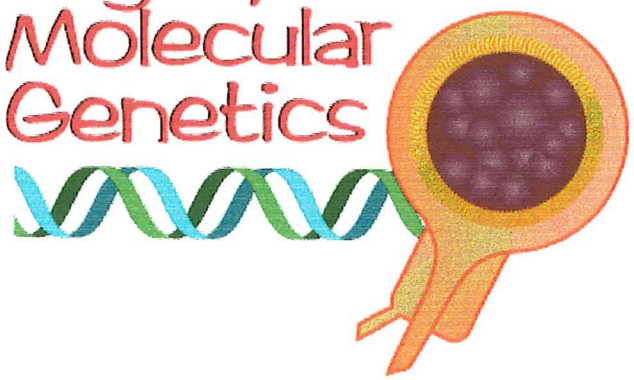
Duncan

Advances in Phytophthora Molecular Genetics



International One Day Symposium

15th August 1998

Heriot-Watt University, Edinburgh

Sponsored by the British Society for Plant Pathology

BSPP

Session 1: The Phytophthora *Genome*

Chair: Francine Govers (Wageningen Agricultural University)

9:00 **Opening Remarks (Francine Govers)**

9:05 **Theo van der Lee (Wageningen Agricultural University)**
AFLP mapping in *P. infestans*: a high density map of an *avr* gene cluster

9:30 **Steve Whisson (The University of Queensland, Brisbane)**
Genetics and cloning of avirulence genes from *P. sojae*

9:55 **Howard Judelson (University of California, Riverside)**
Classical and molecular genetics of mating in *Phytophthora*

10:20 Coffee and Poster viewing

10:55

10:45 **Brett Tyler (University of California, Davis)**
Construction and DNA sequencing of a BAC contig spanning the *Phytophthora sojae* genome

11:15

11:15 **Bruno Sobral (National Center for Genome Resources, New Mexico)**
An information system to support a distributed international *Phytophthora* genome project

11:30

11:40 **Paul Birch (SCRI, Dundee)**
Determining genes involved in the incompatible interaction between *Phytophthora infestans* and potato using a targeted EST approach

12:05 **Discussion "The way ahead in *Phytophthora* genomics" led by Francine Govers**

12:30 On-site buffet lunch followed by coffee and poster viewing

Session 2: Plant-Phytophthora interactions

Chair: Howard Judelson (University of California, Riverside)

2:00 **Pieter van West (University of Aberdeen)**
Antisense and sense mediated gene silencing as a tool to suppress expression of the *inf1* gene in *P. infestans*

2:25 **Franck Panabieres (INRA, Antibes)**
Repetitive DNA sequences from *P. cryptogea:* molecular tools for resolving pathotype diversity and their possible involvement in gene expression

2:50 **Sophien Kamoun (Ohio State University)**
Resistance of *Nicotiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1

3:15 **Discussion "The way ahead in Plant-*Phytophthora* interactions" led by Howard Judelson**

3:30 Tea and Poster viewing

Session 3: Evolutionary and Ecological Genetics
Chair: Jim Duncan (SCRI, Dundee)

4:00 **David Cooke (SCRI, Dundee)**
Whither *Phytophthora*? A revised classification of *Phytophthora* and other oomycetes on the basis of ITS analysis.

4:25 **André Drenth (The University of Queensland, Brisbane)**
Evolution of *Phytophthora* species

4:50 **Steve Goodwin (Purdue University, W. Lafayette)**
Probable sympatric speciation in *Phytophthora* mediated by changes in host specificity

5:15 **Nick Pipe (University of Wales, Bangor)**
New tools for an old problem: advances in the study of the UK *P. infestans* population structure

5:40 **Clive Brasier (Forest Research Agency, Alice Holt)**
Speciation processes and species hybrids

6:05 **Discussion "The way ahead in Evolutionary and Ecological Genetics" led by Jim Duncan**

6:30 Close

6:30 **Open meeting: Ways forward for the '*Phytophthora Genome Initiative*' chaired by Bruno Sobral**

8:00 Evening Meal at Scholars' Restaurant in the Conference Centre

Session 1

The Phytophthora *Genome*

A HIGH DENSITY MAP OF AN AVR-GENE CLUSTER IN *PHYTOPHTHORA INFESTANS*.

