Abstract from the EFGC meeting (4/5-8/08) that mentioned Fusarium.

Novel ways to explore sequenced fungal genomes

Kim Hammond-Kosack, Andrew Beacham, Thomas Baldwin, Martin Urban, Salvador Gazan, John Antoniw
Rothamsted Research, Harpenden, United Kingdom

Complete genomic sequence information is now readily available for many pathogenic and non-pathogenic fungal species and in the near future this will extend to different strains of a single species. For some species this new sequence information has been aligned to an existing genetic map. By exploring and visualising gene content and gene type within the chromosome landscape new information on the evolution of fungal genomes can be obtained.
The sequenced genome of the cereal attacking Ascomycete pathogen *Fusarium graminearum* (teleomorph *Gibberella zeae*) has been explored at various scales using a new visualisation tool in combination with statistics, Affymetrix gene expression data, gene function categories and recombination frequencies.
These combined analyses have revealed that genes coding for specific protein types are non randomly distributed over the four *F. graminearum* chromosomes. Some gene types are located only in centromeric regions, whereas others only reside in sub-telomeric regions, for example *in planta* expressed genes. In addition, regions of high recombination tend to harbour specific protein classes, for example polyketide synthases, whereas other gene types are solely located in regions of low /no recombination. These analyses have also revealed the existence on chromosome 1 of a ~25 kb micro-region rich in homologues of pathogenicity genes and that most of the experimentally verified *F.graminearum* pathogenicity genes do not reside in either sub-telomeric regions or regions with a high recombination frequency.
The four large *F. graminearum* chromosomes are hypothesised to have arisen from a series of earlier chromosome fusion events. The gene content of these genomic sub-regions is unique and reflects this suspected evolutionary path.

Msb2, a putative membrane mucin functioning in signalling and pathogenesis of *Fusarium oxysporum*

Elena Perez-Nadales, Antonio Di Pietro
University of Cordoba, Cordoba, Spain

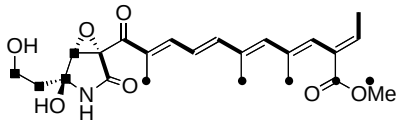
The soilborne vascular wilt fungus *Fusarium oxysporum* infects a wide variety of plant species by directly penetrating roots, invading the cortex and colonizing the vascular tissue. The mitogen activated protein kinase (MAPK) Fmk1 is essential for plant infection. The signalling components upstream of the Fmk1 cascade are currently unknown. We have identified a gene from *F. oxysporum* whose predicted product shows homology with Msb2, a mucin functioning at the head of the filamentation MAPK cascade in yeast. To test whether *F. oxysporum* Msb2 is involved in signalling through the Fmk1 pathway, we produced targeted knockout mutants both in the wild type and the Δ*fmk1* background. The Δ*msb2* strains were still able to grow invasively on fruit tissue and across cellophane sheets, but are less virulent on tomato plants than the wild type strain. Similar to Δ*fmk1* strains, Δ*msb2* mutants also showed reduced secretion of pectinolytic enzymes. In contrast to Δ*fmk1* mutants, growth of Δ*msb2* strains was specifically affected by the cell wall-targeting compounds Congo Red (CR) and Calcofluor White (CFW). Interestingly, a Δ*fmk1*Δ*msb2* double mutant had dramatically impaired growth in the presence of CR and CFW, suggesting that Fmk1 and Msb2 interact genetically to regulate cell integrity in *F. oxysporum*. Western analysis with phospho-ERK antibody revealed similarities in the phosphorylation pattern between Δ*fmk1* and Δ*msb2* strains. These results suggest that *F. oxysporum* Msb2 interacts with the Fmk1 cascade to regulate a number of virulence-related functions as well as response to cell wall stress.

Hybrid polyketide synthase - non-ribosomal peptide synthetases (PKS-NRPS) in fungi

Russell Cox, Colin Lazarus, Andrew Bailey, Thomas Simpson, Zhongshu Song, Laura Halo

University of Bristol, Bristol, United Kingdom

The genomes of filamentous fungi contain numerous gene clusters for the biosynthesis of complex secondary metabolites. These usually include polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS) and terpenoid synthases, but recently we have shown that hybrid PKS-NRPS systems are relatively common and responsible for the biosynthesis of diverse compounds known as acyl tetramic acids. We have worked with PKS-NRPS genes involved in the biosynthesis of Tenellin (*Beauveria bassiana*) and Fusarin-C (*Fusarium venenatum*). These 12Kb ORFs are difficult to manipulate using traditional 'cut and paste' approaches so yeast recombination, combined with GATEWAY *in vitro* recombination has been used to rapidly assemble expression vectors. Expression in *Aspergillus oryzae* has been achieved and the chemical analysis of the compounds produced reveals much about the *programming* of the PKS-NRPS proteins. Co-expression and knockout strategies have revealed more about the post-assembly reactions and have allowed us to probe the wider gene clusters.



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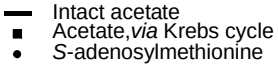

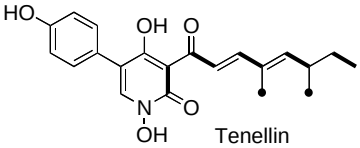

Tenellin

Variation in *TRI1* in thirteen trichothecene-producing species of *Fusarium*: evidence for a complex evolutionary history of a mycotoxin biosynthetic locus

Robert Proctor, Susan McCormick, Nancy Alexander, Anne Desjardins
United States Department of Agriculture, Agriculture Research Service, National Center for Agricultural Utilization Research, Peoria, Illinois, United States

Trichothecenes are mycotoxins produced by several genera of fungi, including some agriculturally important *Fusarium* species. In the two species, *Fusarium graminearum* and *F. sporotrichioides*, that have been examined most thoroughly, trichothecene biosynthetic enzymes are encoded at three loci: 1) the 12-gene, core trichothecene biosynthetic gene (*TRI*) cluster, 2) the *TRI1*/*TRI16* locus, and 3) the *TRI101* locus. In *F. graminearum*, the cytochrome P450 monooxygenase encoded by *TRI1* catalyzes trichothecene C-7 and C-8 hydroxylation, whereas in *F. sporotrichioides*, the enzyme catalyzes only C-8 hydroxylation. Here, nucleotide sequence analysis of 13 *Fusarium* species distinguished four transpecies groups of *TRI1*. Within each group, predicted *TRI1* amino acid sequences are 85-99% identical, and between groups, sequences are only 65-75% identical. *TRI1* sequence variation is not correlated with phylogenetic relationships of *Fusarium* species inferred by maximum parsimony analysis of nucleotide sequences of beta-tubulin, translation elongation factor, and two core *TRI* cluster genes. Sequence analysis of *TRI1*-flanking regions indicated that *TRI1* exists in at least three distinct genetic environments. In contrast to variation in *TRI1* sequence, these genetic environments are highly correlated with inferred phylogenetic relationships of *Fusarium* species. Together, these and other data indicate that the evolutionary history of the *TRI1/TRI16* locus has been complex and has involved ancestral polymorphism as well as gene relocation, inversion and nonfunctionalization.

Global regulation of bikaverin biosynthesis in *Fusarium fujikuroi*

Philipp Wiemann[1], Marita Beyer[2], Hans-Ulrich Humpf[2], Bettina Tudzynski[1]
[1]Institute of Botany, WWU Münster, Münster, Germany, [2]Institute of Food Chemistry, WWU Münster, Münster, Germany

The phytopathogenic ascomycete *Fusarium fujikuroi* produces different secondary metabolites, including gibberellins (GAs) and the red polyketide pigment bikaverin, which was lately shown to act cytotoxically on several human cancer cell lines. The polyketide synthase gene *bik1* was previously shown to be responsible for the first biosynthetic step forming an unmodified nonaketide. Here we present cloning and functional characterization of five new genes, *bik2* to *bik6*; all organized in a bikaverin gene cluster. Furthermore, we studied the complex regulation of these genes in response to different environmental conditions such as nitrogen concentration, pH-value and different growth stages.
The genes *bik2*, *bik3*, *bik5* and *bik6* encode a FAD-dependent monooxygenase, an *O*-methyltransferase, a $Zn(II)_2Cys_6$ transcription factor and a MFS transporter, respectively. Bik4 belongs to the family of NmrA-like proteins but its role in bikaverin production is not clear yet. HPLC-MS analysis of deletion mutants of all *bik* genes showed loss or reduction of the red pigment, demonstrating that they encode proteins involved in bikaverin biosynthesis. Northern blot analysis revealed that expression of all *bik* genes is strongly dependent on the cluster specific zinc finger transcription factor Bik5. Beyond this pathway specific regulation, expression of all genes is repressed by high amounts of nitrogen and basic pH. We show that the general transcription factor AreA is not essential, but might be indirectly involved in nitrogen regulation of bikaverin biosynthesis. Deletion of *pacC*, encoding the Cys_2His_2 zinc finger regulator PACC, resulted in de-repression of the *bik* genes under alkaline pH conditions indicating that PACC acts as a repressor. Furthermore, expression analysis of the bikaverin biosynthetic genes in time course experiments showed a downregulation of these genes after 3 days. Deletion of the "velvet" homologue gene *veA* resulted in an expression of the *bik* genes up to the 10^{th} day. In contrast, overexpression of *veA* let to a downregulation of the *bik* genes even at early time points. Interestingly, the GA genes are regulated by VeA in the opposite way. Our results reveal new aspects of different regulation patterns for genes organized in one cluster in *F. fujikuroi*, responsible for biosynthesis of the cytotoxic bikaverin, and make this gene cluster an excellent model for regulation studies of secondary metabolism in an ascomycete.

