In eukariotic cells, a family of serine/threonine protein kinases (MAPK) are involved in transducting a variety of extracellular signals and regulating growth and differentiation processes. A key link of the MAPK cascade with upstream elements seems to be the STE20 protein, a serine/threonine protein kinase member of the p-21-activated kinase (PAK) family of protein kinases. STE12 is the major transcription factor that binds to the extracellular signal response element found in the upstream region of many responsive genes. We functionally characterized the *ste20* and *ste12* homologs, named *fost20* and *fost12* respectively, in *F. oxysporum*. Gene disruption experiments were developed using Agrobacterium tumefaciens-mediated transformation. Several deletion mutants were isolated by gene replacement in each case. The mutants do not show any indication of growth or sporulation impairment. Infection assays on bean plants were performed resulting that *fost20* mutants had no defect in pathogenicity and/or virulence. However, *fost12* mutants had significatively reduced virulence, in spite of the fungus is able to penetrate into the plant. This results indicate that *fost12* plays a role in plant infection. In order to investigate whether the *fost12* gene is regulated by pH, we analyzed the transcription of the gene under acidic, neutral and alkaline growth conditions. We found expression of *fost12* to be pH regulated, with transcript levels highest at neutral and basic pH. One of the key environmental conditions controlling cell growth and development is ambient pH. Our results suggest that *fost12*, a highly conserved MAPK homolog in *F. oxysporum*, can play a role in pathogenicity co-ordinately with other pathways, such as pH-dependent pathway in fungi.

IXp-26

Gene clusters for secreted proteins in *Ustilago maydis*: Determinants for compatibility

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The basidiomycete *Ustilago maydis* causes smut disease in maize. During its biotrophic phase this fungus penetrates the maize cuticle, establishes an extensive mycelial network within the plant apoplast and finally induces tumours on all aerial parts of its host. Although these stages of fungal development have been described in detail the determinants for compatibility, i. e. gene products which redirect host metabolism, suppress plant defenses and induce tumours, have remained mysterious. To address this issue, we focussed on the predicted secretome because proteins delivered by the fungus could represent effectors for the establishment and maintainance of biotrophic growth. This idea is supported by the observation that a high proportion of the potentially secreted proteins is *U. maydis* specific and more than one quarter of the respective genes is arranged in clusters comprising 3 to 23 consecutive genes. Furthermore, the majority of these clustered genes is specifically upregulated in tumour tissue. On these grounds, deletion strains for each cluster have been generated and four of the resulting mutants showed a dramatic reduction in virulence and are largely deficient in tumour formation while in one case stronger disease symptoms than in wild type infections are observed. Microscopy revealed that the four strongly affected mutants arrest at different stages of biotrophic development. Currently, we identify the genes which are the major contributors to the observed phenotypes and localize the respective gene products.

The bE/bW heterodimer encoded by the *b* mating type locus is required for all stages of pathogenic development of *Ustilago maydis*

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The phytopathogenic fungus *Ustilago maydis* exhibits a dimorphic life style. Nonpathogenic, haploid, yeast like cells have to fuse to form a dikaryotic hyphae that is able to infect corn plants, leading to the formation of tumors. Cell fusion is controlled by the biallelic *a* mating type locus via a pheromone-receptor system. Subsequently, pathogenic and sexual development are controlled by the multiallelic*b*-locus, coding for two homeodomain proteins termed bE and bW. If derived from different *b*-alleles, bE and bW form a heterodimeric complex that functions as a transcriptional regulator. The bE/bW heterodimer is required and sufficient to initiate pathogenic development; however, its requirement for subsequent steps in tumor formation is unknown.

To analyze the role of the *b*-heterodimer during the biotrophic phase of the lifecycle, we used random PCR mutagenesis in order to generate temperature sensitive (ts) *bE* and *bW* derivatives. We identified a *ts-bE2* allele encoding for a protein with a single amino acid alteration (Serin to Prolin) at the border of the homeodomain. At permissive temperature (22°C), a *U. maydis* strain carrying the *ts-bE2* allele in combination with the compatible *bW1* allele is able to infect corn plants at a rate comparable to that of the respective wild-type control. However, at restrictive temperature (31°C), the *ts*-strain is non-pathogenic. Furthermore, tumor development can be stopped at different stages by a shift to the restrictive temperature. Within the tumors, the temperature shift induces the formation of enlarged fungal cells. Currently we are investigating whether the enlargement is caused by a defect in cytokinesis or whether it is triggered by a plant defense reaction.

Our data clearly demonstrate that the *b*-locus is indispensable at all stages for the pathogenic development. We plan to use the *ts-b*-complex to analyze the expression of fungal genes at defined biotrophic stages using DNA microarrays. The *in planta* expression profiles will help to elucidate the molecular mechanisms facilitating the intricate interaction between the fungus and it host during pathogenic development.

IXp-28

The role of reactive oxygen species (ROS) in the *Botrytis cinerea* / host interaction

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Botrytis cinerea, a necrotrophic plant pathogen causing grey mould disease, essentially depends on the ability to kill its host cells. ROS play a major role in defense reactions of plants and animals against pathogens. Phytopathogenic fungi face considerable oxidative stress by the so-called "oxidative burst", an early plant defense reaction. *B. cinerea* can produce ROS himself and thus contribute to the ROS status during the interaction. We focus on signal chain components involved in oxidative stress signalling trying to determine their role either in ROS generation or decomposition. It could be shown for *S. cerevisiae* that the HOG1-mediated MAP kinase pathway is responsible for the reaction to hyperosmolarity. This pathway includes the MAPK HOG1. In *A. nidulans* and *C. albicans* it could be shown that the HOG-homologous genes are involved additionally in oxidative stress response. K.o. mutants of the *A. nidulans* SakA gene (HOG1-homologoues) showed enhanced expression of an NADPH oxidase gene. A HOG-homologous MAP kinase gene (*bcsak1)* was deleted by a gene replacement approach in *B. cinerea*. The mutants have a defect in asexual reproduction and are sensitive to osmotic stress. Furthermore they are completely apathogenic, the by far strongest phenotype observed for this type of mutant. For example *osm1*-mutants of *Magnaporthe grisea* are also sensitive to osmotic stress and show growth defects under hyperosmotic conditions but are still fully pathogenic. Scanning microscope analyses showed that the *bcsak1* mutants are unable to penetrate the plant surface. To analyse the role of the SAPK we performed detailed Northern and phosphorylation studies. *Bcsak1* is upregulated during oxidative stress mediated by H_2O_2 (2mM and 5mM) and osmotic stress, as well as *in planta* 7 dpi. Activation of BcSAK1-protein takes place during osmotic stress and there is evidence for activation of the protein during treatment with higher concentrations of H_2O_2. Phosphorylation could also be monitored during all stages of infection tested (17hpi, 24hpi, 48hpi and 3dpi). The mutants were additionally subjected to cytological analysis with regard to *in vitro*/*in planta* generation of H_2O_2.

Furthermore the identification of upstream and downstream acting components like histidine kinases, transcription factors and ROS-generating and -detoxificating elements like catalases and NADPH-Oxidases is in progress.

Visualizing the infection process of *Fusarium oxysporum* f.sp.*phaseoli* in common bean

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The plant pathogen *Fusarium oxysporum* is a common soilborne fungus with a worlwide distribution. Within the species there is a high level of host specificity with over 120 described formae speciales and races capable of causing vascular wilt diseases of many agricultural crops. This combination of wide range of infection as species and host specificity as formae speciales makes *F. oxysporum* an atractive model for the study of the molecular interactions involved in pathogenicity and/or virulence.

We have isolated and characterized isolates of *F. oxysporum* f.sp. *phaseoli* that show different ranges of virulence towards their host common bean (P. vulgaris L.). Highly virulent strains induce severe symptoms in susceptible plants and most infected plants die in 2-3 weeks. Weakly virulent strains induce a less severe response and most plants do not die even after 4-5 weeks after inoculation. Currently we are investigating the genes that may be involved in the differences in virulence. An interesting problem is whether both kinds of strains move at the same pace inside the plant or there are differences in the progression inside the xylem vessels. To address this question we have obtained transformants of a highly virulent, a weakly virulent and a non-pathogenic strain of *F. oxysporum* f.sp. *phaseoli,* harbouring the GFP coding gene. We have checked that both the transforming DNA and the expression of GFP are stable and that pathogenicity and virulence are not affected.

After inoculation of common bean plants with strains expressing GFP, the infection process was visualized in planta by means of confocal laser scanning microscopy. We have monitorized the advance of mycelium and production of conidia in bean roots and stem for the three types os transformed strains and correlated those data with the progression of the disease. The results obtained suggest that highly virulent strains are more efficient than weakly virulent strains colonizing inoculated plants.

IXp-30

Cladosporium fulvum Nrf1 is a virulence factor that controls *in planta* expression of *Avr9* but no other known effector genes

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Cladosporium fulvum (syn. *Passalora fulva*) is the causal organism of tomato leaf mould (Thomma *et al.*, 2005). During growth on its host tomato, C. fulvum secretes several effector proteins in the leaf apoplast. Eight fungal genes encoding such effector genes have been cloned. The expression of one of these genes, Avr9, has previously been shown to be strongly induced in vitro during nitrogen deprivation, a condition the fungus is expected to encounter while colonizing its host (Pérez-Garcia *et al.*, 2001). This led to the hypothesis that expression of additional effector genes in *C. fulvum* could also be triggered by nitrogen starvation conditions. However, we now show that Avr9 is the only effector gene that is clearly induced in *C. fulvum* during nitrogen deprivation in vitro. In addition, we demonstrate that the nitrogen response regulator Nrf1 only regulates Avr9 expression during infection of the host, whereas none of the other known effectors is significantly controlled by this transcription factor (Thomma *et al.*, 2006). Since the virulence of Nrf1 deletion strains, but not of an Avr9 deletion strain, is significantly reduced, it can be concluded that Nrf1 controls, together with Avr9, additional unidentified effector genes that are required for full virulence of *C. fulvum*.

