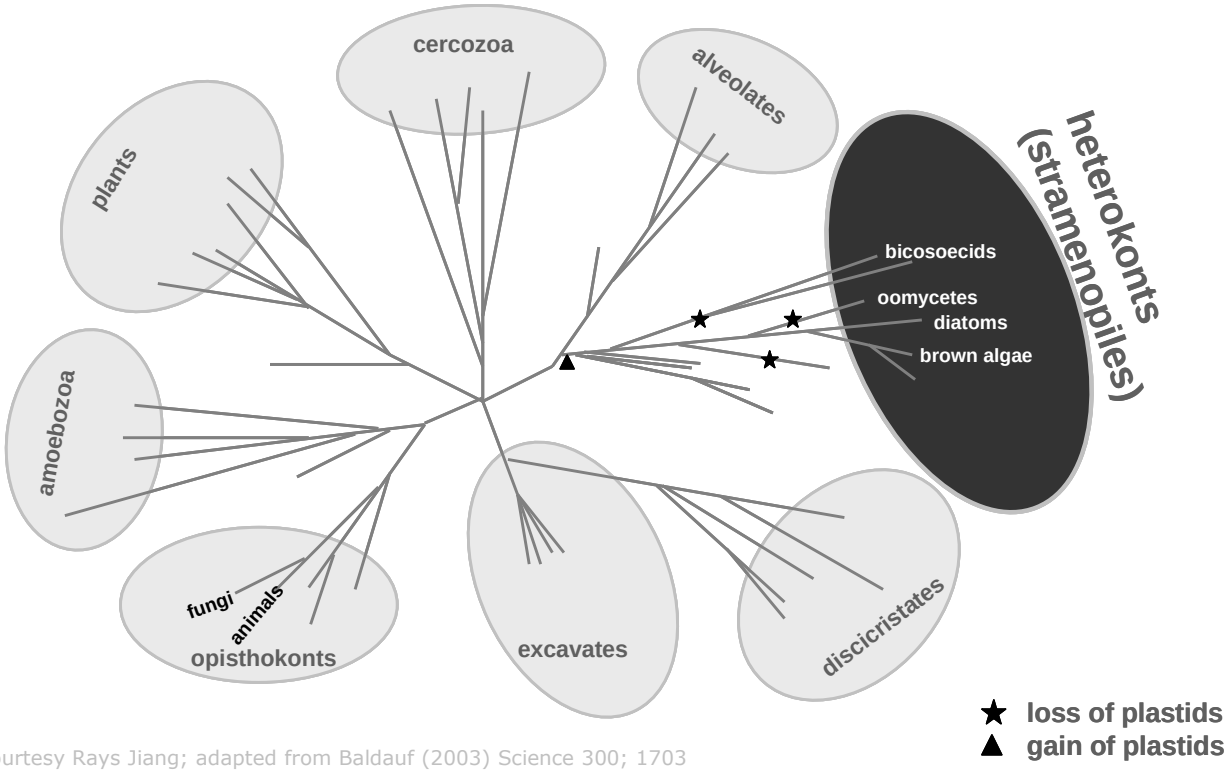
annual meeting

Oomycete

Molecular Genetics Network



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De Wageningse Berg, Wageningen, The Netherlands

May 4-7, 2006

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PROGRAM

Thursday May 4	16:00	**REGISTRATION**		
	19:00	**Dinner**		
Friday May 5	08:30-08:40	**Welcome and Introduction:** Francine Govers		
	08:40-10:00	I. Chair: Francine Govers	Mary COATES	Searching for Avirulence Genes in *Hyaloperonospora parasitica*
			Guido van den ACKERVEKEN	Gene discovery in the Arabidopsis downy mildew pathogen *H. parasitica*
			Nicholas CHAMPOURET	Profiling of R-AVR interactions in wild *Solanum* using RXLR effectors of *Phytophthora infestans*
			Brett TYLER	Role of RXLR effectors in Phytophthora sojae virulence and avirulence
	10:00-10:40	**Coffee and Posters**		
	10:40-12:20	II Chair: Peter Bittner-Eddy	Steve C. WHISSON	Defining the role of the RXLR-EER motif in secreted proteins from *Phytophthora infestans*
			Anne P. REHMANY	Investigating interactions between the downy mildew ATR1^{NdWsB} avirulence protein and Arabidopsis proteins
			Pieter M.J.A. van POPPEL	Identification and characterisation of *Phytophthora infestans* avirulence gene *PiAvr4*
			Miles R. ARMSTRONG	Defence Gene Signalling in the *Phytophthora infestans*-Potato Interaction
			Eleanor M. GILROY	Plant Disease Resistance Involves an Apoplastic Cysteine Protease Cathepsin B
	12:30-14:00	**Lunch**		
	14:00-15:40	III Chair: Steve Whisson	Emma J. ROBERTSON	Functional characterisation of a Kazal-like protease inhibitor from the fish pathogen *Saprolegnia parasitica*
			Elodie GAULIN	Oomycete pathogen-associated molecular patterns (PAMP) : The example of the Cellulose-Binding Domains of secreted proteins
			Klaas BOUWMEESTER	Are lectin receptor kinases involved in the recognition of *Phytophthora* effectors?
			Julia MEITZ	Molecular variation of downy mildew pathogenicity factors
			Sandra CASIMIRO	Transcriptomics of the host-pathogen interaction in crucifer downy mildew: a road to new genes of *Hyaloperonospora parasitica*
	15.40-late	**Wageningen Bevrijdingsfestival** *down town*		

Saturday May 6	08:40-10:00	IV Chair: Pieter van West	Niklaus J. GRUNWALD	Cloning of candidate microRNAs from *Phytophthora sojae* and *P. ramorum*
			Howard S. JUDELSON	Transcriptional networks in *Phytophthora* development
			Catherine R. BRUCE	Proteomic analysis of *Phytophthora infestans* asexual life cycle stages
			Alon SAVIDOR	Expressed Peptide Tags: An additional layer of data for gene prediction
	10:00-10:40	**Coffee and Posters**		
	10:40-12:00	V Chair: Howard Judelson	Claire WALKER	A DEAD-box RNA-helicase is required for normal zoospore development in the potato late blight pathogen *Phytophthora infestans*
			Harold MEIJER	Genome wide analysis of phospholipid signalling genes in *Phytophthora*: novelties and a missing link
			Neil HORNER	Functional characterisation of a putative hemolysine gene of the mycoparasitic oomycete *Pythium oligandrum*
			Andrew J. PHILLIPS	Molecular studies of the Saprolegnia-fish interaction
	12:00-13:30	**Lunch**		
	13:30-15:10	VI Chair: Dave Cooke	Monica BLANCO	Genetic structure of the tobacco blue mold pathogen, *Peronospora tabacina* in North America, Central America and the Caribbean
			Ryan DONAHOO	Hybridization between *Phytophthora capsici* and *P. tropicalis*
			Javier DIEGUEZ-URIBEONDO	"Phylogenetic relationship amoung Saprolegnia diclinaparasitica-isolates"
			John McDOWELL	Segmental Duplications and Intragenic Expansion Mold the RPP7 Resistance Gene Cluster.
			Peter BONANTS	Quantitative multiplex detection of (plant) pathogens
	14:10-15:40	**Coffee and Posters**		
	15:40-16:40	VII Chair: Brett Tyler	Oscar HURTADO	Generation of an inbred line in *Phytophthora capsici* for genome sequencing
			Jean B. RISTAINO	Comparative analysis of the mitochondrial genome organization in *Phytophthora* species and related Straminopiles
			Mike ZODY	Genome sequencing and annotation
	16:40-18:00	**Discussions:** Brett Tyler		
	18:00-19:00	**<u>Keynote</u> Matthias Hahn: 'Nutrient uptake, metabolism and protein transfer into host cells: Trying to understand the role of haustoria in rust biotrophy'.**		
	19:30	**Conference Buffet**		

ABSTRACTS ORAL PRESENTATIONS

Searching for Avirulence Genes in *Hyaloperonospora parasitica*

M. COATES, P. BITTNER-EDDY, M. TÖR and J. BEYNON
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Hyaloperonospora parasitica, an obligate biotrophic oomycete, is the causal agent of downy mildew on Arabidopsis and *Brassica* crops. I am studying the interaction between *H. parasitica* and Arabidopsis. This interaction is a model pathosystem for studying plant disease resistance. In the long run the knowledge gained will be applied to controlling diseases of important food crops, such as brassicas, potato, tomato and soybean. Resistance to *H. parasitica* is by gene for gene resistance, where a resistance gene product and an avirulence gene product must both be present to trigger a response. I aim to identify pathogen genes involved in the infection process, in particular those that induce a hypersensitive response (HR), avirulence genes. To date, 16 candidate avirulence genes have been identified, by screening a suppression subtractive hybridization (SSH) (Bittner-Eddy *et al.*, 2003) and spore cDNA libraries. Full-length sequences of these genes are being obtained. Structural analysis will suggest if they are possible pathogenicity effectors. If so, they will be mapped on segregating, pathogen cross populations to determine if they co-segregate with known avirulence genes.

A cross between the Cala2 and Noks1 isolates of *H. parasitica* has been made. An F2 mapping population of 50 isolates was generated and pathotyped on a set of *Arabidopsis* accessions that give a differential phenotype to each parent, including the accession Columbia, which is known to contain the resistance genes *RPP2a+b*. The RPP2 proteins recognise the *H. parasitica* avirulence protein ATR2, from Cala2, and mounts a HR. Preliminary results suggest at least ten avirulence genes are segregating in this cross, with *ATR2* segregating in a 3:1 avirulent:virulent fashion. This fits a genetic model that a single, dominant gene determines avirulence.

Gene discovery in the Arabidopsis downy mildew pathogen *Hyaloperonospora parasitica*

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Downy mildews are obligate biotrophic pathogens belonging to the oomycetes. After penetration of the host plant downy mildews grow intercellularly forming haustoria in adjacent plant cells. Although haustoria penetrate the plant cell wall they remain separated from the host cell cytoplasm by the plant cell membrane and haustorial wall. Secreted downy mildew proteins are therefore believed to play an important role in the infection process. To identify secreted proteins we have collected Expressed Sequence

Tags (ESTs) from the Arabidopsis downy mildew pathogen *Hyaloperonospora parasitica*. Three sources of mRNA have so far been used: 1. conidiospores of isolate Cala2, 2. seedlings heavily infected with isolate Waco9, and 3. membrane-bound polysomes from isolate Noco2-infected seedlings. A total of >12.000 sequences have been collected so far and were analyzed using a custom bioinformatics pipeline. As we were particularly interested in secreted downy mildew proteins the predicted translation products of the tentative consensus sequences were analyzed by the Signal-P algorithm for the presence of putative N-terminal signal sequences. An alternative method of selection was based on identification of orthologous transcripts in *Phytophthora* species for which two genome sequences and many ESTs are available. The corresponding full-length transcript sequences from *Phytophthora* were subsequently used to search for signal peptides in the predicted proteins. We will report on the different classes of secreted proteins, e.g. hydrolytic enzymes, elicitin-like proteins, NIP-like, inhibitors (Kazal-type protease inhibitors), RXLR proteins, etc. Our current studies are focused on the identification of effector functions by transgenic expression in Arabidopsis, by identification of protein-protein interactions using tagged downy mildew proteins, and by the analysis of the level of DNA polymorphisms for the putative avirulence gene candidates (the RXLR proteins). Based on EST data, *H. parasitica* appears to express a plethora of secreted proteins during the infection process. The challenge for the future is to understand the role and function of these proteins in penetration, haustorium formation and host cell modulation.

