EU-ITN-ARIADNE

FINAL MEETING
ANNUAL MEETING 2013
Asilomar Fungal Genetics Conference 2013

Monday, March 11th 2013

09:00 – 12:00	**Arrival at the Asilomar Conference Grounds**
12:00 – 13:00	**Lunch Crocker Dining Hall**
13:30 – 13:40	**At Kiln**: **Introduction** – Coordinator's Welcome
13:45 – 17:15	**Presentations of the Marie Curie Fellows**
13:45 – 14:00	**Katja Schäfer, Uni Cordoba** **Components of the urease complex govern virulence of *Fusarium oxysporum* on plant and animal hosts**
14:00 – 14:15	**Mennat El Ghalid, Uni Cordoba** **Identification of chemoattractant compounds from tomato root exudate that trigger chemotropism in *Fusarium oxysporum***
14:15 – 14:30	**Vikram Naik, MPI Marburg** **The *Ustilago maydis* MAP Kinase signaling pathway: Identification of direct MAP kinase targets by phospho-peptide enrichment.**
14:30 – 14:45	**Marino Moretti, MPI Marburg** **Functional characterization of the putative cell surface receptor for hydrophobicity, Msb2, in *Ustilago maydis***
14:45 – 15:00	**Sónia Dias, CNB Madrid** **Pheromone-induced G2 cell cycle arrest in *Ustilago maydis* requires inhibitory phosphorylation of Cdk1**
15:00 – 15:15	**Pankaj Mehrotra, Uni Aberdeen** **Yeast-Hypha transition and immune recognition of *Candida albicans* influenced by defects in cell signal transduction pathways**
15:25 – 15:45	Coffee/Tea break
15:45 – 16:00	**Maria Filomena, Uni Aberdeen** **Elevation of chitin is linked with multiparallel mechanisms in response to *C. albicans* cell wall stress**

16:00 – 16:15	**Clara Baldin, HKI Jena** **Signalling pathways in *Aspergillus fumigatus***
16:15 – 16:30	**Elisabetta Marchegiani, INRA** **Role of MAP kinase pathways in the pathogenicity of the wheat pathogen *Mycosphaerella graminicola***
16:30 – 16:45	**Elisabeth Grund, Bayer** **Functional analysis of the Mps1 MAP kinase pathway in the rice blast fungus *Magnaporthe oryzae*.**
16:45 – 17:00	**Miriam Oses Ruiz, Uni Exeter** **Transcriptional regulatory circuits necessary for appressorium-mediated plant infection by *Magnaporthe oryzae***
17:00 – 17:15	**Therese Oskarsson, Carlsberg** **Characterization of the endocytotic proteins Yel1-Arf3-Gts1 in *Ashbya gossypii* and the role of Gts1 in endocytosis, actin localization and filamentous growth.**
17:15 – 17:45	**Open discussion/PI final meeting**
18:00	**Dinner**

Abstract book

Components of the urease complex govern virulence of *Fusarium oxysporum* on plant and animal hosts

Katja Schäfer, Elena Pérez-Nadales, Antonio Di Pietro

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In the soilborne pathogen *Fusarium oxysporum*, a mitogen-activated protein kinase (MAPK) cascade homologous to the yeast filamentous growth pathway controls invasive growth and virulence on tomato plants. Full phosphorylation of Fmk1 requires the transmembrane protein Msb2, a member of the family of signalling mucins that have emerged as novel virulence factors in fungal plant pathogens. A yeast two-hybrid screen for proteins interacting with the Msb2 cytoplasmic tail identified UreG, a component of the urease enzymatic complex. UreG belongs to a set of accessory proteins needed to activate Apo- urease, which converts urea to yield ammonia and carbon dioxide. The *F. oxysporum* genome contains two structural urease genes, *ure1* and *ure2*. Mutants in *ureG* or *ure1* showed reduced growth on urea as the sole carbon and nitrogen source. Lack of urease activity in the mutants resulted in failure to secrete ammonia and to increase the extracellular pH. The Δ*ureG* mutants caused significantly reduced mortality on tomato plants and on the animal model host *Galleria melonella*, while Δ*ure1* mutants only showed reduced virulence on tomato plants. Real-time qPCR analysis of key genes involved in nitrogen uptake and assimilation, as well as in the urea cycle, during infectious growth of *F. oxysporum* in *G. melonella* revealed increased transcript levels of arginase, which converts arginine to urea. Our results suggest a role for the urease accessory protein UreG in fungal virulence on plant and animal hosts.