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Identification of differentially expressed proteins of *Verticillium longisporum* during infection of *Brassica napus* (Rapeseed)

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Verticillium longisporum is a devastating vascular pathogen on rapeseed crops causing wilt disease. It is host specific on the family Brassicaceae e.g. rapeseed (*Brassica napus*). *V. longisporum* is a soil-borne pathogen that infects through the roots and colonizes the vascular system of the plant. The complex communication between *V. longisporum* and rapeseed when it penetrates and colonizes the plant has not been investigated fully yet. It is likely that some proteins of *V. longisporum* may be differentially expressed on receiving possible signals from the host-plant. Such proteins could play an important role during fungal growth or pathogenesis. They represent therefore possible biomarkers for systemic reciprocal effects in fungus-plant interaction and also as potential targets for specific fungicides.

This project aims at the identification of such differentially expressed proteins. It involves quantitative comparison of proteomes of cell extracts of different culture conditions i.e. the fungus cultured with or without xylem sap from *Brassica napus* by 2D-PAGE. Xylem sap extracted from uninfected rapeseed plants and from rapeseed plants infected with *V. longisporum*, was used for induction of differential gene expression in *V. longisporum*. After analysis of 2-D protein gels by PDQuest (BIO-RAD), differentially expressed protein spots were selected. For identification of the trypsin-digested 2-D protein spots ESI-MS/MS was employed. MS/MS analysis software, (Turbo) SEQUEST (Thermo Finnigan) and PEAKS (Bioinformatics Solutions Inc.) were employed for interpretation of MS/MS data.

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Nitric oxide induces the expression of a *Botrytis cinerea* flavohemoglobin coding gene during saprophitic growth

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Botrytis cinerea is a necrotrophic plant pathogen with a wide host range. It colonizes senescent or wounded tissues but it is also able to infect healthy plants. Experimental evidences support the ability of the fungus to exploit the hypersensitive cell response of healthy plant tissues to promote its colonization. In this interaction, the H_2O_2 and nitric oxide ($NO^{\cdot}$) produced by the plant play crucial roles on the activation of the plant cell death and the creation of a potent antimicrobial environment. However, as a necrotroph, *B. cinerea* is able to grow when exposed to this toxic environment.

Flavohemoglobins constitute a group of proteins involved in responding to $NO^{\cdot}$ and nitrosative stress. In bacterial and fungal human pathogens, flavohemoglobins appear to confer protection against $NO^{\cdot}$ toxicity, a role which has also been described recently in the plant pathogen bacteria *Erwinia chrysantemy*. Our interest aims towards the determination of *B. cinerea* mechanisms to survive under nitrosative stress conditions. A flavohemoglobin encoding gene from *B. cinerea* was isolated (*Bcfhg1)* by heterologous hybridization. Genome analysis indicated the lack of additional related sequences in the *B. cinerea* genome. An experimental setup was develop to expose *B. cinerea* to $NO^{\cdot}$ *in vitro* by using the $NO^{\cdot}$ donor DETA (Diethylenetriamine, Sigma). Then, the expression of the flavohemoglobin coding gene was investigated. Northern analysis demonstrated a basal level of expressión of *Bcfhg1* in the absence of $NO^{\cdot}$, but this level of expression increased very quickly after exposure to different levels of $NO^{\cdot}$. Interestingly, in terms of induction of expression of *Bcfhg1*, germinating conidia (grown during 4 hours in liquid medium before exposure) appeared to be more sensitive to $NO^{\cdot}$, as myceluium grown during 12 hours in liquid medium before exposure did not induce the expression of the *Bcfhg1* flavohemoglobin coding gene. Since *B. cinerea* responds to $NO^{\cdot}$ by enhancing the expresion of a flavohemoglobin coding gene, we have decided to undertake a more detailed expression and functional analysis of this gene. To this end, a gene replacement strategy to generate mutants altered in this gene is being carried out. Their analysis will provide information about the relevance of this flavohemoglobin as a factor contributing to fungal survival during the establishment of its interaction with the host plant.

Highly efficient gene targeting in the phytopathogenic fungus *Botrytis cinerea*

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Botrytis cinerea is an aggressive pathogen of the grapevine causing grey mould disease. In the french vineyards, it can be responsible for gain losses ranging from 15 to 40%, depending on the climatic conditions. This fungus is able to infect a broad host range (more than 200 plants) and is one of the most comprehensively studied necrotrophic plant pathogens. Our team contributes to the *B. cinerea* genome project and focuses on its functional analysis. When data issued from the genome sequencing will be available, comparative genomic and transcriptomic analyses will point out new genes potentially involved in the infection process. These genes will be candidate for reverse genetics analysis. Gene targeting by Homologous Recombination (HR) during transformation is possible in *B. cinerea*, but the frequency of correct gene targeting is variable and often low (from 0 to 20%). On the other hand, random integration of exogenous DNA into the chromosome is mainly observed suggesting that Non-Homologous End-Joining (NHEJ) of Double-Stranded DNA Breaks (DSB) is a predominant pathway in *B. cinerea*. In several fungi [1,2,3,4,5,6], the recent inactivation of the KU heterodimer (KU70/KU80), which normally binds DSB and initiates the NHEJ mechanism, resulted in highly efficient gene targeting by HR (from 50 to 100%). These results indicate that *Ku* disrupted strains are efficient recipients for gene targeting. The same strategy was therefore initiated in *B. cinerea* for which two independent mutants at the locus *Bcku70* were obtained by using the "Split-marker" replacement method. Results about the growth, virulence, mutagen sensitivity and targeting frequency of the *Bcku70*- mutants will be discussed.

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Characterization of a new, non-pathogenic mutant in *Botrytis cinerea* with impaired plant colonisation capacity

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Botrytis cinerea is a necrotrophic pathogen that attacks more than 200 plant species. The non-pathogenic mutant A336, obtained via insertional mutagenesis, was characterized (Kunz *et al.* 2006). Mutant A336 is non-pathogenic on leaves and fruits, on intact and wounded tissue while still able to penetrate the host plant. It grows normally *in vitro* on rich media but its conidiation pattern is altered. The mutant does not produce oxalic acid and exhibits a modified regulation of the production of some secreted proteins (acid protease 1, endopolygalacturonase 1). Culture filtrates of the mutant trigger an important oxidative burst in grapevine (*Vitis vinifera*) suspension cells, and the mutant – plant interaction results in the formation of hypersensitive response like necrosis. Genetic segregation analyses revealed that the pathogenicity phenotype is linked to a single locus, but showed that the mutated gene is not tagged by the plasmid pAN7-1. Mutant A336 is the first oxalate deficient mutant to be described in *B. cinere* and it differs from all the non-pathogenic *B.cinerea* mutants described so far.

To further investigate the non-pathogenicity phenotype of mutant A336, we undertook a transcriptome analysis of the mutant and two non-pathogenic progeny from a cross between strains A336 and SAS405 in comparison with the parent wild-type strains Bd90, SAS405 and two pathogenic progeny from the same cross. The most relevant genes, i.e., genes with large variation between the pathogenic and non-pathogenic strains, were selected. Among these genes, four were found to be totally down-regulated in the non-pathogenic strains. One of these genes showed strong homology with the *snodprot1* gene from *Stagonospora nodorum* which is expressed during infection of wheat (Hall *et al.* 1999). Construction of mutant strains of the down-regulated genes, including the *snodprot* homologous gene, is currently underway.

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A cell wall mutant of *Botrytis cinerea*: chitin synthase class III gene (*Bcchs3a*) is necessary for plant infection.

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Botrytis cinerea is a necrotrophic fungus which causes grey mould on a wide range of food plants especially grapevines, soft fruits and vegetable. A number of fungicides, including systemic compounds have been used extensively and serious problems of resistance have arisen. Effective control of *B. cinerea* relies on the development of new strategies. Chitin, an essential ultrastructural constituent of fungal cell walls, could be a suitable target for fongicide. Chitin, a <=-1,4 N-acetylglucosamine polymer, is biosynthesized by a family of chitin synthases. Actually, five chitin synthase genes belonging to class I, III, IV and VI have been sequenced in *Botrytis*. Moreover, genes of class II, V and VII are currently cloned and analyzed (1). Three mutants disrupted in class I, IIIa or IV chitin synthase genes were obtained and characterized (2,3). One of them, the *Bcchs3a* mutant, displays a specific phenotype *in vitro*: the radial growth rate is severely reduced, the hyphal tips are surrounded by an extracellular matrix and the mycelium produced numerous branched hyphae (microscopic studies). These results suggested that this enzyme BcCHS3a functions at the apical tips of the hyphae. *In vivo*, the *Bcchs3a* mutant has a largely reduced virulence on *Vitis vinifera* and *Arabidopsis thaliana* leaves. The *chs3a* gene product could therefore be a good antifungal target in phytopathogenic fungi as previously demonstrated in human pathogens. Other mutants disrupted in class VI of VII are under investigation.

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Gene expression of *Colletotrichum gloeosporioides* Regulated by alkalinization stress

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C. gloeosporioides is an important pathogen of tropical and subtropical fruits. During pathogen colonization the fungi alkalinize the host tissue by secreting significant amount of ammonia. The alkalinization of the host tissue enhanced *pel*B expression, a gene encoding for pectate lyase that affect colonization of fruits and possible other genes that were not identified. As a first stage for identification of the genes expressed during the ammonification process and other environmental stress condition and their contribution to fungal pathogenicity, we prepared cDNA libraries of *C. gloeosporioides* grown at inducing pH *in vitro* and in semi *in vivo* on avocado fruits. The cDNA libraries were printed a macroarray format and were used to determine the differential expression of genes under inducing and non inducing conditions of colonization. The differential expression of *C. gloeosporioides* genes will be discussed in this work and used in the future for gene knockouts for identification of their functionality

Green islands in the interaction of *Colletotrichum graminicola* with *Zea mays*

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Colletotrichum graminicola is a hemibiotrophic pathogen of corn that initially grows biotrophically within infected epidermal cells that remain alive. Later on, its life style changes to necrotrophy and the fungus proliferates also into deeper cell layers. During later stages of pathogenesis C. graminicola normally produces anthracnose spots on its host. Within these dark sunken necrotic areas the fungus produces acervuli that harbour masses of conidia which are embedded in a mucilagenous matrix.