Profiling of R-AVR interactions in wild *Solanum* using RXLR effectors of *Phytophthora infestans*

N. CHAMPOURET[1], C. YOUNG[2], M. LEE[2], S. KAMOUN[2], E. JACOBSEN[1] and V.G.A.A.VLEESHOUWERS[1]
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[2]*Ohio State University, Dept of Plant Pathology, Wooster, USA*

Late blight caused by the oomycete *Phytophthora infestans* is the most destructive disease in potato cultivation worldwide. Understanding of the molecular recognition mechanism of resistance (R) proteins of potato and effectors of *P. infestans* is essential to provide a broad durable resistance in *Solanum*. Previous studies revealed that resistance to *P. infestans* is always associated with the hypersensitive response (HR). This suggests that resistant plants recognize elicitors of *P. infestans*. Favourable candidates of AVR proteins (elicitors) are the extracellular proteins (Pex) containing a RXLR motif. The RXLR motif is conserved in diverse oomycete genera among dissimilar oomycete proteins, some of which have been characterized as avirulence genes (Avr3a). RXLR effectors are thought to be translocated inside the plant cell where they can interact with the intracellular NBS-LRR R proteins, the most common class of R proteins effective to oomycetes. In addition to the known isolated R genes, novel R genes were identified in wild *Solanum* germplasm. In this study, we test these plants responses with a transient expression system based on *Agrobacterium tumefaciens* and potato virus X (PVX) expressing cDNAs of RXLR effectors of *P. infestans*. Finally, the correlation between effector recognition and specific resistance in segregating populations is being examined, and will reveal functional evidence for R-AVR interaction in *Solanum* with the aim to clone the corresponding *R* genes.

Role of RXLR effectors in Phytophthora sojae virulence and avirulence

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The Phytophthora sojae genome contains over 170 genes encoding small secreted proteins with varying degrees of similarity to Avr1b-1. All of these proteins have either the RXLR or a dEER motif common to oomycete avirulence genes and most have both. We hypothesize that some of the genes correspond to one or more of the 13 avirulence genes defined genetically in P. sojae. We further hypothesize that some, if not all, of these proteins have the ability to enter inside plant cells where they act to promote the virulence of the pathogen. We are using stable transformation of P. sojae to test these hypotheses. We have shown, using both gain-of-function transformants and using silencing, that the Avr1b-1 gene is responsible not only for the Avr1b phenotype, but also for the Avr1k phenotype. We have also used stable transformants to test whether the RXLR and dEER motifs are required to confer avirulence, and whether either or both motifs can enable the transport of heterologous proteins such as GFP and avrRpt2 into soybean cells. Finally we are using microarrays to assess the effect of Avr1b-1 over-expression on the soybean defense response. Latest results from these experiments will be presented.

Defining the role of the RXLR-EER motif in secreted proteins from *Phytophthora infestans*

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The potato and tomato late blight pathogen, *Phytophthora infestans*, possesses numerous genes encoding predicted secreted effector proteins. One class of these secreted proteins is characterized by containing both signal peptide and RXLR-EER motifs. This class of effector genes has previously been shown to include the avirulence genes *Avr*3a from *P. infestans*, *Atr*1Nd from *Hyaloperonospora parasitica*, and *Avr*1b from *P. sojae*. The RXLR-EER motif has been proposed as being involved in transport of effector proteins from pathogen to host cell. We are aiming to demonstrate the role of this motif in protein trafficking during infection, using the *Avr*3a gene as a basis for study. Progress towards identifying how and where the RXLR-EER motif acts will be presented.

Investigating interactions between the downy mildew ATR1^{NdWsB} avirulence protein and Arabidopsis proteins

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It is assumed that, since pathogens retain avirulence genes despite their presence triggering resistance in certain plants, avirluence genes must confer a selective advantage on the pathogen in the absence of complimentary resistance genes. The Arabidopsis downy mildew avirulence gene, *ATR1^{NdWsB}*, displays extensive allelic

diversity. This diversity is being used to dissect how the resistance proteins RPP1-Nd and RPP1-WsB detect the products of different *ATR1*^{NdWsB} alleles. Results from yeast two-hybrid experiments suggest that the protein products of different *ATR1*^{NdWsB} alleles target different plant proteins to, presumably, help establish a successful infection.

Identification and characterisation of *Phytophthora infestans* avirulence gene *PiAvr4*

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The oomycete pathogen *Phytophthora infestans* causes late blight and is the major pathogen on cultivated potato. In the past, resistance from wild relatives has been introgressed into the cultivated potato *Solanum tuberosum* and genetic analysis has shown that *P. infestans* and potato interact according to the gene-for-gene model. Proteins encoded by resistance (*R*) genes directly or indirectly recognise the products of *P. infestans* avirulence (*Avr*) genes, resulting in localised cell death called a hypersensitive response (HR).

Through a combined approach of genetic mapping and transcriptional profiling (cDNA-AFLP), we obtained markers linked to avirulence genes in *P. infestans* and these were used for marker landing on a BAC library. In this way we identified a candidate gene for *Avr4*. *PiAvr4* encodes a 287 amino acid protein that has a putative signal peptide and an RXLR domain, a motif found in several oomycete effector proteins identified recently and that might be involved in host cell targeting. AVR4 has no homology to any previously described protein and unlike many fungal AVRs it lacks disulfide bridges.

When *PiAvr4* was transformed to *P. infestans* isolates that are virulent on *R4* potato plants, these transformants were able to elicit a specific response on *MaR4* plants and not on *r0* plants. In a PVX-based expression study, constructs containing *PiAvr4* were able to cause a HR specifically on *MaR4* plants, but not on *r0* and *R3a* plants. We have also analysed the diversity of *PiAvr4* in field isolates to find polymorphisms that are specific for a virulent or avirulent phenotype.

Gene Signalling in the *Phytophthora infestans*-Potato Interaction

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The oomycete *Phytophthora infestans* causes late blight, the potato disease that precipitated the Irish famines in 1846 and 1847. It represents a re-emerging threat to potato production and is one of over 70 species which are arguably the most devastating pathogens of dicotyledonous plants. Until recently, little was known about the molecular bases of oomycete pathogenicity, especially the avirulence molecules that are perceived by host defenses. The recent cloning of the *Avr3a* gene offers an opportunity to study pathogenicity at the molecular level with the twin aims of: 1)

understanding the process of recognition and defence response initiation; and 2) gaining an insight into the effector function of an avirulence gene. To this end we have screened a plant yeast 2 hybrid library for proteins which interact with Avr3a. Progress in this research will be reported.

Plant Disease Resistance Involves an Apoplastic Cysteine Protease Cathepsin B

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To successfully colonize a host plant, biotrophic pathogens must overcome an early defence response known as the hypersensitive response (HR). The HR is a genetically programmed dismantling of the invaded host cell that involves countless proteases. SSH isolated a plant cysteine protease, cathepsin B (CathB) EST from *Phytophthora infestans* challenged potato undergoing *R* gene-mediated HR. To counteract early host defence responses, *P. infestans* has recently been shown to secrete protease inhibitors that almost certainly target defence-related proteases (Tian et al. 2004; 2005). These include cysteine protease inhibitors (EPICs) (Kamoun, *personal communication*) that are believed to function in the plant apoplast. *Nicotiana benthamiana*, a close relative of potato, represents a suitable model for investigating many potato traits and is amenable for rapid functional investigation of candidate genes using virus-induced gene silencing (VIGS). Therefore, the VIGS vector, tobacco rattle virus (TRV), was utilised for investigating the role of cathepsin B during the hypersensitive response (HR). The HR induced with non-host bacteria and agro-mediated expression of *R3a* and *Avr3a* was perturbed in *CathB* silenced *N. benthamiana.* Silencing of *CathB* and co-inoculation of commercial CathB inhibitors suppressed CathB activity, caused an increase in bacterium growth and prevented mitochondrial disruption, a marker of cell death. Fluorescent tagging of CathB with mRFP revealed an apoplastic location. It remains to be tested whether *P. infestans*-derived cysteine protease inhibitors, such as EPICs, could inhibit this key regulatory protease of the plant HR to facilitate infection.

Functional characterisation of a Kazal-like protease inhibitor from the fish pathogen *Saprolegnia parasitica*

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Fish pathogens, such as the oomycete *Saprolegnia parasitica*, cause devastating damage and loss of profit in the fish farming industry worldwide. This pathogen causes Saprolegniosis, a disease where filamentous mycelium grows into the fins and body of freshwater fish. The disease results in slow and sluggish movement of infected fish. Severe infection may result in death of the host. Very little is known of the molecular biology of *S. parasitica*, and pathogenicity of the oomycete is undetermined.

To gain more information about which genes are expressed at the onset of an infection, we constructed an EST library from the pre-infectious stages of the *S. parasitica* life cycle, namely zoospores, cysts and germinating cysts. This resulted in the isolation of an EST sequence with homology to a Kazal-like protease inhibitor.

It is commonly known that the mucus layer of fish forms the first line of defence against pathogens. It is also known that proteases are present in this mucus layer of the fish and are thought to play a major role in preventing pathogen infection. Consequently, we would like to speculate that *S. parasitica* secretes protease inhibitors to counteract the effects from the fish proteases, thus permitting the opportunity for further infection. Here we present experiments to identify corresponding host proteases that may interact with the predicted Kazal-like protease inhibitor. Ultimately these experiments will provide us with a better understanding of the host-pathogen interactions that occur during the initial stages of *S. parasitica* infection. Here we present our latest findings.

OomyceteS pathogen-associated molecular patternS (PAMP): The example of the Cellulose-Binding Domains of secreted proteins

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The cell wall elicitor protein CBEL from *Phytophthora parasitica nicotianae* (*Ppn*) contains two Cellulose-Binding Domains (CBD1 and CBD2) belonging to the Carbohydrate Binding Module I family. CBEL-silencing strains revealed anormal cell wall appositions and incapacity to adhere on cellulose surface (Gaulin *et al.*, 2002). At this time, the mechanism by which CBEL is perceived by the host plant remains unknown. The role of CBDs in eliciting activity was investigated using modified versions of the protein produced in *E. coli* or synthesized *in planta* through the PVX expression system. Altogether the expriments revealed the importance of intact CBDs to induce defence responses. Moreover, tobacco and Arabidopsis leaves infiltration assays using synthetic peptides showed that the CBDs of CBEL are essential and sufficient for its elicitor activity. The characteristic features of the CBDs from CBEL are in agreement with the definition of “pathogen-associated molecular pattern (PAMP)”, used when referring to molecules that are unique to microbes and important for microbial fitness and able to elicit innate immune responses (Nürnberger *et al.,* 2004). Importantly, *in silico* analysis of *Arabidopsis thaliana* and *Oryza sativa* genomes revealed a complete absence of this domain. Data mining for the occurrence of this module in sequenced annotated genomes of filamentous fungi shows that the genome of saprobes such as *Neurospora crassa* contains 2 to 5 times more CBDs than the genome of plant pathogens, like *Magnaporthe grisea* and *Fusarium graminearum* for example. All the oomycetes analysed so far such as *P. sojae, P. ramorum, P. infestans* and *Saprolegnia parasitica* produce proteins harboring Carbohydrate Binding Module I family. Recently we have undertaken the generation of EST collection from the pea pathogen *Aphanomyces euteiches*. This will provide the opportunity to investigate the occurence of this PAMP family in an oomycete phylogenically distant from *Phytophthora* and to perform a comparative analysis.

Gaulin, E., Jauneau, A., Villalba, F., Rickauer, M., Esquerré-Tugayé, M.T., and Bottin, A. (2002). The CBEL glycoprotein of *Phytophthora parasitica* var-*nicotianae* is involved in cell wall deposition and adhesion to cellulosic substrates. J Cell Sci **115,** 4565-4575.
Nürnberger, T., Brunner, F., Kemmerling, B., Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev **198,** 249-266.

Are lectin receptor kinases involved in the recognition of *Phytophthora* effectors?

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Lectin receptor kinases (LecRKs) are wide-spread in higher plants. In *Arabidopsis thaliana* there are 46 LecRKs present. The extracellular domain of LecRKs has features of soluble legume lectins which are believed to be involved in the recognition of sugars. Molecular modeling showed however, that *A. thaliana* LecRKs have a poor conservation of the sugar-binding residues, whereas the hydrophobic binding site is more conserved. This suggests that these receptors unlikely bind simple sugar molecules but could serve in the recognition of small hydrophobic ligands and more complex glycans. We have indirect evidence that one of the LecRKs in *A. thaliana*, here named LecRK79, specifically binds and interacts with the *Phytophthora infestans* effector IPI-O by means of an RGD tripeptide motif present in IPI-O (Gouget *et al*. 2006, Plant Phys. 140: 81). Previously it was shown that IPI-O can disrupt adhesions between the plant cell wall and membrane through its RGD motif. This suggests that LecRK9 is involved in plasma membrane-cell wall adhesions and that these adhesions can be disrupted by IPI-O. Our aim is to analyse the role of LecRK79 in recognition, plant defense, resistance and susceptibility. Induction of expression of *LecRK79* was monitored by using *A .thaliana* $P_{LecRK79}$-GUS fusion lines. The GUS-reporter lines are inoculated with different necrotrophic and biotrophic plant pathogens and treated with signaling compounds such as salicylic acid (SA) and methyl jasmonate (MeJA). In addition, an *A. thaliana LecRK79* knock-out line is used to study phenotypic responses upon infection with different pathogens. The results show an increased expression of *LecRK79* in incompatible interactions and changes in phenotype of the *LecRK79* knock-out line upon infection. A further aim is to analyse the interaction between IPI-O and LecRK, and the recognition of IPI-O in Solanaceous plants.