Identification of chemoattractant compounds from tomato root exudate that trigger chemotropism in *Fusarium oxysporum*

Mennat El Ghalid, David Turra and Antonio Di Pietro

Fusarium oxysporum is a soilborne pathogen that causes vascular wilt disease on a wide range of plant species, including tomato (*Solanum lycopersicum*). The host signals that trigger fungal infection are currently unknown. A chemotropic response of *F. oxysporum* towards tomato root exudate was observed using a plate assay that measures directed growth of fungal germ tubes towards chemoattractants. To purifiy the chemoattractant coumpound(s) from tomato root exudate, we applied a series of purification methods including extraction with organic and inorganic solvents, fractionation by size exclusion and ion exchange chromatography. The compound(s) showing chemoattractant activity were found in the hydophilic fraction, had a molecular weight between 30 and 50 kDa and were sensitive to boiling and treatment with proteinase K, suggesting that they correspond to one or several secreted tomato proteins. Polyacrylamide gel electrophoresis of the active fraction revealed multiple protein bands of the expected size, two of which displayed chemoattractant activity when eluted from the gel. Identification of the active protein(s) by LC-ESI-MS is currently ongoing. Identification of the secreted chemoattractant(s) from tomato roots will advance our understanding of the molecular events that trigger fungus-root interactions.

The *Ustilago maydis* MAP Kinase signaling pathway: Identification of direct MAP kinase targets by phospho-peptide enrichment.

Vikram Naik, Gerold J.M. Beckers, Wolfgang Hoehenwarter and Regine Kahmann.

In the plant pathogenic fungus *Ustilago maydis* three MAP kinase modules have been identified mostly via their homology to genes in *Saccharomyces cerevisiae*. The module consisting of the MAP kinase *kpp2*, the MAP kinase kinase *fuz7* and the MAP kinase kinase kinase *kpp4* controls pheromone signalling and plays an essential role in mating and pathogenicity. Kpp2 is involved in filamentation and appressorium development while the MAP kinase, Kpp6, which also acts downstream of Fuz7, is required for appressorial penetration of plant epidermal cells. Our goal is to identify crucial virulence factors which act directly downstream of the MAP kinases Kpp2 and Kpp6. For this we generated a strain in which MAP kinase signaling can be induced by expressing a constitutively active version of the MAPKK Fuz7 (Fuz7DD) under an inducible promoter in the presence or absence of *kpp2* and *kpp6*. We then used a two-step chromatographic procedure combining phosphoprotein enrichment using $Al(OH)_3$-based metal oxide affinity chromatography (MOAC), followed by tryptic digest of enriched phosphoproteins, and TiO_2-based MOAC for phosphopeptide enrichment. This enabled detection of low abundant phosphorylated peptides using LC-MS/MS and allowed direct identification and site-specific quantification of phosphorylated peptides that differentially accumulated after MAP kinase activation in wild type and mutant cells. LC-MS/MS analysis of the phosphopeptide fraction obtained after the two-step MOAC yielded 111 putative substrates of Kpp2 and Kpp6 MAP kinases in three replicate experiments. Of these 20 differentially phosphorylated proteins were chosen for subsequent functional analyses. We are presently generating deletion mutants of these genes in compatible *U. maydis* strains that carry different *a* and *b* alleles and in a solopathogenic strain. In addition, we are analysing the expression pattern of the chosen genes during the different developmental stages of *U. maydis*. Results on the role of these *U.maydis* genes on signaling and pathogenicity will be presented.