We observed that the fungus generates a different phenotype when the inoculated leaves are senescing. Green islands, a well-known symptom from obligate biotrophes, occur just around the infection sites in otherwise yellow host tissues. In the pathosystem investigated, green islands only appear transiently in respect of space and time and therefore we determined the exact conditions under which the phenomenon occurs. We show that the fungus induces a delay of the shut-down of photosynthesis during senescence. We also show that carbohydrate levels are altered within green islands when compared to the surrounding tissue. In the absence of the fungus, exogenous application of cytokinin results in the same phenotype. In the future, we will analyse cytokinin levels in infected plant tissues and investigate if the fungus may induce green islands by altering host hormone regulation or by producing the hormone itself. Currently, we investigate infection-related morphogenesis at infection sites in green islands in comparison to infection sites leading to necrotic spots.

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Oxidative stress plays an important role in “mal secco” disease caused by Phoma tracheiphila

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"Mal secco" is a pathology of *Citrus* plants caused by mitosporic ascomycota *Phoma tracheiphila*. This pathogen produces a phytotoxic complex called "malseccin", a 93 KDa and a 60 KDa glycoproteins released in *P. tracheiphila* culture filtrates and in the infected host plant (Nachmias *et al.*, 1977; Fogliano *et al.*, 1998). The effects of the malseccin complex on *Citrus* leaves can be evidenced only under light conditions and this factor seems to play a pivotal role in the disease development. Physiological experiments have evidenced that the presence of filtrates of P. tracheiphila grown in presence or absence of lyophilised twigs and leaves (LTL) of tolerant (monachello) or susceptible (femminello) lemon varieties trigger the activity of superoxide dismutase, catalase, glutathione peroxidase involved in oxidative burst defence. The presence in the medium of tolerant *Citrus* LTL induces in the pathogen oxidative stress which affects its ability to produce 60 KDa proteins and polygalacturonase, laccase, Mn dependent peroxidase. On the contrary if *P. tracheiphila* was grown in the presence of susceptible LTL an high stimulation of hydrolytic activities occurred. Considering the 6 peptides sequenced by Fogliano *et al.* (1998) from Pt60 glycoprotein cleaved by trypsin treatment, several couple of degenerated primers were created. The conceptual translation of the several fragments (40 amplicons) obtained showed significant homologies (>50-60%) with iron membrane transporter, NADPH-dependent reductase and monoammine oxidase, enzyme that catalyses the oxidation of ammine and releases as by-products NH4+ and H2O2. A subsequent MALDI-TOF/TOF analysis on extracellular proteins of P. tracheiphila was carried out in order to evaluate the presence of Pt60-like proteins in the SDS-PAGE separated bands. This analysis has shown that a complex of proteins with different functions, among which a monoammine oxidase, is present in the single SDS-PAGE 60KDa band. RT-PCR analysis carried out on P. tracheiphila grown in presence or absence of susceptible and tolerant LTL indicated that the fragment coding for monoammine oxidase is expressed in different conditions. Amongst several factors involved in "mal secco" monoammine oxidase could play a pivotal role together with hydrolytic enzymes in the development of the disesase. The physiological and molecular results highly underline the role of oxidative stress in the *Phoma tracheiphila-Citrus limon* interaction.

Optimization of gene disruptions and foreign DNA integration in *Botrytis cinerea*

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We have addressed two different problems concerning the genetic transformation of *B. cinerea*. First we have studied the influence of the length of the flanking sequences on the frequency of gene disruption by homologous recombination, and second we have devised a method that allows site-specific integration of foreign DNA in the *B. cinerea* genome.

Gene disruptions are usually made by transforming with a construct containing a selectable marker flanked by regions homologous to the target gene. We have used a set of constructs to compare the frequencies of disruption of the endo-ß-1,4-glucanase gene *cel5A* in different conditions. All constructs contained the hygromycin resistance cassette flanked by regions of *cel5A* of varying length, and some times the rest of the plasmid, either circular or lineal. Transformation with a linear plasmid gave a higher transformation frequency, when compared to the circular form, and a higher gene disruption frequency. Flanking regions, homologous to *cel5A*, of 100, 500, 1000 and 2000 bp were all able to generate the disruption mutants at frequencies that were similar for lengths of 500-2000 bp (57-65%), but quite smaller for 100 bp (6%).

The introduction of foreign DNA into the *B. cinerea* occurs at random, so that individual transformants may differ in the integration site of the transforming DNA, and therefore in the genetic background in which transgenes are located. These differences are accentuated by differences in the copy number of the integrating DNA, with the result of, for example, wide differences in the expression of a reporter gene among individual transformants. As a step in the solution of this problem, we have devised a system for site-directed integration in which the recipient strain (B05.HYG-3) contains a 5'-truncated copy of the hygromycin resistance cassette, and is transformed with a circular plasmid (pBSHyg5Sal) containing another truncated copy, now in the opposite end (3'), of the same selection marker. Both truncated copies share a region of the cassette in which homologous recombination can occur after transformation, thus regenerating the whole marker. The system was checked by the expression of the green fluorescent protein.

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The *Botrytis cinerea* endo-ß-1,4-xylanase virulence gene *xyn11A* is expressed preferentially at the hyphal tips

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The phytopathogenic fungus *Botrytis cinerea* secretes a high number of cell wall degrading enzymes during the infection process that have been proposed to play an important role in virulence. Among these, endo-ß-1,4-xylanases promote the initial breakdown of xylan, the major hemicellulosic component of the plant cell wall, by cleaving internal bonds in the polymer backbone. We have previously reported that the *B. cinerea* endo-ß-1,4-xylanase gene *xyn11A* is required for pathogenesis and is induced by xylan, repressed by glucose, and expressed in planta.

Here we report a more detailed *in vivo* study of the expression of the gene by constructing a strain in which the *xyn11A* promoter and terminator control the expression of the green fluorescent protein (GFP). The reporter gene was subjected to glucose repression and xylan induction in the same way as the native *xyn11A* gene. Expression of GFP in this strain showed clear spatial differences along the fungal colonies either in Petri dishes or on onion epidermis: the protein was accumulated at the hyphal tips, in the border of the colony. The synthesis of GFP started from the beginning of infection, and fluorescence could be observed just a few hours after the conidia are laid on the plant, even before germ tubes begin to form. The border of the infection area on onion epidermis shows clearly the autofluorescence of plant cells, which is symptomatic of the hypersensitive response, and its appearance spans as much as ten cells ahead of the green fluorescent hyphal tips. The implications of this type of regulation on the role of Xyn11A on infection will be discussed.

Characterization of four chitinase-encoding genes from the fungus *Clonostachys rosea* (IK726)

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Clonostachys rosea is a widely distributed fungus that often acts as a parasite on other soil fungi. The mycoparasitic activity is thought to be correlated with the secretion of cell wall degrading enzymes, including chitinases. In this work, we identified and characterized four chitinase-encoding genes from the *C. rosea* strain IK726. Conserved motif regions among known exo- and endochitinase amino acid sequences from related fungi were used for design of degenerated primers, which were applied for PCR screening of *C. rosea* genomic DNA. The PCR products had strong similarity with known fungal N-acetyl-b-D-glucosaminidases (exochitinases), and endochitinases and were used to obtain a N-acetyl-b-D-glucosaminidase and three endochitinase genes including their promoter regions by a DNA walking strategy. The genes for the three endochitinases were named according to their predicted molecular mass. The *cr-nag1*, *cr-ech58* and *cr-ech37* ORFs have a length of 1746 bp interrupted by two introns, 1802 bp interrupted by three introns and 1044 bp without any introns, respectively. However, the ORF for *cr-ech42* was not completed. The *cr-nag1*, *cr-ech58* and *cr-ech37* deduced proteins contain 20, 17 and 22 amino acids signal peptide, respectively. Southern analysis showed that all four genes are present as single copy genes in *C. rosea*. Enzymatic assays showed that the chitinase activity of *C. rosea* is specifically repressed in media containing glucose. RT-PCR analysis was performed to confirm that the genes are expressed and to study whether the expression pattern of each gene followed the general chitinase expression pattern found using enzymatic assays. The highest expression of *cr-nag1*, *cr-ech42* and *cr-ech37* were found in media with *Fusarium culmorum* cell walls or chitin whereas almost no expression was detected in media with 1 % glucose. The expression of *cr-ech58* was low in all media tested and seems not to be regulated by glucose. Future work will focus on studies of the role of these four chitinase encoding genes for *C. rosea* interaction with fungal pathogens by studying the expression of the genes using real time RT-PCR analysis.

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Developing molecular tools for studying *Ophiostoma clavigerum*-lodgepole pine interactions

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Lodgepole pine (*Pinus contorta* Douglas var. latifolia Engelmann) is an important commercial species for the BC forest industry. *Dendroctonus ponderosae* (Mountain Pine Beetle, MPB) is one of the most important natural factors in the management of lodgepole pine ecosystems. Due to susceptibility of mature lodgepole pine stands to the recurring attacks by MPB, mature pine forests cannot be held long and therefore even flow and sustained yield management is difficult. The MPB prefers large diameter trees, culling the best and leaving the rest. It is difficult to separate the destructive nature of MPB and staining fungi, due to their close relationship, however, beetle-vectored blue stain fungi contribute significantly to tree mortality. Both bark beetles and fungi trigger a number of tree defense responses. The tree's defense mechanisms include biosynthesis of terpenoids and phenolics, some of which can be deterrent or toxic to the bark beetle and fungus. In order to advance our understanding of lodgepole pine defense responses we are using a recently developed 16.7k cDNA microarray for large-scale gene expression profiling in lodgepole pine trees inoculated with *Ophiostoma clavigerum*. At the same time we are building *O. clavigerum* specific molecular and genetic tools for probing gene expression during growth in its host tree. To better understand the interactions between *O. clavigerum* and lodgepole pine we are probing both the tree and blue-stain fungus. On this poster we will describe our approach and the tools we are developing for studying *O. clavigerum*-lodgepole pine interactions.