Molecular variation of downy mildew pathogenicity factors

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[2]*University of Munich LMU, Germany*
Email: Julia.Meitz@warwick.ac.uk

Downy mildew on brassicaceous hosts is caused by the oomycete *Hyaloperonospora parasitica*. Some infected *Brassicaceae* show disease resistance visible as a hypersensitive response (HR), causing a localised necrosis killing the infected host cell. Some potential novel *H. parasitica* pathogenicity genes, expressed during infection of Arabidopsis thaliana (At), show high levels of amino acid variability between *H. parasitica* isolates, implying positive selection for change. Five of these novel *H. parasitica* (At) genes were assessed for allelic variation by sequencing alleles from up to 16 pathogen isolates. Analyses of levels of variation allowed grouping of genes into those showing diversifying, neutral or purifying selection. One of these genes was shown to be the avirulence gene *ATR13.* I am now attempting to isolate orthologues of these genes from isolates of *H. parasitica* infecting *Brassica oleracea* (Bo) to determine if the same evolutionary pressures are being exerted and if they are recognised by the host. A microsyntenic region between *H. parasitica* (At) and *H. parasitica* (Bo) was identified using a genomic fosmid library. This region is expected to contain a cluster of genes involved in pathogenicity, which carry the RXLR motif. This motif is thought to be required for the translocation of pathogenicity effectors across the extrahaustorial matrix and into the plant host cell. Once the RXLR genes have been identified, they will be assessed for variability between isolates and can potentially be used as markers for *H. parasitica* in field studies. The genes can be tested for HR in a biolistic assay. This would enable further studies on the molecular host pathogen interactions of this pathosystem and potentially into new host resistance mechanisms; these could be exploited in plant breeding to protect crop from downy mildew infection.

Transcriptomics of the host-pathogen interaction in crucifer downy mildew: a road to new genes of *Hyaloperonospora parasitica*

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Crucifer downy mildew is a worldwide spread and economically important disease of brassica crops, caused by the biotrophic oomycete Hyalop*eronospora parasitica*. The disease can lead to severe stunting or death of seedlings in the nurseries or cause leaf damage of adult plants in the field reducing vegetable yield and quality. Chemical control of the disease has adverse environmental effect and may become ineffective due the development of *H. parasitica* to fungicides. There are sources of resistance to *H. parasitica* already identified in *B. oleracea* but the identification and study of pathogenesis-related virulence factors and avirulence genes is crucial for a successful

breeding for resistance. In compatible interactions between *Brassica oleracea* hosts and *H. parasitica* the lack of the *R* gene or the corresponding *Avr* gene leads to the growth of fungal mycelium and sporulation, whereas in incompatible interactions the presence of both *R* and *Avr* genes activates several defence mechanisms, as hypersensitive response, preventing pathogen development. Our main goal has been to study the interaction between *H. parasitica* and its natural cultivated host, *B. oleracea*. We have selected *B. oleracea* stock CrGC3.1 (CrGC, Univ. of Wisconsin, USA), and two *B. oleracea* var. Tronchuda Bailey, 'Couve Coração de Boi' (commercial variety) and 'Couve Algarvia' (ISA 207), which were infected with *H. parasitica* isolates P005 (HRI, Wellesbourne, UK) and P501 (ISA, Portugal) to produce six different phenotypic interactions. Using a DD approach we found 21 exclusively *H. parasitica* cDNAs from 433 sequenced DD clones, 18 encoding for potential new genes. These putative genes are being analysed for their full-length sequence, expression, sequence polymorphism among isolates, presence of known oomycete conserved motifs and identification of new genetic motifs. New putative secreted peptides have already been identified and their potential role in pathogenicity is currently being assessed by agroinfiltration.

Cloning of candidate microRNAs from *Phytophthora sojae* and *P. ramorum*

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A microRNA (miRNA) is a 21-24 nucleotide RNA product of a non-protein-coding gene. miRNAs are the most abundant small RNAs and have to date been described in the animal and plant kingdoms but not for the Stamenopiles. The biogenesis of miRNAs in plants is similar to that in animals in that miRNAs are processed from primary precursors by at least two steps mediated by RNAse II-like enzymes and that the miRNAs are incorporated into a protein complex (RISC). Homologs of argonaute and dicer-like proteins, involved in miRNA processing in plants, were found in both the *P. sojae* and *P. ramorum* genomes. We thus wanted to test the hypothesis that miRNAs exist in *Phytophthora* given that homologs of the miRNA processing machinery were apparently present. To that effect, we conducted a pilot experiment and cloned and sequenced small (18-25bp) candidate RNAs and sequenced 50 clones each from *Phytophthora ramorum* and *P. sojae*. Of the 100 clones sequenced we obtained 7 *P. sojae* and 14 *P. ramorum* candidate miRNAs. Of these, one clone resembled a miRNA based on (1) its adequate clone length of 21-24 bp, (2) a genomic sequence flanking the miRNA that contains highly complementary 20- to 30-nt segments, which is required to form the pre-miRNA hairpin structure, and (3) a pre-miRNA sequence that is conserved in the closely related species *P. infestans* and *P. sojae*. The loop sequence and sequences flanking the stem are much more variable than the miRNA sequence, which is highly conserved among *Phytophthora* sequences. The function of the transcript containing the putative miRNA sequence is not known. Further cloning and sequencing efforts are underway.

Transcriptional networks in *Phytophthora* development

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A two-pronged approach is being employed to identify networks of genes involved in spore development and germination. In one approach, promoters from genes differentially expressed during the spore cycle are analyzed to identify the binding sites for the relevant transcription factors, which can then be purified by affinity approaches and sequenced. To date, transcription factor binding sites have been defined in the promoters of several genes activated during sporulation and zoosporogenesis, such as *PiNifC1, PiPks1,* and *PiCdc14.* In a second approach, transcription factors implicated in developmental regulation based either on their expression patterns or physical association with spore-specific proteins are targeted. Using gene silencing and other methods, the downstream targets of such transcription factors can be identified. For example, this has led to the identification of targets of the PiBzp1 transcriptional regulator, which is required for the normal formation of zoospores and appressoria.

Proteomic analysis of *Phytophthora infestans* asexual life cycle stages

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Phytophthora infestans is a devastating pathogen causing late blight disease in potato and tomato. Understanding the molecular events taking place during the development of infection structures could lead to development of novel control strategies. We compared whole cell protein lysates from *P. infestans* asexual life cycle stages, namely mycelium, sporangia, zoospores, germinated cysts and germinated cysts with appressoria. Proteins were separated by two-dimensional gel electrophoresis then analysed with Phoretix™ software and principal component analysis. Protein spots with differential profiles between life cycle stages were selected for analysis by MALDI-Tof mass spectroscopy. A total of 108 protein spots were identified by peptide mass fingerprinting. Here we present our results.

Expressed Peptide Tags: An additional layer of data for gene prediction

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While genome sequencing is now routine, genome annotation still remains a challenging process. Identification of coding sequences in the genome can be difficult, especially in eukaryotes with complex gene architecture. Here we present a method to assist the annotation process using proteomic data and bioinformatics. Mass spectra of digested protein preparations of three sequenced organisms (*Rhodopseudomonas palustris, Phytophthora sojae, and P. ramorum*) were acquired and searched against a protein database created by a six frame translation of the genome. The identified peptides were mapped back to the genome and compared to the current annotation. Identified peptides were then classified into categories supporting or adding to the current genome annotation. We named the classified peptides Expressed Peptide Tags (EPTs). Special emphasis was placed on EPT analysis for eukaryotic organisms, and the data was manipulated in order to increase confidence in EPT assignment. The well annotated bacterium *R. palustris* was used as a control for the method and showed high degree of correlation between EPT mapping and the current annotation. The eukaryotic plant pathogens *P. ramorum* and *P. sojae*, whose genomes have been recently sequenced and are not extensively annotated, were subjected to the method and showed less correlation between EPT mapping and their current annotation. While the majority of EPTs supported the current annotation, a significant portion suggested modification to current gene calls or identified novel genes.

A DEAD-box RNA-helicase is required for normal zoospore development in the potato late blight pathogen *Phytophthora infestans*

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Zoospores from the potato late blight pathogen, *Phytophthora infestans*, are released from sporangia and play a key role in pathogenicity. Zoospore formation occurs within minutes after a cold-shock and it is considered one of the fastest developmental processes in a biological system. Therefore, we predict that there is a stored pool of "masked mRNAs", which can be rapidly translated during zoosporogenesis. Recently, we isolated a putative DEAD-box RNA-helicase, *rnh1*, from *P. infestans*. RNA-helicases are involved in mRNA processing and translation initiation. Detailed expression studies of *Rnh-1* have indicated a 20-fold increase in young zoospores compared to mycelium. We used RNA-interference to functionally characterize *rnh-1*. Silenced *rnh1* strains produced large aberrant zoospores. These had undergone partial cleavage and often had multiple flagella on their surface. The *rnh1*-silenced zoospores were also sensitive

to osmotic pressure and they often burst upon release from sporangia. We speculate that *rnh-1* may be involved in activation of stored or “masked mRNAs”. Our aim is to isolate the potential stored pool of “masked mRNAs” using polysome profile analysis. Here we present our latest findings.

Genome wide analysis of phospholipid signalling genes in *Phytophthora*: novelties and a missing link

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Phospholipid-based signalling is an important component of the signal transduction network in eukaryotic systems. To date many phospholipid signalling genes have been identified in a variety of organisms. We explored the genome sequences of *Phytophthora sojae* and *Phytophthora ramorum* and made a comprehensive genome-wide inventory of genes involved in the most universal phospholipid signalling pathways. Several genes and gene families were annotated including those encoding phosphatidylinositol (PI) synthase, PI- and PIP kinases, diacylglycerol kinase and phospholipase D (PLD). The most obvious missing link is a gene encoding phospholipase C (PLC). PLC genes are ubiquitous and conserved in all other eukaryotic genomes that have been sequenced. *Phytophthora* either lacks PLC activity or has an alternative PLC enzyme that is structurally different from the universal PLC.

Analyses of the structural features and catalytic domains of the encoded enzymes revealed many novelties in proteins that are usually well conserved. One example is the PIP kinase family. *Phytophthora* contains one copy of the standard type I/II class, and one copy of the standard type III class. In addition, one novel type III class related protein is encoded and there is a gene family (12 members) encoding a novel type (IV) in which the PIP kinase domains is combined with a seven transmembrane domain with homology to G-protein coupled receptors. Another example is PLD. *Phytophthora* has five different PLD subfamilies of which four are unique for *Phytophtora.* Two subfamilies comprise small PLD-like genes with unusual catalytic domains. In the largest of the two the encoded proteins have a signal peptide signature suggesting that they are secreted. One subfamiliy is PXTM-PLD, the homolog of which was recently cloned from *P. infestans.* PXTM-PLD has a domain architecture unknown beyond *Phytophthora* [1]. Implications of these genome discoveries and their putative roles will be discussed.

1. Meijer, H.J.G., Latijnhouwers, M., Ligterink, W., and Govers, F. (2005). A transmembrane phospholipase D in *Phytophthora*; a novel PLD subfamily. Gene *350*, 173-182.