Functional characterization of the putative cell surface receptor for hydrophobicity, Msb2, in *Ustilago maydis*

Marino Moretti, Daniel Lanver, Irina L. Schmidt and Regine Kahmann

Msb2 is a transmembrane mucin protein involved in plant surface sensing in *U. maydis*. *Msb2* deletion mutants are defective in sensing the hydrophobic leaf surface which is prerequisite for the differentiation of infection structures. Consequently, *msb2* mutants are attenuated in virulence (Lanver et al., 2010). The molecular mechanism leading to an activation of Msb2 and the downstream MAP kinase cascade is so far unknown. In yeast Msb2p is processed by the aspartyl protease Yps1p leading to an active cell-associated form and a secreted glycosylated part which has an inhibitory function in the full length protein. In *U. maydis* Msb2 is also processed, but so far there is no evidence that this leads to an activation of surface sensing. Using a yeast mutant lacking five aspartyl proteases we could demonstrate that yeast Yps1p is able to cleave *U. maydis* Msb2. In addition, by using this heterologous system, two *U. maydis* aspartyl proteases were identified that were weakly able to cleave Msb2. The respective genes were deleted in the solopathogenic strain SG200 and its Δ*msb2* derivative expressing Msb2-HA-GFP. Possible phenotypic alterations in virulence as well as in Msb2 processing will be monitored. In addition, a synthetic codon-adapted *YSP1* gene has been introduced in the above-mentioned *U. maydis* strains to analyze the effects of an increment in Msb2 cleavage on biological activity of the protein. Finally, the extracellular domain of Msb2 was subjected to a mutational analysis to identify regions with a presumed positive regulatory function.

Lanver, D., Mendoza-Mendoza, A., Brachmann, A. and Kahmann, R. (2010). Sho1 and Msb2-Related Proteins Regulate Appressorium Development in the Smut Fungus *Ustilago may*dis. The Plant Cell *22*, 2085-2101

Pheromone-induced G2 cell cycle arrest in *Ustilago maydis* requires inhibitory phosphorylation of Cdk1

Sónia Castanheira and José Perez-Martín

Ustilago maydis is a dimorphic basidiomycete that infects maize. In this fungus virulence and sexual development are intricately interconnected. Induction of pathogenicity program requires that two haploid compatible cells fuse and form an infective filament after pheromone signaling. The pheromone is transmitted by a well-known MAPK cascade and as in other fungi its recognition induces a cell cycle arrest in order to syncronyze cell cycle and prepare mating partners for conjugation. *Saccharomyces cerevisiae* and *U. maydis* use a similar MAPK cascade to respond to sexual pheromone and in both cases a morphogenetic response is provided (shmoo and conjugative hypha, respectively). However, while *S. cerevisiae* arrests its cell cycle in G1 in response to pheromone, *U. maydis* does this by arresting at G2. The mechanisms and physiological reasons involved in the pheromone−induced cell cycle arrest in *U. maydis* are unknown. We will introduce our first attempts to characterize the molecular mechanisms behind pheromone−induced cell cycle arrest in *U. maydis*. Our results have indicated that inhibitory phosphorylation of Cdk1 is part of the mechanism of the pheromone-induced G2 cell cycle arrest , as expression of a mutant allele of Cdk1 refractory to inhibitory phosphorylation impairs the cell cycle arrest. We analyzed the transcriptional pattern of cell cycle related genes in response to overactivation of pheromone pathway (using a constitutively activated allele of fuz7, the MAPKK of the cascade) and found that Hsl1, a kinase involved in G2/M transition and Clb2, a mitotic cyclin are downregulated at transcriptional level. We also observed that Hsl1 protein dissapears/delocalizes from the bud neck and Clb2 from the nucleus after inducing the G2 cell cycle arrest. Using chimeric promoter fusions we found that transcriptional downregulation is not important for pheromone-induced cell cycle arrest. We are trying to analyze the pattern of delocalization/degradation of Hsl1 and Clb2 in response to pheromone.