Subcellular localisation of two pathway-specific geranylgeranyl diphosphate synthases in the filamentous fungus *Penicillium paxilli*

Sanjay Saikia, Barry Scott
Centre for Functional Genomics, IMBS, Massey University, Palmerston North, New Zealand

In the filamentous fungi *Penicillium paxilli*, *Neotyphodium lolii*, *Fusarium fujikuroi*, and *Aspergillus flavus* two distinct geranylgeranyl diphosphate (GGPP) synthases, one for the primary metabolism (*P. paxilli* Ggs1-related proteins) and the other for secondary metabolism (*P. paxilli* PaxG-related proteins) are present. The genomes of other filamentous fungi including *A. clavatus*, *A. fumigatus*, *A, nidulans*, *A. niger*, *A. oryzae*, *A. terreus*, *F. graminearum*, and *Neosartoya fischeri* also contain two or more copies of genes homologous to *paxG*, although the secondary metabolite phenotype of most of these fungi are not known. To understand the biological significance of the presence of multiple copies of GGPP synthases across these genera we are using *P. paxilli* as our model organism in conjunction with reporter fusion vectors, deletion and complementation analyses. We found that the presence of *ggs1* failed to complement a *paxG*-deletion mutant strain. These observations suggest that the two metabolic pathways are compartmentalised. To test this, reporter fusion studies were conducted and found that Ggs1-EGFP fusion protein was localised to punctuate structures and EGFP-GRV fusion protein, containing the C-terminal tripeptide GRV of PaxG, was localised to peroxisomes. These and more recent findings will be presented.

Comparative genomics of the transcription factors *ftf1* and *ftf2* in *Fusarium*

Brisa Ramos[2], José J. de Vega-Bartol[1], María A. García-Sánchez[1], Noemí Martín-Rodrigues[3], Arturo P. Eslava[1], José M. Díaz-Mínguez[1]
[1]Centro Hispano-Luso de Investigaciones Agrarias (CIALE), Universidad de Salamanca, Salamanca, Spain, [2]Centro de Biotecnología y Genómica de Plantas (CBGP), Universidad Politécnica de Madrid, Madrid, Spain, [3]Instituto NEIKER, Centro de Arkaute, Vitoria, Spain

We have described a new transcription factor, called *ftf1*, which is likely a virulence factor in *F. oxysporum*, as it is found only in highly virulent strains and is drastically upregulated during infection of the host plant. We have also identified a highly homologous gene to *ftf1*, named *ftf2*, which can be found in *F. oxysporum* strains, either pathogenic and nonpathogenic.
Experimental results obtained in *F. oxysporum* f.sp. *phaseoli* and analyses of the genome sequences of *F. graminearum*, *F. verticillioides* and the recently available *F. oxysporum* f.sp. *lycopersicy*, show that *ftf1* is a multiple copy gene only present in *F. oxysporum*. The four copies in *F. oxysporum* f.sp. *phaseoli* are located in a small chromosome, closely linked to copies of transposon *marsu*, while the nine copies found in the genome of the *lycopersicy* strain are scattered over different chromosomes of the optic map, but also linked to different kinds of transposons, including *marsu* and *Fot* types. On the other hand, *ftf2* is a single copy gene present in the genomes of the three fusaria, the rice pathogen *Magnaporthe grisea* and the nonpathogenic fungi *Neurospora crassa* and *Aspergillus nidulans*. The genomic architecture of the *ftf2* region is very similar in the three fusaria and shows syntheny with the *M. grisea* genome, but syntheny is lost in the nonpathogenic fungi *A. nidulans* and *N. crassa*.
The three *Fusarium* genus members show different host ranges, being *F. oxysporum* a very versatile pathogen with dozens of formae speciales, while *F. verticillioides* is restricted to maize and *F. graminearum* can only infect barley and wheat. The analyses of the genome sequences have the potential to enlighten our understanding of the genetic basis of host specificity. The results here presented show that the, comparatively, larger genome of *F. oxysporum* contains species specific genes (such as *ftf1*) involved in virulence. The linkage between *ftf1* and transposable sequences and the high homology of this gene to *ftf2* suggest that it may have evolved by gene duplication and the resulting copies become dispersed as part of genome reorganizations.

Pathogenicity determinants of *Fusarium graminearum* on wheat ears
Andrew Beacham, John Antoniw, Martin Urban, Kim Hammond-Kosack
Rothamsted Research, Harpenden, United Kingdom

Using a novel bioinformatics approach, we have identified a micro-region of verified pathogenicity gene homologues in the important crop pathogen *Fusarium graminearum*. This region is now being analysed by a combination of bioinformatic and reverse genetics approaches to ascertain its role in the pathogenicity of this species.
Comparative genomics using FASTA alignment, protein identity and property prediction, together with nucleotide repeated element and gene expression analyses have been used to investigate the conservation and properties of the micro-region in other closely and less closely related species. Targeted deletion of single genes using the split marker technique has allowed the determination of the role of each micro-region gene in *F. graminearum* pathogenicity.
The micro-region, which contains 15 genes and spans 25kb in *F. graminearum*, appears highly conserved in the other *Fusarium* species but less so in more distantly related species. It is present in a region of the genome with a low recombination frequency and has a low content of repetitive sequence. The region appears, unlike other fungal gene clusters, not to exhibit coordinated gene expression either during *in vitro* growth or during plant infection. Deletion of the neutral trehalase gene *NTH1* appears to slow infection of wheat ears, while deletion of the *SNF1* kinase gene inhibits spread of the pathogen in wheat.
The micro-region is beginning to appear as a novel type of pathogenicity gene cluster, differing from the virulence-associated biosynthetic and secreted protein clusters identified so far in pathogenic fungi. Further investigation will reveal more about the nature of this unusual pathogenicity region.

Analysis of the *Trichoderma atroviride* transcriptome during mycoparasitism
Barbara Reithner, Enrique Ibarra-Laclette, Alfredo Herrera-Estrella
Cinvestav Campus Guanajuato, Irapuato, Guanajuato, Mexico

Trichoderma spp. have been shown to attack and lyse various plant-pathogenic fungi using a complex mechanism. The way mycoparasitic *Trichoderma* strains recognize their hosts is to a large extent still unknown. But it has already been shown that they grow towards the target

fungi, attach to and lyse the host cell wall by secreting antifungal enzymes and secondary metabolites. The importance of biocontrol and plant protection by *Trichoderma* result from the necessity to reduce the application of chemical fungicides to antagonize plant diseases caused by pathogenic fungi such as *Rhizoctonia*, *Phythium*, *Phytophthora*, *Botrytis*, and *Fusarium*. In the present work we concentrate on the mycoparasitic interaction of *T. atroviride* using plant-pathogens of different phylla to gain information about the host-specific response of *Trichoderma*. Therefore, we used a powerful sequencing system (pyrosequencing by 454 Life Science Technologies) in combination with a new concept of multiplex identifiers to pool genes which are differentially expressed during different stages of confrontation with *R. solani*, *P. capsici* and the non-pathogenic fungus *Neurospora crassa*. This technique allowed us to obtain approximately 250.000 reads in one run of a cDNA mixture of all samples. The assembled contigs and singletons were blasted against the available genomes of *T. reesei*, *T. virens*, *F. graminiarum*, and the genomes of the host fungi and compared between the conditions used. Differences were discovered between the EST's obtained from the plate-confrontationes assays with different plant-pathogenic fungi as well as for the different stages of interactions analyzed. In addition to the qualitative distinction, we can show that differences in gene-expression found with this method are extremely quantitative.
The present study displays an extensive analysis of genes involved in the crosstalk between *T. atroviride* and it's hosts.

Identification of *Fusarium graminearum* proteins differentially expressed during mycotoxin synthesis

Linda Harris, Rebecca Taylor, Audrey Saparno, Barbara Blackwell, Valar Anoop, Steve Gleddie, Danielle Schneiderman, Nicholas Tinker
Eastern Cereal & Oilseed Research Centre, Agriculture & Agri-Food Canada, Ottawa, Ontario, Canada

Fusarium graminearum (*Gibberella zeae* [Schwein.] Petch) produces trichothecene mycotoxins early after plant contact to facilitate host invasion. We have attempted to identify genes, proteins, and pathways that may be induced under conditions that trigger mycotoxin synthesis. A shotgun proteomics approach was used to monitor protein expression changes under aseptic liquid culture conditions conducive to trichothecene production in the absence of contaminating plant proteins. The non-gel-based quantitative iTRAQ technology was used for proteomic profiling of three time course biological replicates. Statistical analysis of a filtered dataset of 435 proteins revealed 130 *F. graminearum* proteins that exhibited significant changes in expression. There was good agreement between up-regulated proteins identified by 2-D PAGE/tandem mass spectrometry and iTRAQ. Seventy-two proteins were significantly up-regulated relative to their level at the initial phase of the time course and this group included predicted secreted proteins, cellular transport proteins, homologs of other fungal virulence proteins, and many conserved hypothetical proteins. We are currently disrupting several genes encoding proteins identified in this study to explore function and contribute to our search for mechanisms of host invasion and novel antifungal targets.