The transcriptional changes of a hemibiotrophic fungal pathogen during successful adaptation to hypersensitive response-like programmed cell death of its plant host

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Many important fungal pathogens of plants are described as being hemibiotrophic in that a significant period of their infection cycle involves biotrophic growth in association with living host tissue followed by a rapid transition to necrotrophic growth as host tissue death occurs. This transition involves a physiological change in both the plant and the pathogen referred to as the "hemibiotrophic switch." Little is known about either the host responses associated with this switch or the specific adaptations made by the pathogen to invoke and/or tolerate it. A major pathogen of agricultural concern on cultivated wheat is the hemibiotrophic fungus *Septoria tritici* (teleomorph *Mycosphaerella graminicola*). We have previously generated a unique *M. graminicola* microarray containing ~2,700 fungal genes. This has been used to identify that the host environment in which the fungus is sporulating at a very late stage of infection is nutrient rich[1,2]. Here we describe the development of an intact plant infection assay to investigate host responses of wheat leaves infected with *M. graminicola* during the hemibiotrophic switch, and use microarray transcription profiling to identify adaptive responses of the fungus to its changing host environment. We show that symptom development on a susceptible host genotype has features reminiscent of the hypersensitive response (HR), a rapid form of host programmed cell death often used by plants to restrict the growth of incompatible pathogens. The initiation and advancement of this host response relieves a nutritional starvation condition in the fungus identified through transcription profiling. Specific physiological adaptations of the fungus are also revealed with respect to membrane transport, chemical and oxidative stress mechanisms and metabolism. These datasets offer a unique insight into the transcriptional changes of a hemibiotrophic fungal pathogen during its successful adaptation to hypersensitive response-like programmed cell death of its host plant and identify many targets for future functional genomic analyses.

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IXp-44

Activity, Expression and Evolution of Transposable Elements in *Magnaporthe*

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Species of *Magnaporthe* are known to possess numerous distinct populations of transposable elements (TEs). Repeat regions in genomes arise through the proliferation of TEs, and these regions have been used to differentiate among apparent epidemic clones of *Magnaporthe*. Previous work has identified a long terminal repeat (LTR) retrotransposon called MAGGY (for MAGnaporthe GYpsy like element) that is specific to rice-infecting isolates of *Magnaporthe*. MAGGY has high variable copy number in all isolates surveyed to date. The recent acquisition of the genome sequence of a laboratory *Magnaporthe* rice-infecting strain and subsequent global transcriptional analyses presented the opportunity to study the relationship between TE genomic demography and expression patterns. The latter microarray studies suggested that MAGGY constituent ORFs, *gag* and *pol*, were highly expressed in conidia, relative to mycelial and appressorial stages of development. Reverse transcription polymerase chain reaction (RT PCR)-based analyses confirmed the pattern of expression observed in microarray experiments. Other TEs also present in high copy number in the *Magnaporthe* genome appear to have a similar pattern of expression. Here, we present representative microarray and RT PCR evidence in support of the conclusion that both type1 and 2 TEs, including MAGGY, MGL, Pot 2, Pot 3, Pot 4, Pyret and Occan are substantially more highly expressed in conidia than in other developmental stages in *Magnaporthe*. These observations and conclusions parallel similar findings in diverse eukaryotes, where TEs are active in germ and progenitor cells. Experiments in progress will determine whether high levels of TE expression truly signal active transposition and resulting observable genomic rearrangement in rice-infecting lineages of *Magnaporthe*. These, as well as our phylogenetic analyses of TEs, promise to offer profound insight into structure, function and evolution of fungal genomes, as well as complex biological phenomena, such morphological development, pathogenesis and speciation.

RNAi as a tool to investigate Verticillium wilt diseases

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Verticillium albo-atrum and *V. dahliae*. are soil-borne plant pathogens that are responsible for *Verticillium* wilt diseases that worldwide occur on many, mainly dicotyledonous, plant species including many economically important crops. The fungus enters the roots of the host plant, grows into the vascular tissue (xylem) and spreads to distal parts of the plant. Infection generally leads to obstruction of xylem vessels, thus hampering water transport and causing wilting. There are currently no fungicides available to cure plants once they are infected and, besides the use of resistant cultivars if available, removal of plants diagnosed with *Verticillium* wilt disease is the most effective means of disease control.

We are studying the interaction of *Verticillium* spp. with two different host plants, tomato and Arabidopsis. Tomato is a natural host of *Verticillium* spp., while Arabidopsis is used as a model plant. *Verticillium* strains have been selected that are pathogenic on both plant species and, with the use of GFP transformants, the infection is characterized on both hosts.

Because ultimately we are interested in genes that play a determinant role in *Verticillium*-host interactions, we have investigated whether we can employ RNA interference (RNAi) as a tool for functional analysis in *Verticillium*. We have designed an inverted repeat construct to knock down GFP expression that, upon introduction into *Verticillium* strains transgenic for *GFP*, indeed resulted in reduced GFP fluorescence. Subsequently, we have designed an inverted repeat construct to target the expression of an endogenous toxin biosynthesis gene in *Verticillium*. Our preliminary data show that introduction of this construct into *Verticillium* results in reduced pathogenicity of the fungus, demonstrating that RNAi can be used in *Verticillium* to investigate putative virulence genes.

IXp-46

A proteomic approach into biological control of sugarcane grubs

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Metarhizium anisopliae is a naturally occurring biological control of many insects including the greyback canegrub (*Dermolepida albohirtum*), a sugarcane pest in Australia. While there have been some gene-based approaches into identifying determinants for biological control and developing improved strains, our study provides a comparative proteomics approach into identifying key proteins produced by *Metarhizium anisopliae* during infection of greyback canegrubs. At the same time, we have developed a proteomic map for the greyback canegrub larvae responsible for significant losses of the crop.

Pathogenicity related proteins were identified by both a liquid culture and solid culture approach. Solid culture approach is thought to give a more realistic view of infection process compared to liquid culture. In order to identify novel target proteins, differential displays of proteomic maps of healthy/infected cuticle (HC/IC) as well as healthy/infected whole grub (HWG/IWG) were produced and analysed using Progenesis image analysis software. Proteomic map of healthy fungus (*Metarhizium anisopliae*) (MY) was also generated successfully. A total of 156 protein spots on HWG, 88 unique spots on IWG, 17 spots from HC and 14 spots from MY were analysed by mass spectrometry. Of these, 61 protein spots from HWG, 40 protein spots from IWG, 15 protein spots from HC and 9 protein spots from MY were confirmed by identification using mass spectrometry. Among the identified proteins were different forms of actin and tropomyosin, an ATP binding protein, arginine kinase, formate dehydrogenalase, enolase, tara like protein isoform and heat shock proteins. Further identification for most of the protein spots has been hindered due to the limited number of suitable/accessible databases. *Metarhizium anisopliae* has been successfully transformed to benomyl resistance using pBENA3, a plasmid containing the *benA3* allele from *Aspergillus nidulans* using particle bombardment as a preparation for introducing genes encoding the identified pathogenesis factors into *Metarhizium*.

Investigating the role of reactive oxygen species (ROS) during *Mycosphaerella graminicola* infection of wheat.

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Mycosphaerella graminicola (anamorph *Septoria tritici*) is a hemibiotrophic ascomycete pathogen of wheat leaves. Infection reduces crop yield via the appearance of chlorotic / necrotic lesions, which reduce the photosynthetically active leaf area. In the early stages of infection, following penetration through stomata, the fungus grows in the leaf intercellular space without producing any specialised feeding structures or causing visible disease symptoms. In the later infection stages, fungal biomass increases, hyphal nutrition becomes necrotrophic and localised host cell death occurs. Septoria leaf blotch disease of wheat is currently regarded as the most economically damaging pathogen of wheat in the UK and Western Europe.

M. graminicola EST microarray datasets have been produced which have compared fungal gene expression under different growth conditions (1,2). High levels of expression of various antioxidant genes were seen during the development of disease symptoms (necrotic lesions) and also in very late stage infected tissue, when compared with earlier stages of infection or during *in vitro* growth. Similarly, microscopy performed on infected leaf material stained for ROS has shown increased levels of both hydrogen peroxide and superoxide during symptom development and in late stage infected leaf tissue. We therefore wished to determine to what extent changes in ROS might influence fungal gene expression. Preliminary microarray data for gene expression under two different oxidative stresses - hydrogen peroxide and superoxide produced from menadione - will be presented. These data will be discussed in relation to genes differentially expressed under non-stressful conditions and during *in planta* infection.

The second and major aim of this project is to create gene deletion mutants of four genes of interest using Agrobacterium-mediated gene deletion. The genes are implicated in the production of and defence against reactive oxygen species during the host-pathogen interaction. Comparing the virulence of these and wild-type strains will provide more information about the role of reactive oxygen species in pathogen attack and survival, host defence, and pathogen development.

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2. Keon J, Rudd JJ, Antoniw J, Skinner W, Hargreaves J, Hammond-Kosack K: (2005) Metabolic and stress adaptation by *Mycosphaerella graminicola* during sporulation in its host revealed through microarray transcription profiling. Molecular Plant Pathology 6: 527 - 540.
This project receives financial support from Syngenta and the Biotechnology and Biosciences Research Council (BBSRC) of the UK. Rothamsted Research receives grant aided support from the BBSRC.

IXp-48

Development of new tools for *Fusarium graminearum*: Cre/lox mediated marker excision and bar-coded insertion mutants

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Testing the virulence function of candidate genes in plant pathogenic fungi is often hampered by two main problems. A) Due to genetic redundancy more than one gene disruption may be required to affect virulence. B) The variability of the infection process makes it difficult to reliably detect small differences in virulence.

To solve the first problem we are developing the *Cre/lox* system for fungi, to allow removal of the transformation marker and its subsequent reuse. The *Cre* recombinase gene was cloned behind the *Trichoderma reesei* xylanase 1 promoter and placed together with a hygromycin B resistance marker between repeated *loxP*-sites. This cassette should be stable on glucose medium, but allow self-excision on xylose medium.

In order to avoid confounding of data by failed inoculations into wheat heads ("disease escape"), we are developing competitive virulence assays. In brief, two unique 20 bp tags (uptag and downtag sequences) are introduced during the gene disruption. The distribution of tags is coordinated by MIPS (FGDB). The tag sequences can be amplified from infected plant tissue by unique primers, labelled and hybridzed to immobilized tag sequences. Shifts in the relative amount of tags reamplified by flanking primers should reveal differences in virulence between mutants present in the mixed inoculum. We are currently performing reconstitution experiments with *tri5* and other mutants of *Fusarium graminearum*.

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Ethylene perception by *Botrytis cinerea*

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Ethylene regulates several developmental processes in plants and plays an important role in plant-pathogen interactions. We currently investigate possible effects of ethylene in the pathogen *Botrytis cinerea* during the infection on tomato. *B. cinerea* is a necrotrophic fungus that is thought to take advantage of the ethylene released by the plant. Sensing ethylene by the fungus does not affect the hyphal development, but could activate genes involved in the pathogen attack.