Yeast-Hypha transition and immune recognition of *Candida albicans* influenced by defects in cell signal transduction pathways

Pankaj Mehrotra, Rebecca A. Hall, Jeanette Wagener and Neil A.R. Gow

During the infection process *C. albicans* has to respond to various stresses imposed by the host environment including oxidative and osmolarity stress generated by phagocytic cells such as macrophages and neutrophils, and also the cell wall stress agents such as exposure to caspofungin and other antifungal antibiotics. These stress responses area orchestrated through the activation of multiple stress pathways including the cAMP-PKA, several MAPK pathways and the Ca^{2+}-calcineurin pathway influence the cell wall shape and composition. We are investigating the effect of the activation or inhibition of these pathways on immune recognition mechanisms. We therefore determined the importance of the MAPK, cAMP-PKA and Ca-calcineurin pathways on the fungal innate immune response by examining uptake, phagocytosis, and cytokine profile induced by mononuclear and polynuclear lymphocytes in response to a library of mutants in each of the above pathways under stressed and non-stressed conditions. We find that the activation and inhibition of these pathways plays a important role in remodeling of cell wall and hence the immunological profile. For example, deletion of *TPK1* and *CNA1* resulted in lower pro-inflammatory cytokine production. Immune- recognition was also affected by the exposure of *C. albicans* signaling mutants with Calcofluor-white, caspofungin , oxidative and osmotic stress and changes in temperature. These results suggest that stress signaling pathways act in a co-ordinated fashion to regulate yeast-hypha morphogenesis and the changes in the cell wall which in turn affects the immunological signature of the cell. Thus exposure to different microenvironments significantly modifies the immunological response to fungal cells, suggesting that responses to local stresses makes the fungal cell surface is a moving target for immunological surveillance.

Elevation of chitin is linked with multiparallel mechanisms in response to C. albicans cell wall stress

Maria Filomena and Neil A.R. Gow

The role of the MAPK, Ca2+/calcineurin and cAMP/PKA signal transduction pathways in regulating the *Candida albicans* cell wall stress response was investigated. A library of mutants lacking receptors, signalling elements and transcription factors were screened for alterations in the ability to respond to a range of cell wall stressing agents, including $CaCl_2$, Calcofluor White and caspofungin. Pre-treatment of wild-type cells with $CaCl_2$ and CFW, activates the Ca^{2+}/calcineurin and PKC pathways, leading to an increase in chitin content, and reduced susceptibility to caspofungin. Although elevation of cell wall chitin content often resulted in decreased sensitivity to caspofungin, we show here that some strains with increased chitin levels remained sensitive to caspofungin. The results show that elevation of chitin is a common property of a range of mutants that are affected in coordinating cell wall stress pathways, but that multiple mechanisms are likely to operate in maintaining the robustness of the *C. albicans* cell wall.

Signalling pathways in *Aspergillus fumigatus*

Clara Baldin, Vito Valiante, and Axel A. Brakhage

The saprophytic fungus *Aspergillus fumigatus* is emerging as one of the most important airborne human pathogens, with a very high mortality rate in immuno-compromised hosts. The great ability of this fungus to survive in different environments stimulated research on the regulation of the signalling pathways, with the aim to identify the mechanisms that allow *A. fumigatus* to adapt to different stresses.

The present study is focused on Tor signalling pathways and in particular on Tor kinase, the core protein of the pathway. Tor (Target of rapamycin) is an evolutionary conserved nutrient sensing protein kinase that regulates growth and metabolism in all eukaryotic cells. Tor pathway has been studied mainly in model organisms such as *Saccharomyces cerevisiae* and *Caenorabditis elegans*, and it was always reported as a major hub that intagrates multiple signals and relays them toward the regulation of a number of downstream pathways.

Tor has not been yet characterized in *A. fumigatus*. Unlikely *S. cerevisiae*, *A. fumigatus* has only one Tor kinase protein and our results demonstrate that this protein is essential for the fungus. Conditional mutants have been generated, testing different inducible promoters. Best results were obtained with the endoxylanase promoter from *Penicillium chrysogenum* (Zadra *et al.*, 2000). Fine regulation of Tor production showed that not only the absence of this protein is lethal for *A. fumigatus*, but also the overexpression leads to some toxic effects. However, our sophisticated conditional lethal mutant allows us to gain insight into the physiology of Tor kinase in filamentous fungi.