Survey of mRNA isoforms in *Fusarium verticillioides* by ESTs: alternative splicing is part of the story

Daren Brown[1], Robert Butchko[1], Li-Jun Ma[2]
[1]USDA-ARS, Peoria, IL, United States, [2]The Broad Institute of MIT, Cambridge, MA, United States

The filamentous fungus *Fusarium verticillioides* is a pathogen of maize and synthesizes a number of economically important toxins including fumonisins. Fumonisins cause a variety of animal diseases and have been shown to cause cancer in some animals. Contaminated maize and maize products lead to substantial losses to farmers each year. The transcriptional and posttranscriptional regulatory mechanisms controlling fungal pathogenesis and toxin production are poorly understood. Analysis of over 87,000 *F. verticillioides* ESTs identified a total of 578 genes with one or more mRNA isoforms and is equivalent to 6.3% of the genes represented. The most common isoform found correspond to alternatively spliced transcripts. Alternative splicing (AS) in higher eukaryotes play a critical role in expanding gene function by

increasing protein diversity and by affecting mRNA stability. The different AS forms (ASF) include intron retention (73%), alternative donor or acceptor sites (25%) and exon skipping (2%). The percentage of genes with ASFs and the ratio of different ASFs is substantial different than that found in mammals and plants. Preliminary analysis of predicted open reading frames found that 28% of the AS events could generate altered proteins while 54% introduce frame shifts or stop codons and 16% were in 5' or 3' non-coding sequences. Transcript isoforms were also found that reflect multiple transcriptional initiation start as well as termination sites. And finally, we found sets of antisense RNA transcripts of which some have ASFs including retained introns and alternative donor and acceptor sites. Analysis of the *Fusarium* transcriptome indicate a complexity previously not described and the continued study of ASFs and their possible function will aid our understanding of fungal gene regulation.

Insight into the molecular requirements for pathogenicity of *Fusarium oxysporum* f. sp. *lycopersici* through large-scale insertional mutagenesis

Caroline Michielse, Ringo van Wijk, Linda Reijnen, Ben Cornelissen, Martijn Rep
University of Amsterdam, Amsterdam, Netherlands

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 10,209 random insertion mutants were generated using T-DNA of *Agrobacterium tumefaciens* as an insertional mutagen.
All transformants were screened for loss of pathogenicity on tomato. This led to the identification of 20 non-pathogenic and 86 reduced-in-virulence mutants. The genomic regions flanking the T-DNA were isolated by TAIL-PCR. In total 129 potential pathogenicity genes were identified of which several known pathogenicity genes, such as class V chitin synthase, Zn(II)2Cys6 transcription factor FOW2, carbon catabolite derepressing protein kinase SNF1 and mannose-6-phosphate isomerase. Based on the putative function of the proteins identified, several general and specific processes seem to play a role in pathogenicity, e.g. certain metabolic pathways, peroxisome biogenesis and protein mannosylation.
Complementation of three insertion mutants confirmed a role for peroxisomes and a probable cell wall mannosidase in pathogenicity.

The role of the fungal f-box protein Frp1 in colonisation of tomato roots by *Fusarium oxysporum* f.sp. *lycopersici*

Wilfried Jonkers, Ben Cornelissen, Martijn Rep
University of Amsterdam, Amsterdam, Netherlands

Microbial pathogens have to break plant defence systems 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, 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.
We found that the Δ*frp1* mutant has a defect in assimilation of certain carbon sources which might be related to the loss of pathogenicity. The mutant shows decreased growth on certain root exudate components, cell wall components and C_2-carbon sources like ethanol and acetate, as well as fatty acids. The mutant is unable to induce expression of genes required for assimilation of these carbon sources: the Isocitrate lyase gene (*ICL*) encoding an enzyme of the glyoxylate cycle and the cell wall degrading enzyme genes polygalacturonase 1 (*PG1*), exo-polygalacturonase (*PGX*), and pectate lyase (*PL*). A gene knock out of *ICL* (Δ*icl*) turned out to be not required for pathogenicity, but did resemble the phenotype of Δ*frp1* strain on certain carbonn sources.
Microscopic studies with a wild type and *Δfrp1* mutant strain transformed with GFP revealed the inability of the mutant to form a hyphal network around the roots of tomato seedlings.
We conclude that the F-box protein Frp1 is required for assimilation of the available nutrients from the plant and, therefore, for development of its pathogenic phase. Unravelling the

biological and molecular role of Frp1 and its target proteins will provide more insight into the first stages of the infection process of pathogenic fungi.

Siderophore biosynthesis in *Epichloë festucae* and its role in maintaining a mutualistic interaction with its host grass *Lolium perenne*

Melle Steringa[1], Albert Koulman[1], Michael Christensen[1], Wayne Simpson[1], Karl Fraser[1], Richard Johnson[1], Iain Lamont[2], Linda Johnson[1]
[1]AgResearch Limited, Palmerston North, New Zealand, [2]University of Otago, Dunedin, New Zealand

Epichloë and *Neotyphodium* fungal endophytes form mutualistic associations with cool-season grasses, considerably increasing the survival of their hosts during abiotic and biotic stress. Elimination of extracellular siderophore biosynthesis by disruption of the encoding nonribosomal peptide synthetase (NRPS) gene *sidN* from the obligate grass symbiont *E. festucae* has previously been shown to perturb symbiosis with its host *Lolium perenne* (perennial ryegrass). Plants inoculated with the *sidN* mutant were severely stunted and the fungus exhibited excessive hyphal branching. These phenotypes are likely to be due to a disturbance of iron homeostasis within the symbiotum as a result of loss of siderophore production. To further investigate the role of iron and siderophore biosynthesis in grass-endophyte interactions, other genes putatively involved in siderophore biosynthesis are functionally being characterised in this study.
The intracellular siderophore ferricrocin was previously identified from *E. festucae* mycelial extracts by LCMSMS, alongside a NRPS gene with high similarity to the ferricrocin NRPS genes *NPS2* and *sidC* from *Fusarium graminearum* and *Aspergillus nidulan*s respectively. Targeted gene replacement of this putative *E. festucae sidC* orthologue eliminated ferricrocin biosynthesis. However, plants inoculated with the *sidC* mutant were not stunted and hyphae *in planta* did not show excessive branching under light microscopy, showing that ferricrocin is less critical than the extracellular siderophore in maintaining symbiosis. Currently, experiments are under way to determine whether absence of ferricrocin has more subtle effects on grass-endophyte interactions. Studies include Transmission Electron Microscopy (TEM) to investigate hyphal ultrastructure of the *sidC* mutant *in planta*, and *in vitro* growth assays to explore the potential involvement of ferricrocin in resistance to oxidative stress. A double targeted gene replacement of both *sidC* and *sidN* is currently being created to determine how elimination of biosynthesis of both siderophores affects symbiosis. In addition, a targeted gene deletion of a putative *E. festucae urbs1* orthologue from *Ustilago maydis,* encoding a GATA-type transcription factor that regulates siderophore biosynthesis, will further elucidate the role of siderophore biosynthesis and iron in maintaining these mutualistic grass-endophyte symbioses.

RNAi silencing of the virulence gene *ftf1* of F*usarium oxysporum* causes a reduced pathogenicity

Jose Javier de Vega Bartol[1], Bart P. H. J. Thomma[2], Jose Maria Diaz-Minguez[1]
[1]Centro Hispano-Luso de Investigaciones Agrarias (CIALE), University of Salamanca, Salamanca, Spain, [2]Laboratory of Phytopathology, Wageningen University, Wageningen, Netherlands

Fusarium oxysporum is a soil-born fungus that causes vascular wilt disease by penetrating the plant roots and colonizing the plant xylem vessels. We have isolated and described a new transcription factor (*ftf1*) that is likely a virulence factor, as its distribution is limited to highly virulent strains of several formae speciales of *F. oxysporum*, and its expression is drastically upregulated in planta. *ftf1* is found in multiple copies, the number varying in different formae speciales (four copies in f.sp. *phaseoli* and up to nine in the sequenced strain of f.sp. *lycopersici*). This feature makes it very difficult to assess the role of *ftf1* by standard knock-out methodology.
In order to check whether *ftf1* is required for fully pathogenic *F. oxysporum* strains, an RNA interference (RNAi) vector was constructed to target expression of the virulence gene. Upon *Agrobacterium tumefaciens*-mediated transformation of this construct into *F. oxysporum* several transformants were obtained. We analyzed these transformants for a) growth in rich

medium, b) virulence in infection essays conducted under greenhouse conditions, and c) *ftf1* expression by means of quantitative RT-PCR. Preliminary results on tomato indicate that some transformants clearly showed reduced virulence in planta when compared to the wild type infection control, and a significative reduction of in planta expression of *ftf1*. We are currently investigating the effect of the silencing the *ftf1* gene in strains of f.sp. *phaseoli*. The experimental confirmation of the power of gene silencing for abolishing the expression of multi-copy genes would make this tool very valuable for functional analysis in *Fusarium*.

VeLB, a member of the velvet family, is a virulence factor in the vascular wilt fungus *Fusarium oxysporum*

Manuel Sánchez López-Berges, Concepción Hera, Antonio Di Pietro
University of Córdoba, Córdoba, Spain

Forward genetic screens are efficient tools to dissect complex biological processes such as fungal pathogenicity. A transposon tagging system was used to search for novel virulence genes in the vascular wilt fungus *Fusarium oxysporum*. A strain exhibiting reduced virulence on tomato plants carried a transposon insertion 80 bp upstream of the open reading frame FOXG_00016. The predicted gene product is a small protein with homology to *Aspergillus nidulans* VelB, a member of the velvet family involved in regulation of fungal development and secondary metabolism. Targeted knockout and functional complementation of the insertion mutant with the wild type *velB* allele confirmed its role in virulence of *F. oxysporum*. The □*velB* strains exhibited developmental alterations such as reduced aerial mycelium and abnormally shaped microconidia. A search in the *F. oxysporum* genome database revealed the presence of two additional members of the velvet gene family, named *veA* and *velC*. These results suggest a novel role of velvet-type proteins in fungal virulence on plants.