The genome of *B. cinerea* contains a histidine kinase receptor, which belongs to the two component phosphorelay signaling system and is structurally similar to ETR1, the best characterized ethylene receptor in plants. In order to study the role of this gene, called *Bchhk5,* in ethylene perception, gene replacement mutants were made. The mutants are neither affected in growth *in vitro* nor in virulence. Studies on transcriptional activation of ethylene responsive genes in *Bchhk*5 mutants are in progress.

Arabidopsis thaliana ETR1 is a dominant negative receptor. Its heterologous expression in *B. cinerea* could prevent or reduce ethylene sensing by the fungus. Mutants were made carrying the ETR1 transmembrane domain or the full-length receptor. Characterization of these mutants is in progress.

IXp-50

Lipid mobilization in vacuoles of mature appressoria is essential for pathogenicity in the rice blast fungus *Magnaporthe grisea*

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The rice blast pathogen *Magnaporthe grisea* penetrates the host cuticle by mechanical force, generated by an enormous turgor of up to 8 Mpa in its appressoria. Glycerol accumulates in the appressorium to high concentrations and is believed to generate hydrostatic pressure by drawing water into the cell[1]. The biochemical source for the synthesis of glycerol in the appressorium is unknown, but the process must involve mobilisation of storage carbohydrates from the conidium and their degradation followed by polyol synthesis inside the maturing appressorium. One efficient way to generate glycerol inside the appressorium would be the degradation of triacylglycerol, which is exported as lipid droplets from the conidium to the incipient appressorium during infection-related morphogensis[2]. Upon maturation of the infection structure, lipid droplets enter the central vacuole in which they undergo degradation. The mechanism by which the lipid enters the vacuolar lumen has been identified as an autophagocytotic process by light and transmission electron microscopy[3].

In order to substantiate the role of autophagocytosis in lipid uptake by the vacuole, a reverse genetics approach was chosen, in which a gene homologous to the *Saccharomyces cerevisiae* gene *AUT1* was selected as knock-out candidate. *AUT1* is essential for autophagocytosis in *S. cerevisiae* and *aut1* null mutants in this species showed decreased survival rates during starvation[4]. Expression studies revealed that the *AUT1* homologue in *M. grisea, MgAUT1*,was not only expressed under starvation, but also during germination, appressorium formation and maturation.

In *DMgAUT1* mutants lipid droplets were not transported into the central vacuole and therefore could not be degraded during appressorium maturation. Vegetative growth of *DMgAUT1* mutants was unaffected on complete media but significantly reduced on nitrogen-limited media. Differentiation processes, such as conidiation, condial germination and appressorium formation were not affected in the mutants. However, they showed significantly reduced pathogenicity on intact leaves of either rice or barley as host plants. Consistent with a reduced penetration frequency in the mutant, cytorrhisis experiments revealed that appressoria of the mutants had reduced turgor levels.

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[4] Schlumpberger *et al.*, J. Bacteriol.,1997, 179, 1068-1076.

The H+-ATPase *LmPMA1* is involved in pathogenicity of *Leptosphaeria maculans* on oilseed rape

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Leptosphaeria maculans, the causal agent of stem canker, is the major pathogen of oilseed rape all over the world. In order to decipher the fungus infection strategies, and in parallel to the current genome initiative (see Balesdent *et al.* communication), a collection of 3000 *Agrobacterium*-mediated transformants has recently been generated and characterized in our laboratory. Here, we describe the phenotypic and functional characterization of one non-pathogenic mutant, m210. M210 is morphologically similar to the wild type isolate *in vitro* and shows no growth or sporulation defect. It induces a typical hypersensibility reaction on susceptible oilseed rape leaves and is totally inefficient to colonize the stem. Formal genetic studies performed on a progeny of 54 isolates showed an exact co-segregation between the m210 phenotype and the selection marker, thus confirming the efficient tagging of the mutant. T-DNA border sequencing allowed us to localize its insertion within the promoter of one gene, 274 bp upstream the start codon. The insertion results in the deletion of 7 bp of the promoter region. This gene is homologous to the highly conserved fungal gene *PMA1*, which encodes the predominant and essential plasma membrane H^+-ATPase. The basic function of this protein in fungal cells is to create an electrochemical proton gradient, which drives the uptake of nutrients by secondary active transport systems and regulates the intracellular pH. The *Leptosphaeria maculans* H^+-ATPase possesses all the characteristics common to other fungal H^+-ATPases (conserved catalytic and transmembrane domains). Quantitative RT-PCR analyses showed that *LmPMA1* is expressed at a high level and in a constitutive way *in vitro* (germinating conidia, mycelia) and *in planta*. In m210, the T-DNA insertion induced a 50% reduced expression of *LmPMA1* in *in vitro* growing mycelium but not in germinating conidia, compared to the wild type. We suggest that the separation of transcriptionnal regulation boxes from the gene start by the T-DNA insertion led to a deregulation of the expression in m210. In fungi, the expression of the H^+-ATPase is under the control of environmental pH and morphogenic development. We are currently investigating the influence of these two factors on *LmPMA1* expression, both in the wild type and mutant strains.

IXp-52

A fungal gene identified from *Neotyphodium lolii* is expressed only *in planta* and regulates the biosynthesis of a putative non-ribosomal peptide synthetase secondary metabolite

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Endophytes, belonging to the genus *Neotyphodium*, live symptomlessly within the intercellular spaces of cool-season grasses, and confer a number of biotic and abiotic advantages to their hosts. We identified a novel endophyte gene (designated Nc25) that is expressed only *in planta*, is one of the most abundantly expressed fungal transcripts in endopyte-infected grasses and which is distributed and highly expressed in a wide range of endophyte/ grass associations. Nc25 is novel and shows no homology to sequence databases or fungal genome initiatives. Characterisation indicates that it encodes a small protein that is predicted to be secreted. Furthermore, we have performed a gene deletion of Nc25. Re-introduction of the deletion strain into perennial ryegrass showed no visible effect on the symbiosis but surprisingly an unknown compound detected only in infected plants was eliminated. Even more surprising, the compound is possibly (as inferred from LC-MS-MS data) the product of a non-ribosomal peptide synthetase (NRPS), and is therefore unrelated to the predicted peptide product of Nc25. We hypothesize that Nc25 may regulate the putative NRPS biosynthetic pathway and are investigating this using Affymetrix chips arrayed with endophyte and symbiosis enriched ryegrass ESTs to determine how Nc25 affects global gene expression. In addition we are interested in the biological function of this secondary metabolite during symbiosis and in particular whether it has bioactivity that may confer abiotic or biotic advantages to the host plant.

Carbon exchanges during pathogenesis of *B. cinerea*: preponderance of mannitol during development and infection

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The strategy developed by necrotrophic pathogens is mainly based on the production of toxins and cell wall-degrading enzymes. Successful invasion depends on their ability to penetrate plants and evade defensive mechanisms. But to complete their life style in planta, pathogenic fungi must also be able to gain nutrients from plant cells. Our purpose is to characterize metabolic interactions between plants and the necrotrophic polyphage pathogen *Botrytis cinerea*, by using NMR spectroscopy to monitor cellular metabolism. We characterized the metabolic profiles of each partner (*B. cinerea* and sunflower cotyledons as plant host) and followed the evolution of the metabolites during the course of infection. Natural abundance 13C- and 31P- NMR spectra showed the progressive exhaustion of plant carbohydrates stores (fructose and saccharose) in favour of mannitol of fungal origin. The continuous presence of mannitol during the development stages of*B. cinerea* was assed by TLC and HPLC experiments. During an osmotic stress, quantification of polyols revealed a decreasing level of mannitol at the expense of glycerol, suggesting that mannitol accumulation was not an osmoprotective response in *B. cinerea*. The metabolic pathway for mannitol biosynthesis and catabolism in fungi takes place through the mannitol cycle which involves two pathways. Expression analyses of two genes involved in these pathways, mannitol 1-phosphate dehydrogenase (MPD) and mannitol dehydrogenase (MTD) revealed that both ways are functional and sequentially expressed during sunflower cotyledons infection. Inactivation of *mpd* and *mtd* genes is being currently underway and will contribute to establish the role of mannitol during development and pathogenesis of *B. cinerea*.

IXp-54

A putative transcriptional regulator Mstu1 involved in control of fungal developments and appressorium maturation in the rice blast fungus *Magnaporthe grisea*

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APSES proteins are fungal transcriptional regulators defined by similarities in their basic loop-helix-loop DNA binding domain, and are involved in regulating an array of functions related to fungal development. Examples are found in Asm-1, a *Neurospora crassa* APSES protein involved in spore germination, mycelial growth, conidiation and female fertility, and StuA, an *Aspergillus nidulans* APSES protein required for conidiophore morphogenesis and sexual reproduction. In *Saccharomyces cerevisiae* and *Candida albicans*, the function of APSES proteins is associated with pseudo-hyphal growth. We have cloned the gene of a novel member of APSES proteins from *Magnaporthe grisea* and analyzed its function using gene knockout mutants. Using conserved sequences present in StuA, we first identified nucleotide sequences encoding a putative APSES protein homolog of *M. grisea* from the annotated gene data base (http://www.broad.mit.edu), and named the gene *MSTU1* (Magnaporthe StuA - like). The *mstu1* mutants showed slow mycelial growth on oatmeal medium and were female sterile during sexual reproduction. Unlike *A. nidulans stuA* , *mstu1* did not exhibit deficiency in conidiophore developmental pattern or conidial morphology, although a reduction in conidiophores and conidia was observed. On hydrophobic surface, *mstu1* conidia germinated normally, and although appressorium formation was slightly delayed in the early hours (< 6 hours) after the start of the incubation, the number of appressoria formed 24 hours after incubation were comparable to that produced by the wild type. After incubating the mutant conidia on plant epidermal cells for 48 hours, a reduction in penetration was observed. Results from spray infection assays showed the mutants were dramatically reduced in pathogenicity towards rice. Interestingly, when inoculated through wounds, *mstu1* formed lesions on the rice leaves. *M. grisea* uses turgor pressure in appressorium to penetrate plant cells. To generate turgor, glycerol is generated from conidial reserves, such as glycogen and lipids, and is accumulated in appressorium. Since results from inoculation assays indicated that *mstu1* might have functional defects in appressorium, we observed degradation of conidial reserves. Cytological analyses indicated that *mstu1* was greatly delayed in glycerol generation from conidial reserves. Taken together, our results demonstrated that Mstu1 is a novel member of APSES proteins and regulated fungal developments and appressorium maturation.

