3rd

Botrytis Genome Workshop
25-27 September 2008
Tenerife, Canary Islands, Spain

ULL | Universidad de La Laguna
Departamento de Bioquímica y Biología Molecular

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Bayer CropScience ensures through innovation sustainable agriculture and profitable growth for nutrition and prosperity.

Fungicides avoid or abolish fungal plant diseases, which could affect severely harvest outcome and quality. There is a trend for a worldwide increase of the fungicide market - particularly in fruits and vegetables. Bayer is a global market leader in the agrochemical sector in providing plant protection products for agriculture worldwide.

Develop a new fungicide into a registered plant protection product means to end with a product which is not only good in controlling the fungal diseases, but also safe for the user, the environment and the consumer. There is a strong commitment for Bayer CropScience to develop new products that can fit with these requirements, and is planning to invest a total of EUR 3.4 billion in research and development between 2008 and 2012.

To meet the various challenges of fungal diseases, fungicides come in various forms. They are used to protect seeds and crops or to stop initial infestation as a part of integrated pest management. Bayer CropScience a broad range of active ingredients in our fungicide portfolio to control various fungal diseases in all major crops. Our clear goal is to gain market leadership through innovation and new tailor-made solutions.

The need for innovation in new fungicides comes from different issues: resistance management as a continuous challenge, the need for using fully the productivity potential of crops (yield and quality), and the need to strength enhanced food safety. Innovation is also needed for the control of new diseases or shifts in plant disease epidemics, and to provide innovative offers from the competition.

Bayer CropScience offers to the farmers good quality products to control botrytis as Fenhexamid. Fluopyram, the new developed fungicide for botrytis and other diseases control provides best protection with lowest application rates, and offers control of newly emerging diseases, e.g. brown spot on pears (stemphylium), free trade flow assured, broad label with possible use in essentially all crops and a new option for resistance management.

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Thursday 25 September 2008

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9:10 **Nicholas J. Talbot**. What have we learned from sequencing fungal and oomycete genomes?
9:50 **Marc-Henri Lebrun**. Whole genome sequencing of the fungal plant pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*.
10:20 **Christina Cuomo**. Comparative genomics of *Sclerotinia sclerotiorum* and *Botrytis cinerea*.

11:00 Coffee break.

11:30 **Michael R. Thon**. Gene Family Evolution in *Botrytis cinerea* and *Sclerotinia sclerotiorum.*
11:50 **Linda Kohn**. Evolution on three scales: A Leotiomycete-eye view of *Botrytis* and *Sclerotinia*, a mind's eye-view of ecological speciation and MAT-locus evolution, and a genome-wide SNP-view of recombination in *Sclerotinia sclerotiorum*.
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15:20 **Francisco J. Fernández-Acero**. A proteomic approach to cellulose degradation by *B. cinerea*.

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16:40 **Géraldine Mey**. Mechanisms of regulation of amino acid transport and metabolism in phytopathogenic fungi.
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Third session

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10:30 **Juan Luis Turrión-Gomez**. Nitric oxide production in *Botrytis cinerea*.

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12:00 **Jens Heller**. Identification of BcSak1 regulated genes during oxidative stress and functional characterization of *bop1*, as one target gene of BcSak1

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16:50 **Nora Temme**. The transcription factor Bap1 or the role of H_2O_2 degradation during plant infection in *Botrytis cinerea.*

17:10 **Leonie B. Kokkelink**. The small GTPases BcRAS1 and BcRAC are linked to the stress activated MAPK cascade in *Botrytis cinerea.*

17:30 **Sabine Fillinger**. Expression analysis of stress-related genes in signal transduction mutants.

17:50 **Michaela Leroch**. Complex signalling pathways that control germination and infection of *Botrytis cinerea*.

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11:20 **Matthias Hahn**. About ABC transporters and map-based cloning: Molecular mechanisms of multidrug resistance in *Botrytis*.
12:00 **Alexis Billard**. Fenhexamid resistance in the phytopathogenic fungus *Botrytis cinerea*.
12:20 Concluding remarks.
12:30 Free time / Genome paper meeting.

13:00 Lunch.

First session

COMPARATIVE GENOMICS

What have we learned from sequencing fungal and oomycete genomes?

Nicholas J Talbot

School of Biosciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter, EX4 4QD, United Kingdom. E-mail: N.J.Talbot@exeter.ac.uk.

Fungi and oomycetes are the causal agents of many of the world's most serious plant diseases and are unique among the microbial pathogens in being able to breach the intact surfaces of host plants, rapidly establishing infections that can have disastrous consequences for large-scale agricultural production. The ability to cause plant disease is not a common trait among the many saprotrophic and mutualistic fungal species, but it is a very widespread one, occurring throughout the fungal kingdom. The availability of genome sequences from a wide range of fungal and oomycete pathogens is, for the first time, allowing an analysis of the evolution and diversification of fungal pathogenicity traits. There are currently genome sequences from 12 plant pathogenic fungi and 2 oomycetes that are publicly available, out of a total of more than 40 fungal genomes that have now been sequenced and many more that are in progress. The seminar will bring together some observations based on analysis of the fungal and oomycete genomes that have been reported so far and dicsuss the evolution of phytopathogenicity in microbial eukaryotes and the features present within predicted gene inventories that are common to plant pathogenic species and which may be fundamental to their biology. In addition, the deployment of functional genomic strategies to investigate the biology of plant infection by the model plant pathogen *Magnaporthe oryzae* will be discussed and how these methods are changing the way in which the plant-fungal interaction can be explored.

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Soanes, D.M., Alam, I., Cornell, M., Wong, H.M., Hedeler, C., Paton, N.W., Rattray, M., Hubbard, S.J., Oliver, S.G., and Talbot, N.J. (2008) Comparative genome analysis of filamentous fungi reveals gene family expansions associated with fungal pathogenesis. *PLoS ONE* **3:** e2300 doi:10.1371/journal.pone.0002300

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Soanes, D.M., Richards, T.A., and Talbot, N.J. (2007) Insights from sequencing fungal and oomycete genomes: What can we learn about plant disease and the evolution of pathogenicity? *Plant Cell* **19:** 3318-26.

Cornell, M., Alam, I. Soanes, D.M., Wong, H.M., Hedeler, C. Paton, N.W., Rattray, M.R., Hubbard, S.J., Talbot, N.J. Oliver, S.G. (2007) Comparative genome analysis across a kingdom of eukaryotic organisms: specialization and diversification of the fungi. *Genome Research* **17:** 1809-1822

Whole genome sequencing of the fungal plant pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*

Joëlle Amselem[1,2], Alain Billault[3], Mathias Choquer[1], Arnaud Couloux[2], Christina Cuomo[4], David DeCaprio[4], Martin Dickman[5], Sabine Fillinger[1], Elisabeth Fournier[1], James Galagan[4], Corinne Giraud[1], Chinnappa Kodira[4], Linda Kohn[6], Caroline Levis[1], Evan Mauceli[4], Cyril Pommier[2], Jean-Marc Pradier[1], Emmanuel Quevillon[2,8], Jeffrey Rollins, Béatrice Ségurens[3], Adeline Simon[1], Muriel Viaud[1], Jean Weissenbach[3], Patrick Wincker[3] and Marc-Henri Lebrun[8]

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Botrytis cinerea and *Sclerotinia sclerotiorum* are destructive fungal pathogens of many economically important crops provoking grey and white mould respectively. The Broad Institute has released the assembly of a 4-5 x genomic sequence from *B. cinerea* strain B05-10 (TMRI/Syngenta) as well as the 7-8 x genomic sequence of *S. sclerotiorum* (Broad Institute). In parallel, the French national sequencing center (Genoscope) has sequenced the genomes of grapevine and of two of its pathogens: Stolbur phytoplasm and *B. cinerea* strain T4. A large collection of ESTs (> 80 000) have been obtained for both *B. cinerea* and *S. sclerotiorum*. Automatic gene prediction with *ab initio* and similarity softwares have been applied to the three genomes revealing 14.000 to 16.000 potential gene calls for both species. Comparative analyses of both *B. cinerea* strains and of *B. cinerea vs. S. sclerotiorum* has revealed a common set of 8400 gene calls, 1200 of which are absent from other fungal genomes. In addition both species present a high degree of sequence similarity (80 % average protein sequence identity among orthologues), and of synteny at the contig level. These two species significantly differ in their repetitive DNA content, since *S. sclerotiorum* has more repetitive elements (7.7 %) than *B. cinerea* (3 - 4 %). This difference is likely related to the expansion of some families of transposable elements (TEs) in *S. sclerotiorum*. TEs seem to have been subject to RIP in both species although at higher levels in *B. cinerea.* The comparison between *B. cinerea* and *S. sclerotiorum* offers an interesting opportunity to compare the genomes of two closely related necrotrophic plant pathogens helping to understand evolutionary trends that have shaped their genomes. This comparison has highlighted differences between *B. cinerea* and *S. sclerotiorum* in secondary metabolism gene clusters, transcription factors and mating type loci, while sets of secreted proteins and metabolic enzymes are almost similar. These findings suggest that both species have evolved different strategies to behave as non specialized necrotrophic plant pathogens.

Comparative genomics of *Sclerotinia sclerotiorum* and *Botrytis cinerea*

Christina Cuomo[1], Chinnappa Kodira[1], Zehua Chen[1], Evan Mauceli[1], James Galagan[1], Bruce Birren[1], Joelle Amselem[2,3], Muriel Viaud[2], Marc-Henri Lebrun[2-4], Jeffery Rollins[5], Linda Kohn[6], and Martin Dickman[7]

[1]Broad Institute of MIT and Harvard, Cambridge, MA, USA,[2]INRA-BIOGER,Versailles, France, [3]INRA-Unité de Recherche Genomique-Info, Versailles, France, [4]UMR CNRS-UCB-INSA-BCS, Lyon, France, [5]Department of Plant Pathology, University of Florida, Gainesville, FL, USA, [6]Department of Botany, University of Toronto, Ontario, Canada, [7]Inst for Plant Genomics and Biotech, TAMU, College Station, TX, USA.

The sequencing and annotation of *Sclerotinia sclerotiorum* and two strains of *Botrytis cinerea* provides an extraordinary opportunity to study the genome evolution of two closely related necrotropic fungal pathogens. The 8X assembly of *S. sclerotiorum,* which is 38Mb, is comprised of 36 scaffolds, which we mapped to 16 chromosomes using an optical map. We predicted 14,522 predicted genes using an automatic annotation process. We have also generated more than 64,000 EST sequences from five different cDNA libraries representing different developmental stages (mycelia, sclerotia, stipe, infection cushion) and a library from infected Brassica; three other libraries (oxidative stress, apothecia, and infected tomato) will be constructed and sequenced. A 5X assembly of the Syngenta AG sequence of *B. cinerea* from strain B05.10, which is 39Mb, was subjected to similar automated annotation, producing 16,448 genes. We have revised these gene sets to flag ~2,500 dubious gene calls in each genome, which include probable transposons and low confidence predictions. We are using the revised gene sets to evaluate the synteny between the two genomes, and to identify regions which appear unique to each. Such regions include potential secondary metabolism gene clusters, and examples are found in both genomes. To look at common features of *Botrytis* and *Sclerotinia*, we are building gene families using OrthoMCL with selected sequenced Ascomycetes, to identify expanded, unique or missing gene families with respect to both *Sclerotinia* and *Botrytis*.