Comparative study of tomato root colonization by *Fusarium oxysporum* f. sp. *lycopersici* wild type strain and the non-pathogenic knock-out mutants for class V and class VII chitin synthase genes

Magdalena Martin-Urdiroz[1], M. Isabel G. Roncero[1], Jose Antonio Gonzalez-Reyes[2], Carmen Ruiz-Roldan[1]
[1]Dpto. Genetica, Edif. C5. Universidad de Cordoba, Cordoba, Spain, [2]Dpto. Biologia Celular, Fisiologia e Inmunologia, Edif. C6. Universidad de Cordoba, Cordoba, Spain

Chitin synthases (CHSs) are the enzymes implicated in the synthesis of chitin, the main structural component of the fungal cell wall. These enzymes constitute a great family of isozymes grouped into seven classes. Furthermore, each fungal species contains a number of CHSs belonging to different classes. In the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* the CHS genes of classes V (*chsV*) and VII (*chsVb*) have been shown to play an important role in the pathotypic behaviour towards tomato plants (*Lycopersicon esculentum*). The construction of single (Δ*chsV* and Δ*chsVb*) or double (Δ*chsV*Δ*chsVb*) knock-out mutants showed that ChsV and ChsVb are required for full virulence of this pathogenic fungus.
To establish the differences in the infection process of the non-pathogenic strains of *F. oxysporum* compared to that of the wild-type strain, transmission electron microscopy (TEM) images were analyzed. TEM of sectioned tomato root samples inoculated with the wild-type strain, showed that all tissues were invaded by the pathogen 48 hours after inoculation. However, the non-pathogenic strains (Δ*chsV*, Δ*chsVb* and Δ*chsV*Δ*chsVb*) were unable to colonize the cortex and the vascular bundles in the upper section of the root 48 hours after inoculation. This difference joined to the fact that the non-pathogenic strains did not induce host cell changes in the inoculated roots compared with roots inoculated with the wild-type strain, suggest that other mechanisms of plant defense are involved in the interaction between the non-pathogenic strains of *F. oxysporum* and tomato roots. Thus, complex responses protecting plants from pathogen involve intensive generation of reactive oxygen species (ROS) that can be used for rapid inhibition of pathogens. However, due to the damaging nature of ROS, plants deploy an enzymatic antioxidative system to prevent damage to host cells. Studies of the variation in the expression profile of the antioxidant enzymes ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase as well as determination of guaiacol peroxidase and ascorbate peroxidase activities, are currently being

performed in tomato plants in response to inoculation with pathogenic and non-pathogenic strains of *F. oxysporum*.

The infection biology of *Fusarium graminearum* single gene deletion strains

Neil Brown[1], Thomas Baldwin[2], Martin Urban[1], Allison van de Meene[1], Kim Hammond-Kosack[1]
[1]*Rothamsted Research, Harpenden, Hertfordshire, United Kingdom,* [2]*INRA, Renne, France*

F. graminearum causes a globally important disease on wheat that has both economic and health implications. Single gene knockout mutants are constantly being generated by the Fusarium community. However, the exact point of fungal arrest for each mutant at the cellular level remains to be determined. Therefore, the objective of this project is to create a fate map of the *F. graminearum* virulence gene mutants and to fully characterise their infection biology *in planta*.
Characterisation of the spread of the wild type or mutant pathogen throughout the ear was assessed using light, scanning electron and transmission electron microscopy. Histochemical staining and X-ray microanalysis was used to identify specific compounds or elements in infected and healthy tissue. The onset of DON mycotoxin production and its final distribution was determined via immuno-localisation.
A type I topoisomerase was the first mutant to be assessed. Initial observations suggested that the *top1* gene deletion strain infection was blocked at the rachis node. A study of the anatomy of the healthy rachis node has shed light on this under-explored tissue type. In the case of the *top1* mutant infection, there appears to be a plant defence response hindering hyphal progress into the rachis.
Observations from both the wild type and mutant infections may be correlated with gene expression profiles of the specific region via Laser Capture Microdissection. Comparisons between the transcriptome of the wild type and mutant infections should provide the detailed analysis required to assign function to these genes.

Tomatinase from F*usarium oxysporum* f. sp. *lycopersici* is required for full virulence on tomato plants

Yolanda Pareja-Jaime, M. Isabel G. Roncero, Carmen Ruiz-Roldán
Universidad De Cordoba, Cordoba, Spain

Saponin detoxification enzymes from pathogenic fungi are involved in the infection process of their host plants. *Fusarium oxysporum* f. sp *lycopersici*, a tomato pathogen, produces the tomatinase enzyme Tom1, which degrades α-tomatine to non-toxic derivates. To study the role of *tom1* gene in the virulence of *F. oxysporum*, we performed targeted disruption and overexpression of the gene. The infection process of tomato plants inoculated with transformants constitutively producing Tom1 resulted in an increase of symptom development. By contrast, tomato plants infected with the knockout mutants showed a delay in the disease process, indicating that Tom1, although not essential for pathogenicity, is required for the full virulence of *F. oxysporum*. Total tomatinase activity in the disrupted strains was reduced only 25%, being β_2-tomatine the main hydrolysis product of the saponin *in vitro*. *In silico* analysis of *F. oxysporum* genome revealed the existence of four additional putative tomatinase genes showing identity to tomatinases belonging to family 3 of glycosyl hydrolases, which might be the responsible for the remaining tomatinase activity in the *Δtom1* transformants. Our results indicate that detoxification of α-tomatine in *F. oxysporum* might be carried out by several tomatinase activities, suggesting the importance of these enzymes during the infection process.

Colonisation of barley roots with DsRed expressing *Fusarium avenaceum* and GFP expressing *Fusarium culmorum*-competition and effect on toxin production

Thomas Johansen[1], Morten Nedergaard Grell[1], Johan Åhman[1], Stefan Olsson[1], Michael Hansen[2], Henriette Giese[1]
[1]*University of Copenhagen, LIFE, Department of Ecology, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark,* [2]*University of Copenhagen, LIFE, Department of Plant Biology, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark*

Fusarium culmorum and *Fusarium avenaceum* are infamous for causing root rot, seedling and head blight in cereal species. The latter disease has attracted the greater attention as the fungi are producers of mycotoxins that are deposited in the grain. The fungi are soil born and studies were carried out to register in real time the colonization process of these fungi in barley roots. Two reporter strains: *F. culmorum* expressing the green fluorescent protein (GFP) and *F. avenaceum* expressing the red fluorescent protein (DsRed) were generated. A sterile root system was used for the application of macroconidia from the fungi individually or together to determine any competitive effects. Infection of agar embedded barley roots allowed us for the first time to obtain Laser Scanning Confocal Microscopic recordings of *F. culmorum* and *F. avenaceum* hyphae growing inside root cells. The primary region of entry appears to be the root hair zone. Both fungi grow in the intercellular space and can directly enter living plant cells. The fungi do not appear to be antagonistic but *F. culmorum* appear to have a faster growth rate than *F. avenaceum*. Secondary metabolite profiles for *F. culmorum*, *F. avenaceum* and the combination of the two species under infection of barley roots were obtained by RP-HPLC. Aurofusarin were produced by *F. culmorum* under barley root infection while the same peak was missing in the *F. avenaceum* infection assay. The result was confirmed by qualitative RT-PCR on the *PKS*12 gene. In addition *PKS*6, *PKS*8, *NPS*2 and *NPS*6 were expressed in *F. culmorum* after 7 day of infection of barley roots.

The role of autophagy in *Fusarium graminearum* during infection of cereals

Lone Josefsen, Jan Svensson, Stefan Olsson, Henriette Giese
[1]*University of Copenhagen, Department of Ecology, Frederiksberg, Copenhagen, Denmark,* [2]*University of Copenhagen, Department of Plant Biology, Frederiksberg, Copenhagen, Denmark*

Fusarium graminearum is a fungus that infects cereal crops. The infection is a very rapid process that starts with spore germination and invasion of the plant tissue where it turns into a necrotrophic infection that reduces crop quality and cause yield losses.
Recycling of nutrients is a strategy for fungi to survive the stress and starvation it experiences prior to plant infection. Autophagy is one of the primary mechanisms for recycling of resources within fungal mycelia. It is dependent on the vesicular enclosement of portions of the cytosol resulting in the formation of autophagosomes (double layered vesicles). The autophagosomes in turn fuse with vacuoles, which contain digestive enzymes that degrade the contents of the autophagosome and make nutrients available for new production of proteins and organelles.
Autophagy has in other plant pathogens been shown to be essential for the infection process. It is thought that autophagy is necessary for turnover of organic matter and optimizing the energy needed for pathogenecity. Using homologous recombination and an *Agrobacterium*-mediated transformation technique we have produced a *F. graminearum* strain where the Atg8-gene essential for the autophagic process is interrupted. On nutrient rich media the ΔAtg8-mutant and the WT show identically growth patterns. However, on low nutrient media the mutant performs poorly compared to the WT, indicating that that the mutant has lost the ability to recycle internal resources. Plant infection assays show that the ΔAtg8-mutant is able to infect plant tissue; however, there is a delay in the time of infection and visible symptoms compared to the WT.