Functional characterisation of a putative hemolysine gene of the mycoparasitic oomycete *Pythium oligandrum*

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We are interested in *P. oligandrum* effector proteins that are involved in the mycoparasitic interaction between *P. oligandrum* and *P. infestans*. One approach we have used to identify effector proteins is to analyse expressed sequence tags (ESTs) created from two cDNA libraries. One library was created from mycelia of *P. oligandrum* to gain an initial insight into gene expression in the vegetative stage of this organism. A second cDNA library was created from the interaction between *P. oligandrum* and *P. infestans*. Comparison of the two data sets should provide us with a glimpse of genes that may play a role during the mycoparasitic interaction.

Detailed expression analysis of a select group of genes identified from the EST libraries by real-time quantitative PCR revealed up-regulation of a putative hemolysine homologue. Gene expression was found to be specifically increased in the early stages of the interaction. The complete open reading frame of the hemolysine gene was determined and cloned in a FLAG-expression vector of *E. coli* in order to functionally characterise the hemolysine protein employing various biochemical assays. Here we present our latest findings.

Molecular studies of the Saprolegnia-fish interaction

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Oomycetes of the genus *Saprolegnia* are responsible for devastating infections of fish. The disease (Saprolegniosis) is characterised by visible white or grey patches of filamentous mycelium on the body or fins of freshwater fish and is of particular problem to aqua-cultural businesses. Previously, the impact of Saprolegniosis was minimised by the use of an organic dye, malachite green. However, the use of this compound has been banned around the world, resulting in a dramatic recrudescence of *Saprolegnia* infection. Despite the huge economic importance and documented re-emergence of this pathogen, very little molecular work is being undertaken to investigate *Saprolegnia*; or indeed any animal-pathogenic oomycete.

We aim to investigate the molecular mechanisms, which enable *Saprolegnia* to successfully infect fish, the molecular processes that suppress host defenses during infection, and the nature of the pathogen/host interaction. We believe that these processes must be regulated, at-least partially, by proteins secreted by *Saporolegnia*, both prior to and during the infection stages. Preliminary data supports this hypothesis, as work in our laboratory generated an EST library from *Saprolegnia parasitica* and revealed the secretion of a diverse set of proteins with similarities to various proteases, cell attachment proteins, and protease inhibitors.

We will be further analysing the available EST sequence information and applying recently developed oomycete (molecular) research techniques. The function of candidate virulence genes from *S. parasticia* will be investigated, using detailed expression analysis during developmental stages, RNA-interference, *in vitro* cell attachment assays, and virulence tests. We will shortly initiate mircoarray hybridisation studies to investigate the host response upon *Saprolegnia* infections.
Advances in oomycete biology and host immunity have potential impact for the global aquaculture industry, and new research will hopefully lead to novel control strategies that increase fish health and reduce disease losses. Here we present our latest findings.

Genetic structure of the tobacco blue mold pathogen, *Peronospora tabacina* in North America, Central America and the Caribbean

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Tobacco blue mold, caused by the oomycete pathogen *Peronospora tabacina* is a highly destructive pathogen of tobacco seed beds and production fields in tobacco producing areas globally. The disease has been responsible for multi-million dollars losses to growers and resistance to the commonly used fungicide metalaxyl has made management increasingly difficult. Blue mold can be extremely severe in tobacco transplant production houses and movement of infected plants to the field can lead to widespread disease occurrence. The main objective of the work is to study the genetic structure of populations of *P. tabacina* using a collection of samples from North America, Central America and the Caribbean.

We developed a PCR primer called Ptab that can be used to specifically identify the blue mold pathogen in infected tissue. We have also developed diagnostic PCR-RFLP (restriction fragment length polymorphisms) fingerprints that allow us to distinguish all the major tobacco leaf pathogens.

We are using gene sequencing of specific mitochondrial, nuclear and ribosomal genes to study the genetic structure of populations and to differentiate among strains of *Peronospora tabacina* from epidemics that occur locally in NC, within the US, in the Caribbean basin, and in Central America.

We amplified the mitochondrial P3 region containing the genes rpl5, rpl14 and tRNA's; P4 regions containing cytochrome c oxidase subunit 1 gene (Cox1); cytochrome c oxidase subunit 2 gene (Cox2) and NADH (NADH dehydrogenase subunit 1 gene). Some portions of the nuclear Ras gene (Piypt1 gene), Beta tubulin (β tub), and some regions of the nuclear large subunit (LSU) ribosomal DNA. Gene sequencing is underway to study the genetic structure of the sample populations, determine the source of inoculum for epidemics in North Carolina, and to determine if the pathogen has undergone sexual recombination.

Hybridization between *Phytophthora capsici* and *P. tropicalis*

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Phytophthora capsici and *P. tropicalis* are closely related species that were until recently considered to be a single species, *P. capsici*. *Phytophthora tropicalis* is found on woody species such as cacao, black pepper, and macadamia whereas *P. capsici* is generally found on annual vegetables. Distinction between these two species is based on hosts, sporangia shape, the presence/absence of chlamydospores, and ITS sequences. In this study we show that sexual recombination can occur between these two species. Crosses were made between a *P. tropicalis* isolate recovered from a nursery grown Rhododendron in Tennessee and an isolate of *P. capsici* recovered from pumpkin in Tennessee. Progeny were tested using AFLP and SNP analysis and 42 recombinant progeny recovered. Chlamydospores were observed for all of the recombinant progeny. Mating type segregated as 28 A1, 11 A2, and 3 sterile isolates. Crosses back to the parents as well as among sibs are being conducted and the utility of interspecific crosses for dissecting traits such as chlamydosporogenesis and preference for perennial vs. annual hosts will be discussed.

Segmental Duplications and Intragenic Expansion Mold the *RPP7* Resistance Gene Cluster.

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The Arabidopsis *RPP7* gene determines race-specific resistance to the Hiks1 isolate of the downy mildew pathogen *Hyaloperonospora parasitica*. Map-based cloning placed *RPP7* on Chr. 1, within a cluster of eight CC-NBS-LRR genes that are monophyletic, but interspersed with other genes. The *RPP7* cluster belongs to a clade of nine CC-NB-LRR loci, dispersed on Chromosomes 1 and 5, that includes the *RPP8*/*HRT1*/*RCY1* locus. These nine loci apparently radiated from a single copy ancestral sequence, through segmental duplications and/or ectopic recombination. In striking contrast to *RPP7*, the other eight loci in this clade exist as singletons in the Col genome. The *RPP7* cluster expanded through a series of single-gene and segmental duplications, the most recent of which generated two pairs of identical CC-NB-LRR genes. Little similarity exists between promoters of genes in the *RPP7* cluster, except between genes that were recently duplicated. The *RPP7* transcribed region has a very atypical length of ~19 Kb. Most of the gene is comprised of long introns within short 5' and 3' UTRs. The *RPP7* transcript undergoes a complicated pattern of alternative splicing in the 5' and 3' UTRs. Alternative splicing does not affect the structure of the RPP7 protein, which is similar in length and structure to RPP8 (52% identity). The functional significance of alternative splicing in the 5' and 3' UTRs is currently unclear, but could impact mRNA stability and/or translation**.** Intragenic recombination has affected the

protein coding sequence of *RPP7* by expanding the LRRs. Taken together, these features suggest that segmental duplications and intragenic recombination have generated extensive diversity during the evolutionary history of the *RPP7* cluster.

Phylogenetic relationship among *Saprolegnia diclinaparasitica*-isolates

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The complex *Saprolegnia diclina-parasitica* comprises severe parasites on aquatic animals such as fish, amphibians, crustaceans, etc. We have investigated for the first time the phylogenetic relationship among isolates of the *Saprolegnia diclina-parasitica*-complex based on internal transcribed spacer of nuclear ribosomal DNA sequences (ITS nrDNA). We have generated 128 new ITS sequences of Saprolegnia from a wide range of hosts, and environments, and of worldwide distribution. Bayesian and MP phylogenetic analyses reveal that the isolates of *S. diclina-parasitica* complex belong to three phylogenetically distant clades. Morphological studies show that both presence of long hairs of secondary cysts and high frequency of indirect germination are specific characters for a clade that we named *S. parasitica*. Our data support a previous proposal by Beakes *et al*. (1994) of assigning the name S. parasitica to strains pathogenic to salmonids. However, we also found that the *S. parasitica* clade comprises a number of non-pathogenic strains as well as isolates from other wide range of other hosts. These results emphasise the need of taking special care when assigning species based on classical taxonomic criteria, i.e. morphological traits of sexual structures.

Quantitative multiplex detection of (plant) pathogens, including *Phytophthora* species, based on PRI-lock probe technology and the OpenArray platform

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A concept is described for quantitative multiplex detection of (plant) pathogens, including several *Phytophthora* species, in which several pathogens will be detected real-time in one sample. After the extraction of genomic DNA we introduce a ligation step with target specific probes. In this step we make use of PRI-lock probes, linear oligonucleotides in which the 5' and 3' ends of the probes will hybridize in immediate juxtaposition on a DNA or RNA target strand and can be covalently joined by a DNA ligase, converting the probe to a circular molecule only when both end segments correctly recognize nearby target segments. Ligation reactions permit easy differentiation among similar target sequence variants, as mismatched probes are poor substrates for ligases.

After ligation, circularized probes can be PCR amplified in each well of the BioTrove OpenArray platform. This platform uses wells with a volume of only 30

nanoliter. On one slide 1024 reactions can be followed real-time on the NT-Cycler of BioTrove. In this way quantitative data can be obtained. Target specific set of primers are preloaded in each well, if the corresponding sequences have been included in the non-target complementary segment of the PRI-lock probes. The orientation of the primers is chosen in such a way that only originally circularized PRI-lock probes will be amplified.

PRI-lock probes combined with the OpenArray platform are promising for multiplex DNA and RNA diagnostic analyses, since the targeted sequences and the PCR amplifiable target probes are independent. The use of pre-selected codes in the PRI-lock probes makes universal PCR conditions and universal TaqMan detection assay possible and is easily modifiable and extendable to include other, even newly-emerged pathogens.

The OpenArray platform of BioTrove offers also applications in the quantitative multiplex analysis of gene expression. Examples and possibilities of this new technology will be presented and discussed.

Generation of an inbred line in *Phytophthora capsici* for genome sequencing

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Phytophthora capsici (Leonian) is an important plant pathogen with a broad host range including eggplant, tomato, pepper, pumpkin, cucumber, squash, and snap bean. *Phytophthora capsici* regularly completes an outcrossing sexual cycle in natural populations and individuals carry extensive variation within their genomes. Genome sequencing is underway for *P. capsici* and will be accomplished using both a traditional shotgun strategy and a novel high throughput sequencing platform. In order to reduce heterozygosity and facilitate assembly we have developed an inbred line of *P. capsici* as follows: (i) 200 recombinant isolates (F_1 progeny) were recovered from a cross between an isolate recovered from cucumber in Michigan (isolate OP97) and pumpkin in Tennessee (isolate LT263), (ii) an F1 progeny isolate was then backcrossed to LT263 and a total of 72 recombinant isolates recovered (backcross 1, BC_1), and (iii) a BC_1 progeny isolate was crossed with LT263 and 73 recombinant isolates were recovered (backcross 2, BC_2). For each generation (F_1, BC_1, and BC_2), pathogenicity on cucumbers, mating type and growth rate were assessed. A progeny isolate from the BC_2 that sporulated well and retained pathogenicity was selected for genome sequencing. To estimate the impact of inbreeding we analyzed the F_1, BC_1, and BC_2 progeny with a single AFLP primer combination (Eco+CC/Mse+CA) and out of 21 polymorphic markers present in the F_1, six (~30%) changed to the homozygous state in the final inbred isolate.