Gene Family Evolution in *Botrytis cinerea* and *Sclerotinia sclerotiorum*

Michael R. Thon and Serenella A. Sukno

Centro Hispano-Luso de Investigaciones Agrarias (CIALE), University of Salamanca

To better understand the role of gene family expansion and contraction in the adaptive evolution of *B. cinerea* and *S. sclerotiorum*, we performed a genome-wide comparative analysis of gene family content. Protein sequences from seven fungal genomes were clustered and all clusters containing five or more proteins were analyzed with the CAFE software package to identify statistically significant changes in gene family size. Since we are interested in the evolution of the Leotiomycetes, we identified the gene families that showed a significant size changes along the branch that represents the divergence of the Leotiomycetes from the lineage shared by the non-Leotiomycete, *Stagonospora nodorum*. We identified 20 gene families with statistically significant *p*-values ($P < 0.01$), of which seven represent gene family expansions, and 13 represent gene family contractions. Three of the seven families that show evidence for expansion have roles in carbohydrate metabolism, suggesting that changes in carbohydrate metabolism occurred during the evolution of this lineage of fungi.

We have also performed a detailed analysis of the G-protein coupled receptor (GPCR) gene families. We used InterProScan and BLASTP to identify GPCRs in the genomes of *Botrytis cinerea* and *S. sclerotiorum*. Proteins belonging to six different GPCR families were identified. The PTHll-like proteins make up the largest family of GPCRs, and is comprised of 56 copies in *B. cinerea* and 48 copies in *S. sclerotiorum.* The *M. grisea* PTH11 protein functions in substrate sensing for appressorium development. The large and variable size of this family suggests that it is evolving rapidly, and coupled with its hypothetical role in external substrate sensing, suggests that this family has an important role in the adaptation of these species to their environment.

Evolution on three scales: A Leotiomycete-eye view of *Botrytis* and *Sclerotinia*, a mind's eye-view of ecological speciation and MAT-locus evolution, and a genome-wide SNP-view of recombination in *Sclerotinia sclerotiorum*

Linda M. Kohn

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The relationship of *Botrytis* and *Sclerotinia* to the Sclerotiniaceae and Rutstroemiaceae and to the Leotiomycetes will be briefly presented. In collaboration with E. Fournier (INRA-Versailles) we have constructed a genealogy with a solid foundation of 11 loci: actin (*ACT*), calmodulin (*CAL*), chitin sythase 1 (*CHS1*), ras protein (*RAS*), internal transcribed spacer region of the rDNA repeat (*ITS*), intergenic spacer region of the rDNA repeat (*IGS*), translation elongation factor 1-alpha *(EF-1α*), heat shock protein 60 (*HSP60*), glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*), ubiquitin chain assembly factor (*FG1020*), and a putative ATP-dependent RNA helicase (*MS547*). It is evident that *Botrytis* and *Sclerotinia* evolved from a common ancestor in a close timeframe; branch lengths are short and directionality cannot be determined. Necrotrophy appears to be the fundamental state in the Sclerotiniaceae, with at least two shifts to biotrophy, but this is being tested with a set of trophic-type defining criteria to be screened via *in planta* expression studies.

A perspective on speciation in *Botrytis* and *Sclerotinia* will be presented, with consideration of the evidence for heterothallism and dual-mating in the former, and homothallism and heterothallism in the later.

On a finer scale, we have used the set of 500 SNPs dispersed on all 16 linkage groups developed from the genome isolate and an Australian isolate at the Broad Institute (C. Cuomo). Forty regions were screened for SNPs, on four linkage groups, in 19 isolates representing four *S. sclerotiorum* populations (as determined by populations genetic, phylogenetic, and coalescent methods with multilocus sequence data in previous studies). In this sample, which did not include the Australian isolate, approximately 75% of the Broad SNPs were present, plus additional SNPs not identified in the Broad screen. There was strong evidence of both inter- and intra-chromosomal recombination. Both these data and other, indirect tests indicate a history of recombination in this species, although not necessarily recent. This is interesting given the clonal signature of many population samples and the failure to find segregants among sibling ascospores. These SNPs are now the tool of choice for population-scale studies in *S. sclerotiorum*.

Carbohydrate-active enzymes in *Botrytis* and *Sclerotinia* genomes: expert annotation and analysis

Pedro M. Coutinho[1], Etienne Danchin[1,2], and Bernard Henrissat[1]

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Carbohydrate-active enzymes (CAZymes) are responsible for the degradation, the biosynthesis and plasticity of complex carbohydrates and glycoconjugates. While some CAZymes are heavily involved in housekeeping aspects dealing particularly with N- and O-glycosylation, energy storage, and cell-wall biosynthesis and remodelling in most organisms, others mediate the interaction with carbohydrates present in the environment, a feature particularly important for interaction with the host in phytopathogenic fungi. We identified and annotated the CAZyme sets from the Leotiomycetes *Botrytis cinerea* and of *Sclerotinia sclerotiorum* deduced from the respective genomes by comparison of the respective sets of 'best protein models' against libraries derived from CAZy (1), and other genomes previously annotated by our team.

The genomes from these two necrotrophic Ascomycetes contain rich sets of glycoside hydrolases and transglycosidases, that are however smaller those found in saprophytes and phytopathogens from the Eurotiomycetes and Sordariomycetes groups. A pectin-degrading machinery is found in both organisms, with an important content in family GH28, only overtaken by known *Aspergilli* genomes (2,3). Significantly, and when compared to Sclerotinia, a slightly larger set of pectin-degrading enzymes is consistently found in *Botrytis* for several families. This pectin-degrading capacity together with a low hemicellulose-degrading capacity observed in the two genomes contrast significantly with what is observed for other Ascomycetes, among known phytopathogens (4), and saprophytes (2,3,5). Interestingly a very large set of GH71 with candidate α-1,3-glucan degrading activity is observed. The importance of this set is emphasized by the multiplicity of ancillary CBM24 α-1,3-glucan binding modules attached to these GH71 enzymes that have no equivalent in other known genomes. The impact of this large α-1,3-glucan-degrading capacity in phytopathogenicity is unknown at present.

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2. Pel HJ, et al. (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nature Biotechnol. **25**: 221-231.

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Second session

GENE FAMILIES

Secreted protein-encoding gene families in *Botrytis cinerea*

Jan van Kan

Wageningen University, Laboratory of Phytopathology, Binnenhaven 5, 6709 PD Wageningen, The Netherlands

Infection of host plants by *Botryis cinerea* is mediated by numerous enzymes and metabolites that the pathogen secretes at the host-fungus interface.

I will present an overview of the secreted protein-encoding genes that were identified in the genome sequences of *B. cinerea* strains B05.10 and T4, and then focus on the family of aspartic proteinases (APs), comprising 14 genes. The structural features of the different AP enzymes will be discussed. Functional analysis by gene replacement has been performed for 6 gene family members, i.e. *Bcap*1-*Bcap*5 and *Bcap*8. Results of these functional analyses will be presented.

Preliminary analysis of *Botrytis cinerea* secretome during first stages of infection

José J. Espino[1], Gerardo Gutiérrez-Sánchez[2], Nélida Brito[1], Punit Shah[2], Ron Orlando[2], and Celedonio González[1]

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The extracellular proteome, or secretome, of phytopathogenic fungi is presumed to be a key element of its infection ability. Especially interesting constituents of this set are those proteins secreted at the beginning of the infection, during the adherence and germination of conidia on the plant surface, since they may play essential roles in the establishment of a successful infection. Identification of the proteins secreted by *B. cinerea* at an early stage could be, therefore, a good approach to understand the interaction between this fungus and its hosts.

We have devised a method to prepare and isolate the proteins secreted by *B. cinerea* during the germination of conidia, in conditions that resemble the plant environment. Basically, *B. cinerea* conidia are germinated for 16 hours in a synthetic medium enriched with low-molecular weight plant compounds, those able to cross a dialysis membrane, and the secreted proteins are then collected by a double precipitation protocol. 2D electrophoresis of the precipitate showed more than 50 proteins, mainly in the acidic region, with molecular weights in the range of 10-60 kDa. Germination of the conidia in different vegetable extracts produced secretomes with different compositions. Peptide mass fingerprint of the spots in 2D-electrophoresis, as well as liquid chromatography-tandem mass spectrometry of tryptic fragments, were both used to identify individual components of the secretome, and the results will be reported.

A proteomic approach to cellulose degradation by *B. cinerea*

F.J. Fernández-Acero[1], T. Colby[2], A. Harzen[2], U. Wieneke[2], C. Garrido[1], M. Carbú[1], I. Vallejo[1], J. Schmidt[2] and J. M. Cantoral[1]

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At present, *Botrytis cinerea* has been adopted as an important model system in molecular phytopathology, although the molecular basis of the infection remains unclear. Proteomic approaches have revealed significant information about the infective cycle of several pathogens, including *B. cinerea* (1, 2, 3). Only a few papers have reported a proteomic approach to the fungus itself, and a proteomic overview of *B. cinerea* has been lacking. The main aim of this study is to make available to the scientific community a proteomic database containing a significant number of identified proteins from *B. cinerea* during cellulose degradation. In brief, three biological samples were used, and the extracted proteins were independently separated by 2-D PAGE to obtain the proteome map from *B. cinerea.* More than 300 proteins spots were selected for MALDI TOF/TOF MS analysis, resulting in 267 positive identifications, most representing unannotated proteins. Identified proteins were then classified into categories using the PANTHER classification system, showing the relevance of protein metabolism and modification process and oxidoreductase activity. This proteomic map of *B. cinerea* will be a useful basis for exploring the proteins involved in the infection cycle, which will in turn provide new targets both for crop diagnosis and focused fungicide design.

1. Fernández-Acero, F. J., Jorge, I., Calvo, E., Vallejo, I.*, et al.*, Two-dimensional electrophoresis protein profile of the phytopathogenic fungus *Botrytis cinerea*. *Proteomics* 2006, *6*, S88-S96.

2. Fernández-Acero, F. J., Jorge, I., Calvo, E., Vallejo, I.*, et al.*, Proteomic analysis of phytopathogenic fungus *Botrytis cinerea* as a potential tool for identifying pathogenicity factors, therapeutic targets and for basic research. *Arch. Microbiol.* 2007, *187*, 207–215.

3. Fernández-Acero, F. J., Carbú, M., Garrido, C., Vallejo, I.*, et al*., Proteomic Advances in Phytopathogenic Fungi. *Curr. Proteomics* 2007, 79-88.