Role of MAP kinase pathways in the pathogenicity of the wheat pathogen *Mycosphaerella graminicola*

Elisabetta Marchegiani, Julie Vallet, Siân Deller, and Marc-Henri Lebrun

Mitogen-activated protein kinases (MAPKs) are essential components of fungal signaling pathways involved in different developmental processes and are required for host plant infection. *Mycosphaerella graminicola*, the causal agent of *Septoria tritici* leaf blotch (STB) of wheat, has three MAPK pathways that are all required for infection (*MgFUS3* , *MgHOG1, MgSLT2*; Cousin *et al.*, 2006; Mehrabi *et al.*, 2006a, Mehrabi *et al.*, 2006b). We showed that *Mgfus3* null mutants are non-pathogenic on intact wheat leaves (paint brush inoculation), but highly-reduced in pathogenicity when infiltrated into leaf tissues by syringe injection (reduced necrosis, low number of pycnidia). This suggests that *MgFUS3* is involved in fungal penetration, host colonization and pycnidia formation. *Mghog1* null mutants have pathogenicity defects similar to *Mgfus3* null mutants. This result highlights that the role of *HOG1* in pathogenicity on plants differs among fungi (Segmüller *et al.*, 2007). *Mgslt2* null mutants are fully non-pathogenic on inoculated wheat leaves either by paint brush inoculation or injection. This phenotype is unusual among *slt2* null mutants from other fungi. Therefore, *Mycosphaerella graminicola* MAPK pathways may have evolved to control regulatory networks differing from other fungal plant pathogens. To identify which genes are under the control of the *MgSLT2* signaling pathway, we are developing different transcriptomics analyses. Expression profiling relies on the comparison of transcriptomes of *Mgslt2* null mutants and wild type strains grown under conditions corresponding to either an active or an inactive *SLT2* pathway. Additional transcriptomics analyses will be performed using an allele encoding a conditionally active MAPKK expressed under the control of an inducible/repressible promoter. Genes whose expression requires an active *SLT2* MAPK will be further studied for their role in development and infection using reverse genetics.

Functional analysis of the Mps1 MAP kinase pathway in the rice blast fungus *Magnaporthe oryzae*.

Elisabeth Grund, Cemile Ant, Marie-Josèphe Gagey, Valérie Toquin, Roland Beffa, Nathalie Poussereau, and Marc-Henri Lebrun

Signaling pathways are important in coordinating fungal cellular processes required for stress resistance, development and pathogenicity. The project concerns the study of the Mps1 MAP kinase pathway of the rice blast fungus *M. oryzae*, involved in cell wall integrity, sporulation and pathogenicity. *MPS1* is the orthologue of yeast *SLT2* that encodes a MAP kinase activating the transcription factors Rlm1, Swi4 and Swi6. The null mutants Δ*mps1*, Δ*swi4*, Δ*swi6* and Δ*rlm1* were constructed in *M. oryzae* by gene replacement and phenotyped. Sensitivity of *M. oryzae* to cell wall degrading enzymes and cell wall inhibitors was found to be dependent on pH. Indeed, *M. oryzae* cell walls display a resistance to enzymatic degradation at pH 5, while they are sensitive at pH 6. Δ*mps1* lose this pH 5 induced cell wall resistance, but is as sensitive as wild type at pH 6. *M. oryzae* is also highly resistant to calcofluor at pH 5 (10x) compared to pH 6. Δ*mps1* loses this pH 5 induced calcofluor resistance, but is as sensitive as wild type at pH 6. *M. oryzae* is more sensitive (20x) to Nikkomycin Z (chitine synthase inhibitor) at pH 5 than pH 6, while sensitivity to Aculeacin (glucan synthase inhibitor) is independent of the pH. However, Δ*mps1* is as sensitive as wild type to these inhibitors at both pH. We conclude that the pH 5 induced resistance of fungal cell wall to degrading enzymes and calcofluor requires the Mps1 Pathway. This also suggests that the Mps1 pathway is strongly activated at pH 5 compared to pH 6. To test this hypothesis, we are assaying the phosphorylation status of Mps1 at different pH as well as under several stress conditions and developmental stages to know when the pathway is activated. Additionally we constructed an activated allele of Mkk1, the MAPKK upstream of Mps1, placed under the control of either its own promoter (a) or the repressible *pNIA1* promoter. These transformants will be used to assess the effect of controlled activation of the Mps1 pathway on *M. oryzae* cellular functions. The different conditions of Mps1 pathway activation will be used for a comparative transcriptomic analysis of wild type and Δ*mps1* mutants.