Gene regulation by the homeodomain transcription factor PTH12 in *Magnaporthe grisea*

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Rice blast disease caused by *Magnaporthe grisea* is the most devastating disease of cultivated rice worldwide. The fungus can also cause a similar disease on a wide variety of grasses including economically important crops such as barley, wheat and millet. Disease is initiated through the development of a sophisticated infection structure called the appressorium. Appressoria development is induced by surface hydrophobicity and soluble cutin monomers. Sweigard identified the *pth12* gene, which encoded a putative homeodomain transcription factor, by REMI mutagenesis and suggested the gene to be involved in the appressorium maturation. To characterize the function of PTH12 in detail and to identify possible downstream target genes, we first recreated the *pth12* gene mutant in *M. grisea* 70-15 by gene disruption. The *pth12* disruptant formed irregularly shaped swollen structures at the germ tube tip in response to surface hydrophobicity and soluble cutin monomer. Furthermore, the abnormal appressorium-like structure was not melanized from which emerged new hyphae. A drastic reduction in pathogenicity was also observed in both barley and rice infection assays. To investigate genes regulated by PTH12, transcriptional profiles between wild-type and the *pth12* disruptant were compared using the Agilent *M. grisea* whole genome oligo DNA microarray. Spores from both wild-type and the *pth12* disruptant were inoculated on appressorium-inductive hydrophobic surface and total RNA was extracted at 3 and 6 hr. By 6 hr of incubation, approximately four hundred genes were found to be differentially expressed. Functional categorization of those genes will be discussed.

IXp-56

Molecular tools for the study of Fusarium ear blight caused by *Fusarium graminearum* and *Fusarium culmorum*.

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Fusarium ear blight (FEB) is an important disease of cereal crops caused by many species in the genus *Fusarium*. The most common species causing FEB are *Fusarium graminearum* and *Fusarium culmorum*. As well as effecting the yield and quality of grain *Fusarium* species contaminate the crop by producing trichothecene mycotoxins which are toxic to both animals and humans. We are currently developing new tools to allow us to study these important pathogens *in planta.* These include the creation of transgenic *Fusarium* strains which constitutively express either the GFP (green fluorescent protein) or GUS (ß-glucuronidase) reporter proteins and the creation of a *F. graminearum* transgenic strain of PH-1 (sequenced strain) harbouring a *TRI5 promoter:GUS* reporter construct. We have also recently started to utilise the plasmid-based split marker deletion method (Catlett *et al.* (2003), FGN, 50, 9-11) to construct targeted gene deletions in *Fusarium*. For this method two DNA constructs are prepared, each containing a flank of the target gene and two thirds of the selectable hygromycin resistance gene. During protoplast transformation homologous recombination between the overlapping hygromycin gene regions and the flank regions and their genome counterparts result in targeted gene deletions. The split-marker approach has allowed us to speed up the generation of constructs for transformation and will be used to generate single gene mutant isolates with reduced pathogenicity. These can then be subsequently transformed with the reporter gene constructs to study their altered infection process. We hope to use the transgenic strain of PH-1 carrying the *TRI5 promoter:GUS* reporter construct to infer the characteristics of the transcription of the *Tri5* gene by examining (a) when *Tri5* expression is induced *in vitro* and *in vivo*, (b) where in the fungus and plant is *Tri5* expression switched on and (c) how much *Tri5* product and mycotoxin is produced.

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Cloning and characterization of *AvrLm6*, a second "lost in the middle of nowhere" avirulence gene in the Dothideomycete *Leptosphaeria maculans*

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The Dothideomycete *Leptosphaeria maculans* causes stem canker, the most devastating disease of oilseed rape (*Brassica napus*) worldwide. Genetic studies demonstrated gene-for-gene relationships between *L. maculans* and *B. napus*. Most of the avirulence genes were found to be genetically clustered in the genome; with two main clusters : *AvrLm1-2-6* and *AvrLm3-4-7-9*. Map-based cloning of the *AvrLm1-2-6* cluster of avirulence genes was initiated and recently led to the identification of *AvrLm1*. *AvrLm1* occurs as a solo gene located in the middle of large stretches of A+T-rich composite and degenerated retrotransposons. The ensuing chromosome walk towards *AvrLm6* resulted in delineation of a 562 kb BAC clone contig in an avirulent isolate. The contig was fully sequenced and four candidate genes for *AvrLm6* were identified on the basis of sequence polymorphisms between the avirulent isolate sequenced and a virulent isolate. Assays for complementation of the virulent isolate with the different candidates were performed and one gene was found to fully restore the avirulent phenotype on a range of *Rlm6* oilseed rape genotypes. Identification of *AvrLm6* was confirmed by using RNA interference (RNAi). Decreasing expression of *AvrLm6* resulted in virulence of the corresponding transformants towards *Rlm6* oilseed rape genotypes, whenever the expression was reduced by more than 55 %. *AvrLm6* was found to be located in the same genome context as *AvrLm1,* since it is a solo gene surrounded by 85 kb of degenerated repeats on its 5' side and 48 kb on its 3' side. *AvrLm6* is an orphan gene that encodes a small cystein-rich protein (6 cystein residues) potentially secreted. Comparison of *AvrLm1* and *AvrLm6* expressions by quantitative RT-PCR revealed that both genes are constitutively expressed and are highly over-expressed during the primary leaf infection, suggesting that *AvrLm1* and *AvrLm6* could be co-regulated.

IXp-58

The influence of nitrogen source and pH on *pel*B expression and PL secretion during pathogenicity of *C. gloeosporioides*

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Colletotrichum gloeosporioides is a filamentous ascomycete phytopathogen that attacks many fruits at preharvest and postharvest stages. During pathogen colonization the fungus accumulates ammonia in the host, which creates the alkaline pH environment necessary for the expression and secretion extracellular lyase enzymes, including pectate lyase. Pectate lyase (PL) has been implicated as a virulence factor of *C. gloeosporioides* in avocado fruit, and its expression is strongly affected by alkalinization and ammonia accumulation. New data suggest that pH is not the only environmental factor modulating *pelB* expression. Increasing concentration of nitrogen sources enhanced PL secretion. On the contrary developments of nit- mutants, that can not utilize KNO3 as nitrogen source, did not accumulate NH4, secrete PL to the media and showed reduced pathogenicity on avocado fruits.

These results suggest that ambient pH alkalinization resulting from ammonia accumulation and the availability of ammonia as a nitrogen source are independent regulating *pelB* expression, pectate lyase secretion and virulence of *C. gloeosporioides.* This indicates that pH and nitrogen both contribute during pathogenicity of *C. gloeosporioides.*

The *Fusarium graminearum* Gpmk1 MAP kinase regulates the secreted lipase FGL1, a novel virulence factor required for infection of cereals

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Mitogen activated protein (MAP) kinases regulate virulence in several pathogenic fungi e.g. *Claviceps purpurea, Cochliobolus heterostrophus, Magnaporthe grisea*, and *Fusarium graminearum*. Up to now it is unclear which virulence factors are regulated via this signal transduction pathway. We reported that Gpmk1 MAP kinase disruption mutants of *F. graminearum* are apathogenic and cannot infect wheat spikes (1). Additionally, we showed that Gpmk1 MAP kinase disruption mutants exhibit an altered induction of several hydrolytic enzymes (2), among them the secreted lipase FGL1. This lipase is a major virulence factor of *F. graminearum* (3). Here, we show the regulation of the FGL1 gene in dependence on the MAP kinase Gpmk1 in culture and during plant infections. We constructed Gpmk1 MAP kinase deficient *F. graminearum* strains that express the FGL1 gene constitutively under the control of the gpdA promoter. These mutants are not longer apathogenic on wheat, but in contrast to the wild type strain, they cannot spread out through the entire spike.

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IXp-60

Allele variation in the *Phytophthora infestans* effector gene *ipiO*

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The oomycete *Phytophthora infestans* is the causal agent of potato late blight. In the past we identified a number of *P. infestans* genes that are specifically activated during a compatible interaction. One of these *in planta* induced genes, named *ipiO,* encodes a secreted protein of 131 amino acids that has no obvious homology with known proteins. *ipiO* is not expressed in mycelial cultures but is highly expressed in the periphery of water-soaked lesions and in healthy looking plant tissue surrounding the lesion (van West *et al.*, FGB 23: 126-138). Also prior to infection, in particular in germinated cysts, *ipiO* expression in high. The IPI-O protein contains two intriguing motifs in the N-terminal part. One is an RGD tripeptide, a motif that is found in several extracellular proteins in mammalians, which act as ligands of integrins and play a role in cell-cell interactions and cell attachment. Plants lack integrins but in recent years evidence accumulated that also plants have RGD-binding proteins in their cell membranes (see e.g. Gouget *et al.*, Plant Physiol 140: 81-90). The second motif is RXLR, a motif that is shared by five recently identified oomycete avirulence (AVR) proteins and thought to play a role in the translocation of AVR proteins into plant cells. Database mining has revealed that RXLR is also present in a large group of very diverse secreted proteins found in *Phytophthora* and other oomycete plant pathogens, and this motif is now considered as a hallmark of oomycete effectors. Although the *ipiO* expression pattern supports a role for IPI-O in pathogenesis, IPI-O may also function as an AVR factor that is recognized by Solanaceous plants thereby acting as an elicitor of defense responses. To further investigate the role of IPI-O as effector in the potato - *P. infestans* interaction, we determined *ipiO* allele variation in a variety of isolates collected over the years and in different geographical locations, in particular in Europe and Central- and South-America. Also IPI-O homologues in closely related *Phytophthora* species with a different host range were analysed. Several allele variants of *ipiO* were cloned and their expression was studied. The various alleles will be tested for their activity as effector or elicitor of defense responses on Solanaceous plants.