Investigating secondary metabolism genes in *B. cinerea* and *S. sclerotiorum* genomes reveals a high potential for sesquiterpenes biosynthesis in the grey mould fungus

Muriel Viaud[1], Isidro Collado[1], Jean-Marc Pradier[1], Cristina Pinedo[1], Mathias Choquer[1], Bérengère Dalmais[1], Pascal Le Pêcheur[1], Guillaume Morgant[1] & the « *Botrytis/Sclerotinia* genome consortium »

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Comparative genomics between the closely related Leotiomycetes species *B. cinerea* and *S. sclerotiorum* may unravel the differences that could be associated with their necrotrophic lifestyles and their broad host ranges. Secondary metabolism genes were investigated by looking for conserved domains of the relevant « key enzymes » *i.e.* Terpene Synthases, Polyketide Synthases (PKS), Non-Ribosomal Peptide Synthases (NRPS) and Dimethylallyl Tryptophan Synthetases (DMATS). Genomic data revealed that *B. cinerea* and *S. sclerotiorum* have 42 and 28 key enzymes respectively, and share only 19 of them. Most genes are organized in physical clusters, and species-specific clusters correspond to synteny breaks suggesting recent cluster gains or losses.

The most significant difference is an enrichment of sesquiterpene cyclases (STC) genes in *B. cinerea* (6 *versus* 1 in *S. sclerotiorum*). The distribution of *STC1-6* genes was further investigated in other *Botrytis* species that, in contrast to *B. cinerea*, have narrow host ranges. A typical « patchy » distribution and an enrichment in *B. cinerea* and its closest related species were revealed.

Functional analysis of the STC genes has been initiated by gene inactivation. STC1/CND15 was shown to be the cyclase necessary for the first step of biosynthesis of botrydial from farnesyl pyrophosphate (FPP). Pathogenicity tests confirmed that this toxin acts as a strain-dependant virulence factor (1). Indeed *STC1* inactivation in T4 led to a defect in colonization while the same mutation in the B05-10 strain had no effect on virulence. The lack of botrydial synthesis in B05-10 *stc1/cnd15* mutants seems to be compensated by the overproduction of the polyketide type toxin botcinic acid (a toxin not produced in T4 strain).

The STC2 amino acid sequence is similar to the trichothecene cyclase TRI5. Biochemical characterization of *stc2/tri5* null mutants did not allow yet to identify the corresponding metabolite. However, these mutants also showed a reduced colonization on bean and tomato leaves.

Altogether genomic and functional data suggest that the polyphagous fungus *B. cinerea* has acquired a high potential for sesquiterpenes biosynthesis, that at least two STCs are involved in virulence and that botrydial and botcinic acid may have a redundant function in virulence

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Mechanisms of regulation of amino acid transport and metabolism in phytopathogenic fungi

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How pathogenic fungi fulfill their nutritional needs during the interaction with their host remains poorly documented. The low concentrations of several amino acids (cysteine, methionine, proline, tryptophan, histidine and arginine) in the plant leaf apoplast may not be sufficient to support the parasitic growth (Solomon and Oliver, 2002). Thus phytopathogenic fungi may first mobilize their storage compounds to synthesize their own amino acids during the early stages of the infection process, then use the subsequent enzymatic degradation of the host constituents to release simple compounds used to support further their development *in planta*. The metabolism of amino acids in parasitic fungi may thus be redirected and adapted to the different stages of plant infection. The metabolic orientations and their control mechanisms required for the development cycle progression have not been well characterized so far.

In this context, our studies focus on the regulation of amino acid transport and metabolism with a special emphasis on the pathways involving the protein kinase TOR and the sulfur regulator MetR. The gene encoding the monomeric GTPase Rheb (Ras Homologue Enriched in Brain), known to activate the kinase TOR and to be involved in the regulation of arginine and lysine uptake in *Saccharomyces cerevisiae and Schizosacharomyces pombe*, was inactivated by RNA interference in the necrotrophic fungus *Botrytis cinerea*. The implication of Rheb in the control of amino acid acquisition was appreciated using (1) complementation experiments of a mutant strain of *S. cerevisiae*, (2) analysis of amino acid uptake/metabolism by reversed chromatography using HPLC and transport kinetic assays, (3) quantification of the transcripts encoding amino acid transporters in the mutant strains. Knock-out mutants of the gene encoding the bZIP transcription factor MetR orthologue of the *S. cerevisiae* Met4p, which controls the expression of the genes encoding the sulfate reduction pathway leading to methionine, cysteine and glutathione biosyntheses, were generated by gene replacement in the hemibiotrophic rice pathogen *Magnaporthe grisea*. Trophic requirements of the deleted strains is being appreciated using (1) nutritional complementation experiments, (2) evaluation of the expression level of the putative target genes of MetR by qPCR, (3) quantification of the intracellular amino acids and sulfur compounds by reversed chromatography and (4) transcriptomic approaches.

Mutants of MetR and Rheb are currently generated respectively in *B. cinerea* and *M. grisea*. The comparative analysis of the results obtained with both fungi will allow a better understanding of the mechanisms which control amino acid and sulfur metabolism into pathogenic fungi and their role in the control of fungal development.

Botrytis secretes arabinan-degrading activities that may play a role in pathogenesis

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Botrytis cinerea secretes a wide range of hydrolytic enzymes that degrade complex polysaccharides, including pectins. Pectin constitutes nearly one third of the cell wall polysaccharides in the model plant *Arabidopsis thaliana* and arabinan is a major pectic polysaccharide that is relatively well conserved among plants. It has previously been reported that *Botrytis* secretes arabinan degrading enzymes during the infection of apples; however, exactly what roles these activities and arabinan play during infection are still unknown.

To address this problem, we first studied the presence of arabinan-degraging activities *in vitro*. A plate assay using AZCL-conjugated arabinan showed that arabinan-degrading activities were absent in cultures in PDB medium, but were present in soy-peptone cultures as well as the culture supernants of *Botrytis*. Crude protein extracts from *Arabidopsis* leaves infected with *Botrytis* also showed the presence of arabinan-degrading activities. The culture supernatants were shown to generate arabinooligosaccharides with the degree of polymerization one through six when mixed with de-branched arabinan for varying periods, as measured by high-pressure aninon exchange chromatography. Taken together, these results indicate that *Botrytis* secretes both endo-arabinanase and arabinofuranosidase during infection. Genomic loci with significant sequence similarities to *Aspergillus* endoarabinanase and arabinofuranosidase were found in the genome sequence of *Botrytis (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html).*
Constrution of knock-out mutants of each of these loci are in progress.

In order to identify the function of arabinan in plants, *Arabidopsis* wild type and arabinan-deficient mutant (*arad1*) were subjected to infection assays with *Botrytis*. Our group has previously shown that *arad1* mutants are defective in arabinan biosynthesis and contains ca. 30% wild-type level of arabinose in the pectic rhamonogalacturonan I fraction (Harholt et al., 2006). The assays showed that *arad1* mutants were more susceptible to *Botrytis* infection as compared to wild type. These restuls clearly demonstrate that pectic arabinan plays a role in delaying *Botrytis* infection in *Arabidopsis.*
Conclusion: Our results indicate that arabinan forms a barrier against *Botrytis* and *Botrytis* overcomes this barrier by inducing arabinan-degrading enzymes and degrading arabinan.

Third session

PATHOGENICITY

Pathogenicity determinants in *Botrytis cinerea*.

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Concerted efforts of the rapidly growing *Botrytis* community have made *B. cinerea* to one of the best-investigated fungal pathogens; it is becoming the model system for the study of necrotrophy. A large set of knock out mutants and the increasing use of "omics" approaches helped to improve our understanding of the complex and highly variable life-style of this pathogen; still our knowledge is limited. I will give a short overview on the candidate gene approach (cell-wall degrading enzymes, toxic metabolites, etc.); only very few true pathogenicity factors have been identified/confirmed so far by this approach. Also the status of forward genetic approaches (T-DNA insertional mutagenesis) will be reviewed. A major topic will be the role of the ROS status for the interaction (as seen from the fungal side), i.e. the impact of ROS scavenging and generation on differentiation and virulence in *Botrytis*.

Sclerotinia sclerotiorum subverts host pathways by inducing programmed cell death for disease development

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Accumulating evidence supports the idea that necrotrophic plant pathogens interact with their hosts in a much more subtle manner than originally considered and that signaling between the necrotroph and the host plays a significant role in the lifestyle of these pathogens. *Sclerotinia sclerotiorum* is a necrotrophic ascomycete fungus with an extremely broad host range (>400 species). Our data indicate that modulation of reactive oxygen species (ROS) are key components in the *S. sclerotiorum* interaction with plant hosts. Recently we have shown that fungal secreted oxalate (OA) can function as an elicitor of programmed cell death (PCD) in the plant host and independently recapitulates *S. sclerotiorum* disease symptoms. OA also induces increased ROS levels in the plant which correlate with, and are necessary for, PCD and disease. Thus, via OA, *S. sclerotiorum* can subvert and trigger host cell death regulatory machinery as a means to pathogenic success. Mutants defective in oxalic acid, NADPH oxidase activity or superoxide dismutase, are non-pathogenic and surprisingly, appear to induce a hypersensitive-like response (HR) when inoculated to plants. These observations suggest that these mutants, unlike the wild type fungus, are actively recognized by the plant and our preliminary data suggest a defense response is induced by these mutants. In an attempt to identify plant genes that regulate cell death during compatible (wild type) or "incompatible" (mutant) interactions, results of high throughput gene silencing (VIGS) screens to identify suppressors of *Sclerotinia* induced PCD and suppressors of mutant induced "HR"-like resistance will be discussed. These studies will advance our knowledge of how *Sclerotinia*, and quite possibly other necrotrophic pathogens, co-opt plant cell death pathways for successful colonization of the host.

Oxaloacetate Acetyl Hydrolase is responsible for oxalic acid production in *Botrytis cinerea* and required for lesion expansion on some, but not on most host plants.

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Botrytis cinerea secretes oxalic acid that may act as a general pathogenicity factor. In various isolates of *B. cinerea* the level of oxalic acid production has been correlated with virulence.

We studied the regulation of oxalic acid production in the strains B05.10 and T4. Both strains show differential virulence on various hosts, including *A. thaliana*, and display different levels of acidification of the culture medium. B05.10 responded strongly and T4 weakly to alkalinization by increasing the expression of oxaloacetate acetylhydrolase (OAH), the key enzyme in oxalic acid production. This correlated with medium acidification during the experiment: the pH is restored to pre-induction levels for B05.10 within hours, while very small changes are observed for T4. These data suggest that pH-dependent regulation of pathogenicity factors is different between the strains B05.10 and T4, which could explain some of the variation in virulence.

We studied the effect of deletion of OAH in B05.10 on media acidification, calcium oxalate crystal deposition and pathogenicity on various hosts. Remarkably, acidification of the medium is not affected in the mutants: parent strain and mutants responded similarly to medium alkalinization. We are testing the level of various organic acids in the culture medium to verify the source of this acidification.