Comparative analysis of the mitochondrial genome organization in *Phytophthora* species and related Straminopiles

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The mitochondrial genome organization of Oomycete species *Phytophthora infestans, P. sojae*, and *P. ramorum* were compared to mitochondrial genomes of *Saprolegnia ferax*, and other Straminopiles (section Heterokonta) including the autotrophic species in the Bacillariophyceae (*Thalassiosira pseudonana*), the Chrysophyceae (*Chrysodidymus synuroideus*, *Ochromonas danica*), and the Phaeophyceae (*Laminaria digitata, Pylaiella littoralis*, *Dictyota dichotoma, Fucus vesiculosus*, *Desmarestia viridis)* and the heterotrophic species in the Bicosoecida (*Cafeteria roenbergenesis*). We performed whole mitochondrial genome analyses with nucmer (in the Mummer 3.0 package) and have displayed the results with mummerplot and customized synteny software to evaluate genome organization. *P. ramorum* and *P sojae* contain a similar ~13kb inversion in the mitochondrial genome when compared to the genome of *P. infestans* but otherwise share nearly identical sets of genes. Sequence divergence within genes and changes in relative gene order were observed when comparing the *Phytophthora* species to the closely related Oomycete *S. ferax.* This was also observed when comparing the genomes of the Chrysophytes, *Chrysodidymus synuroideus* and *Ochromonas danica.* Among the 5 species represented in the Phaeophyceaeae, broad differences in gene order and sequence divergence were observed that mirror the results from the Oomycetes and Chrysophytes. These differences observed at the whole mitochondrial genome level indicate that genera within the Stramenopiles are more divergent than single gene phylogenies would indicate. Supporting this observation, there is little to no conservation of gene order and sequence homology among genera in the Stramenopiles indicating that these mitochondrial genomes have either undergone rapid divergence or originated from different mitochondrial lineages. Whole mitochondrial genome gene geneaologies will be developed in order to understand molecular evolution in the autotrophic and heterotrophic Straminopiles.

Genome sequencing and annotation

M. ZODY
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ABSTRACTS POSTERS

1 Function of the avirulence gene ATR13 from Hyaloperonospora parasitica

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We have cloned the avirulence gene *ATR13* from the obligate biotrophic oomycete, Hyaloperonospora parasitica and shown that, like its partner resistance gene *RPP13*, it exhibits extreme levels of amino acid polymorphism consistent with diversifying selection. This suggests that both plant and pathogen are locked in co-evolutionary conflict where, as pathogen attempts to evade detection, the host develops new detection capabilities. *ATR13* encodes a 187 amino acid protein with no homologies to other proteins. It has a series of domains; an N-terminal signal peptide sequence, RxLR motif thought to be involved in protein transport into the plant cell, a leucine heptad repeat, 4 highly conserved 11aa repeats and a C-terminal domain. Examination of *ATR13* alleles from a range of H.parasitica isolates show variation both in amino acid sequence and in the number of 11aa repeat units. We have used a biolistic transient assay to determine the domains required for recognition by the *RPP13-Nd* allele. We have shown the C-terminal domain to be the region of recognition by *RPP13-Nd*. We have used a site-directed mutagenesis approach to determine which amino acids are required for recognition by the *RPP13-Nd* allele. We are currently looking for proteins which interact with ATR13 in a bid to determine the molecular basis of the recognition event. ATR13 must be required for the growth of *H.parasitica* on Arabidopsis and our studies of the avirulence protein will provide insight into the role of the identified domains in biotrophic pathogenicity aswell as disease resistance specificity.

2 Developing a vaccine against the fish pathogen *Saprolegnia parasitica* in salmonids

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Saprolegnia parasitica is an economically important fish pathogen causing devastating losses in fish-farming worldwide. The resulting disease, Saprolegniosis, is characterised by filamentous mycelial growth on the fins and body of the fish. A global ban on the *Saprolegnia* control agent malachite green in 2001, has resulted in *S. parasitica* infections becoming a re-emerging problem. It is therefore essential to find a new and effective control strategy. One possible solution is fish vaccination and so the aim of this project is to design a vaccine against *S. parasitica*. We initially plan to generate a DNA vaccine. Therefore we are constructing a cDNA library from infected salmonid tissue and will sequence up to 5000 clones. Bioinformatics analysis of the EST library should

identify genes encoding secreted proteins from *S. parasitica*. Such proteins are potential vaccine candidates as they may play a crucial role in a successful infection and are the first molecules that could be interacting with host cells. By expressing the corresponding genes in salmonids through an expression vector that is injected in the fish, it may be possible to identify a gene that induces an immune response in the host. Ultimately, this work may lead to the development of a commercial protein vaccine that has the potential to alleviate the problems caused to the aquaculture industry due to *S. parasitica*. Here we present our latest findings.

3 Proteomic analysis of cell surfaces and secretome of *Aphanomyces euteiches*

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When a pathogenic microorganism comes to interact with a host plant, effectors involved in adhesion or colonization are secreted into the medium or retained at the cell surface. The cell wall itself is an essential compartment representing a target for antimicrobial compounds. The proteomic approach makes it possible to directly identify proteins produced in a specific situation or cellular compartment, as well as some post-translational modifications that are important for biological activity (proteolytic cleavage, glycosylation...). This approach was chosen to analyze the cell surfaces and the secretome of *Aphanomyces euteiches*, a parasite causing seedling damping off and root rot of legumes such as Alfalfa and Pea. Identification of proteins will be based on two EST libraries currently being analyzed, one library corresponding to mycelium cultivated in a glucose-yeast extract medium, the other library generated from mycelium in early condition of infection (mycelium in contact with host roots). In a comparative proteomic approach, three situations will be analyzed : the first one where the parasite is in the presence of plant host roots, the second one where it undergoes starvation after transfer into water, and the third one where it grows actively in glucose-yeast extract medium (control situation). In a systematic approach, supernatants of zoospore suspensions in the motile state or in the course of germination after encystment will be analyzed to identify the produced proteins. Lastly, parasite cell walls will be prepared, biochemically characterized, and the associated proteins will be analyzed. First results of this work will be presented.

4 Investigating the costs of metalaxyl resistance

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Mutations that confer resistance to a fungicide are generally believed to lower the fitness in the absence of the chemical. However, this relationship has not been found consistently among comparisons between field isolates of *Phytophthora infestans* for resistance to metalaxyl (Gisi and Cohen 1996). Differences in the genetic backgrounds

and compensatory mutations of these field isolates can confound the fitness effect. An experimental evolution approach should be used to investigate this phenomenon by comparing a resistant and a sensitive genotype with a similar genetic background. Such experiments have been performed in several experiments but only by transferring fast growing pieces of mycelium. By serial transfer of zoospores, we generated a number of genotypes that clearly showed an increased growth rate on several concentrations of metalaxyl compared to the sensitive control. Our results suggest that resistance to metalaxyl can emerge quickly on sub-lethal concentrations of metalaxyl and that the fitness of resistant genotypes does not show the expected response in the absence of metalaxyl.

5 Gene discovery in the Arabidopsis downy mildew pathogen *Hyaloperonospora parasitica*

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Downy mildews are obligate biotrophic pathogens belonging to the oomycetes. After penetration of the host plant downy mildews grow intercellularly forming haustoria in adjacent plant cells. Although haustoria penetrate the plant cell wall they remain separated from the host cell cytoplasm by the plant cell membrane and haustorial wall. Secreted downy mildew proteins are therefore believed to play an important role in the infection process. To identify secreted proteins we have collected Expressed Sequence Tags (ESTs) from the Arabidopsis downy mildew pathogen *Hyaloperonospora parasitica*. Three sources of mRNA have so far been used: 1. conidiospores of isolate Cala2, 2. seedlings heavily infected with isolate Waco9, and 3. membrane-bound polysomes from isolate Noco2-infected seedlings. A total of >12.000 sequences have been collected so far and were analyzed using a custom bioinformatics pipeline. As we were particularly interested in secreted downy mildew proteins the predicted translation products of the tentative consensus sequences were analyzed by the Signal-P algorithm for the presence of putative N-terminal signal sequences. An alternative method of selection was based on identification of orthologous transcripts in *Phytophthora* species for which two genome sequences and many ESTs are available. The corresponding full-length transcript sequences from *Phytophthora* were subsequently used to search for signal peptides in the predicted proteins. We will report on the different classes of secreted proteins, e.g. hydrolytic enzymes, elicitin-like proteins, NIP-like, inhibitors (Kazal-type protease inhibitors), RXLR proteins, etc. Our current studies are focused on the identification of effector functions by transgenic expression in Arabidopsis, by identification of protein-protein interactions using tagged downy mildew proteins, and by the analysis of the level of DNA polymorphisms for the putative avirulence gene candidates (the RXLR proteins). Based on EST data, *H. parasitica* appears to express a plethora of secreted proteins during the infection process. The challenge for the future is to understand the role and function of these proteins in penetration, haustorium formation and host cell modulation.

6 *In vitro* sporulation and stability of drug-resistant and silenced transformants of *Phytophthora parasitica*

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Genetic transformation of oomycetes has been thoroughly studied only in *Phytophthora infestans*, where stable transformants can be obtained through various procedures, sometimes involving the isolation of single-zoospore derivatives. However, no detailed study of the sporulation capability of these transformants has been reported. Here we show that the antibiotics hygromycin B or geneticin used for selection of transformed protoplasts inhibit zoospore production in *Phytophthora parasitica* transformants. In addition, single-zoospore resistant isolates obtained from the transformants give rise to zoosporic derivatives that are mostly drug-susceptible. The resistance gene in strains that are drug-resistant is associated with high molecular-weight DNA that may not be linked to chromosomal DNA, and loss of the resistance phenotype observed in most zoosporic derivatives is correlated with loss of the resistance gene. However, in transformants where the resident gene encoding the CBEL elicitor has been silenced, silencing is not reversed when transgenic sequences are lost. This shows that the instability of *P. parasitica* transformants does not hamper functional studies in this organism.

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7 The role of an *Arabidopsis* Lectin Receptor Kinase in plant-pathogen interactions

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Lectin receptor kinases (LecRKs) are wide-spread in higher plants. In *Arabidopsis thaliana* there are 46 LecRKs present. The extracellular domain of LecRKs has features of soluble legume lectins which are believed to be involved in the recognition of sugars. Molecular modeling showed however, that *A. thaliana* LecRKs have a poor conservation of the sugar-binding residues, whereas the hydrophobic binding site is

more conserved. This suggests that these receptors unlikely bind simple sugar molecules but could serve in the recognition of small hydrophobic ligands and more complex glycans.

Plants can recognize effectors produced by pathogens and this recognition may result in defense responses. We have indirect evidence that one of the LecRKs in *A. thaliana*, here named LecRK79 specifically binds and interacts with such a pathogen effector (Gouget *et al*. submitted). Our aim is to analyse the role of LecRK79 in plant defense, resistance and susceptibility. Induction of expression of *LecRK79* is monitored by using a $P_{LecRK79}$-GUS fusion as a reporter in transgenic *A .thaliana* lines. The GUS-reporter lines are inoculated with different necrotrophic and biotrophic plant pathogens and treated with signaling compounds such as salicylic acid (SA) and methyl jasmonate (MeJA). In addition, an *A. thaliana LecRK79* knock-out line is used to study phenotypic responses upon infection with different pathogens. The results show an increased expression of *LecRK79* in incompatible interactions and changes in phenotype of the *LecRK79* knock-out line upon infection.

8 Identification of the catalytic triad of the *Phytophthora sojae* 42 kDa transglutaminase by site-directed mutagenesis

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Transglutaminases (TGases) (R-glutamyl-peptide:amine-γ-glutamyltransferase, EC 2.3.2.13) catalyze an acyl transfer reaction between peptide-bound glutamine residues and primary amines including the ε-amino group of peptide-bound lysine residues. Formation of TGase-mediated intra- or intermolecular isopeptide bonds results in irreversible protein cross-linking. TGase activity has been implicated in a multitude of physiological activities in animals, plants and bacteria. In *P. sojae*, a 42-kDa Ca^{2+}-dependent transglutaminase was identified that exhibited similar biochemical and enzymological characteristics as animal papain-like TGases (1,2). Immunocytochemical analyses *in situ* localized the protein to hyphal cell walls of *P. sojae* growing *in* as well as *ex planta* (3). A surface-exposed epitope within the *P.sojae* TGase (Pep-13) was shown to act as a pathogen-associated molecular pattern (PAMP) in eliciting plant innate immune responses (1,4).

Single amino acid substitutions in the P.sojae 42 kDa TGase allowed the identification of the Cys, His and Asp residues that form the catalytic triad in the active site. *Phytophthora* TGases form a monophyletic gene family. PCR experiments designed to detect homologous genes in species of the genus Pythium failed. Unrooted phylogenetic trees constructed from the sequences of the *P.sojae* and *P.ramorum* genome show that two groups of TGases apparently evolved at different rates. Limited but significant sequence similarity between *Phytophthora* and mammalian TGases was observed in the regions adjacent to the catalytic cysteine residue (WY/FGICHAW) which is indicative of convergent evolution of TGases in different phylae.