Phases of fungal development and gene expression in *Fusarium graminearum* during crown rot disease development in wheat

Amber Stephens[1], Donald Gardiner[1], Kemal Kazan[1], Rosemary White[1], Alan Munn[2], John Manners[1]
[1]*CSIRO, Brisbane, Queensland, Australia,* [2]*University of Queensland, Brisbane, Queensland, Australia*

Fusarium graminearum (Fg) can cause head blight (FHB) and crown rot (CR) diseases in wheat. CR occurs at the stem base of the wheat plant, causing major reductions in grain yield and has been less intensively researched than FHB. Real-time quantitative PCR analysis of fungal genomic DNA relative to plant genomic DNA was used to estimate Fg biomass during CR disease development and using this method three distinct phases of infection were identified. Phase 1 was a statistically significant increase in fungal biomass that occurred in

the first 2 days post-inoculation (dpi) of the stem base with Fg macroconidia. Phase 2 was a subsequent significant decrease in fungal biomass for a period of approximately 14 dpi, and this was followed by phase 3 where a massive increase in fungal biomass was observed for the period 14 - 45 dpi. Histological characterisation of Fg colonisation during these three phases of infection revealed that in phase 1 the spores germinated on the stem surface at the point of inoculation forming a superficial hyphal mat. In phase 2, infecting hyphae had moved down from the point of inoculation to the crown area of the wheat seedling and had penetrated and colonised the epidermis of the outer leaf sheath while in phase 3 a major colonisation of the internal tissues of the crown had occurred. Fungal gene expression at 2, 14 and 35 dpi was examined using the Affymetrix GeneChip system comprising 22,000 Fg gene probe sets. Results showed 1,839 Fg genes were significantly up regulated in planta compared to vegetative mycelia, including some known FHB virulence genes (e.g. TRI5 and TRI14). Fungal genes differentially regulated between the phases were identified. These results indicate that CR disease development requires a coordinated process that involves distinct phases of infection and associated fungal gene expression programs.

Transformation of progesterone by a progesterone-induced multienzyme hydroxylating system in *Fusarium oxysporum*

Anna Poli, Helena Lenasi
University of Ljubljana, Ljubljana, Slovenia

The ability to hydroxylate steroidal substrates including progesterone was demonstrated in several fungi including members of the genus *Fusarium*. Hydroxylated compounds are more water soluble, and easily removed from the mycelia, if surrounded by water: the meaning of the hydroxylation process is most likely to be detoxification. In the present study we investigated the progesterone-response in the vascular wilt fungus *Fusarium oxysporum*. Specifically, the hypothesis that a multienzyme hydroxylating system could be induced by the presence of the steroid was tested.
Progesterone was applied as inducer to the liquid growth medium for 120 min; following washes of the mycelium the steroid was supplied as a substrate for another 30 min. Steroids were extracted from mycelium and medium, respectively. The content of progesterone and progesterone transformed products was measured by HPLC and identified by gas chromatography/mass spectrometry.
Progesterone was shown to induce steroid hydroxylation: the content of hydroxylated product significantly increased from 1.5 ± 0.63% (control) to 9.42 ± 0.63% (induced). Progesterone was mainly transformed into 15α-hydroxyprogesterone, which was mostly present in the medium. In order to test whether mitogen activeted protein kinase MAPK, G proteins and histidine kinase were involved in the signling leading to the induction of the enzyme system the following mutants were studied: *Δfmk1* (MAPK), *Δfgb1* (G protein subunit β) , *Δfh1* (histidine kinase) and *Δfmk1/Δfgb1* (MAPK and G β double mutant). G β proteins seem to be involved in progesterone signaling: no steroid hydroxylation occurred in Δfgb1. Exogenous cAMP restored the ability of this mutant to transform progesterone, indicating the the signaling is mediated by cAMP-PKA pathway.
In the presence of progesterone fungal growth is inhibited, as revealed by toxicity tests; therefore, we can assume that the biological role of progesterone-induced enzymes is most likely transformation of toxic progesterone into a less toxic product.

Quantification of trichothecene biosynthetic genes during the growth cycle of *Fusarium sporotrichioides* in culture

Nancy Alexander, Susan McCormick
National Center for Agricultural Utilization Research, Peoria, IL, United States

Trichothecene mycotoxins are secondary metabolites produced by several species of phytopathogenic fungi and are potent inhibitors of protein biosynthesis. The genes involved in the biosynthetic pathway of T-2 toxin in *Fusarium sporotrichioides* have been characterized and are located in four identified loci. We wished to determine when these genes were turned on in the metabolic cycle and in what quantity. RNA was harvested at 8, 12, 16, and 24 h after inoculation with spores of *F. sporotrichioides* in a defined, liquid medium. Reverse transcriptase was used to form cDNA and quantitative PCR was performed using primers

specific to selected trichothecene biosynthetic genes. Trichothecene genes were transcribed between 12 and 16 h post inoculation, typical of secondary metabolite genes, while basic metabolic genes were detected earlier at 8 h. As the expression of the trichothecene genes rise, the expression of the basic housekeeping genes declines. *FsTri4* and *FsTri5* are the most highly expressed trichothecene genes, with 7-fold greater expression than *FsTri1, FsTri13,* and *FsTri15*. *FsTri15*, a gene involved in the negative regulation of the pathway, has a delayed profile. Together, these and other data indicate that the level of expression of each of the trichothecene pathway genes is unique. This may lead to a novel method for controlling the expression of this pathway with the ultimate goal of reducing the presence of these mycotoxins in our food and feed supply.

Effects of solute and matric potential stress, growth and *FUM1* gene expression in *Fusarium proliferatum*

Patricia Marín[1], Miguel Jurado[1], Naresh Magan[2], Covadonga Vázquez[3], Mª Teresa González-Jaén[1]

[1]Department of Genetics, University Complutense of Madrid, Jose Antonio Novais 2, 28040 Madrid, Spain, [2]Applied Mycology Group, Cranfield Health, Cranfield University, Silsoe, Bedford MK45 4DT, United Kingdom, [3]Department of Microbioloy III, University Complutense of Madrid, Jose Antonio Novais 2, 28040 Madrid, Spain

Fusarium proliferatum is considered one of the most important fungal species capable to produce fumonisins during host plant colonization besides *F. verticillioides*. These compounds are toxic to human and animals and represent a source of risk for health especially in corn-based commodities. Both species belong to the so-called *Gibberella fujikuroi* species complex and, although they may occur simultaneously in maize, they have differences in host range. The occurrence of fungal species and their mycotoxin production are influenced by ecophysiological factors, in particular water stress. The objective of this work was to study the effect of water stress, solute and matric potential, on growth rate and fumonisin gene expression in two strains of *F. proliferatum* isolated from a maize field in Spain. The effect of ionic and non-ionic solute water stress and matric potential, which indicates the water stress during growing in soils, was analysed by measuring *in vitro* mycelial growth rates. The expression of *FUM1* gene, involved in fumonisin biosynthesis, was quantified by a species specific Real Time RT-PCR protocol. The results obtained indicated a reduction in growth, in particular in response to matric potencial stress, while *FUM1* gene expression was enhanced, in particular when caused by non-ionic solute in both species. The temporal kinetic performed to examine the effect of ionic solute stress on growth and *FUM1* expression suggested that water stress might be a critical factor affecting fumonisin accumulation by *F. proliferatum* under natural conditions, in particular when water stress is progressively increasing during kernel maturation. On the other hand, the results also indicated that *F. proliferatum* may represent a similar risk for fumonisin production than *F. verticillioides* in natural conditions. The gene expression studies by Real Time RT-PCR have proven to be a valuable tool to gain knowledge of the ecophysiological basis for fumonisin gene expression and enable better control strategies to be developed during the life cycle of these toxigenic fungi.

Real-time PCR assay for detection and quantification of A*spergillus carbonarius* in grapes: comparison between SYBR®Green and TaqMan®

Amaia González, Belén Patiño, Jéssica Gil-Serna, Covadonga Vázquez, María Teresa González-Jaén

University Complutense of Madrid, Madrid, Spain

Aspergillus carbonarius is the main species responsible for the production of Ochratoxin A (OTA) in grapes and wine. This mycotoxin is one of the most common naturally occurring contamination in agroproducts and is toxic to both humans and animals. The purpose of this work was to develop a specific qPCR assay for identifying and quantifying *A. carbonarius* genomic DNA occurring on contaminated grapes in order to predict the potential OTA risk.
Nucleotide sequences obtained in our lab and from the GeneBank were aligned to design a specific primer pair QCARBOF/QCARBOR from the Internal Transcribed Region ITS. A FAM labelled TaqMan® probe was also designed for the TaqMan® assays. The specificity and sensibility of the primer/probe combinations were tested on a number of *A. carbonarius* strains

and on mixes in different proportions of genomic DNA from *A. carbonarius* and other *Aspergillus* species commonly found on grapes, *Fusarium* sp., *Penicillium* sp. and *Alternaria* sp. None of the other species alone gave a positive result with this PCR primer set either using Sybr®Green or TaqMan®. No inhibition was even observed in the mixes of different genomic DNAs.
To test the ability of the designed primers to detect *A. carbonarius* in grapes, the QPCR assays were coupled with a fungal enrichment and a DNA extraction method for grapes. Different lots of commercially grapes were contaminated with a spore suspension (10^2 and 10^6 spores/ml) and DNA was extracted after 0, 8, 16 and 24 hours of incubation. The results indicated that the critical qPCR amplification product was clearly observed for grapes contaminated by 10^6 spores without incubation using either SYBR®Green or TaqMan®.
These quantitative analyses demonstrated that both reporters SYBR®Green or TaqMan® are equally valid, but we recommend SYBR®Green as is less expensive, easy to use and gave more sensibility than TaqMan®.The qPCR assays presented in this work are based on ITS sequence and therefore are more sensitive than primers based on single copy sequences.