Diverse signals and signalling pathways control germination and infection of *Botrytis cinerea*

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Germination of *Botrytis cinerea* conidia was found to be controlled by different signals, including nutrient levels, surface hydrophobicity and surface hardness. Analysis of mutants defective in a Gα3 subunit of the heterotrimeric G protein and in the MAP kinase cascade Bst11-Bst7-BMP1 revealed at least two signalling pathways that control germination. While Gα3 controls, in a cAMP dependent fashion, germination in the presence of limited amounts of a carbon source, the BMP1-MAP kinase cascade is partly involved in carbon source sensing but essential both for germination induced by hydrophobic surface contact, and for penetration of the host surface. The Gα3 -deficient mutant also showed delayed infection of plant tissue. Penetration of these mutants is delayed due to a defect in surface sensing: Germlings of the Gα3 mutant showed a more extended superficial growth and a lower frequency of penetration attempts. Addition of cAMP restores the surface sensing of the mutant to wild type levels. These data show for the first time a complex signaling network in a phytopathogenic fungus that controls not only different pathways of germination but also early events of infection, including surface recognition and penetration. We are currently analysing the role of further signaling components and their interaction during early development of *B. cinerea*.

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Colonization of maize roots by *Colletotrichum graminicola*

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Colletotrichum graminicola (Ces.) G. W. Wils. causes anthracnose stalk rot, top die-back and leaf blight of maize. The pathogen survives in residue on the soil surface, in buried residue, and as sclerotia (stromata) inside stems on the soil. Little is known about its role as a root pathogen though it has been suggested that *C. graminicola* can infect maize roots in the field. Recent literature suggests that many fungi that are commonly regarded as causal agents of foliar diseases can also cause systemic infection of their hosts by invading roots. We are investigating the importance of root infections of *C. graminicola* on the anthracnose disease cycle. Maize seeds were grown in vermiculite that had been inoculated with mycelial agar plugs of *C. graminicola* isolate M1.001BH. Three weeks after sowing, the roots of the seedlings were washed, sectioned, and visualized with light and fluorescent microscopy. Lesions on the roots were not observed, however fungal hyphae could be found colonizing the surface of the roots and invading epidermal cortex and vascular tissue. Structures, typically formed by root pathogens but not previously reported for *C. graminicola* on roots, including hyphopodia and microsclerotia were observed. Certain epidermal and cortical cells become infected from intercellular hyphae while surrounding cells are uninfected, resulting in a mosaic pattern of infection. Interestingly, conidia were formed in acervuli on the root surfaces but were also found filling epidermal cells and root hairs. Preliminary data indicate that root infections cause significant stunting of both roots and above ground portions of seedlings and that the fungus can spread from the roots to aerial tissues. These observations suggest that root infection may be an important component of the maize anthracnose disease cycle.

Fusarium graminearum infects wheat by specific suppression of plant defense

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The (1,3)-β-glucan callose is known for its involvement in plant defense during pathogenic attack. Callose is a main component of papillae that are considered to delay pathogenic spreading. We analyzed callose concentration, callose synthase activity and glucan sythase-like (GSL) gene expression during fungal infection. Wheat spikes were inoculated with *F. graminearum* wild type and a lipase-deficient mutant (Δfgl1) that is strongly reduced in virulence (1). To observe non-host reaction, wheat spikes were inoculated with *Pyrenophora teres* wild type, a barley-leaf pathogen. Callose synthase activity correlated to callose concentration of the examined tissue. There was a constant decrease in callose synthase activity as well as in total amount of callose in spike tissue during infection with the *F. graminearum* wild type. An induction of callose synthase activity was shown for the Δfgl1 strain and the *P. teres* infection of spikes. A regulation at transcriptional level can be excluded from expression analysis of GSL genes. GFP-tagged wild type-like and Δfgl1 *F. graminearum* strains enabled a detailed observation of mycelium growth in the infected wheat spike. Whereas *F. graminearum* wild type spread from the inoculated spikelet directly into the rachis, the lipase-deficient mutant was arrested at the transition zone of rachilla and rachis. The addition of free fatty acids (FFA) to spikelets 3 days post-inoculation significantly increased the virulence of the Δfgl1 strain since mycelium growth was partly restored in the rachis. The inhibition of callose synthase activity by FFA was shown in an in vitro assay. Thus, the release of FFA due to the fungal lipase activity is necessary for full infection the host plant.

(1) Voigt *et al.* (2005) Plant Journal 42: 364-375

IXp-64

The emopamil-binding protein of *Botrytis cinerea*: role in pathogenicity and phylogenetic analysis

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Botrytis cinerea is the ascomycete responsible for the grey mould disease, affecting more than 200 dicotyledonous crop plants and ornamentals. Our group is investigating the molecular mechanisms of pathogenicity of *B. cinerea* by combining macroarray transcriptomic approaches and reverse genetics. The expression profiling of 3032 fungal genes during the infection of *A. thaliana* permitted the identification of 27 *in planta*-induced genes[1]. These genes are now systematically inactivated in order to test their implication in the infection process.

In this context, the BcPIE3 gene, highly expressed during the early stages of infection, was disrupted in two different wild-type strains of *B. cinerea*, T4 and B05.10. The ΔBcPIE3 mutant strain of T4 was severely altered in pathogenicity: After the appearance of primary lesions, the infections were blocked. On the contrary, the aggressivity of the BcPIE3 mutant strain of B05.10 was not affected, suggesting that the BcPIE3 protein is a strain-dependent vuirulence factor.

BcPIE3 protein shares significant similarity with mammalian proteins within a conserved region called emopamil-binding domain. Emopamil Binding Domain Proteins (EBDPs) belong to a superfamily of transmembrane proteins of the endoplasmic reticulum that bind a series of structurally diverse ligands. There are two types of EBDPs, called EBP and EBP-like. In mammals and plants, EBPs have a Δ8- Δ7 isomerase activity, involved in the biosynthesis of sterols and are able to bind fungicides and sigma ligands. EBP-like proteins have been only identified in vertebrates[2]; the human EBP-like protein, which is the only studied so far, lacks both the sterol isomerase and the ligand binding activity. EBP-like proteins have therefore a yet-to-be-identified function.

A biochemical study was undertaken in order to identify the function of the BcPIE3 protein. Moreover, the analysis of the *B. cinerea* and other fungal genomes revealed the presence of two proteins possessing the emopamil-binding domain.The phylogenetic relationships among fungal, vertebrate and plant EBDPs will be discussed.

1. Gioti *et al.*, (2006). J. Mol. Biol. (in press).
2. Moebius *et al.*, (2003). Biochem. J., 374; 229-237.

clp1 is essential for proliferation *in planta* during pathogenic development of *Ustilago maydis*

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Ustilago maydis is a phytopathogenic basidiomycete which is able to infect all aerial parts of its host plant *Zea mays*. After fusion of two haploid, yeast-like sporidia, the fungus switches from its saprophytic form to the biotrophic filamentous dikaryon. This switch and the subsequent steps in pathogenic development are controlled by the multiallelic *b*-mating type locus, encoding two unrelated homeodomain proteins bE and bW. The two proteins can form a heterodimeric complex, controlling genes required for pathogenicity via its function as a transcriptional regulator. *clp1* is one of more than 300 b-dependently regulated genes identified in a microarray approach. The encoded protein shows similarities to the Clp1 protein of *Coprinopsis cinerea* that is required and sufficient to trigger clamp formation [1]; however, the function of the protein is still unknown. In *U. maydis*, *clp1* is absolutely required for pathogenicity. Mutant strains are able to penetrate the plant surface but fail to proliferate in planta. Although the *clp1*-transcript is abundant rapidly after *b*-induction, the nuclear localization of Clp1 can first be observed at the point of plant penetration. The following steps of pathogenic development include the release of the b-induced cell-cycle arrest followed by massive fungal proliferation comprising the formation of clamp-like structures. Contrary to *C. cinerea*, formation of clamp-like structures is in *U. maydis* probably independent of the pheromone-pathway resulting in unfused clamp-cells and nuclear movement through a septal pore. To gain further insight into the function of Clp1, we performed ascreen for interacting proteins using the Y2H-system. Among 22 putative interacting proteins, we identified bW, Rbf1 and the so far unknown bZip transcription factor Cib1 (Clp1 interacting bZIP). The *rbf1* gene encodes a transcription factor essential for pathogenicity, regulating more than 90% of the b-dependent genes. Deletion of *cib1* leads to a phenotype strikingly similar to that of *clp1* strains. *Δcib1* strains are able to grow filamentous but development stops soon after penetration of the leaf surface. Therefore we suppose the function of Clp1 as transcriptional modulator leading to cell-cycle release and proliferation in planta. Additionally, this theory is supported by the finding that simultaneous induction of an active b-heterodimer and *clp1* strongly inhibits b-dependent filament formation. Although expression of the *b* genes is not altered *clp1* induction seems to counteract b-dependent transcriptional regulation.
These findings place Clp1 in the centre of the regulatory circuits that lead to the establishment of the biotrophic growth-phase.

[1] Inada *et al.* Genetics. 2001 Jan;157(1):133-140.

IXp-66

Exploring possible links between vegetive hyphal fusion and plant pathogenicity in the vascular wilt fungus *Fusarium oxysporum*

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Vegetative hyphal fusion (anastomosis) is an ubiquitous phenomenon in filamentous fungi, although the biological role of this process remains poorly understood. Recent studies in the model fungus *Neurospora crassa* have established that cell fusion during anastomosis and mating both require a mitogen-activated protein kinase (MAPK) pathway orthologous to the yeast Fus3/Kss1 pathway. This conserved MAPK cascade is also required for pathogenicity in a large number of biologically diverse plant pathogens, suggesting a possible evolutionary link between the signalling events that underly vegetative hyphal fusion and plant infection. To address this hypothesis, we have studied the role in vegetative hyphal fusion of Fmk1, the orthologous MAPK of the vascular wilt fungus *Fusarium oxysporum*. *fmk1* mutants are non-pathogenic on tomato plants and defective in a number of pathogenicity-related functions. We found that *fmk1* mutants are unable to undergo vegetative hyphal fusion similar to *N. crassa mak-2* mutants. To further study the possible relationship between anastomosis and pathogenicity, we have cloned and knocked out the *F. oxysporum fso* gene, an orthologue of *N. crassa so,* encoding a WW domain protein required for vegetative hyphal fusion. Similar to *fmk1* mutants, the *fso* mutants do not form heterokaryons, suggesting a conserved role of Fso in hyphal fusion between *Neurospora* and *Fusarium*. However, in contrast to *fmk1* strains, *fso* mutants still are able to perform a number of pathogenicity-related functions such as secretion of pectinolytic enzymes or invasive growth on plant tissue. Based on these results we propose that Fso mediates hyphal fusion by acting either downstream of Fmk1 or via an Fmk1-independent pathway. Thus, while plant pathogenicity and vegetative hyphal fusion both require the Fmk1 MAPK cascade, the two processes can be separated genetically.