Theo Van Der Lee, Antonino Testa[1] and Francine Govers

Laboratory of Phytopathology, Wageningen Agricultural University and Graduate School Experimental Plant Sciences, Binnenhaven 9, 6709 PD Wageningen, The Netherlands. [1] permanent address: Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, University of Napels, via Università 00, 80055 Portici, Italy.

Background and objectives

The heterothallic oomycetous plant pathogen *Phytophthora infestans* is the causal agent of potato late blight. Vertical resistance to late blight is based on a gene-for-gene interaction between pathogen and host. We expect this interaction to be the result of active defence responses of the host initiated upon recognition of a race-specific elicitor produced by the pathogen. We want to characterize the main fungal components of this race-specific incompatibility and set out to clone the avirulence (*avr*) genes of the pathogen.

Material and methods

We choose a map based cloning strategy, using the powerful AFLP DNA fingerprint technology [1] to deal with the relatively large genome size (250 Mb) of *P. infestans*. AFLP DNA fingerprints were generated from 83 F1 progeny from a cross between two Dutch *P. infestans* field isolates (NL80029 x NL88133). Part of this progeny was also tested for virulence on a differential set of potato lines carrying different major R-genes.

Results and conclusions

In the F1 progeny there is segregation for virulence on potato plants carrying the R1, R2, R3, R4, R10 and R11 resistance gene. We demonstrated that in this cross avirulence on potato plants, carrying the R3, R4, R10 and R11 resistance gene, is a dominant trait. *Avr3, Avr10* and A*vr11* appeared to be closely linked and we positioned this *avr* gene cluster on linkage group VIII of the AFLP linkage map previously generated with the same progeny [2]. We pooled DNA from avirulent and virulent progeny and performed a Bulked Segregant Analysis (BSA) to identify AFLP markers linked to the *Avr3, Avr10, Avr11* gene cluster. We tested 25.000 AFLP fragments and identified 18 linked markers. Twelve of these markers co-segregate with the *Avr* gene cluster.

1. Vos P, Hogers R, Bleeker M, Reijans M, van der Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M, 1995. Nucleic Acids Research 23, pp.4407-4414.
2. Van der Lee T, De Witte IJ, Drenth A, Alfonso C, Govers F, 1997 Fungal Genetics and Biology 21, pp.278-291.

GENETICS AND CLONING OF AVIRULENCE GENES FROM *PHYTOPHTHORA SOJAE*

SC Whisson, KJ May, A Drenth, DJ Maclean and JAG Irwin

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Phytophthora sojae causes a destructive root and stem rot of soybean. Thirteen dominant resistance genes (*Rps* genes) exist in soybean. Detection of low levels of outcrossing among isolates of the homothallic *P. sojae* using molecular markers has allowed the generation of F_1 hybrids and subsequent F_2 populations, and the study of the genetic basis of avirulence. Construction of four genetic crosses (race 1/7, race 7/25, race 7/16, race 17/25) has enabled the genetics of avirulence to be determined and confirmed for eleven avirulence-*Rps* gene interactions. Avirulence was found to be a single dominant allele for *Avr*1b, 1d, 1k, 3b, 4, and 6. Segregation of *Avr*3c best fit a 9:7 ratio with avirulence dominant. Virulence towards *Rps*1c was determined to be dominant in all crosses in which this allele segregated. *Avr*1a, 3a, and 5 were determined to be dominant (avirulence) in some crosses but recessive (virulent) in others. Avirulence genes *Avr*1b and 1k, and *Avr*4 and 6 cosegregated absolutely in all crosses.

The segregation data for all avirulence genes, together with segregation data for molecular markers in all crosses was used to construct a single genetic linkage map comprising 264 molecular markers and ten avirulence genes. The closest linkage of a marker to an avirulence gene was 2 cM between a RAPD marker and cosegregating avirulence genes *Avr*4 and *Avr*6. This RAPD marker was used as a starting point for a chromosome walk towards cloning these genes.

Three overlapping cosmids which span the genomic region containing the *Avr*4/6 locus were identified. The chromosome walk was determined to encompass 67.3 kb representing 10.1 cM. Northern analysis of *P. sojae* RNA expressed under a range of nutritional regimes revealed low levels of expression for at least three genes in the region represented by the chromosome walk. Two genes were expressed constitutively and one was expressed in the absence of an organic nitrogen source.