On medium amended with calcium carbonate, formation of characteristically shaped calcium oxalate crystals (eight-faced bi-pyramids) was observed with the wild type strain but such crystals were completely absent with the mutants. During infection on various hosts it was also possible to observe the formation of crystals in the lesions caused by B05.10 after staining with trypan blue, whereas the crystals were absent in lesions caused by the ΔOAH mutants.

On tomato leaves and fruits, cucumber fruits, apples and carrots no significant difference in virulence between B05.10 and the ΔOAH mutant strains could be observed. On *A. thaliana* we could not observe any difference in germination and initial lesion formation between wild type and mutants. However, a clear difference in lesion expansion was measurable: even if initial water soaked lesions were formed after 2 to 3 days in the mutant strain, further expansion of these lesions was severely limited. Microscopical observation of the lesions revealed complete absence of crystals close to the outgrowing hyphae of the mutants whereas crystals were present in lesions caused by wild type. Similar results were obtained on cucumber plants (*Cucumis sativa* cv Wisconsin), with strongly reduced expansion of the initial lesions.

We can conclude that while acidification in *B. cinerea* is not exclusively dependent on oxalic acid production, oxalic acid high efficacy as chelating agent and strong organic acid is required for virulence on some hosts, thus contributing to the broad host range of *B. cinerea*.

Nitric oxide production in *Botrytis cinerea*

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NO is a gaseous signalling molecule known to play crucial roles in the establishment of plant-pathogen interactions by orchestrating, together with hydrogen peroxide (H_2O_2), the activation of the plant hypersensitive response (HR). The HR, while effective against biotrophs, seems to be unable to keep necrotophs under control. In the case of *B. cinerea* interactions, experimental evidences indicate that the pathogen even exploits the plant HR to favour infection and colonization. As NO is essential for the activation of the plant HR, it could be considered a factor contributing to make the life of *B. cinerea in planta* easier. However, NO has a strong antimicrobial activity. Both facts highlight the importance of investigating the NO metabolism by the fungus in order to understand the behaviour of the pathogen *in planta*. Analysis of NO metabolism by *B. cinerea* can be approached from two perspectives: (1) is the fungus able to detoxify the NO produced by the plant?, and (2) does the fungus produce NO?

We have previously demonstrated that *B. cinerea* has a single flavohemoglobin coding gene (*Bcfhg1*) whose expression is enhanced by exogenously added NO but only in young mycelium (germlings). This flavohemoglobin has a very high affinity for NO and constitutes the main, if not the only, NO detoxification system of *B. cinerea*. Although the enzyme confers protection against nitrosative stress to *B. cinerea*, deletion of the coding gene does not cause any obvious alteration in the fungus, neither during saprophytic growth nor during infection.

We now focus our work on the NO production by *B. cinerea*. Using the dye DAF-2DA we have detected specific NO staining covering the total volume of the cell in about 50% of the cells analyzed and in all the developmental stages of the fungus, from germlings to mature mycelium. NO staining has also been observed around the spores and mycelium in liquid cultures, indicating that the fungus secretes NO into the surrounding medium. Further evidence of the production of NO by *B. cinerea* during saprophytic growth comes from the analysis of the variations in NO concentration in liquid media cultures following the addition of 2 µM NO in water. When spores, germlings or mycelium up to 14 hours old (precultured in liquid minimal medium) were used, enzymatic degradation of NO was observed. However, older mycelium (precultured during 16 hours or longer) responded by producing NO. These data demonstrate that at least the "old mycelium" is able to react to the signal of external NO by increasing the levels of NO that the fungus itself produces.

In planta accumulation of NO is detected in the fungal mycelium and in higher amounts than in plant cells. NO production by *B. cinerea* is detected during interactions with tomato, tobacco and *Arabidopsis thaliana*. The time after inoculation when accumulation of NO is highest depends on the plant host, but it seems to be maximal during the development of spreading lesions.

Taking into consideration all these observations it is tempting to argue that *B. cinerea* has adapted mechanisms which could provide the fungus with tools to modulate the plant HR for its own benefit.

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The necrotizing activity of *Botrytis cinerea* endo-ß-1,4-xylanase Xyn11A

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The ability to elicit the hypersensitive response in the host tissue surrounding the infection is a key strategy for necrotrophic fungi such as *Botrytis cinerea*. A fungal enzyme able to elicit necrosis of plant tissues is the endo-ß-1,4-xylanase *(1)*. We have previously shown that the endo-ß-1,4-xylanase Xyn11A from *Botrytis cinerea* contributes significantly to virulence *(2)*. We have expressed Xyn11A in *Pichia pastoris*, characterized its enzymatic activity, and shown that it is able to elicit necrosis when infiltrated in tomato leaves. Interestingly, we observed a correlation between the virulence of *B. cinerea* on three different tobacco cultivars, and the necrotizing activity of Xyn11A on them. Single-site mutations in the enzyme's active site eliminate the xylanase activity but not its necrotizing ability, indicating that both properties are independent and may reside in different domains. Moreover, these mutant enzymes lacking xylanase activity are able to complement the reduced infectivity of a xyn11A mutant. By sequence comparisons and the study of the predicted structure of Xyn11A, we generated a hypothesis about the nature of the elicitor domain. The putative elicitor peptide has been expressed in *E. coli* either alone or fused to GFP. The necrotizing activity of these proteins is being studied and will be reported.

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The ABC-transporter BcatrB protects *Botrytis cinerea* against camalexin and is a virulence factor on *Arabidopsis*.

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Arabidopsis thaliana produces the phytoalexin camalexin in response to abiotic and biotic stress. Here we studied the mechanisms of tolerance to camalexin in the fungus *Botrytis cinerea,* a necrotrophic pathogen on *A. thaliana*. *B. cinerea* inoculated on WT *A. thaliana* plants yields smaller lesions than on camalexin-deficient mutants. Exposure of *B. cinerea* to camalexin induces the expression of several transporter genes, among which BcatrB, an ABC transporter that functions in efflux of fungitoxic compounds. The accumulation of the known BcatrB substrate ^{14}C-fludioxonil is increased by treatment with camalexin, indicating that the two compounds compete for active efflux and that BcatrB can transport camalexin. A *B. cinerea* strain lacking functional BcatrB is more sensitive to camalexin *in vitro* and less virulent on WT plants but is still fully virulent on camalexin mutants. Pre-treatment of *A. thaliana* with UV-C leads to increased camalexin accumulation and substantial resistance to *B. cinerea.* UV-C-induced resistance does not occur in camalexin-deficient *A. thaliana* mutants. This is the first time that an ABC transporter is demonstrated to be a virulence factor by increasing tolerance of the pathogen towards a phytoalexin, combined with complete restoration of virulence on host plants lacking this phytoalexin.

Identification of BcSak1 regulated genes during oxidative stress and functional characterization of *bop1*, as one target gene of BcSak1

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ROS play a major role in defense reactions of plants and animals against pathogens. Phytopathogenic fungi face considerable oxidative stress by the so-called "oxidative burst", an early plant defense reaction. *B. cinerea* can produce ROS itself and thus contribute to the ROS status during infection. We focus on signal chain components involved in oxidative stress signaling trying to determine their role either in ROS generation or decomposition. For *S. cerevisiae* it was shown that the HOG1-mediated MAP kinase pathway is responsible for the reaction to hyperosmolarity and oxidative stress. The gene encoding the HOG1-homologue of *B. cinerea* (BcSak1) was already functionally characterized (1).

The stress-activated mitogen-activated protein kinase (MAPK) BcSak1 of *B. cinerea* is transcriptionally upregulated and activated during oxidative stress mediated by H_2O_2. It seems that BcSak1 is partially involved in the oxidative stress response. Additionally in *B. cinerea* the stress-activated MAPK cascade seems to be necessary for essential differentiation programs like conidiation, sclerotia development and pathogenicity. Reactive oxygen species are proposed to play a part in such differentiation processes. Macroarray hybridization was performed to identify target genes of BcSak1 that are expressed differentially in the wild type strain and the *Δbcsak1* mutant during normal and especially oxidative stress conditions. Several ROS scavenging genes encoding e.g. a glutathione peroxidase, catalases, glutathione S-transferases, a thioredoxin and redoxins are clearly regulated by BcSak1. Genes involved in signaling, stress response and differentiation processes were additionally found to be regulated by the stress-activated MAP kinase of *B. cinerea*.

The transcription of the gene *bop1* which encodes a eukaryotic homologue of archaeal rhodopsins is clearly induced during oxidative stress in the wild type but not in the *Δbcsak1* mutant. Opsins belong to a class of retinal binding proteins with seven transmembrane structures that function as light-responsive ion pumps or sensory receptors. Expression analyses showed that the transcription of *bop1* is H_2O_2 but not light-dependent. We knocked out the gene *bop1* and characterized the deletion mutant. Extensive analysis of *Δbop1*-strains did not reveal obvious defects in light-regulated processes under normal laboratory conditions. The sensitivity towards different stressors like H_2O_2 or menadione and the pathogenicity of the mutant is also not affected.

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Bioinformatics and functional analyses of *Botrytis* IAP-like protein

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The IAP family of proteins are negative regulators of apoptosis that had been identified in various insects and vertebrates. Members of this group of proteins are characterized by the existence of three BIR domains, which are zinc-fingers with a zinc binding motif of Cys-Cys-His-Cys (C2HC). IAP proteins often contain an N-terminal RING domain, and may also include domains such as CARD, NACHT, and LRR. A second group of BIR domain-containing proteins exists that can be distinguished functionally from IAPs. Apart from their BIR domains these "IAP-like" proteins are otherwise highly variable in size and structure and are present in a more diverse group of organisms. Yeast has a single IAP-like homolog callesd Bir1p, which is a homolog of the human survivin protein. Here we report on the bioinformatic analysis of IAP and IAP-like proteins in fungi and on the partial characteriation of *B. cinerea BcIAP1*.

Search of fungal databases for BIR domain-containing proteins revealed a single IAP-like homologus protein in most (but not all) species. The fungal IAP-like homologs contain either one or two BIR domain. The fungal BIR domains were clustered in two separate groups representing BIR first repeat and BIR second repeat in each protein. This shows that there exists greater sequence similarity between identically positioned domains from different proteins, rather than between differently positioned domains from the same protein. An additional group of IAP-like proteins have been reported in plants; these proteins contain two repeats of a BIR Like Doman (BLD). Proteins containing the BLD have been found in fungi, hwoever not in all species.

We have identified both BIR and BLD-containing putative proteins in the *Botrytis* genome and isolated the genomic and cDNA clones of the *Botrytis* BIR-containing gene (*BcBIR1*). Knockout mutats were generated, hwoever all attempts to purify homokaryons failed, suggesting that *BcBIR1* is an essential gene. Over expression isoaltes were generated and partially characterized. These isolates have several phenotypes including enhanced germination and biomass production. Isolates also accumulated less ROS than wild type in dense cultures. These and other phenotypes might suggest association of *BcBIR1* with stress response. Further details including pathogenicity assays will be reported and discussed.

Enolase, a cAMP regulated, multifunctional protein is upregulated during cold stress in *B. cinerea*.