Suppression of wheat plant defense by a successful *Fusarium graminearum*

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The fungal pathogen *Fusarium graminearum* is the most common causal agent of Fusarium head blight (FHB) of small grain cereals and of cob rot of maize in both temperate and in semitropical areas throughout the world. The threat posed by this fungus is due to yield decreases and mycotoxin contamination. We generated *F. graminearum* mutants deficient in specific virulence traits i.e. ΔTRI5 strains, deficient in trichothecene mycotoxin production and ΔFGL1 strains, deficient in secreted lipase activity. Both disruptions led to a strong reduction of virulence. For histological analysis of the infection patterns in wheat, wild-type and knockout mutants were marked by constitutively expression of GFP. The wheat rachis is utilized by the wild type as avenue to colonize adjacent spikelets. In the absence of the trichothecenes, the fungus is blocked by the development of heavy cell wall thickenings in the rachis node. This plant defence reaction is suppressed by the trichothecene mycotoxins. The loss of FGL1 function results in a plant callose formation in the rachis. This barrier prevents *F. graminearum* from spreading out through the rachis of the inoculated wheat spikelet, too. By using the described fungal virulence mutants we could identify two different plant defence reactions, each of them sufficient for wheat plant resistance. The suppression of both resistance reactions by *F. graminearum* is necessary for successful fungal invasion.

Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K-H., Felk, A., and Maier, F.J.: Infection patterns in barley and wheat spikes inoculated with wild type and trichodiene synthase gene disrupted *Fusarium graminearum*. PNAS 102(46), 16892–16897 (2005).
Voigt, C.A., Schäfer, W., and Salomon, S.: A secreted lipase of *Fusarium graminearum* is a novel virulence factor during infection of cereals. The Plant Journal 42, 364–375 (2005).

IXp-68

Comparative analysis of expressed sequence tags (ESTs) generated from four specific-stages of *Phakopsora pachyrhizi*

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The fungal pathogen *Phakopsora pachyrhizi* causes soybean rust, the most devastating foliar disease responsible for significant losses of soybean crop in Africa, Asia, Australia and South America. The pathogen was recently found for the first time in the continental U.S.A. and has spread to more than 110 counties in the Southeastern states and Texas, becoming a major thrat to the U.S. soybean production.

Here, we present a comparative analysis of 44.000 expressed sequence tags (ESTs) generated from four specific-stages of *Phakopsora pachyrhiz*, funded by the USDA/Agricultural Research Service and the US Department of Energy.

Ph signalling in *Botrytis cinerea*

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During plant infection, necrotrophic fungi like *B. cinerea* are known to secrete a large panel of lytic enzymes that participate in pathogenesis through at least the degradation of the plant polymers. The production of some of these enzymes has been shown to be under the control of several environmental signals that emanate mainly from the chemical composition of the medium (nutrients and pH). For each of these enzymes is tuned to target a particular substrate and to function at a given optimal pH, it is expected that one or more signal transduction pathways will regulate the transcription of each of their corresponding genes. The pH-signalling pathway in fungi consists of seven proteins of which two are predicted to reside in the cell membranes (PalH and PalI). Neutral or alkaline pH is sensed by the cell via un unknown mechanism and it then triggers an activation of the pathway that leads to the proteolytic activation of the transcriptional regulator PacC. Active PacC migrates to the cell nucleus and binds to target genes to either increase or decrease their expression. Through the study of pacC and palH deletion mutants, our work investigates the importance of pH signalling in the pathogenicity of B. cinerea and a special interest is taken in the pH control over the fungus secretome.

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Fusarium oxysporum Rho1 regulates cell wall synthesis, hyphal growth and virulence on tomato plants

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The family of *RHO* genes is implicated in the control of cellular morphogenetic events in eukaryotes. The Rho family GTPase Rho1 has been shown to control polarity establishment and cell wall deposition in *Saccharomyces cerevisiae*. In yeast, biosynthesis of β-1,3-glucan, a structural component of the cell wall is the result of the activity of at least a two-component complex, the catalytic subunit Fks1p and the GTP-dependent regulatory subunit Rho1p. In the present study a gene encoding a putative Rho1 orthologue was cloned from the vascular wilt pathogen *Fusarium oxysporum.* The *rho1* gene encodes a predicted 195 amino acid polypeptide with homology to fungal Rho1 proteins. The *Δrho1* mutant lacking a functional copy of *rho1* was viable and grew similar to the wild type strain in submerged culture. However, the *Δrho1* strain exhibited severly restricted colony growth on solid substrates, increased sensitivity to Calcofluor white and Congo red and cell lysis. Strains carrying ectopic copies of the constitutively active rho^{G14V} allele or the dominant negative rho^{E40I} allele were generated in a *Δrho1* background. None of the two mutated alleles restored the wild type growth phenotype in the *Δrho1* mutant, whereas introduction of a wild type *rho1* allele did. The β -1,3-glucan synthase and chitin synthase activities were determined in *Δrho1*, rho^{G14V}, rho^{E40I} and wild type strains, using membrane fractions as an enzyme source. Glucan synthase activity was increased in rho^{G14V} and decreased in *Δrho* and rho^{E40I} strains relative to wild type. In contrast, chitin synthase activity was increased in *Δrho1* and rho^{E40I}, and decreased in rho^{G14V}. [^{32}P]-ADP-ribosylation withy *Clostridium botulinum* C3 exotoxin detected a 22 kDa protein with a pI of 6.2. The *Δrho1* mutants showed dramatically reduced virulence on tomato plants. Our results indicate that Rho1 controls cell wall synthesis, hyphal growth and virulence in *F. oxysporum*.

Genetic analysis of the role of a small extracellular protein of an apoplast-colonizing plant pathogenic fungus

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Septoria lycopersici is a hemibiotrophic, non-obligate fungal pathogen of tomatoes. *S. lycopersici* is closely related to several other economically important fungal pathogens including *Mycosphaerella graminicola* (the causal agent of wheat leaf blotch) and *Septoria passerinii* (the causal agent of speckled leaf blotch of barley). These pathogens share the distinct infection mechanism of hyphal colonization of the apoplastic space. Fungi which colonize the apoplastic space differ from other fungal plant pathogens in that they do not form specialized infection structures such as appressoria or haustoria. The fungal spores of apoplast-infecting fungi germinate on the leaf surface. Hyphae then grow until they find an open stomata or other opening such as a wound. Once in the apoplastic space, the hyphae will proliferate until the space is filled. It has been suggested that these fungi primarily utilize the secretion of fungal virulence proteins into the host apoplastic space to promote interaction with the plant cell. In order to better understand pathogenesis, we are using a genetic approach with emphasis on the proteins which are secreted into the apoplastic space by *S. lycopersici*.

One previously identified *S. lycopersici* secreted protein is SEP (small extracellular protein). The crystal structure of the SEP protein has been solved, and the structure is that of a beta-trefoil fold protein. *S. lycopersici* mutants which lack SEP have been generated using *Agrobacterium*-mediated transformation. These mutants have no obvious growth or conidiation defects and are still able to cause wildtype levels of disease. However, these mutants do display a subtle increase in the amount of hyphal branching observed *in planta*. This phenotype is not seen when hyphal growth is observed *in vitro*. Complementation analyses have shown that the *in planta* hyphal growth phenotype is due to the absence of the SEP protein. This study suggests that the SEP protein may play a role in the interaction between *S. lycopersici* and its host plant tomato.

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A secondary metabolite is involved in recognition of the blast fungus *Magnaporthe grisea* by resistant rice cultivars

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Recognition of the plant pathogenic fungus *Magnaporthe grisea* by resistant rice cultivars is controlled by interactions between fungal avirulence genes (*AVR*) and corresponding rice resistance genes (*R*). Fungal *AVR* genes encode small secreted peptides except *ACE1* from *M. grisea*. This *AVR* gene encodes for a polyketide synthase fused to a non-ribosomal peptide synthetase, an enzyme involved in the biosynthesis of a secondary metabolite. *ACE1* is specifically expressed in mature appressoria during penetration of the fungus into host plant leaves. Functional analysis of *ACE1* promoter led to the identification of a 58 bp region required for its appressorium-specific transcription that contains a *Ste12* and a fungal binuclear zinc finger transcription factor binding site. Assessment of their role in the control of *ACE1* expression is currently performed using site-directed mutagenesis. The protein Ace1 is only detected in the cytoplasm of appressoria and does not seem to move into infectious hyphae differentiated inside host plant tissues. *Ace1-ks0*, a non-functional *ACE1* allele obtained by site-directed mutagenesis of an amino acid of the polyketide synthase KS domain essential for its enzymatic activity, is unable to confer avirulence. This result suggests that the avirulence signal is not Ace1 protein, but the secondary metabolite synthesized by Ace1. In order to characterize this metabolite, we have performed a metabolic profiling of *M. grisea* appressoria by LC-MS-MS differentiated on onion epidermis. Fungal metabolites were detected but none was specific of avirulent isolates. Alternatively, *ACE1* has been expressed under the control of a constitutive promoter. New metabolites produced by these transgenic strains are currently analyzed. 14 genes predicted to encode enzymes involved in secondary metabolism, including two enoyl-reductases and a binuclear zinc-finger transcription factor are located close to *ACE1* within a region of 70 kb. These genes displayed the same penetration-specific expression pattern as *ACE1*, defining a cluster of co-expressed genes that could define a new biosynthetic pathway. The inactivation of these genes in an avirulent isolate is underway to evaluate their role in the biosynthesis of the metabolite recognized by resistant rice cultivars.

Böhnert, H. U. *et al.* (2004). A Putative Polyketide Synthase/Peptide Synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. *The Plant Cell*, **16**:2499-2513.