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Regulation of secondary metabolism in *Fusarium fujikuroi*: alteration of nitrogen-regulated pathways in carotenoid overproducing mutants

L. Roberto Rodríguez-Ortiz, M. Carmen Limón, Javier Avalos
Department of Genetics, University of Sevilla, Sevilla, Spain

The ability of *Fusarium fujikuroi* (*Gibberella fujikuroi* MP-C) to produce gibberellins, growth-stimulating plant hormones, makes this fungus an outstanding model in secondary metabolism. Additionally, *F. fujikuroi* produces bikaverins, reddish polyketides with antibiotic properties, and carotenoids, health-promoting terpenoid pigments. Gibberellins and bikaverins are produced upon exhaustion of the nitrogen source, a regulation mediated by the AreA protein. In contrast, carotenoids are primarily regulated by light, which induces the transcription of the structural genes of the pathway by a WC-independent mechanism (Estrada et al, FGB, in press), currently under investigation. Little information is available on the regulatory genes for carotenogenesis. We have investigated the effect of nitrogen availability on the biosynthesis of carotenoids and the expression of structural genes of the pathway and we have compared it with the effect on bikaverin and gibberellin production and the expression of key genes for each pathway. The experiments were extended to four independent carotenoid overproducing mutants (abbreviated *carS*), a phenotype attributed to the loss of a key-repressor protein. As expected, the mutants produced higher amounts of carotenoids under the culture conditions tested, but nitrogen starvation produced a partial derepression of carotenogenesis in the wild type. Expression of gene *ggs1*, putatively responsible for the synthesis of geranylgeranyl pyrophosphate (GGP) for carotenoid production, was derepressed in the *carS* mutants. Two of them produced less gibberellic acid and three produced less bikaverins than the wild type. These results correlated with reduced mRNA levels of key genes for both pathways in the mutants (*ggs2* and *cps/KS*, coding for GGPP and kaurene synthases for gibberellin biosynthesis, and *pks4*, coding for bikaverin polyketide synthase). Unexpectedly, expression of the gene *niaD* (nitrate reductase) was very high in minimal medium with an organic nitrogen source (asparagine) in the four *carS* mutants compared to the wild type. However, the expression of the partner gene in nitrate assimilation, *niiA*, was not affected. Taken together, our results suggest a regulatory connection between the function of the putative CarS protein and AreA-mediated processes in *F. fujikuroi*. The effect of the *carS* mutations on other nitrogen-regulated processes, such as conidiation, will be also analyzed.

Regulation of bikaverin and fusarin production in *Fusarium fujikuroi*

M. Carmen Limón, Violeta Díaz-Sánchez, Bina J. Mehta, Javier Avalos
Department of Genetics, University of Sevilla, Sevilla, Spain

Fusarium fujikuroi (*Gibberella fujikuroi* MP-C) is used for the production of gibberellins, terpenoid hormones that induce growth and regulate various stages of development in plants. This fungus synthesizes many other secondary metabolites, mainly mycotoxins and pigments.

Among them are some polyketides, such as bikaverins and fusarins. Regulation of bikaverin and gibberellin biosynthesis by nitrogen has been thoroughly investigated in *Fusarium* but little is known about their regulation by carbon source. On the other hand, little information is available on the control of fusarin production. Our main interest is to understand the regulation of the biosynthesis of these compounds in *F. fujikuroi*.
Gibberellin production was not affected significantly by different concentrations of a variety of carbon sources. However, bikaverin production was enhanced when sucrose was the only carbon source. The high production in sucrose required a minimal amount of the sugar, but did not change appreciably above this threshold along a wide range of concentrations. Bikaverin synthesis was repressed when glucose coexisted with sucrose in the medium. The former identification of the gene coding for the polyketide synthase of bikaverin in *F. fujikuroi*, *pks4*, allows studying the regulation of the pathway at mRNA level. The stimulation of bikaverin production by sucrose was not paralleled by an induction of pKS4 mRNA.
We have obtained a collection of mutants affected in the production of bikaverins. Mutants are different with regards to their regulation, morphology and secretion of these polyketides. Some mutants produced bikaverins under any culture condition whereas others produced them only under nitrogen starvation. Some mutants were affected morphologically, being the best producers the most affected. Outstandingly, some mutants excreted most of these pigments into the culture media meanwhile others accumulated them mainly in the mycelium.
Fusarin biosynthesis depended on incubation temperature and nitrogen availability but it was not affected by light. The effect of these regulatory signals is being also investigated in mutant SG62, formerly described as a fusarin overproducer. Two different wild type strains, IMI58289 and FKMC1995, strongly differed in their capacity to produce fusarins, indicating a variability of this trait in this species.

Cloning and heterologous expression of fungal type I polyketide synthases in *Saccharomyces cerevisiae*

Michaela Peruci[1], Angela Csziferský[1], Franz Berthiller[2], Rainer Schuhmacher[2], Rudolf Mitterbauer[1], Gerhard Adam[1]
[1]*Institute of Applied Genetics and Cell Biology, Department of Applied Plant Sciences and Plant Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria,*
[2]*Center for Analytical Chemistry, Department for Agrobiotechnology (IFA-Tulln), University of Natural Resources and Applied Life Sciences, Tulln, Austria*

Fungal polyketides constitute a large family of secondary metabolites endowed with a high degree of structural diversity and various biological activities. Despite their apparent structural diversity, polyketides share a common mechanism of biosynthesis. Polyketides are synthesized by sequential reactions catalysed by enzymes called polyketide synthases (PKSs) by repetitive Claisen condensations of an acyl-coenzyme A (CoA) starter with malonyl-CoA elongation units in a fashion reminiscent of fatty acid biosynthesis. Both the extender unit of malonyl-CoA and the growing poly-b-ketone intermediates are covalently tethered to the ACP subunit of PKSs in an acyl thioester linkage to the phosphopantetheinyl moiety during polyketide biosynthesis. The latter prosthetic group is introduced posttranslationally by the phosphopantetheinyl transferase (PPTase), which transfers the 4'-phosphopantetheine moiety of CoA to the highly conserved Ser residue of apo-ACP, converting the enzyme into holo-ACP. Phosphopantetheinylation is absolutely necessary for polyketide biosynthesis, without which the PKS is nonfunctional.
It was shown, that the 4‘ phosphopantetheinyl transferease of *F. gramineaum* (*FgPPT1*) is necessary for full virulence on wheat (Peruci *et. al*. in preparation). The aim of our study was to establish a heterologous expression system for polyketide synthases which allows easy screening for 4'-phosphopantetheinyl transferases inhibitors. We expressed type I PKSs in a genetically modified yeast strain allowing detection systems of pigment formation or the estrogenic activity of b-ZOL. For that purpose we have coexpressed *FgPPT1* with the PKSs responsible for nor-rubrofusarin production *FgPKS12* or the two PKSs required for ZON biosynthesis, *FgPKS4* and *FgPKS13*. In case of *FgPKS12*, we also had to coexpress the structural gene for a putative methyltransferase *aurJ* to obtain nor-rubrofusarin.
Obviously, all three heterologous PKS genes are expressed and posttranlationally activated. Detectable amounts of nor-rubrofusarin and ß-ZOL were produced in liquid culture. Efforts to

increase the expression levels and to establish suitable screening methods for PPTase inhibitors are ongoing.

Global players involved in nitrogen regulation in *Fusarium fujikuroi*

Bettina Tudzynski, Birgit Schönig, Dominik Wagner, Sabine Teichert
Institut für Botanik, Westfälische Wilhelms-Universität Münster, Schlossgarten 3, D-48149 Münster, Germany

In *F. fujikuroi*, the global nitrogen regulator AreA does not only control the utilization of alternative nitrogen sources, but also the expression of gibberellin (GA) biosynthetic genes. Using microarrays of the closely related species *F. verticillioides*, we identified a set of genes down-or up-regulated in the *areA* mutant, but also a set of genes which are nitrogen-regulated in an AreA-independent manner. However, not much is known about regulatory proteins, affecting AreA activity. In contrast to *A. nidulans*, the AreA binding protein NMR does not play a major role in regulating AreA activity in *F. fujikuroi*. The deletion of *nmr* did not result in a significant deregulation of AreA target genes, but only in a slightly earlier expression of these genes suggesting that other regulators must exist which inhibit AreA activity under nitrogen sufficient conditions. One candidate is the *F. fujikuroi* homologue of the *A. nidulans meaB* gene. Deletion of *meaB* led to a slight up-regulation of several nitrogen-regulated genes such as the GA- and bikaverin biosynthesis genes and the GS-encoding gene *glnA* demonstrating that MeaB negatively affects the expression of these genes. A differential hybridization of macroarrays with cDNA from the wild-type and the *meaB* mutant revealed a set of MeaB target genes. A MeaB-GFP fusion clearly showed that MeaB is translocated to the nucleus under nitrogen-sufficient conditions. To find out if MeaB and NMR act in the same pathway as it has been shown in *A. nidulans*, or if both factors act independently from each other, we generated *nmr meaB* double knock-out mutants and compared the expression of nitrogen-regulated genes in the wild-type with that in all mutants. In addition to these regulators, we are searching for signalling components acting upstream of AreA. We show that MepB, one of three ammonium permeases, seems to be involved in sensing and transduction of the nitrogen signal. Δ*mepB* mutants revealed severe growth defect on low ammonium concentrations suggesting that MepB is the major permease. AreA target genes such as GA- biosynthetic genes or several permease genes, which are normally repressed by ammonium, are de-repressed in Δ*mepB* mutants. Overexpression of *mepC*, encoding a second ammonium permease, in Δ*mepB* restored the growth but not the regulation defect. Based on our recent results, we propose a model for the nitrogen regulation network in *F. fujikuroi*.