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Botrytis cinerea is a necrotrophic fungal plant pathogen that can survive, grow and infect crops under cold stress. In an attempt to understand the molecular mechanisms leading to cold tolerance of this phytopathogen, we identified an enolase, *BcEnol-1*. The enolase protein from *B. cinerea* BO5.10 shows high homology to yeast, *Arabidopsis* and human enolases (72 %, 63 % and 63%, respectively). *BcEnol-1* encodes a 48 kDa protein as identified by Western analysis using human α-enolase antibody. Northern analysis confirms that *BcEnol-1* is highly upregulated when *B. cinerea* mycelium was shifted from 22^{o}C to 4^{o}C. In order to understand its regulation during cold stress, *BcEnol-1* expression was studied in *B. cinerea* mutants *viz*. *Δbcg1* (mutant of *B. cinerea* for *bcg1)*, *Δbcg3* (mutant of *B. cinerea* for *bcg3)* and *Δbac* (mutant of *B. cinerea* for adenylate cyclase). A decrease in enolase expression in these mutants was observed during cold stress. Expression could be restored with the exogenous addition of a cAMP analogue to the *Δbac* mutant. These results support a role for the cAMP mediated cascade in enolase activation. Recombinant enolase protein was also found to bind to the promoter elements of fungal regulatory proteins belonging to Zinc-C_6 family and calpain like proteases. These results support a multifunctional role for BcEnol-1 that includes DNA binding. Overall, our results lead to the conclusion that enolase from *Botrytis* is upregulated under cold conditions, influenced by cAMP and acts putatively as a transcriptional regulator for cold-mediated gene expression.

Fourth session

SIGNALING AND DEVELOPMENT

Genome-wide transcriptome profiling of early sclerotial and apothecial disc development

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The generation of expressed sequence tags (ESTs) and the assembled, sequenced genome of *Sclerotinia sclerotiorum* (Lib.) de Bary opens the potential for comprehensively analyzing transcript expression dynamics during key developmental and pathogenic stages of its lifecycle. The automated gene annotation processes conducted by the Broad Institute identified 14,522 predicted genes. These predicted gene sequences were used as "seeds" in a sequence comparison cluster analysis with greater than 74,000 EST sequences derived from numerous axenic development stages and infected plant tissues. From this analysis, 4,827 unseeded sequence assemblies were derived. These sequences, in addition to 79 EST sequences identified as orphan sequences with canonical splice sites by the Broad Institute, the 14,522 predicted gene sequences, 1,012 randomly chosen predicted gene sequences in reverse, complementary orientation and seven soybean plant gene sequences were used to design probes for microarrays. The Agilent 4 by 44k platform using long oligo (60-mer) probe sequences was chosen for microarray design with two unique probes representing each "gene" sequence included on each array. Two hybridization experiments have been completed with these arrays. Both experiments involved competitive hybridizations comparing transcripts from two stages of development with eight biological replications for each comparison. The first hybridization compared transcripts from etiolated apothecial stipes produced in complete darkness (CD) with those from etiolated stipes exposed to 6 hrs of UV-A (3.5 µmoles/m^{-2}/s). The second hybridization compared transcripts from cultures of vegetative hyphae (VEG) grown on potato dextrose agar (PDA) with those from PDA cultures containing sclerotial initials (SCL). Signal intensities for "genes" represented by two probes were analyzed by Anova and statistical significance assigned by F-test. A Bonferroni multiple testing correction cutoff of 0.001 was used to assign statistical significance to differential expression between treatments. Using these criteria, the apothecial stipe CD versus UV-A treatment hybridizations identified 134 (0.8%) of the examined "genes" as up-regulated >2 fold in UV-A and 204 (1.2%) as down-regulated >2 fold in UV-A. In the VEG versus SCL hybridizations 1262 (8%) of the "genes" were identified as up-regulated >2 fold in SCL, and 1115 (7%) were identified as down-regulated >2 fold in SCL. Mapping the differential expression values of probes representing the Broad Institute predicted genes set to the chromosomes predicted by the optical map and supercontig assemblies has revealed several clusters of co-regulated genes that represent metabolic pathways and other unknown peptides. Utilizing expression values from both data sets (CD vs. UV-A and VEG vs. SCL), expression profile patterns have also been generated. This analysis has allowed us to identify genes that are up-regulated specifically in a single developmental stage. Together, these global transcriptome analyses have identified many potential regulators, metabolic pathways and structural components of key multicellular developmental stages of the *S. sclerotiorum* lifecycle.

Signalling pathways and their role in developmental processes and virulence of *Botrytis cinerea*

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In the past years, several mutants of different signalling components have been created and characterized. Comparative analysis of all these mutants regarding their growth characteristics, conidiation, sclerotia formation, virulence, toxin and oxalic acid production, protease secretion, stress resistance and expression profiles gives us the possibility to get more and more information on functions of, and interconnections between different signalling pathways. Thus, we were able to show that the Gα subunit BCG1 does not only regulate the cAMP but at least one additional, the Ca^{2+}/calcineurin pathway (Schumacher et al., 2008a, b).

Of special interest are several significant differences in functions and interconnections of conserved signalling pathways between *B. cinerea* and other pathogenic fungi. Thus, the cAMP-dependent signalling pathway is essential for pathogenicity in appressoria-forming fungi such as *Magnaporthe grisea* and *Colletotrichum lagenarium*, but in *B. cinerea* it does only play a role in late infection stages. Furthermore, while the deletion of the regulatory subunit of the PKA resulted in a constitutively active PKA in fungi such as *N. crassa*, mutants of the catalytic and regulatory subunits of the PKA, Δ*bcpka1* and Δ*bcpkaR*, displayed almost identical phenotypes. They both show strong growth defects on all media, totally lost their PKA activity and show dramatically increased cAMP levels suggesting that the deletion of *bcpkaR* results in degradation of the catalytic subunit(s) (Schumacher et al., 2008c).

Based on specific phenotypes of a whole group of mutants including their expression profiles, we will give a preliminary model of interconnected signalling processes.

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Heterotrimeric G protein-mediated Signalling in *B. cinerea* – A Never-ending Story –

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Several signalling cascades have been shown to be involved in *B. cinerea*-host interaction: the mitogen-activated kinase (MAPK) cascades are essential for penetrating the host tissue, while heterotrimeric G proteins and the cAMP pathway play the major role during colonization of plant tissue (invasive growth) and subsequent asexual reproduction. The Gα subunit BCG1 regulates the transition from primary to spreading infections by affecting (at least) two signalling cascades acting in parallel: the cAMP cascade and the Ca^{2+}/calcineurin pathway.

To further investigate heterotrimeric G protein-mediated signalling, we studied the role of the Gβγ dimer in regulation of both signalling pathways by deletion of the gene encoding the Gβ subunit BCGB1 in the wild-type and the Δ*bcg1* background, and by generation of mutants expressing a constitutively active BCG1 (*bcg1*^{G42R}). Δ*bcgb1* single mutants displayed increased aerial mycelium formation, produced almost no conidia, were unable to develop sclerotia, were retarded in the infection process and stopped at the stage of spreading lesion formation. The increased cellular cAMP levels in Δ*bcgb1* strains and the similarity of the strains to the *bcg1*^{G42R} mutants suggest that BCGB1 is a negative regulator of Gα/cAMP signal transduction.

While only BCG1 functions in regulation of production of oxalic acid, proteases and phytotoxins (botrydial and botcinins) in a cAMP-independent manner, both G protein subunits BCG1 and BCGB1 are involved in regulation of conidial germination, surface sensing, cell polarity and penetration. Δ*bcg1*, Δ*bcgb1* as well as Δ*bcg1*Δ*bcgb1*-derived conidia displayed inappropriate germination rates in water (without inductive signal!), and formed unusual elongated, thin and less branched germ tubes/hyphae on all tested surfaces (glass slides, polypropylene foil and bean leaf epidermis). A conceivable scenario how BCG1 and BCGB1 can function together in the same (cAMP-independent) pathway is that the inactive heterotrimeric G protein, consisting of the BCG1 subunit and the Gβγ dimer, acts as general repressor (mediated via unknown signalling components) of germination. Consequentially, the deletion either of BCG1 or BCGB1 would prevent the formation of the "repressor complex" and thereby result in de-repression of germination.

Does the Gα subunit BCG1 affect Bcmp1 expression via PacC-dependent pH-regulation?

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Heterotrimeric G-proteins play an important role during the infection of host plants by *B. cinerea*. Deletion mutants of *bcg1*, encoding one of three G alpha encoding genes, are able to penetrate the host tissue and produce small primary lesions, but are disabled in further invasion of the plant tissue. An *in planta* SSH-approach was used to identify genes whose expression on the host plant was specifically affected in Δ*bcg1* mutants. Among the BCG1 target genes were those which were expressed via the activation of the cAMP cascade (e.g. xylanase genes), while others, e.g. a set of protease genes, secondary metabolite biosynthesis genes and the oxalic acid biosynthesis gene *bcoaha*, are expressed in a BCG1-dependent, but cAMP-independent manner. For secondary metabolite biosynthesis genes, e.g. the botrydial genes, it has been shown that they are regulated by BCG1 via the Ca^{2+}/calcineurin-pathway.

As both oxalic acid-synthesis and -degradation are needed to provide an optimal pH environment for lytic enzyme activities, and Δ*bcg1* mutants are not able to acidify the culture media, an indirect effect of BCG1 on pH regulation is likely. This suggestion is supported by the observation, that Δ*bcg1* mutants possess a strongly downregulated expression of *bcoaha in vitro* and *in planta*. Therefore, Δ*bcg1* mutants fail to change ambient pH by secretion of oxalic acid, consequently it is likely that this results in a different gene expression pattern of pH-dependent genes.

To study the regulation of one of the BCG1 target genes in more detail, we chose the gene *bcmp1* encoding one of the two metalloproteases of *B. cinerea*. As *bcmp1* is neither regulated via the cAMP-pathway nor by the Ca^{2+}/calcineurin-pathway, we speculate that the regulation of this gene might be pH-dependent. This prediction is supported by the presence of five potential PacC-binding motifs (one single and two tandem copies) in the promoter sequence of *bcmp1*. As PacC is the global transcription factor regulating the expression of a set of pH-controlled genes in fungi, its involvement in *bcmp1* regulation would link BCG1 signalling with pH-regulation: BCG1 controls the production of oxalic acid and therefore the activity of PacC and by this the expression of PacC target genes.

To confirm the PacC-dependent expression of *bcmp1*, promoter studies with GUS-reporter gene-constructs were performed. The full length promoter, fused to the *uidA*-gene, showed strong reporter gene expression. Furthermore, subsequently shortened promoter fragments and fragments with specifically mutated PacC-binding motifs, were fused to the *uidA*-gene. Following quantification of GUS-activity in all transformants is on the way to prove the potential role of PacC in regulation of *bcmp1* expression *in planta* and *in vitro*.

In addition to that, the gene encoding Bcmp1 was cloned and deleted to analyse the role of this metalloprotease in virulence.