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9 Characterization of Glucanase Inhibitor Protein gene from *Phytophthora cinnamomi* by Asymmetric PCR

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Plants employ a wide variety of strategies to defend themselves from pathogenic microbes. In response to pathogenic attack, plants typically secrete β-endoglucanases. These enzymes hydrolyse β-(1-3),(1-6)-linked glucans in the pathogen cell wall, and both weaken the cell wall of the attacking microorganism and generate low molecular weight glucan oligosaccharide fragments that act as molecular signals leading to the activation of plant defence responses. We report the sequencing and characterization of a class of proteins, termed glucanase inhibitor proteins (GIPs), that are secreted by the oomycete *Phytophthora cinnamomi*, a pathogen of *Castanea*, and that specifically inhibit the endoglucanase activity of their plant host, thereby suppressing the degration of glucans in the oomycete cell wall and the release of oligoglucoside elicitors.

A BLAST search of the databases revealed that Gip sequence are homologous to the trypsin class of Ser proteases but are proteolytically non-functional because one or more residues of the essential catalytic triad is absent. However, specific structural features are conserved that are characteristic of protein-protein interactions, suggesting a mechanism of action that as not been very described previously in plant pathogen studies.

High-efficiency thermal asymmetric interlaced (HE-TAIL) PCR is a modified thermal asymmetric interlaced (TAIL) method for finding unknown genomic DNA sequences adjacent to known sequences. We obtained a small sequence of 300pb by amplification using degenerated primers designed based on homology in the open reading frames of others GIPs (*Phytophthora sojae*), and the full length gene sequence was obtained by flanking this known sequence with asymmetric PCR.

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10 Characterization of the *Phytophthora cinnamomi* lipase activity

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Phytophthora cinnamomi is soilborn pseudofungus, associated to the Oomycetes with ink disease of chestnut.

Lipases (triacylglycerols hydrolases) are important enzymes in fat metabolism, catalyzing the breakdown of triacylglycerols to free fatty acids and glycerol. Owing to the very low solubility of their natural substrates, this hydrolysis is catalyzed at the interface between an insoluble substrate and the aqueous phase in which the enzyme is solubilized. This feature distinguishes them from esterases, which preferentially catalyze the hydrolysis of soluble esters in water. Lipases constitute a ubiquitous group of enzymes able to catalyze a number of different reactions, many of them of industrial interest (stereoselective hydrolysis, transesterification, etc.). The objective of the present study was to evaluate the growth of *P. cinnamomi* in the lipids content medium. Additionally it was researched the presence or absence of lipases.

The analyse of the results, proved that the growth of the *P. cinnamomi* decreased exponentially and the lipids content increased in the extracelular medium, for concentrations upper the 1 and 2% (w/v), for tributyrin and for olive oil, respectively. The maximum concentration for the grown was 3% for the tributyrin and 10% for the olive oil.

The determination of the lipase activity was effectuated in the semi solid medium. The quantification was result for the quotient between the square of the diameter of the clear halo to the return of the colonial and the square of the diameter of the colonial.

In this study, was verified that to the contrary of the growth, the lipase activity was upper for the concentration of lipids of 2 and 3%. In this work, we will also study the lipase quantification by spectrophotometric methods.

11 Phylogenetic relationships of a new species of *Phytophthora* closely related to *Phytophtora infestans* in the Andean Highland of Ecuador

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Recent studies suggest that *Phytophthora infestans* sensu lato in the Andean Highlands of South America has higher genetic diversity than previously described. In Ecuador, three clonal lineages (US-1, EC-1, EC-3) were found on different host species in the genus *Solanum*. Another lineage, EC-2, has both A1 and A2 mating types and belongs to Ia and Ic mitochondrial DNA (mtDNA) haplotype, respectively. Moreover, the EC-2 lineage was found in association with different plant genera beside *Solanum*. Isolates of *P. infestans* sensu lato EC-2 and EC-3 lineages fit the morphological description of *P. infestans* but are quite different from any genotypes of *P. infestans* described to date, raising questions about their taxonomic status and relatedness to isolates of *P. infestans* associated with potato and tomato. Hence, the objective of this study was to

elucidate the phylogenetic relationship of *P. infestans* sensu lato with other close related *Phytophthora* species,particularly the Ic Clade. We sequenced intron 1 of a nuclear single-copy Ras gene and a portion of mitochondrial-encoded Cox1 gene for the phylogenetic analysis. Our results shows that the EC-2 lineage associated with the Ic mtDNA haplotype isolated from *S. tetrapetalum* form a distinct branch in the same clade with *P. infestans* and other closely related species including *P. mirabilis*, *P. phaseoli* and *P. ipomoeae* and is identical to *P. andina* isolated from *S. muricatum* in Ecuadorian.

12 BTH molecular response assessment in petunia, potato and tomato

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Induced resistance by chemicals such as benzothiadiazole BTH (Syngenta Inc) mimics the biological activation of Systemic Acquired Resistance (SAR) by necrogenic pathogens, taking the place of salicylic acid (SA) in the SAR signal pathway, inducing the same molecular markers and range of resistance. Previous work in our laboratory found that BTH activates resistance against late blight caused by *P. infestans*, on petunias and tomatoes while it did not activate resistance against the same pathogen on potatoes, suggesting that the spectra of resistance activated by BTH are very crop and pathogen specific. Our current research seeks to understand the molecular mechanism by which BTH mimics the SAR response and further understanding why BTH works in some plants and not others. To address this question we used microarray technology to identify the genes expressed in response to BTH in petunias, tomatoes and potatoes. Knowledge of the differences of BTH effect on these plants will have important implications to practical disease management and will increase our understanding of disease resistance mechanisms in plants.

13 Gene transcription profiling in *Phytophthora sojae* during early-stages of soybean infection

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Phytophthora sojae is a soil-borne plant pathogen that causes root and stem rot in soybean plants resulting in an estimated $300 million annual losses in North America. Despite significant recent progress in identifying genes involved in the interaction of *P. sojae* and soybean plants, the entire spectrum of genes is still unknown. The available genome sequence of *P. sojae* and *Phytophthora ramorum* (sudden oak death syndrome) reveals over 170 secreted proteins with similarity to the avirulence Avr1B, thus providing a long list of candidate genes with potential roles in pathogenicity. We are conducting experiments to identify genes in *P. sojae* induced during infection, more specifically during early-stage of infection, and to characterize their roles in pathogenesis.

Towards this goal, and as part of an USDA-funded collaborative research project on the functional genomics of *P sojae* we are using a whole genome microarray (Affymetrix GeneChips) to assay the mRNA levels in *P. sojae* at 0h, 3h, 6h and 12h post-infection. We will describe progress in using these arrays to identify genes that are involved in pathogenicity of *P. sojae*

14 Population biology of the oomycete *Aphanomyces euteiches*: a hierarchical analysis of diversity, selfing and genetic differentiation

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Relatively little is known about the population biology of the legume pathogen *Aphanomyces euteiches* Drechs. *A. euteiches* is a soilborne pathogen causing Aphanomyces root rot of several legumes including alfalfa, bean, lentil and pea. Our objectives were to assess the degree of diversity, selfing and population differentiation in *A. euteiches* contrasting populations within and among two geographically separated fields with a history of pea production. Molecular genotyping relied on amplified fragment length polymorphism analysis. Samples of *A. euteiches* recovered from two fields in northeast Oregon and western Washington confirm previous reports of moderately high genetic diversity in populations of *A. euteiches* at the regional scale, but reveal higher than expected genotypic diversity within individual soil samples. Populations of *A. euteiches* were significantly differentiated at the soil sample, field and regional level. While most variation was seen at the regional scale, considerable variation was present at the level of the soil sample. The population structure appears to be patterned by regular selfing via oospores, a mixed reproductive system including both asexual and sexual reproduction, with occasional migration of novel genotypes or outcrossing.

15 Comparative genomics of the ATR13 locus

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Hyaloperonospora parasitica is an obligate biotrophic oomycete that causes downy mildew in *Arabidopsis thaliana*. ATR13, a known avirulence gene in *H. parasitica*, interacts with the RPP13 resistance gene product in *A. thaliana*, triggering a hypersensitive response from the host. A 200 kb BAC contig spanning the ATR13 locus was recently identified and sequenced. Another region of interest is BAC P1202, which contains 2 genes from the SSH (Suppression Subtraction Hybridisation) library of genes expressed during infection. A bioinformatics approach has been taken to annotate both of these regions by using gene predictor software and BLAST. Comparative genomic analysis with the genomes of *P. sojae* and *P. ramorum* was also performed to identify any syntenic loci. There is noticeable synteny between the ATR13 locus and scaffold 42 of *P. sojae* and scaffold 63 of *P. ramorum*. The syntenic regions showed conservation of several genes including an Acetyl CoA carboxylase, a GTPase and an Acyl transferase. There were also clear syntenic regions between the P1202 BAC and

scaffold 9 of *P. sojae* and scaffold 2 in *P. ramorum*. The main focus of future work will be to take advantage of the developing *H. parasitica* genome sequence to carry out bioinformatic analyses in relation to novel putative pathogenicity effectors. I will group and sub-categorise the genes based on protein domain structure and attempt to define new protein motifs conserved amongst such effectors. This will then be extended to comparative studies with related crop pathogens *P. sojae* and *P. ramorum*.

16 Genetic and molecular characterization of *Phytophthora infestans* in Tunisia reveals the presence of the A2 mating type

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Phytophthora infestans, the causal agent of late blight, is the most devastating disease of potato world-wide including Tunisia where this crop is present in fields almost all the year (late season, early season and season). Since *P. infestans* is a heterothallic species, with two mating types, A1 and A2 which are necessary for sexual reproduction, our first objective was to identify the mating type of the pathogen in the country. The presence of both types may generate new genotypes of the pathogen that could be more aggressive and lead to oospore formation for long term conservation of the pathogen. Isolates of *P. infestans* were collected from potato fields located in different regions during the 2004/2005 season. A molecular approach was used in order to identify the mating type. Genomic DNA isolated from infected tubers or pure culture was amplified with the specific primers W16-1 and W16-2 and subsequently digested with *Hae*III restriction enzyme. The patterns obtained could precisely differentiate between A2 and A1 mating type. Our study revealed the presence of the A2 mating type for the first time in Tunisia, which represented about 12.5 % of the population analysed. All the A2 isolates identified were collected from the same region, called Cap-bon, which is situated in the north east of Tunisia. On another hand, since we observed that the fungicide metalaxyl lost of its efficacy in some fields, we evaluated the resistance to the chemical. In vitro tests were carried out by applying three concentrations of the systemic fungicide (1ppm, 5ppm and 10ppm). The sensitivity of the isolates was evaluated by comparing the percentage of mycelial growth inhibition to the control. This test revealed the presence of resistant genotypes of *P. infestans* to metalaxyl in Tunisia. The origin of the A2 type and the resistance genotype to metalaxyl are currently under investigation.

17 Characterization of a novel protein kinase specifically expressed in *Phytophthora infestans* during mating

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Our laboratory has been studying the roles of several regulators of protein phosphorylation, including kinases and phosphatases, in developmental transitions in *Phytophthora infestans.* One such kinase, *PiMatk2*, is expressed specifically during mating. Phylogenetic analyses revealed that the kinase belongs to the calcium/calmodulin-dependent family of serine/threonine kinases. However, unlike members of the family characterized previously from non-stramenopile clades, the PiMatk2 protein contains catalytic and regulatory domains at its C and N-termini, respectively, which is opposite to the orientation found in other eukaryotic groups. However, similar domain orientations were detected in homologues from *P. sojae, P. ramorum,* and the apicomplexan *Plasmodium.* Preliminary studies have confirmed that PiMatk2 binds Ca^{2+} and the role of this cation in regulating kinase activity is being studied. Yeast two-hybrid has identified a number of possible interactors which are now being evaluated using *in vitro* pull-down assays. Functional analysis of the *PiMatk2* gene or its interacting proteins are likely to shed light on the role of this kinase in mating.