Role of *Fusarium oxysporum* white collar 1 photoreceptor on carotenogenesis, UV resistance, hydrophobicity and virulence on mammalian hosts

Carmen Ruiz-Roldán[1], Victoriano Garre[2], Josep Guarro[3], Marçal Mariné[3], M. Isabel G. Roncero[1]
[1]*Universidad de Córdoba, Córdoba, Spain,* [2]*Universidad de Murcia, Murcia, Spain,* [3]*Universidad Rovira y Virgili, Reus, Tarragona, Spain*

Light regulates developmental and physiological processes in a wide range of organisms, including filamentous fungi. *Fusarium oxysporum*, the causal agent of the vascular wilt disease in a wide variety of crops and an emerging human pathogen, contains a putative photoreceptor Wc1, the orthologe to WC-1 of *Neurospora crassa.* Isolation and characterisation of the *wc1* gene revealed that the predicted protein contained all the characteristic domains present in other fungal photoreceptors, including a conserved LOV domain, two PAS dimerization domains, a nuclear localization sequence and a Zn-finger DNA binding domain. *Wc1* is expressed constitutively at very low levels and does not regulate expression of *wc2* gene. Targeted disrupted mutants showed that this gene is involved in formation of aerial hyphae when grown on solid medium under white light. Defects in aerial hyphae development could be related to altered hydrophobocity, since Δ*wc1* mutants showed altered colony surface hydrophobicity under white light, as well as differential expression pattern of the hydrophobin gene *hyd1*. Δ*wc1* mutants were also affected in induction of carotenogenesis by light, indicating that this gene is involved in this process. Additionally, Wc1 contributes to photoreactivation after UV treatment and controls light induced up-regulation of the photolyase gene *phr1.* Pathotypic behaviour of Δ*wc1* mutants on tomato plants was unaltered, indicating that this gene is dispensable for pathogenicity on host plants. By

contrast, mutation of *wc1* impairs virulence on immunodepressed mice. These results demonstrate that light perception in *F. oxysporum* plays important roles in the development and behaviour of this ascomycete fungus.

Identification and phytotoxical properties of fungi genus *Trichoderma* from ancient lands and antagonistic activity by *Trichoderma koningii* against *Fusarium oxysporum* in experiences *in vitro*

Hector Alejandro Cabrera Fuentes, Farida Alimova, Rezeda Tukhbatova, Elina Rafailova
Kazan State University, Kazan, Tatarstan, Russian Federation

Different species of the genus *Trichoderma* are employed in different areas of human activity. We acquired more than 400 stems of fungi from the genus *Trichoderma,* from burial grounds dating 800-600 BC, on the territory of the Republic of Tatarstan, Russia. The objective of this study was to look for stems, perspective for the manufacture of biological products and evaluate *Trichoderma* species as potential biocontrol agents to reduce the impact of the *Fusarium oxysporum*. In order to reach this goal we identified, classified and systematized allocated stems with the use of morphological and molecular-genetic methods and investigated their physiological-biochemical properties. Descriptions of the appearance of colonies and their morphology have been recorded in a before-stated work developed by identifiers Samuels, Chaverri, Bissett. Molecular-genetic research was carried out with use of method PCR (RAPD-analysis). On the basis of the received results investigated stems have been related to species: *Tr. longibrachiatum, Tr. koningii, Tr. harzianum, Tr. asperillum, Tr. citrinoviride, Tr. atroviride, Tr. oblongisporum, Tr. spirale.* Classification of these stems was done with the help of an earlier specially developed program. Also have been investigated antagonistic activities and phytotoxical properties, enzymatic activity of the complex exohydrolase (xylanase, protease and cellulase activity) of allocated stems.Morphological analysis revealed the 2 types of colony (II and VI). Their kinetic parameters were also determined: slow growth isolates and fast growth isolates were calculated as 0.01-0.07 mm/h and 0.1 mm/h. We have also investigated antagonistic activities. As a result of our work, selected stems of *Trichoderma koningii* with a positive influence on the growth of rye and wheat with prospects in biotechnology have been identified.

Fast and efficient single step construction of replacement vectors by USER friendly cloning, for targeted gene replacement in fungi

Rasmus J.N. Frandsen, Jens A. Andersson, Henriette Giese
Institute for Ecology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark

Functional genetics in filamentous fungi have always been dependent on the isolation or construction of mutant strains. The genome sequencing of over 40 fungi genomes has increased the need for faster and more efficient methods to construct targeted replacement and overexpression mutants. To accommodate this we have developed a new vector system that allows single step construction of vectors for targeted gene replacement, thereby cutting vector construction time from ten to only three days and removing half of the required work load. The vector system is dependent on the Uracil-Specific Excision Reagent cloning technology (USER Friendly™), which in its commercial version offers high efficient directional cloning of a single PCR amplicon. However, our research shows that USER friendly™ cloning technology also can be used for the simultaneous directional cloning of several PCR amplicons and vector fragments, with a cloning efficiency of 85 %, thus allowing single-step construction of replacement vectors. In addition to the increased speed and reduced workload, the single-step construction strategy also offers greater freedom of operation with respect to the placing of the homologous recombination flanks, as it is independent of restriction enzymes.
The new vector system includes vectors for targeted gene replacement (pRF-HU2), promoter exchange (pRF-HU2E), ectopic overexpression (pRF-HUE) and general purpose cloning (pRF-HU). All are compatible with both protoplast and *Agrobacterium tumefaciens* mediated transformation technologies. The system has been used to analyse putative polyketide gene clusters in *Fusarium graminearum*.

PCR and spectral imaging based assays for the early detection of aflatoxigenic fungi on maize kernels

Antonella Del Fiore[1], Silvia Serranti[2], Alessandra Ricelli[3], Massimo Reverberi[2], Annadele Fabbri[2], Giuseppe Bonifazi[2], Corrado Fanelli[2]
[1]ENEA CR Casaccia, Roma, Italy, [2]Univesità La Sapienza, Roma, Italy, [3]CNR, Roma, Italy

Fungi are a real issue for the cereal industry. Some fungal species, such as *Aspergillus flavus* and *A. parasiticus*, can produce under suitable conditions, aflatoxins, secondary metabolites which are toxic for human and animals. Many methods have been utilized to measure fungal contamination on cereals. The aim of this work is to use a PCR based method and a spectral imaging based method to detect aflatoxigenic fungi on maize. In the experiments were used two aflatoxigenic strains, *A. parasiticus* NRRL 2999, *A. flavus* NRRL 3357, and one non aflatoxigenic strain of *A. parasiticus* NRRL 11096. An *A. Niger* strain and other strains of *Aspergillus spp*., *Penicillium sp*. and *Fusarium sp*. isolated from maize were also analyzed. Maize hybrids employed in food industry, were used in the PCR-based assay. Genomic DNA was extracted from pure fungal strains, not inoculated and inoculated maize kernels. A primer pair was designed on the coding portion of *omt*-1 gene which encodes for the enzyme O-methyltransferase II necessary for the last step of aflatoxin biosynthesis. The DNA extracted was used as template for PCR amplification with the primer pair selected. PCR amplification generate a fragment of 1254 bp length in *A. flavus* and *A. parasiticus*. Amplification operative conditions were optimized on pure fungal strains and confirmed on maize. DNA amplification was achieved only with DNA from fungal strains of *A. parasiticu*s and *A. flavus* and from maize inoculated with *A. flavus* or *A. parasiticus*, never with DNA of other fungal strains. Specificity was confirmed with DNA extracted from different fungal strains. These results indicate that this PCR-based method could be able to discriminate maize kernels infected with *A. flavus* and *A. parasiticus* from un-infected ones; this method could be used like early detection system, for aflatoxigenic strains in maize. Several studies have tried non-destructive, spectral methods to detect fungal contamination on cereals. At this concern we present a method, based on the spectral imaging, to detect fungal contamination on maize. A desktop spectral imaging system, ImSpectorTM, V10 were used in the work; two aflatoxigenic species were used, *A. parasiticus*, and *A. flavus*. They are inoculated on maize and were imaged on day 7 of growth. Changes in reflectance of maize were observed during fungal growth. This approach could be a rapid method of detecting the aflatoxigenic fungi on cereals.

Non-dermatophytic molds as a causative agents of onychomycosis in Tehran

Shahindokht Bassiri-Jahromi, Ali Asghar Khaksar, Golnar Sadeghi, Farnoosh Asghari, Ali Najafi
Pasteur Institute of Iran, Tehran, Islamic Republic of Iran

Back ground: Non dermatophytic onychomycosis is a fungal infection of fingernails or toenails caused by moulds and yeasts. Onychomycosis is an opportunistic fungal disease and is usually caused due to impaired barrier functions in healthy individuals, for example, trauma in nail. In the last few years the number of cases of non-dermatophytic onychomycosis has greatly increased, mainly in Europe, associated with cases of immunodepression and in immunocompetent subjects.
The aim of our study was to evaluate the incidence, the clinic characteristics and predisposing factors of non-dermatophytic onychomycosis.
Material and Methods: A total of 1268 patients' samples including toe and finger nails clippings and subungual debris were collected at the Medical mycology Laboratory of Pasteur Institute of Iran, Tehran from March 2005 through March 2006. Specimens were obtained from clinically suspected fungal infections of toe and finger nail by mycosis, and primary care physicians. The etiologic agents of onychomycosis were established after repeated cultural examinations. All collected specimens were analyzed by direct microscopy and culture. Microscopic examination of these specimens was carried out in potassium hydroxide solution (20%) with demethyle sulfoxide (4%). These specimens were cultured on sabouraud glucose agar with chloramphenicol and sabouraud glucose agar with chloramphenicol and cyclohexamide. Cultures were incubated at 25ºc for up to 28 days and checked twice weekly for growth.

Results: The patients with onychomycosis were 410 cases , of whom 47 cases (11.5%) were non-dermatophytic onychomycosis. . We found that Aspergillus spp. were the more responsible etiologic agents of non dermatophytic agents of onychomycosis, resulting in a total of 28 patients (59.6%). The toe nail was more affected (72.3%). Non-dermatophytic onychomycosis were 11.5% of all ungual infections. In our study other causative agents were *Acromonium spp., Fusarium spp., Trichosporum spp. Scopolariopsis spp.* and *Geotrichom spp*.
Conclusion: In our opinion such studies should be performed in every country in order to determine the major fungal species responsible; such information is extremely useful in the treatment of nail onychomycosis. Early diagnosis and accurate therapy are important for the resolution of onychomycosis. Knowing the exact pathogens is important and has implications in therapy and prognosis.