Screening of a cDNA library with the three cosmids from the chromosome walk identified 35 cDNA clones which represent nine sequence groups. Of these nine groups, six are present on the three cosmid contig; the other three groups presumably arising from cross hybridisation with sequences within the cloned DNA of the chromosome walk. Mutations between the parental strains in the coding sequences causing amino acid substitutions have been identified for four of the six cloned genes. PCR primers specific to these mutations have been synthesised to accurately locate the cloned genes in the region of *Avr*4/6. This mapping process has narrowed the number of candidate genes for *Avr*4/6 to two genes. Transformation experiments to determine if any of the cloned genes function as *Avr*4 or *Avr*6 are in progress.

CLASSICAL AND MOLECULAR GENETICS OF MATING IN *PHYTOPHTHORA*

Howard S. Judelson, Thomas A. Randall, and Anna-Liisa Fabritius.

Department of Plant Pathology, University of California, Riverside, California 92521 USA

A genetic model was developed to indicate how mating type is inherited in heterothallic species of *Phytophthora.* Studies of *P. infestans* and *P. parasitica,* aided by DNA markers linked to the mating-type locus, indicated that the A1 type is determined by heterozygosity *(A/a)* and the A2 by homozygosity *(a/a)* at that locus. In most isolates of *P. infestans,* however, this simple model is complicated by genetic abnormalities that include distorted segregation and nonrandom assortment of alleles. Heteromorphic and hypervariable regions exist near the mating type locus; these may, in theory, explain why such non-Mendelian behaviours are observed. Chromosomal variability may also explain our observation that the frequencies of recombination near the mating type locus vary substantially in different strains. Evidence for translocations and duplications of the mating type locus were also identified. A contig spanning the chromosomal interval that contains the mating type locus is being assembled using libraries of genomic DNA. For such studies, an ordered bacterial artificial chromosome library (ca. 15,000 clones, 5-genome equivalents) and cosmid libraries are being used. Both types of libraries were constructed in vectors that contain selectable markers for *P. infestans* transformation; the ability to transform both BACs and cosmids have been demonstrated. To locate the mating type locus within the contig, fine-structure genetic mapping, complementation assays, and searches for expressed sequences are being pursued; candidate genes are now being examined. One gene shows a mating type-specific pattern of expression and resides in a region of chromosomal heteromorphism. Studies of the mating type locus are being integrated with studies of differential gene expression during mating. Mating-specific, vegetative-specific, and mating type-specific genes from *P. infestans* have been identified through PCR-based subtraction cloning. Several genes appear to be conserved in other members of the genus, including homothallic species. These studies should lead to an understanding of the basis and evolution of the mating systems present within *Phytophthora.*

CONSTRUCTION AND DNA SEQUENCING OF A BAC CONTIG SPANNING THE *PHYTOPHTHORA SOJAE* GENOME.

F Arredondo[1], WX Shan[1], A Chan[1], P Hraber[2], M Waugh[2], B Sobral[2] and BM Tyler[1].

[1]Department of Plant Pathology, University of California, Davis, CA95616, USA and
[2]National Center for Genome Resources, Santa Fe, NM87505, USA

The more than 40 species of the oomycete *Phytophthora* cause serious diseases of a huge range of crop and ornamental plants. We are characterizing genes that control recognition, host specificity and pathogenicity in the soybean pathogen *Phytophthora sojae*. To facilitate isolation of such genes from *P. sojae* by map-based cloning, we are constructing a BAC contig of the entire genome of this organism, using a novel hybridization fingerprinting strategy. We have constructed a library of 7680 BACs of average size 55kb, spanning the 62 Mb genome 7 times. We are hybridizing the BACs with unique mixtures of random probes, most of them repetitive. We will use the subset of probes hybridizing to each BAC to identify overlapping BACs. Computer software has been developed to collect, simulate and analyze the data. At present we have probed the library with 49 of the mixtures. Each mixture hybridized to around 300 - 500 BACs resulting in 20,200 hybridization hits to BACs in the library. 19% of the BACs so far have received the minimum number of hits needed to establish statistically significant overlaps (5 each). Of these BACs, 34% have been placed into contigs, which at present average 8 BACs per contig. These results suggest about 180 probe mixtures will be sufficient to enable us to assemble the BACs into less than 100 contigs spanning >95% of the genome. We have tested the authenticity of three of the contigs by *Hin*dIII digestion. The contigs contained 30, 13 and 7 BACs respectively and spanned 640 kb, 250 kb and 160 kb respectively. All three contigs proved to be bona fide contigs, validating our strategy.