The transcription factor Bap1 or the role of H_2O_2 degradation during plant infection in *Botrytis cinerea*

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Reactive oxygen species (ROS) are present in all aerobic environments where they cause cell damage when interacting with cellular components. They are also part of normal cellular processes like signal transduction or pathogen defence.

In the interaction of the necrotrophic pathogen *Botrytis cinerea* ROS are present during the early stages of infection and can even be detected 2 dpi in primary lesions. Attacked by the pathogen the host plant produces ROS in the so called oxidative burst to defend against the fungus. Therefore an effective fungal detoxification system with catalases and peroxidases should be essential for the survival on the host. However, expression of several H_2O_2 degrading enzymes is not induced in the primary lesions of *B. cinerea* on bean leaves and starts in axenic culture at very high H_2O_2 levels (2.5 - 5 mM) that might not be present on the plant tissue. Expression of these ROS degrading enzymes is mainly regulated by the transcription factor Bap1 (*Botrytis* activator protein). Macroarray analyses with the wild type B05.10 and the Δ*bap1* deletion mutant with or without 10 mM H_2O_2 revealed more than 90 genes that are upregulated under ROS exposure in the wild type B05.10 with at least 7 genes directly involved in ROS detoxification. Induction of these genes could not be detected in the Δ*bap1* deletion mutant in these experiments. The transcription factor Bap1 as well as some of its target genes essential for the survival on H_2O_2 are of minor or no importance for host invasion indicating that ROS degradation might be not essential for the infection.

However, characterisation of other proteins possibly downstream of stress-activated MAP kinase BcSak1 or ulterior involved in the ROS regulation process is on its way and will enlighten the ROS signalling network that seems to involve a number of general conserved signalling processes.

The small GTPases BcRAS1 and BcRAC are linked to the stress activated MAPK cascade in *Botrytis cinerea*

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Up to the present, many signaling components were identified and characterized in *Botrytis cinerea*, albeit many interconnections between different signaling pathways stay unclear. It is of great importance to understand the relationships between known pathways to further analyze pathogenicity, differentiation and secondary metabolite production of *B. cinerea*.

We currently characterize small GTPases of the Ras superfamily in *B. cinerea*: BcRAS1, BcRAS2, BcRAC and BcCDC42 and their influence on other signal transduction mechanisms. Recently, it was shown that the small GTPase BcRAS2 is linked to cAMP signaling in *B. cinerea* (1).

Furthermore, we investigate MAPK cascades, in particular the pathway including the stress activated kinase BcSAK1 (2). BcSAK1 is involved in scavenging reactive oxygen species (ROS) that may act as messenger molecules besides their role as stress producing agents. In mammalian systems it is known that there is a ROS-dependent crosstalk between the SAK cascade and the small GTPases RAS and RAC (3).

By northern blot analyses we could show that a set of genes is identically regulated by BcSAK1, BcRAS1 and BcRAC. Western blot analyses are performed to prove the link between the small GTPases and the MAPK cascade. Together with additional results concerning other ROS scavenging/producing proteins like transcription factors and NADPH oxidases, we develop a model of RAS dependent ROS signaling.

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Expression analysis of stress-related genes in signal transduction mutants

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Hog1-like fungal signal transduction cascades are involved in diverse cellular functions, such as adaptation to various stresses, fungicide resistance, development and, in some cases, virulence. The homologous pathway of the plant pathogenic ascomycete *Botrytis cinerea* was previously characterized *via* the inactivation of the sensor histidine kinase Bos1 (1), the downstream MAP kinase (MAPK) Sak1 (2), as well as their relationship (3). Bos1 represses Sak1 phosphorylation in the absence of external signals through a yet unknown mechanism. The perception of an external signal, e.g. dicarboximide fungicides, high osmolarity, releases the repression leading to Sak1 phsophorylation. Both kinases co-ordinately control the adaptation to high ionic osmolarity, macroconidia development and plant penetration. Other functions are controlled by Bos1 independently of Sak1. They include superoxide tolerance, adaptation to high neutral osmolarity, and susceptibility to three families of fungicides (dicarboximides, phenylpyrroles and aromatic hydrocarbons). Cell wall integrity, appressoria- and sclerotia development are probably controlled by two parallel signalling cascades both regulated by the Bos1 HK.

We performed RT-PCR analysis on selected sets of genes, potentially involved in the mutant phenotypes, in the *bos1*- and *sak1* mutants subjected to various stress conditions. Expression patterns of most Bos1-Sak1 controlled genes under standard conditions corroborate the negative control of Sak1 phosphorylation. We show that most but not all phenotypes can be related to differential gene expression suggesting that the Bos1-Sak1 cascade acts through posttranslational control of several target enzymes besides controlling the transcription of other target genes.

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Complex signalling pathways that control germination and infection of *Botrytis cinerea*

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Germination of *Botrytis cinerea* conidia can be induced by several chemical and physical stimuli. Analysis of mutants defective in the Gα3 subunit of the heterotrimeric G protein and in the MAP kinase cascade Bst11-Bst7-BMP1 revealed at least two signalling pathways that control germination. While the Gα3 subunit controls, in a cAMP dependent fashion, germination on hydrophilic surfaces (e.g. glass) in the presence of limited amounts of a carbon source, the Bst11-Bst7-BMP1-mutants showed that this MAP kinase cascade is partly involved in carbon source sensing but essential both for germination induced by hydrophobic surface contact (e.g. polypropylene), and for penetration of the host surface. Further components of this MAP-kinase cascade around BMP1 could be identified.

First of all Bst50, an adaptor protein of the kinase complex, which shows the same phenotype in comparison to the MAPK cascade mutants, when it is deleted. Furthermore we found that the transcription factor Bst12, also seems to play an important role in this signalling cascade. Using qRT-PCR we searched for BMP1 dependent activated and down-regulated genes and found out, that e.g. the transcription of the cell surface sensor protein Msb2 seems to be negatively regulated by BMP1.

In order to identify molecular sensors involved in surface recognition, we constructed deletion mutants of the *msb2* gene, which is a putative homolog of a yeast cell surface protein involved in filamentous growth and osmoregulation. Similar to yeast Msb2, BcMsb2 contains a large extracellular domain of about 1450 amino acids, a single transmembrane domain and a short cytoplasmic tail. When germinated on hydrophobic surfaces, mutants deleted in *msb2* showed increased numbers of germ tubes as well as increased elongation of the germ tubes in comparison to the wildtype. The increased elongation of germ tubes both on hydrophobic as well as on hydrophilic surfaces appears to be due to a reduced surface recognition, which is recognized by decreased pseudoappressorial swellings. Because of reduced penetration efficiency, the *msb2* mutants showed delayed lesion formation on intact leaves and flowers. Thus, Msb2 seems to be a cell surface sensor, that is involved in the regulation of initial stages of germ tube appearance and surface penetration.

Our further studies are aimed to identify further target genes of the BMP1 MAP-kinase cascade using qRT-PCR and microarray analyses as well as to clarify the role of the sensor protein Msb2 in the signalling cascade.

Fifth session

RESEARCH TOOLS / FUNGICIDE RESISTANCE

The fungal genomic annotation platform

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Novel fungal genomes are continuously sequenced producing an ever increasing amount of new genome sequence. To face the challenge of their rapid annotation, we have developed an efficient fungal genomic annotation platform (http://urgi.versailles.inra.fr/projects/FungalPlf/) that has been tested and validated for the annotation of *Botrytis cinerea* genome sequence [1]. We developed and use three pipelines for (i) gene structure annotation, (ii) transposable element identification and annotation and (iii) gene functional annotation. The gene prediction pipeline is based on *ab initio* and similarity gene finding softwares, results are then gathered and combined by the Eugene annotation program [2]. The transposable element annotation pipeline [3] allows their *de novo* detection and the structural annotation of their genomic copies. Our functional annotation pipeline is based on various methods of pattern matching and motif recognition [4], intracellular targeting prediction methods, and comparative genomics with other fungal genomes.

We recently developed a roundtrip between our genomic databases on the one hand, and two interfaces for visualisation (with GBrowse) and manual annotation editing (with Apollo), on the other hand. These databases and interfaces are part of the GMOD project and are set up and improved at URGI in the framework of the GnpAnnot project (A platform of structural and functional annotation supported by comparative genomics dedicated to plant and bio-agressor genomes, http://urgi.versailles.inra.fr/projects/GnpAnnot/).

Two other species are currently annotated using this platform: the fungal causal agent of the stem canker on oilseed rape, *Leptosphaeria maculans* 'brassicae' (Lmb) and the barley powdery mildew fungus *Blumeria graminis*.

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Network inference following gene expression profiling in host and pathogen during *Botrytis cinerea* infection

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The host-pathogen interaction during a *Botrytis cinerea* infection is a highly variable and dynamic system. Despite significant differences in pathogen virulence and host susceptibility even for a single host species, the broad host range of *Botrytis* seems to suggest a common series of interactions leading to successful infection. We are using a systems biology approach to elucidate the gene regulatory networks operating in *Arabidopsis* after infection with *B. cinerea*. We have conducted high-resolution time series expression profiling of infected and uninfected *Arabidopsis* leaves over 48 hours. This time course expression data covers both primary lesion initiation as well as subsequent lesion expansion. Over 6000 genes have been identified, which are differentially expressed over time in infected compared to mock-inoculated leaves. We have used a variational Bayesian state space modelling approach developed within our group to generate transcriptional regulatory models for subsets of these genes. The resulting network models reflect many of the regulatory interactions known to occur during plant defence and provide a basis for the development of rational and experimentally testable hypotheses. Network validation is being carried out by altering expression of key hub genes and monitoring the effect on predicted target genes.

As a prelude to obtaining *B. cinerea* expression profiles from the same time course samples, we have conducted a macroarray study to analyse *Botrytis* gene expression during the initial stages of lesion formation and latent lesion expansion in both *Arabidopsis* and tomato. This has identified genes differentially expressed during the different stages of infection. Genes commonly and uniquely expressed on the different hosts have also been identified. Gaining insight into both host and pathogen gene expression during infection will aid in determining mechanisms of the interaction occurring between the host and pathogen facilitating disease progression or inhibition. This may in turn reveal key genes or gene networks which make this fungus such a successful pathogen.

Efficient Gene Replacement and Direct Hyphal Transformation in *Sclerotinia sclerotiorum*

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Homologous recombination is required for gene-targeted procedures such as gene disruption and gene replacement. Ku80 is part of the nonhomologous end-joining DNA-repair mechanism in many organisms. We identified and disrupted the Ku80 homologue in *Sclerotinia sclerotiorum* and generated heterokaryon mutants enriched with Ku80-deficient nuclei (ssku80). Sclerotial formation and pathogenicity of ssku80 mutants were normal on tomato fruits. The frequencies of homologous recombination in these strains were much higher than those of the wild type when transformed with a cna1 (encoding calcineurin) replacement construct. We coupled the increase in homologous recombination with a direct BIM-LAB-mediated transformation procedure, which utilizes compressed air to assist the transforming DNA in penetrating fungal hyphae of *S. sclerotiorum*. We found this method to be efficient and reproducible, and to not alter the mutants' fitness. We also demonstrated the first case of direct transformation of sclerotia. In this study, nourseothricin was introduced as a selectable marker in *S. sclerotiorum*. The described tools and procedures will improve our ability to study gene function in *S. sclerotiorum* and are most likely to be adaptable for use in other plant pathogens.