18 Population resource and genomic sequence of the vegetable pathogen *Phytophthora capsici*

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In 2005 the joint NSF-USDA Microbial Sequencing Program and the Joint Genome Institute Community Sequencing Program approved proposals to generate genome sequence for the vegetable pathogen *Phytophthora capsici*. *Phytophthora capsici* presents a uniquely tractable genetic system due to the ease with which it can be crossed, it's wide host range, and the high level of naturally occurring genetic variation. An overview of the project objectives will be presented including: (i) the novel sequencing platform that will be used for a large portion of the sequencing (ii) the rationale for the five isolates selected for sequencing and (iii) the expected outcomes and resources that will be made available via this project.

19 Identification of genes expressed in *Hyaloperonospora parasitica* race NoCo2 during infection process

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The oomycete *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*) is an obligate biotroph that infects *Arabidopsis thaliana* and Brassica species. One of the major problems working with obligate biotrophs is to identify genes expressed by the pathogen during infection. Different races of *H. parasitica* infect different ecotypes of Arabidopsis. For example, the race NoCo2 is virulent on Col-0 but not La-er. Only two avirulence genes in *H. parasitica* have been cloned-specific alleles of ATR1 and ATR13 (*Arabidopsis thaliana* recognised) cause a hypersensitive response in combination with specific alleles of RPP1 and RPP13 (Rehmany *et al*, 2005; Allen *et al*, 2005). Our aim is to identify genes expressed by *H. parasitica* race Noco2 during infection.

We are interested in avirulence genes including ATR5, which when expressed by Noco2 in an incompatible reaction, causes RPP5-dependent resistance. We have developed an effective method for selecting *H. parasitica* cDNA from total cDNA of infected leaves by selection with biotinylated *H. parasitica* genomic DNA. This protocol enriches for *H. parasitica* cDNA in infected leaves from 1% to about 80%. We have identified some candidate genes containing the RXLR motif and are in the process of testing the functions of these candidate genes.

20 Cloning of three late blight *R* genes from a major resistance gene cluster on Chromosome 4 of potato

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The oomycete *Phytophthora infestans*, the causal agent of late blight remains the most important pathogen in major potato producing regions of the world. Since its start, around the turn of the twentieth century, breeding for resistance to late blight in potato has hardly been successful in an agronomic sense. Introgressed *R* genes from the wild *Solanum* species *S. demissum* were rapidly overcome and breeding for field resistance, also known as partial or quantitative resistance, by using race-nonspecific sources of resistance, has turned out to be difficult. Disease management is therefore currently based on the application of fungicides. However, with the onset of the genomics era, breeders are reconsidering their breeding goals. The reservoir of unknown R genes present in primitive varieties and in wild Solanum species is presumed to be large. Molecular isolation of novel *R* genes from the *Solanum* gene pool and introduction of a combination of these genes into existing varieties through genetic modification (GM) is currently considered the fastest means of introducing potentially durable resistance into modern potato varieties. Here we report on the cloning of three novel late blight *R* genes: *Rpi-abpt, Rpi-blb3* and *R2-like*. Using several different approaches, high-resolution genetic maps of the three R loci have been generated. Although marker order and allelic conservation was observed, varying race-specificities and origin

differences indicate that the three *R* genes are distinct alleles of a single *R* gene cluster on Chromosome 4 (Park *et al.,* 2005, MPMI **18**, 722-729.

21 The genome of *Phytophthora cinnamomi* an overview

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The genus *Phytophthora* belongs to the oomycetes in the eukaryotic stramenopile lineage and is comprised of over 65 species that are all destructive plant pathogens on a wide range of dicotyledons. *Phytophthora cinnamomi* is a destructive and widespread soil-borne pathogen that infects woody plant hosts and it has been estimated to directly affect over 2000 of the 9000 native plant species in the south west of Western Australia. In the north east of Portugal and in the Spanish adjoin region *P. cinnamomi* has been reported to affect chestnut-tree (*Castanea sativa*) and cherry-tree (*Prunus avium*).

While many data exist about the genome *Phytophthora infestans* and *Phytophthora sojae* those related to *P. cinnamomi* are scanty. By digestion of genomic DNA from strain *Phytophthora cinnamomi* PR120 we have constructed a DNA library with an insert size average of 6-12 kb in *Escherichia coli* XL1 Blue. We have sequenced 8 clones comprising 40,000-50.000 bp. We have found 15 putative protein-coding sequences and three tRNA. Database similarity analysis reveals the presence of genes conserved mainly in the other oomycetes.

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22 Isolation and characterization of the endo-1,3-beta-glucanase gene in *Phytophthora cinnamomi* associate to the ink disease of *Castanea sativa* Mill.

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The genus *Phytophthora* belongs to the oomycetes in the eukaryotic stramenopile lineage and is comprised of over 65 species that are all destructive plant pathogens on a wide range of dicotyledons. *Phytophthora cinnamomi* is a destructive and widespread soil-borne pathogen that infects woody plant hosts. It requires moist soil conditions and warm temperatures to be active, but damage caused by the disease most often occurs in summer when plants are drought stressed. *P. cinnamomi* is one among the most destructive species of *Phytophthora*, and has been associated with the decline of

several forestry, ornamental, and fruit industries as well as over 900 woody perennial plant species.

Endo-1,3-beta-glucanase (EC 3.2.1.39) catalyzes the hydrolysis of 1,3-beta-D-glucosidic linkages in callose, lamanarin, and various carbohydrates found in the cell wall of plants and fungi. 1,3-beta-glucanases are included among the family of pathogenesis-related proteins because many forms are rapidly induced during fungal invasion. It is generally thought that glucanases play a role in plant defense by digesting wall components of the fungal pathogen [2].

We obtained a fragment with 1231pb of the endo-1,3-b-glucanase gene by amplification using conserved primers and the full length gene sequence was obtained by flanking this known sequence with asymmetric PCR. This fragment was obtained from genomic DNA of *P. cinnamomi* isolated for us in Trás-os-Montes (Portugal) and associate to the ink disease of *Castanea sativa* Mill.

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23 Mechanisms of Virulence and Avirulence in the Biotrophic Interaction Between Potato and the Late Blight Pathogen *Phytophthora infestans*

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Despite the historical and economic importance of the potato and tomato late blight pathogen *Phytophthora infestans*, mechanisms required for causing disease are not very well understood. Recently an avirulence gene from *P. infestans*, *Avr3a* (Armstrong *et al*., 2005, *PNAS* **102**:7766-7771), and a protein motif (RXLR) (Rehmany *et al*, 2005, *Plant Cell* **17**:1839-1850), were identified; the RXLR motif is present in more than 50 predicted secreted proteins from *P. infestans*, including AVR3a. One of the objectives of my PhD is to analyse gene expression profiles and function of *P. infestans* sequences encoding proteins with the RXLR motif (RXLR sequences). Their expression was determined throughout infection using real time reverse transcriptase PCR (RT-RT-PCR). Six distinct expression profiles were observed, from pre-infection to necrotrophic stages of infection. Expression of *Avr3a*-like orthologues was also investigated and revealed isolate-dependent differences.

24 Evolutionary insights from the expansion of the ABC transporter superfamily in the Oomycete genome.

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The release of a draft sequence of the *Phytophthora sojae* and *Phytophthora ramorum* genomes has provided us with the first opportunity to examine the diversity of the ABC transporter family in two important plant pathogens of the Kingdom Stramenopiles. The oomycete genomes of *P sojae* and *P ramorum* contain 134 and 135 genes respectfully, slightly more than that reported for the model plant *A. thaliana* (131). The majority of *P. sojae* and *P. ramorum* sequences from most families could be grouped into orthologous pairs. Thus it seems unlikely that the increased host range of *P ramorum* can be attributed to either the increased number or diversity of ABC transporters. Import type ABC transporters have previously thought to be restricted to prokaryotic genomes, but the oomycetes also contain two sequences with homology to bacterial sequences involved with iron and molybdenum uptake. Homologues to these sequences are also present in the diatom genome. Sheps et al (2004) noted that a phylogenetic analysis of membrane transporters from *C elegans*, human, *S. cerevisiae*, and fly showed only low levels of orthology within gene families. In our studies we have noted that phylogenetic comparisions of ABC subfamilies from the oomycetes, with plants and human sequences show oomycete transporters falling in separate resolved nodes. Moreover genetic variability within oomycete subfamilies is greater than that seen in other eukaryotic kingdoms. Thus the oomycetes appear to have taken maximum advantage of the architecture of these proteins to catalyze the transport of compounds across membranes. Functional analysis of ABC transporters must therefore rely on characterization of individual genes rather than comparative studies with transporters from other kingdoms

25 Development of EST resources for the soilborne pathogen *Phytophthora* parasitica

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Phytophthora parasitica (syn. = *nicotianae*) is a soilborne oomycete pathogen of great importance worldwide. It exhibits an overall broad host range, but some isolates are specialized towards a single host. Characterizing the pathogenicity determinants of *P. parasitica* will help to identify general virulence factors as well as mechanisms involved in host specificity. Initial research into understanding the molecular basis for development and pathogenicity in *P. parasitica* has centered on expressed sequence tag (EST) sequencing of a cDNA library synthesized from *in vitro* vegetative growth. In addition, we developed a *P. parasitica*/tomato pathosystem to characterize genes expressed during late stages of infection. More than 6,500 ESTs were generated from these two libraries, leading to the identification of 3,405 unisequences. Comparison

between the two EST data sets indicated striking differences in the abundance of many unigenes, revealing global changes in gene expression during plant infection. Preliminary results confirmed these observations. Large-scale studies of expression patterns of those genes are now undertaken and functional analyses are actually developed through genetic transformation and complementation strategies.

26 The *Phytophthora sojae* necrosis inducing protein, PsojNIP, is cleaved by a soybean protease in infected tissues

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Phytophthora sojae is a hemi-biotrophic pathogen that causes stem and root rot of soybean. Transcripts encoding the 26 kDa necrosis inducing protein, PsojNIP, are highly expressed during the necrotrophic phase of disease development, possibly to accelerate host cell death during this stage of infection. To measure levels of PsojNIP protein present in infection sites, western blots were performed using PsojNIP-specific antibodies. These results show that the accumulation of PsojNIP occurs late during infection, but that most of the protein is proteolytically cleaved into smaller fragments. Experiments indicate that this proteolysis is not an artefact of the extraction process. Such proteolytic processing is not observed with PsojNIP extracted from axenically grown *P. sojae* tissues, such as mycelia, suggesting involvement of a soybean protease. Incubation of purified, recombinant PsojNIP with cell-free extracts from uninfected soybean tissues results in an identical proteolytic processing of the recombinant protein, as observed with the native protein from infection sites.This PsojNIP-specific proteolytic activity from soybean is heat labile, inhibited by EDTA, and may be purified by gel filtration and ion exchange chromatography. Thus, our results suggest that host proteases may represent a counter-defence strategy to pathogen-secreted necrosis inducing proteins.

27 The mechanosensitive channel MscL in *Phytophthora infestans*

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Mechanosensitive (MS) channels have been thoroughly investigated in bacteria and are known to mediate solute release from the cytoplasm during hypoosmotic conditions. Thus they play a major role in osmoregulation protecting the bacterial cell from lysis when challenged with a hypoosmotic shock. During hyperosmotic conditions compatible solutes such as glycine, betaine, K-glutamate, and proline are accumulated in bacteria, plants, and nematodes. Proline was also found to be important for osmoregulation in sporangia of *P. nicotianae* during zoospore release.

Employing bioinformatics approaches, we found evidence for the presence of a homologue of the mechanosensitive channel of large conductance, MscL, in the genome of *P. infestans*. Analysis of EST data and amplification from cDNA suggests that possibly two homologues of this channel are present in *P. infestans*.

The existence of MS channels in an oomycete is interesting since the mechanism of osmoregulation in these organisms is poorly understood. To investigate the role of the potential channel in *P. infestans*, fragments containing the gene of interest have been cloned. Upon obtaining the full open reading frame, its function will be characterised physiologically as well as electrophysiologically and its localisation and expression profile investigated using immuno-co-localisation studies and quantitative real-time PCR. Furthermore we intend to generate silenced mutants to functionally characterise the MS genes. Here we present our latest findings.