With the long term goal of sequencing the entire genome of *P. sojae* and selected sequences from other *Phytophthora* species, such as *P. infestans* we have established the *Phytophthora* Genome Initiative (PGI) in collaboration with *P. infestans* researchers. We have begun preliminary sequencing of a 200 kb BAC contig spanning two avirulence genes from *P. sojae*. Sequencing of the first 60 kb BAC is nearly complete, and software has been developed for automatic processing, annotation and publishing of the sequence data via the web. In the region sequenced so far the gene density is extremely high. 16 firm matches to the sequence databases have been obtained, even though only 50% of all *P. sojae* genes are expected to show sufficient conservation to obtain matches. Several pairs of genes are less than 300 bp apart. These observations prompt the speculation that in *P. sojae*, functional genes are located in high-density gene islands dispersed among clusters of repetitive sequences, which are known to constitute around 50% of the genome.

AN INFORMATION SYSTEM TO SUPPORT A DISTRIBUTED INTERNATIONAL *PHYTOPHTHORA* GENOME PROJECT

B Sobral

National Centre for Genome Resources, New Mexico.

The *Phytophthora* Genome Initiative (PGI) is a collaboration among several molecular biology laboratories and the National Center for Genome Resources (NCGR). In the early, pilot stages of this initiative, two labs (Brett Tyler's lab in the Plant Pathology Department at UC Davis and Francine Govers' lab in the Phytopathology Department at Wageningen Agricultural University) prepare DNA clones for sequencing. A third laboratory (Center for Agricultural Biotechnology at the University of Maryland Biotechnology Institute, under the supervision of Don Nuss) runs the sequencing reactions and transfers resulting data to NCGR's ftp site.

The main role of NCGR in this effort is to provide bioinformatics support. Broadly defined, bioinformatics support includes vector screening, homology annotation, data base support, interim reports on the status of preliminary data, and, ultimately, contig assembly, gene prediction, support for graphical viewing of genetic maps, and integration of genetic and physical maps. A second important role for NCGR in this project is to provide a nexus for the geographically distributed collaborators. In other words, we meet some of the organizational needs of the group by providing a central web site to facilitate communication among group members, as well as provide centralized access to the sequence data. Future goals include addition of host species genomic information, in particular for soybeans, potatoes, and tomatoes.

The software development group at NCGR has developed an analysis "pipeline" in which raw data are processed with minimal human oversight. Inputs to the pipeline are raw sequence data in the form of sequence text files and chromatogram trace files from an ABI Auto-Sequencer. Products (outputs) of the pipeline are a data base which contains sequence and homology data and, ultimately, an assembled and annotated representation of the *Phytophthora sojae* genome. Analysis of the *P. sojae* genome will be used to investigate plant pathogen interactions via genetic and physical maps of soybeans and potatoes, ESTs from soybeans and potatoes, and integration and comparisons across map types and species. In general, the output from one step of the pipeline serves as input to the next step.

DETERMINING GENES INVOLVED IN THE INCOMPATIBLE INTERACTION BETWEEN *PHYTOPHTHORA INFESTANS* AND POTATO USING A TARGETED EST APPROACH

Paul RJ Birch, Anna O Avrova, Rachel Toth, Gary D Lyon and James M Duncan

Department of Fungal and Bacterial Plant Pathology, Scottish Crop Research Inst., Invergowrie, Dundee DD2 5DA, UK