Transcriptome of *Botrytis cinerea* and *Sclerotinia sclerotiorum*.

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Avaibility of *Botrytis cinerea* and *Sclerotinia sclerotiorum* genome sequences [1] allows genome-wide functional approaches. Among the post-genomic strategies, study of transcriptome in different physiological or genetic backgrounds conditions is a gateway to understand the function of genes.

EST from different libraries as well as preliminary macroarray studies give a first idea of gene expression in a range of conditions. To explore more widely the transcriptome of the both fungi, Nimblegen whole genome oligonucleotide arrays where designed. The *Sclerotinia* 1-plex array contains 190130 probes covering 14801 genes (about 13 probes per gene). For *Botrytis*, two designs were defined: (i) a 1-plex design containing 186218 probes covering 20889 genes (about 9 probes per gene); (ii) a 4-plex design containing 62478 probes covering 20885 genes (about 3 probes per gene). For the 3 designs, 2 copies of each probe are placed on the array.

The data resulting from hybridization of these chips will be investigated in different ways: (i) to point out clusters of differentially expressed genes dependent on conditions of experimentation (ii) to compare the behaviour of the genes in the two fungi (iii) to correct the structural annotation.

Quality controls of the designs and first hybridization experiments will be presented.

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Use of replicative genomic libraries to clone fungicide resistance alleles

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Telomere sequences have been found to enhance transformation frequencies of fungal plasmids. Such plasmids do not integrate into the genome; they may be replicated independently. We constructed two derivatives of the pFAC1 plasmid, which transform *B. cinerea* at high frequencies (1). Our plasmids pFACR5 and pFB2N both give rise to transformation frequencies 500-1.000 times higher than integrative plasmids. They are replicated under selective pressure but unstable without selection. We analyzed conformation and copy number of both plasmids in *B. cinerea* transformants.

Integration of a fenhexamid resistance allele into the pFB2N vector induces fungicide resistance in the transformants showing that dominant alleles can be selected from replicative plasmids (2). Genomic libraries in the pFB2N vector were used to transform the B05.10 strain. Transformants analysis showed a ratio of recombinant plasmids and an average insert size comparable to the initial *E. coli* library. The replicative plasmids are found at roughly 1 copy/genome.

After the construction of a 3-4X genome coverage B05.10 transformant pool, harboring the replicative library generated from a fenhexamid resistant strain, we screened the transformants for fenhexamid resistance. Results will be presented. Perspectives and limitations of the system will be discussed.

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About ABC transporters and map-based cloning: Molecular mechanisms of multidrug resistance in *Botrytis*

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Botrytis cinerea grey mold causes great losses in economical important in vegetable, fruit and flower crops, and often needs to be controlled by several fungicide treatments per year. Common resistance phenotypes are due to mutational changes of fungicide target sites or increased degradation. Resistance against chemically unrelated drugs (multidrug resistance, MDR), is often observed in cancer cells or microbial human pathogens (e.g. *Candida*), but not well-described in phytopathogenic fungi. In French and German vineyards, *B. cinerea* strains with MDR phenotype have been observed to constitute rapidly increasing populations, accounting for up to 50% (France) and 30% (Germany), respectively, of all field strains. MDR strains can be classified into three groups (MDR1, MDR2, MDR3), according to their resistance spectrum. Genetic and molecular evidence indicate that mutations in just two regulatory genes (*reg1* and *reg2*) are responsible for MDR development. Uptake experiments with ^{14}C-labeled fungicides reveal reduced uptake (increased efflux) activity of MDR strains, when compared to sensitive strains. Based on the *B. cinerea* genome sequence, the expression of genes encoding 29 ABC-type and 18 MFS-type efflux transporters was analysed. In MDR1/3 strains, several genes, in particular *atrB*, were upregulated, whereas in MDR2/3 strains only one gene (*mfsM2*) was found to be overexpressed. Knock-out of *atrB* in MDR1 strains led to a complete loss of the MDR1 phenotype, whereas deletion of *mfsM2* showed only a small decrease in fungicide resistance. Promoter::GUS fusions were constructed, which confirmed fungicide-dependent expression of *atrB*, and allowed the identification of a putative transcription factor binding site in the *atrB* promoter. Using microsatellite-based mapping of MDR1x MDR2 F1 progeny strains, a map-based cloning approach is currently followed in order to identify the (probably regulatory) genes *reg1* and *reg2* in which the mutations leading to MDR in *B. cinerea* have occurred.

Fenhexamid resistance in the phytopathogenic fungus *Botrytis cinerea*

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Fenhexamid (hydroxyanilide family) is one of the most active fungicides used against *Botrytis cinerea* on vineyards. Sensitivity monitoring, carried out on *B. cinerea* samples from vineyards, showed occurrence of three phenotypes of strains resistant to fenhexamid when tested in laboratory. These three phenotypes, called HydR1, 2 and 3, are highly resistant at mycelium growth stage. At germ tube elongation stage, HydR1 strains which correspond to the naturally resistant species *B. pseudocinerea* and HydR2 strains are sensitive to slightly resistant to the product, whereas HydR3 strains exhibit a high resistance level. Therefore, molecular characterisation of these strains will allow a better understanding of *B. cinerea* resistance mechanisms towards the fungicide. Biochemical experiments (Debieu et al., 2001), suggest that the 3 keto reductase (*erg27*) involved in C4 demethylation during ergosterol biosynthesis acts as a target for this compound. Sequence analysis, enzyme inhibition measurements and reverse genetics (Fillinger et al., 2008) showed mutations in the *erg27* gene in the HydR3 strains. In contrast, the cause of fenhexamid resistance in HydR2 strains remains unknown.

An inducible RNAi approach was initiated for the functional analysis of the *erg27* gene in *B. cinerea* and to validate this enzyme as target of fenhexamid. In addition, a site-directed mutagenesis approach of the *erg27* gene was carried out in the fenhexamid sensitive B05.10 Δ*ku70* strain in order to evaluate the effect of each mutation on fenhexamid susceptibility and on the enzyme affinity for the compound. Methodology of the gene replacement and first results will be presented.

POSTERS

P1
Botrytis Goes Crazy
– about calcium, calcineurin, CRZ1 and magnesium –

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Recently, we showed that the subunit BCG1 of a heterotrimeric G protein is an upstream activator of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. The transcription factor BcCRZ1 ("CRaZy" – the calcineurin-responsive zinc finger) acts as mediator of calcineurin function and induces gene expression after its dephosphorylation by calcineurin and subsequent translocation to the nucleus. Replacement of *bccrz1* affects vegetative growth (no growth on minimal medium!), hyphal morphology, conidiation, sclerotia formation, cell wall and membrane integrity as well as the resistance to different stressors (extreme pH, oxidative stress, Li^{+}). While BcCRZ1 is almost dispensable for conidium-derived infection, Δ*bccrz1*-derived hyphae are significantly impaired in the ability to penetrate the intact host surface. Interestingly, Mg^{2+} supplementation restores growth rate, conidiation, and penetration, and improves cell wall integrity but has no impact on sclerotium formation and the hypersensitivity to H_2O_2.

In accordance with the model in which BcCRZ1 is activated when cytosolic Ca^{2+} levels are increased, high Ca^{2+} concentrations in the medium are lethal for Δ*bccrz1* mutants (even in presence of Mg^{2+}) as the appropriate signal transduction via BcCRZ1 is blocked. However, high Ca^{2+} concentrations also affect growth of the wild-type: more aerial hyphae, less conidia and no sclerotia are formed under the respective cultivation conditions.

Contrary to our previous expectations that the deletion of calcineurin subunit-encoding genes might be lethal in *B. cinerea*, we recently succeeded in the isolation of calcineurin A (*bccnA*) null mutants. As expected, mutants are severely impaired in growth: strains grow very slow, are only viable on grape juice medium and do not produce conidia. The fact that the Δ*bccnA* phenotype is more severe than that of *bccrz1* mutants corroborates our hypothesis that additional downstream targets of the calcineurin phosphatase complex exist.

The study of Ca^{2+}-dependent signal transduction is continued by generating mutants displaying modified calcineurin signalling, either by expressing a truncated form of BcCNA (without autoinhibitory domain) or by deletion of the calcineurin regulator BcRCN1. However, the availability of microarrays in the near future will allow the genome-wide analysis of Ca^{2+}-dependent gene expression and will thereby gain more insights into the role of Ca^{2+}/calcineurin/BcCRZ1-mediated signal transduction in life and virulence of *B. cinerea*.

P2
Control of gray mould caused by *Botrytis cinerea* by enhancing defense mechanisms of the host plant

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The control of plant disease which could be achieved by the use of chemicals, genetic approach or farming practices remains insufficient for the expectations of the humans and the environment. That's why it is crucial to find alternatives for efficient disease control by reducing negative effects on environment. Thus we evaluated the potentialities of liquid and solid products on one pathogen that lead to economically important disease on crops which is caused by *Botrytis cinerea*. The target crops are strawberry and tomato considered as best plants hosts to the pathogen. Our objective is to evaluate the efficacy of the products and their mode of action by *in vivo* and *in vitro* tests. Some products proved to be effective by direct or indirect action on the microbe while the systemic products remain more effective. On the other hand, for the plants, the best results were obtained by the strawberry rather than tomato. It is certainly clear that the results obtained suggest that the products tested have beneficial effects not only on resistance to the diseases and inhibition of the development of the pathogen but also on the growth and the production of the plant. However, more investigations are needed to use these products on the suitable targeted crop and period of application.

P3
Characterization of the cytochrome b gene in laboratory mutants of *Botryotinia fuckeliana* resistant to QoI fungicides

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The molecular basis of resistance to the respiration inhibitors QoI fungicides was investigated in laboratory mutants of *Botryotinia fuckeliana* (de Bary) Whetz. (anamorph *Botrytis cinerea* Pers.) exhibiting *in vitro* reduced sensitivity to trifloxystrobin. Mutants were obtained by selecting spontaneous mutations from two wild-type reference strains (SAS56 and SAS405) on a medium amended with 1-3 mg l^{-1} trifloxystrobin and 2 mM salicylhydroxamic acid (SHAM), a specific inhibitor of the cyanide-resistant alternative respiratory pathway.

Several point mutations leading to resistance to QoI fungicides in several phytopathogenic fungi have been described in the mitochondrial *cytb* gene coding for the cytochrome b target protein. The most frequent mutation occurs at the codon 143 causing the amino acid change from glycine to alanine (G143A) and conferring a high level of resistance.