Sequence-based identification, aggressiveness and fumonisin production of a population of *Fusarium* species causing bakanae disease of rice in the Philippines

Christian Joseph Cumagun[1], Carlos Padilla[1], Evelyn Gergon[2], Francois Van Hove[3], Maria Teresa Gonzàlez-Jaén[4], Patricia Marin[4]
[1]Crop Protection Cluster, College of Agriculture, University of the Philippines Los Baños, Los Banos, College, Laguna, Philippines, [2]Philippine Rice Research Institute, Munoz, Nueva Ecija, Philippines, [3]Mycothèque de l'Université catholique de Louvain, Louvain-la Neuve, Belgium, [4]Department of Genetics, Faculty of Biology, Universidad Complutense de Madrid, José Antonio Nováis, Madrid, Spain

Forty *Fusarium* strains were isolated from rice stems, shoots and grains in the Philippine provinces of Nueva Ecija and Laguna. All isolates were identified as *Fusarium fujikuroi* based on the elongation factor -1 α sequence except three isolates which were identified as *F. proliferatum*, *F. sacchari* and *F. oxysporum*. Based on PCR amplification of *MAT* (mating type) specific sequences, the 37 *F. fujikuroi* segregated 10:27 of *MAT-1* and *MAT-2,* respectively. Only five isolates produced fumonisins in liquid culture; concentrations, estimated by Enzyme Linked Immunosorbent Assay (ELISA), ranged from 0.025 ppm to 0.238 ppm. High Performance Liquid Chromatography (HPLC) analysis of 20 isolates revealed seven isolates as fumonisin producers with production ranging from 0.86µg/g -210 µg/g. Amplification of a partial sequence of the *fum1* gene (a key gene in fumonisin biosynthesis) is ongoing. No association between fumonisin production and aggressiveness of isolates under lab and greenhouse conditions were observed.The production of fumonisins of *F. fujikuroi* in rice implies the need to explore a larger population of this pathogen to promote food safety

Aggressiveness, vegetative compatibility groups, mating type assessment and fumonisin production of *Gibberella fujikuroi* (*Fusarium verticillioides*) from corn in the Philippines

Christian Joseph Cumagun[1], Mabel Vallena[1], Ma. Leizel Vargas[1], April Alviar[1], Francoise Munaut[2], Francois Van Hove[2]
[1]Crop Protection Cluster, College of Agriculture, University of the Philippines Los Baños, Los Baños, College, Laguna, Philippines, [2]Mycothèque de l'Université catholique de Louvain (BCCMTM /MUCL), Université catholique de Louvain, Louvain-la Neuve, Belgium

Gibberella fujikuroi (*Fusarium verticillioides*) is one of the most important fungal pathogens of corn worldwide. The pathogen produces mycotoxin fumonisins that are potentially harmful to human and animal health. Twenty isolates of *G. fujikuroi* from Laguna, Philippines were characterized at the molecular level by mating population (MP) and mating type (*MAT*). The identity of the 12 isolates was confirmed by sexual crosses with standard tester strains. Eleven isolates were *MATA-1* [and] one *MATA-2*. All the isolates were further characterized by fertility and vegetative compatibility group (VCG) in the laboratory and tested for their stalk rot aggressiveness on 'Super Sweet' corn variety under field conditions across two environments using the toothpick inoculation method. In two greenhouse trials, inhibition of seedling emergence, seedling height, fresh and dry weight were also determined. Fumonisin production was analysed by High Performance Liquid Chromatography (HPLC). Analysis of fertility revealed that six out of 12 MPA isolates were hermaphrodites. Significant (P = 0.05) differences in aggressiveness toward corn of some isolates were observed for both experimental locations while vegetative compatibility grouping by pairing *nit* mutants

identified 19 vegetative compatibility groups for this population with a genotype diversity of 0.95. All isolates were pathogenic to corn seedlings and mature plants compared to non-inoculated control. Fumonisin production of the isolates was not associated with their fumonisin production. Majority of the MPA isolates, 9 out of 11 analyzed, were fumonisin producers whereas 3 out of 5 analyzed MPA isolates are non fumonisin producers.

Philigenetic analysis and fumonisin production of *Fusarium fujikuroi* strains isolated from rice in the Philippines

Patricia Marín[1], Christian Cumagum[2], Alejandra Cruz[1], Covadonga Vázquez[3], Carmen Callejas[1], Mª Teresa González-Jaén[1]

[1]Department of Genetics, University Complutense of Madrid, Jose Antonio Novais 2, 28040 Madrid, Spain, [2]Crop Protection Cluster, University of the Philippines Los Baños, College, Laguna, 4031, Philippines, [3]Department of Microbiology III, University Complutense of Madrid, Jose Antonio Novais 2, 28040 Madrid, Spain

Fumonisins are important mycotoxins which often contaminate several cereals such as maize, wheat or rice, although they can also occur in a wide variety of other commodities. Fumonisins are responsible of serious chronic and acute diseases in human and animals and their presence is under regulation in more than 100 countries. Fumonisins production is basically limited to the members of the formely so-called *Gibberella fujikuroi* species complex. Although *Fusarium verticillioides* and *F. proliferatum* are the main fumonisin producers, other related species have been also reported. *F. fujikuroi* has been described as a maize and rice pathogen causing important agricultural losses. However, little information is available about the phylogenetics of this species and its ability to produce fumonisins. In this work, we studied 23 strains isolated from rice in the Philippines and we performed a philogenetic analysis using the partial sequence of the Elongation Factor 1 alpha (EF-1α) including isolates belonging to closely related species. Fumonisin producion was analysed in seven-day-old cultures grown in fumonisin-inducing medium by an ELISA-based method and by Real Time RT- PCR using primers for *FUM1* gene, a key gene in fumonisin biosynthesis. The results indicated the ability of F*. fujikuroi* isolates to produce fumonisin at low levels in the conditions tested and a good agreement between results obtained by ELISA and Real Time RT-PCR.

Characterization of toxigenic *Fusarium* species in China

Theo A.J. Van der Lee, X. Xu, C. Huang, L. Yang, D. Yu, Z. Zhang, J. Feng, C. Waalwijk
Plant Research International, Wageningen, Netherlands

The cereals wheat, maize, and barley are among the world's most important food and feed crops, yet quantity and quality of the production is threatened by Fusarium Head Blight (FHB) caused by a complex of *Fusarium* species. The Chinese climate is very conducive for FHB and disease pressure in several regions in China is among the highest in the world. Consumption of food and feed contaminated with the secondary metabolites produced by *Fusarium* species poses a great threat as these mycotoxins can cause serious illnesses and immune-suppression in humans and animals. The EU has recently set limits for several of these mycotoxins. The *Fusarium* genus is represented by a large number of evolutionary related but relatively diverse groups of organisms. Molecular research has shown that isolates that are morphologically similar represent different species (O' Donnell, 2004). We characterized over 2500 single spore isolates collected from maize, wheat and barley from many different provinces in China using diagnostic primers for the different species and chemotype as well as SSR markers. We found *F. asiaticum* to be the dominant species collected along the Yangtze River from wheat and barley samples. In the Northeast (Liaoning, Jilin, Heilongjiang) *Fusarium graminearum sensu stricto* was the dominant species on maize, but more to the West (Inner Mongolia, Henan) *F. boothii* and *F. meridionale* were more prevalent. In addition to a clear distinction for the different species based on geographic origin and host, we also found that isolates collected from barley from the downstream valleys of the Yangtze River are mainly deoxynivalenol (DON) producers, while the majority of isolates collected in the isolated mountainous provinces of Sichuan and Yunnan produce nivalenol (NIV). Our data suggest a recent displacement of the *Fusarium* population in the valleys of the Yangtze River.

O' Donnell, K., T. J. Ward, et al. (2004). Fungal Genetics and Biology. **41**(6): 600-623.

Msb2, a putative membrane mucin functioning in signalling and pathogenesis of *Fusarium oxysporum*

Elena Perez-Nadales, Antonio Di Pietro
University of Cordoba, Cordoba, Spain

The soilborne vascular wilt fungus *Fusarium oxysporum* infects a wide variety of plant species by directly penetrating roots, invading the cortex and colonizing the vascular tissue. The mitogen activated protein kinase (MAPK) Fmk1 is essential for plant infection. The signalling components upstream of the Fmk1 cascade are currently unknown. We have identified a gene from *F. oxysporum* whose predicted product shows homology with Msb2, a mucin functioning at the head of the filamentation MAPK cascade in yeast. To test whether *F. oxysporum* Msb2 is involved in signalling through the Fmk1 pathway, we produced targeted knockout mutants both in the wild type and the Δ*fmk1* background. The Δ*msb2* strains were still able to grow invasively on fruit tissue and across cellophane sheets, but are less virulent on tomato plants than the wild type strain. Similar to Δ*fmk1* strains, Δ*msb2* mutants also showed reduced secretion of pectinolytic enzymes. In contrast to Δ*fmk1* mutants, growth of Δ*msb2* strains was specifically affected by the cell wall-targeting compounds Congo Red (CR) and Calcofluor White (CFW). Interestingly, a Δ*fmk1*Δ*msb2* double mutant had dramatically impaired growth in the presence of CR and CFW, suggesting that Fmk1 and Msb2 interact genetically to regulate cell integrity in *F. oxysporum*. Western analysis with phospho-ERK antibody revealed similarities in the phosphorylation pattern between Δ*fmk1* and Δ*msb2* strains. These results suggest that *F. oxysporum* Msb2 interacts with the Fmk1 cascade to regulate a number of virulence-related functions as well as response to cell wall stress.