This talk will describe the use of a recently developed PCR-based technique , suppression subtractive hybridisation (SSH), to generate a cDNA library enriched for sequences induced specifically in a *Phytophthora infestans*-resistant potato cultivar undergoing the hypersensitive response (HR). Of 100 partial cDNAs sequenced from this library and submitted to international databases, 42 showed similarity to 35 genes, of which 31 were from plants. Of these, 14 were previously characterised as either defense-, stress- or senescence-associated. The former included *prb-1*-like genes associated with HR in tomato and tobacco, and basic chitinase (PR3), previously shown to associate with actin during the potato HR. A sequence not previously reported in plants showed 75-81 % protein similarity to all known serine palmitoyltransferases (SPTs). SPT catalyses the first step in the synthesis of sphingolipids, major signalling molecules involved in cell differentiation and apoptosis. Putative products of a number of other sequences may also play a role in programmed cell death including protein degradation (ubiquitin, ubiquitin carrier protein, cysteine protease), DNA degradation (cyclophilin), metal ion chelation (metallothioneins) and signal transduction (phosphatidylinositol-4-phosphate 5-kinase).. An additional PCR-based technique, cDNA-amplified fragment length polymorphism (cDNA-AFLP), was used as an independent test of the SSH and to compare profiles of gene expression between resistant and susceptible cultivars, either infected or healthy. The results suggested active, distinct processes, at the level of gene expression, in both phenotypes. The use of SSH and cDNA-AFLP in tandem will allow targeted isolation of genes associated with a specific tissue or metabolic process.

Session 2

Plant*-Phytophthora *Interactions

ANTISENSE AND SENSE MEDIATED GENE SILENCING AS A TOOL TO SUPPRESS EXPRESSION OF THE *INF1* GENE OF *PHYTOPHTHORA INFESTANS.*

P Van West[1], S Kamoun[2], JW Van T'Klooster and F Govers

Department of Phytopathology, WAU, Binnenhaven 9, 6709 PD, Wageningen, The Netherlands. Graduate School of Experimental Plant Sciences.
[1]Department of Molecular & Cell Biology, University of Aberdeen, Institute of Medical Sciences, Aberdeen AB25 2ZD, Scotland.
[2]Department of Plant Pathology, Ohio State University, Wooster, OH 444691, USA.

Background and objectives

Most *Phytophthora* and *Pythium* species produce 10 kDa extracellular protein elicitors, generally termed elicitins. Elicitins induce a hypersensitive response in a restricted number of plants, particularly in the genus *Nicotiana* within the *Solanaceae* family. Elicitins are thought to act as avirulence factors that restrict the host-range of the pathogen by triggering plant defence responses [1]. *Phytophthora infestans*, the causal agent of the potato late blight disease, produces an elicitin named INF1. A cDNA clone encoding INF1 was isolated and characterised [2]. The aim of this study was to engineer *P. infestans* mutants deficient in the production of INF1. Such mutants will help us to determine the role of elictin in host specificity.

Results and conclusions

Since *P. infestans* is a diploid organism and homologous integration of plasmids has not been demonstrated yet, we adopted a gene silencing strategy to inhibit *inf1* expression. *P. infestans* was transformed with constructs carrying strong oomycete promoters fused to the *inf1* coding sequence in both antisense and sense orientation. Expression of both the integrated transgenes and the native *inf1* gene was analysed and the production of extracellular INF1 protein was determined. It appeared that up to 20% of the transgenic *P. infestans* transformants failed to produce *inf1* mRNA and, as a consequence, were INF1 deficient. Genomic Southern blot analysis of the *inf1* silenced transformants demonstrated that the endogenous *inf1* gene was not mutated or deleted. In addition, we found that the silenced phenotype remained stable during several *in vitro* and *in planta* growth conditions in time. Therefore, these INF1 deficient transformants can be used to determine the role of the elicitin protein in host specificity, unambiguously.

To unravel the mechanism of silencing, we analysed genomic DNA of the silenced transformants for methylation, but found no hyper-methylation of the endogenous and transgene *inf1* sequences. Moreover, nuclear run-on assays showed that the silencing phenomenon is not based on high *inf1* mRNA turn-over. Further experiments to unravel the silencing mechanism will be presented.

1. Yu, L.M. *Proc. Natl. Acad. Sci. USA* 92: 4088-4094
2. Kamoun, S., van West, P., de Jong, A.J., de Groot, K.E., Vleeshouwers, V.G.A.A. and Govers, F. *Mol. Plant-Microbe Interact.* 6:15-25

REPETITIVE DNA SEQUENCES FROM *P. CRYPTOGEA*: MOLECULAR TOOLS FOR RESOLVING PATHOTYPE DIVERSITY AND THEIR POSSIBLE INVOLVEMENT IN GENE EXPRESSION

Franck Panabières

Institut National de la Recherche Agronomique, Station de Botanique et de Pathologie végétale, F-06606 Antibes cedex, France.