Specific primer pairs were designed on the nucleotide sequence of *B. fuckeliana cytb* available in the EMBL database (accession number AB262969, http://www.ebi.ac.uk) and used in PCR reactions to amplify and sequence the whole target gene in representative sensitive strains and their derived laboratory-resistant mutants. Likewise, the alternative oxidase *aox* gene was amplified and sequenced starting from the BC1G-05703.1 sequence obtained from the *Botrytis cinerea* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broad.mit.edu).

No point mutations were detected neither in the nucleotide sequences of the *cytb* gene nor in those of the *aox* gene of the tested mutants. Other gene(s) underlying different mechanisms of resistance could be involved, as observed in other phytopathogenic fungi.

Moreover, sequence analysis of the *cytb* gene revealed differences in its structure among *B. fuckeliana* strains that were not related to tryfloxistrobin resistance. In the strain SAS56 and its mutants, but not in the strain SAS405 and its mutants, a 1197-bp sequence flanked downstream the codon 143. ClustalW alignment of such sequence revealed a high homology with conserved regions of group I introns, possessing self-splicing activity. The presence of such type of introns at the same position has been previously reported in other fungi, and it has been supposed that it prevents the occurrence of the G143A mutation conferring resistance to QoI fungicides (1).

Additional PCR experiments showed that sequences of similar size occur in the *cytb* gene in about 17% of *B. fuckeliana* isolates from different countries and host plants.

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P4
Chemical and anatomical features of grape berries associated with ontogenic resistance to *Botrytis cinerea*

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Grey mould (*B. cinerea*) is a major airborne grapevine disease that can drastically reduce yield and wine quality. The main objective was to investigate potential determinants involved in the dynamics of fruit susceptibility to the pathogen. First, a laboratory bioassay allowed us to assess the ontogenic resistance of undamaged berries (cv. Sauvignon blanc) at six stages from bunch closure to fruit overmaturity. Different pathogenic parameters were assessed: incidence of infected berries, severity of rot expansion and sporulation intensity. Significant differences in *B. cinerea* development were shown between the berry stages and between the *B. cinerea* strains tested. The increasing susceptibility of grape berries during ripening was confirmed. Second, at the different stages, various anatomical parameters were assessed including i) cluster compactness, ii) mean fresh masses of bunch, berry and skin iii) water content in the skin (%). Moreover, berries were analyzed chemically considering the berry juice composition (sugar content and organic acid concentration) and, for the skins, by assessing total phenol content, tannin concentration and pectic compounds soluble in water. Because exosmosis increases during fruit ripening and modifies the availability of water at the surface of grape skin, external free water was assessed by measuring water activity (a_w) at the different berry stages. Using principal component analysis, the relationships were investigated between the anatomical and chemical fruit characteristics and the time course of berry susceptibility. A significant and negative linear regression was established between a_w at the skin surface and the berry susceptibility level. The temporal variations in the different fruit parameters and their correlation with the ontogenic resistance to the pathogen have been established.

P5
Genetic and cytological dissection of germination and penetration of Botrytis cinerea penetration of *Botrytis cinerea*

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For *B. cinerea* conidia, appropriate timing of germination and host surface sensing are critical steps during the early phase of pathogenic development. Germination efficiency is dependent on the quality of the surface below the conidium, as well as on the amount and quality of nutrients. Germ tube outgrowth is highly oriented in order to ensure rapid contact of the germ tube tip to the surface. A surprisingly large number of *B. cinerea* mutants have been shown to be affected in each of these steps, which underlines the molecular complexity of the underlying mechanisms. By using light and scanning electron microscopy as well as video microscopy, behaviour of wild type and mutant spores during germination can be investigated, allowing a detailed molecular and phenotypic dissection of the early infection process.

P6
Multiple fungicide resistance and regulation of drug efflux transporters in *Botrytis cinerea*

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Botrytis cinerea is a world-wide occuring plant pathogen which usually needs to be controlled by fungicides. Continuous chemical treatments lead to the selection of resistant strains in the fungal population. Fungicide resistance occurs most often by mutations of the fungicide target proteins, but increased fungicide degradation or reduced uptake due to membrane modifications have also been observed (Leroux et al., 2002). Another type of resistance, termed multi drug resistance (MDR), is a great medical problem for chemical treatment of cancer or microbial infections. MDR in cancer or microbial cells is often caused by mutations leading to the overexpression of ABC- or MFS-type membrane efflux transporters. Due to their low substrate specificity, overexpression of these socalled MDR transporters can result in the increased export and thereby reduced sensitivity to many different natural or synthetic drugs.

In French vineyards, *B. cinerea* strains with low to moderate levels of resistance to chemically different fungicides (MDR phenotype) have been observed for the first time 15 years ago. Since the late 1990's, MDR strains constitute a rapidly increasing population which, in 2006, accounted for 50% of the total population of the Champagne region. In Germany, more than 20% MDR strains were found in 2006 in the Palatine region. A joint analysis of the French and German MDR strains revealed that all strains could be classified into three MDR groups according to their fungicide resistance spectrum. Several lines of evidence indicate that MDR3 strains represent the progeny of natural crosses between MDR1 and MDR2 strains: (i) MDR3 strains occur with a much lower frequency than MDR1/2 strains in the field; (ii) the spectrum of fungicide resistance of MDR3 strains is the composite of the fungicide resistance of MDR1 and MDR2, respectively.

Uptake experiments with ^{14}C-labeled fungicides were performed with germlings of strains with a MDR1, MDR2 or MDR3 phenotype. While MDR1 and MDR3 strains showed reduced uptake of ^{14}C-fludioxonil, MDR2 and MDR3 showed reduced uptake of 14C-triadimefon and cyproconazol. This result is a clear evidence for a functional correlation between MDR and increased, energy-dependent efflux transport activity.

Gene expressing studies revealed that in MDR1 and MDR3 strains, the gene encoding the ABC transporter BcatrB is constitutively upregulated, while in MDR2 strains overexpression of several unknown transporter genes was observed. To confirm that overexpression of efflux transporters is responsible for the MDR phenotype, the *BcatrB* gene was knocked out in two MDR1 strains. All mutants had indeed completely lost their MDR1 phenotype: They showed high ^{14}C-fludioxonil uptake rates and were highly sensitive to fungicides.

Our current work focuses on the mechanisms of regulation of MDR-related efflux transporters, and in particular towards the identification of the regulatory genes the mutations of which lead to the MDR phenotypes.

Reference: Leroux P, Fritz R, Debieu D, Albertini C, Lanen C, Bach J, Gerdt M, Chapeland F. (2002) Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest. Manag. Sci.* **58**:876-888.

P7
The Influences of Abiotic Stresses on Expression of Zinc Finger Protein Gene in Rice (*Oryza sativa* L.)

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We investigated the expression pattern of zinc finger protein gene in rice under different abiotic stresses. This gene contains an open reading frame (ORF) encoding a zinc-finger protein of 171 amino acids and is induced after different stresses, namely salinity, drought and cold. Phylogenetic analysis based on deduced amino acid sequences of *Os*ZFP cDNA revealed significant sequence similarity to the zinc finger protein from a very diverse family of plant species. Southern blot analysis of rice genomic DNA suggests that *Os*ZFP is encoded by a multiple copy gene. Steady state *Os*ZFP transcript levels were found to increase in response to several stresses imposed by salinity, drought and cold. A rice zinc-finger protein gene, *Os*ZFP, encoding the C_2H_2-type zinc-finger transcription factor was isolated from rice (*Oryza sativa* L.) by RT-PCR. The expression profiling indicated that *Os*ZFP was constitutively expressed in leaves and roots. The Northern blotting showed *Os*ZFP was strongly induced by high-salinity and drought, but slightly regulated by low temperature (4°C). These results suggested that the *Os*ZFP may play an important role in rice responses to salinity, drought and cold as a transcription factor.

P8
Expression of genes responsible for cell cycle regulation and stress tolerance in *Lycopersicon esculentum*

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Tomato growth, development and stress tolerance are the result of the activity of some genes. There are some cyclins, cyclin dependent kinase which regulate cell division of tomato plant. Calcineurin B- like protein, one of calcium binding protein genes involve in stress tolerance of plant. We studied a pattern of gene expression of cyclin A2; SGN-326225, Cyclin A3; SGN-U322407 and B2 type cyclin dependent kinase; SGN-U323897. Expression study of Calcineurin B-like protein (AJ717347) is going on under different stress and normal conditions. Genomic DNA digestion was done with *Eco*RI, *Hind* III and *Xba* I and blotted with cyclin genes labeled with α-^{32}P dCTP radioactive probe. Multiple copies of genes were exhibited in the tomato genome and it was confirmed by southern using full-length and 3'UTR probe separately. Northern analysis of different cyclins was also done to know the expression level of cyclins in different tomato tissues such as old leaves, young leaves, flowers, roots and stems. It was evident that cyclin genes were abundant in young leaves compared to other tissues. Expression level of Cyclin A3 and cyclin A2 was also higher in roots. We are generating transgenic tomato plant in order to look over the role of these cyclins and calcineurin B like protein genes in plant growth, development and stress tolerant signaling.

P9
The MABI1 key play root meristem maintenance in *Arabidopsis*

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Plants have the ability to maintain growth and development throughout their entire life. This is made possible by their ability to maintain groups of division-competent cells known as meristems. In the *Arabidopsis thaliana* root meristem, the stem cells surround a small group of hardly ever dividing cells; it called the quiescent centre, and give rise to distal (columella), lateral (lateral root cap and epidermis) and proximal (cortex, endodermis and stele) cell types(1). Ablation studies give us clear answer that short range signals from the quiescent centre keep only the directly abutting stem cells undifferentiated(2). *WOX* gene expression dynamics during early embryo patterning(3). But maintained quiescent centre formation mechanisms still unknown. Understanding the mechanisms underlying meristem maintenance has great scientific and agronomic/horticultural values.

This study aims at elucidating the mechanisms for root meristem maintenance in the model plant *Arabidopsis thaliana*. It is based on an observation we made mutant known as *mabi1* (for root meristem ABA *i*nsensitive 1). We have found that in *mabi1* was unable to maintain the function of root meristems.

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P10
Characterization of the dihydroflavonol 4-reductase (*DFR*) and chalcone synthase (*CHS*) genes from tomato (*Solanum lycopersicum* L.)

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The full-length DFR (1140 bp, GenBank accession no. Z18277) and CHS (1170 bp, GenBank accession no. X55194) cDNAs were obtained by RT-PCR. Both the *SlDFR* and *SlCHS* genes exist as multiple copies in the tomato plant. *SlDFR* transcript was strongly expressed in young leaves and *SlCHS* was expressed in young leaves and shoots. From 5 days post-anthesis to the onset of ripening, *SlCHS* expression decreased gradually; however, *SlDFR* transcript expression did not change significantly during early development of tomato fruit. *SlDFR* expression was induced in response to mannitol and cold treatment at 24 h. *SlDFR* and *SlCHS* expression decreased gradually after treatment with H_2O_2, suggesting that the *SlDFR* gene may function in the response to abiotic stress.

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