Transcriptional regulatory circuits necessary for appressorium-mediated plant infection by *Magnaporthe oryzae*

Miriam Oses-Ruiz, Darren M. Soanes, and Nicholas J. Talbot

Rice blast disease is caused by the fungus *Magnaporthe oryzae* and is the most destructive disease of cultivated rice. The pathogen elaborates a specialized infection structure called the appressorium. The morphological and physiological transitions that lead to appressorium formation of *M. oryzae* during plant infection are stimulated through perception of environmental signals including surface hydrophobicity and hardness, and the presence of cutin monomers and leaf surface waxes. The fungus perceives and internalizes these stimuli by a variety of intracellular MAP kinase signaling pathways. The homeobox and C2/H2 Zn finger domain transcription factor, MST12 (ScSte12 homogue) is part of the PMK1 MAP kinase signalling pathway, which is required for appressorium formation and invasion. The Mst12 null mutant is able to form completely normal melanised appressoria but it is non pathogenic. The Mst12 null mutant is unable to form a penetration peg and therefore to cause disease in the rice plant. To understand the mechanism of the penetration peg formation, we have recently carried out genome-wide comparative transcriptional profiling analysis for *mst12* null mutant using RNA-seq and HiSeq 2000 sequencing. In this way, we will show the transcriptional signature associated with penetration peg differentiation in the rice blast fungus. Moreover we will show the set of genes that are likely to be MST12 regulated and therefore help define the regulatory circuits necessary for appressorium-mediated plant infection by plant pathogenic fungi.

Characterization of the endocytotic proteins Yel1-Arf3-Gts1 in *Ashbya gossypii* and the role of Gts1 in endocytosis, actin localization and filamentous growth.

Therése Oskarsson, Klaus Lengeler, and Jürgen Wendland

Endocytic vesicle formation and regulation thereof is performed by a complex protein machinery, coordinating every detail of the endocytic process from initiation and pit formation to vesicle scission and uncoating.

We have used the filamentous fungi *Ashbya gossypii* to study three proteins that are involved in uncoating of vesicles in clathrin-mediated endocytosis. We deleted the corresponding genes encoding the GTP-binding protein Arf3 and its regulators the Guanine nucleotide Exchange Factor Yel1 and the ArfGAP protein Gts1 using PCR-based gene targeting methods. We then characterized these mutant strains under various conditions. While no deletion-specific phenotypes could be observed in the *Δarf3* and *Δyel1*, the *Δgts1* strain shows several severe mutant phenotypes. Deletion of *GTS1* results in a strong growth defect and renders mycelia with severe endocytotic deficiencies indicated by distinctly reduced endocytic rates, and large immobile vacuoles. Other phenotypic observations in *A. gossypii Δgts1* strains indicate that Gts1 may have additional functions other than regulating the activity of Arf3. We have observed effects of Gts1 on temperature stress resistance, actin localization and polar- as well as filamentous growth. The importance of *GTS1* for polarized hyphal growth leads us to studying the *GTS1* homolog of the human fungal pathogen *Candia albicans* in an effort to elucidate its role for the yeast-to-hyphal transition in this dimorphic fungi.