28 PcF PROTEIN FROM *P. CACTORUM* ELICITS DIFFERENTIAL EXPRESSION OF PR PROTEIN FAMILIES IN TOMATO

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The signaling mechanisms underlying the outcome of plant-pathogen interactions involve in most cases both pathogen-secreted proteins and plant-derived membrane receptors: their interaction is responsible for both signal perception and transduction inside the host plant cell (1). The protein PcF [*Phytophthora cactorum-Fragaria*], a novel factor secreted by *Phytophthora cactorum* (*Phc*), has been recently isolated and characterized, including cloning and sequencing of the full-length cDNA (GenBank Acc. AF354650) (2). Mature PcF is a 52-amino acid protein (pI = 4.4, MW 5,622), comprising a 4-hydroxyproline residue and 6 cysteines bridged intramolecularly, which triggers plant reactivity on both strawberry and tomato, and activation on tomato of the marker-enzyme Phenylalanine Ammonia Lyase (PAL) (2,3). Even though the biochemical mechanism remains to be investigated, such a biological activity suggests the involvement of PcF in the *Phc*-mediated plant pathogenesis.

In this study we focused on the PcF-tomato interaction, as a model system, to assay through Real-Time PCR the transcriptional induction of some plant-defense related genes, known to be upregulated in response to different biotic and abiotic stresses (4). A recombinant homologue of the PcF protein was obtained after extracellular overexpression in *P. pastoris* (3). To test its bioactivity (2), sets of four root-excised tomato seedlings were incubated in duplicate eppendorf tubes containing 10 mg pure protein in 200 ml distilled water; duplicate sets of controls in distilled water were run in parallel. At time-intervals (3 h, 6 h, 30 h), both treated and control samples were harvested, frozen in liquid nitrogen, and stored at -80 ℃ for subsequent analysis. Both treated and control specimens were utilized for PAL activity evaluation on crude extracts (3), and for total RNA extraction. After reverse transcription of 5 mg RNA from each sample, a quantitative mRNA evaluation was performed through Real-Time PCR. The tomato nucleotide sequences for PAL and PR proteins, available in data bases, were used for primers design.

Previous experiments have established that both native and recombinant PcF proteins trigger morphological symptoms on leaf and activate marker-enzyme PAL on

tomato seedlings (3). Here we present data showing that this activation involves the transcriptional induction of the corresponding PAL gene. Both PAL activity and mRNA expression peaked at 3-6 h after treatment. However, the PAL activity level remained high after 30 h, while the PAL gene is only transiently expressed. The observed PcF-mediated PAL induction in our whole plant system is consistent with the fact that (i) PcF is perceived by tomato as an elicitor, and (ii) the induction of gene expression occurs, after interaction with a putative receptor, via a signal transduction pathway. Furthermore, the results show that PcF treatment induces the expression of several members of PR families, namely PR3, PR4, PR5, PR7, and PR9, with similar time course but different levels of relative expression. This result appears to be consistent with the fact that in our whole plant system, PcF is perceived by tomato seedlings as an elicitor, possibly after interaction with a putative plant receptor. On the other hand, genes corresponding to PR1, PR2 and PR6 families are not induced, showing that the effect caused by PcF is at least in part selective. This lead us to further investigate on other gene expression patterns known to be related to plant pathogenesis, like Hypersensitive Response (HR), oxidative burst, Systemic Acquired Resistance (SAR), Programmed Cell Death (PCD), currently underway in our laboratory.

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29 Towards cisgenic late blight resistant potato varieties; cloning and characterization of relevant *R* genes from wild *Solanum* species

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The necessity to develop potato varieties that possess durable resistance against the oomycete pathogen *Phytophthora infestans* is increasing as more virulent and pesticide resistant strains of the pathogen are rapidly emerging. Isolation of resistance (*R*) genes from wild *Solanum* species and subsequent introduction of a combination of these genes as cisgenes into existing potato varieties using marker free plant transformation technologies is currently the fastest means of exploiting potentially durable resistance present in the *Solanum* gene pool. This approach opens the way to efficient *R* gene pyramiding or polyculture strategies. We have identified high levels of resistance in several wild *Solanum* species. Based on resistance assays with a diverse set of *P. infestans* isolates and apparent 1:1 segregation of resistance in initial intraspecific F1 mapping populations (n=50) we are currently pursuing map-based cloning and candidate gene approaches to isolate 10 novel late blight *R* genes from a diverse set of wild *Solanum* species. Marker saturation of the different *R* loci is being achieved through BSA and NBS profiling. Markers that cosegregate with resistance in large intra- or interspecific F1 mapping populations (n=2500) will be used to screen BAC libraries that are being generated for the relevant parental genotypes. Candidate RGA's derived from BAC clones spanning the individual *R* loci will be subjected to complementation analyses in a susceptible potato variety. Ultimately, the cloned *R* genes will be used to develop cisgenic late blight resistant potato varieties.

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

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

31 Differential screening identified genes differentially expressed between low virulence and high virulence *Phytophthora sojae* near-isogenic lines

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In order to explore the molecular mechanisms involved in the virulence variation of *Phytophthora sojae*, a low virulence isolate Pmg2 was inoculated on a resistant soybean cultivar successively. After 14 times successively inoculation, a high virulence progeny termed Pmg2-vir with less oospores production was obtained. DNA fingerprints analysis indicated there was no big scale change on DNA level between Pmg2 and Pmg2-vir. Suppression subtractive hybridization (SSH) approach was developed to

investigate the differences of genes expression between Pmg2 and Pmg2-vir in the early stage of soybean infection. Among a total of 323 selected clones, 74 putative unigenes were obtained with characters of high expression in Pmg2-vir. Bioinformatics analysis revealed the encoding proteins involved in energy production, protein biosynthesis, signalling, cell-wall biogenesis and transcription regulation et al.. Macro-array screening was verified by Virtual Northern blotting of six selected clones. Four clones were selected for expression analysis of temporal pattern based on results from the dot-blot screens and virtual Northern blots using RT-PCR measure. Possible genetic mechanisms involved in these phenomena are discussed.

32 A pathogen-secreted cysteine protease inhibitor is implicated in a cascade of protease-protease inhibitor interactions between tomato and *Phytophthora infestans*

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Plant proteolytic machinery plays important roles in plant defense. Plant pathogens have co-evolved protease inhibitors for counterdefense. Previously, we described a diverse family of extracellular Kazal-like serine protease inhibitors (EPI1-EPI14) from the oomycete plant pathogen *Phytophthora infestans* [1-3]. Among them, EPI1 and EPI10 inhibit and interact with the pathogenesis-related P69B subtilisin-like serine protease of the host plant tomato suggesting that protease inhibitors represent an important virulence strategy of *P. infestans*. Here, we describe a new inhibitor protein, EPIC2, that belongs to a family of putative extracellular protease inhibitors with cystatin-like domains from *P. infestans*. The *epiC2* gene is relatively fast evolving within *P. infestans* populations and is upregulated during infection of tomato, suggesting a role during *P. infestans*-host interactions. *In vitro* experiments showed that EPIC2 was degraded by P69B subtilase. However, the Kazal-like inhibitor EPI1 protected EPIC2 from degradation by P69B. Coimmunoprecipitation experiments revealed that EPIC2 interacted with a novel papain-like extracellular cysteine protease named PIP1. PIP1 is a pathogenesis-related protein closely related to RCR3, an apoplastic cysteine protease required for tomato *Cf-2*-dependent defense response against *Cladosporium fulvm* expressing AVR2. Protease activity profiling [4] using a synthetic cysteine protease inhibitor E64 and its biotinylated analog DCG-04 showed that purified PIP1 bound specifically to both inhibitors in a competitive manner suggesting PIP1 is a cysteine protease. Competitive binding assays using EPIC2 and DCG-04 revealed that EPIC2 blocked the binding of DCG-04 to purified PIP1, providing biochemical evidence that EPIC2 is a cysteine protease inhibitor. Together, our results suggest that EPIC2 inhibition of plant cysteine proteases could be part of complex cascades of host protease inhibition initiated by EPI1 in the plant apoplast during infection. We propose that a likely defense mechanism of plant protease P69B is to degrade pathogen-derived proteins including EPIC2. EPI1 protects EPIC2, which in turn inhibits proteases such as PIP1 to protect degradation of *P. infestans* virulence proteins required for suppression of plant defense mechanisms. Future experiments will aim at identifying the pathogen targets of PIP1 and how PIP1 contributes to plant defense.

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33 The use of microsatellite markers to examine European *P. infestans* populations via the EUCABLIGHT database.

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Despite intensive study of *P. infestans* populations many questions remain. Part of the challenge has been the lack of objective data for powerful population genetic analysis and sets of population data collated in a common format. To resolve the first issue considerable effort has been devoted to the development of a set of microsatellite markers suited for high throughput analysis of populations at local and international scales. The second issue has been addressed via an EU-funded Concerted Action project 'EUCABLIGHT' with the task of examining the population structure of European *P. infestans* populations. Standard protocols ranging from sampling to genotyping have been designed, tested and published and the exploitation of objective micro-satellite data is playing a significant part in the project. A database containing information on *P. infestans* collections in many European countries (www.eucablight.org) has been constructed and contains data on over 13,600 isolates from 20 European countries. Data is entered using a PC-based custom entry tool and is processed and presented using a series of web interfaces that allow key parameters of the population to be examined on a range of spatial and temporal scales. In this paper an update on the utility of SSRs, their use in the EUCABLIGHT project and future plans will be presented.

34 Towards map-based cloning of *ATR2* and/or *ATR5* gene(s) from *H. parasitica*

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Arabidopsis-Hyaloperonospora pathosystem has been established as a laboratory model in the 1990s. Although downy mildew of Arabidopsis is not a destructive disease of an economically important crop plant, an enormous research effort has been devoted to this system. Since then, more than twenty major genes for resistance (*RPP* genes) have been identified and some of them have been cloned and characterized. In addition, a great deal of progress has been made in identifying some of the downstream

signaling components. A small progress on the pathogen side has also been made where two avirulent genes have been cloned. However, efforts to sequence the entire genome of the pathogen are underway. We have been using this model to understand compatibility and incompatibility at the molecular and cellular level. Isolation of the *ATR* gene is a prerequisite to reveal how defense response is activated and how incompatibility can arise from the innate immune system. Genetic analysis of the cross between Cala2 and Noks1 isolates of *H. parasitica* indicated the segregation of *ATR2* from Cala2 and *ATR5* from Noks1 as a single dominant gene. We have taken a map-based approach to clone these genes using bulk segregant analysis with AFLP method. Genetic evidence indicates that Emoy2 (the isolate used for the reference genome of *H. parasitica*) expresses an effector that that is recognized by *RPP5*. We will use bulks from the original Emoy2 X Maks9 cross to determine whether this effector is likely to be encoded by an Emoy2 allele of *ATR5*.

35 Profiling of R-AVR interactions in wild *Solanum* using RXLR effectors of *Phytophthora infestans*

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Late blight caused by the oomycete *Phytophthora infestans* is the most destructive disease in potato cultivation worldwide. Understanding of the molecular recognition mechanism ofresistance (R) proteins of potato and effectors of *P. infestans* is essential to provide a broad durable resistance in *Solanum*. Previous studies revealed that resistance to *P. infestans* is always associated with the hypersensitive response (HR). This suggests that resistant plants recognize elicitors of *P. infestans*. Favourable candidates of AVR proteins (elicitors) are the extracellular proteins (Pex) containing a RXLR motif. The RXLR motif is conserved in diverse oomycete genera among dissimilar oomycete proteins, some of which have been characterized as avirulence genes (Avr3a). RXLR effectors are thought to be translocated inside the plant cell where they can interact with the intracellular NBS-LRR R proteins, the most common class of R proteins effective to oomycetes. In addition to the known isolated R genes, novel R genes were identified in wild *Solanum* germplasm. In this study, we test these plants responses with a transient expression system based on *Agrobacterium tumefaciens* and potato virus X (PVX) expressing cDNAs of RXLR effectors of *P. infestans*. Finally, the correlation between effector recognition and specific resistance in segregating populations is being examined, and will reveal functional evidence for R-AVR interaction in *Solanum* with the aim to clone the corresponding *R* genes.

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