We have identified three different families of repetitive DNA sequences in the genome of *P. cryptogea*, a main pathogen of ornamental crops, and surveyed for their distribution among *Phytophthora* spp.

A first family, called the 2C4 family, exhibits a discontinuous distribution among *P. cryptogea*, as it is present in only certain genotypes. Restriction analysis suggests that 2C4 copies are dispersed. RFLPs generated by 2C4 can serve as genotype-specific DNA fingerprints.

The 1B10 family is a high copy number family composed of 1.3 kb elements. Unlike 2C4, the 1B10 elements are highly conserved (90-95%) and reveal few polymorphism. However a *Bam*HI digestion of *P. cryptogea* DNA generates 1.3 kb monomers in certain, but not all genotypes, in a strict correlation with the presence of 2C4 copies.

The 1B10 and the 2C4 families are restricted to *P. cryptogea*. The co-occurrence of monomeric structures of *Bam*HI elements and 2C4 sequences among genotypes appears to be associated with the ability of the fungus to colonize Gerbera, a common host of *P. cryptogea*. Therefore these two families might be used to resolve pathotype diversity.

The third class of repetitive sequences, called PCIS (Phytophthora Cryptogea Insertion Sequence) represents a high copy number family. It exhibits insertion polymorphism, as well as a very low sequence divergence. Unlike what is observed with 2C4 and 1B10 families, a highly similar PCIS copy has been characterized in the genome of *P. cinnamomi*. Several PCIS copies have been characterized in *P. cryptogea* at the nucleotide level. One copy, located in the elicitin intergenic region, is flanked by terminal direct repeats, and probably represents a mobile element. Other copies lack terminal repeats, but are associated with another repetitive element, that constitute a larger structure. As composite elements, they interrupt coding regions, such as a gene encoding a potassium channel. The consequence of such insertions on gene expression will be discussed.

RESISTANCE OF *NICOTIANA* TO *PHYTOPHTHORA INFESTANS* IS MEDIATED BY THE RECOGNITION OF THE ELICITOR PROTEIN INF1

S Kamoun [1,3], P Van West [2,3], and F Govers [3]

[1] Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, USA
[2] Department of Molecular and Cell Biology, University of Aberdeen, Scotland
[3] Department of Phytopathology, Wageningen Agriculrural University, The Netherlands

Most *Phytophthora* and *Pythium* species produce 10 kDa extracellular protein elicitors, generally termed elicitins. Elicitins induce a hypersensitive response in a restricted number of plants, particularly in the genus *Nicotiana* within the Solanaceae family. Elicitins are thought to act as avirulence factors that restrict the host-range of the pathogen by triggering plant defense responses [1,2]. *Phytophthora infestans*, the causal agent of the potato late blight disease, produces an elicitin named INF1. A cDNA clone encoding INF1 was isolated and characterized [3]. In virulence assays with different *P. infestans* isolates, five INF1 responsive *Nicotiana* species displayed resistant responses. In all interactions, following inoculation with *P. infestans* zoospores, cyst germination and penetration of a *Nicotiana* epidermal cell was observed. This was followed by a localized necrotic hypersensitive response that varied in timing and severity between different *Nicotiana* spp. The objective of this study is to determine whether INF1 functions as an avirulence factor that governs resistance in interactions between *P. infestans* and *Nicotiana*. To determine whether INF1 functions as an avirulence factor in interactions between *P. infestans* and *Nicotiana*, we engineered *P. infestans* mutants deficient in INF1 production. Since *P. infestans* is a diploid and homologous recombination of introduced DNA was never detected, we adopted a gene silencing strategy to inhibit INF1 production. *P. infestans* was transformed with a construct carrying the oomycete promoter *ham34* fused to the *inf1* coding sequence in antisense orientation. Several transformants totally deficient in *inf1* mRNA and INF1 protein were obtained. The silenced state of the *inf1* gene was shown to be mitotically stable under various conditions in vitro and in planta suggesting that the mutant strains can be used in functional analyses. In virulence assays, INF1 mutants remained pathogenic on the host plants potato and tomato. However, in contrast to wild-type and control transformant strains, INF1 mutants induced disease lesions and extensive sporulation when inoculated on *Nicotiana benthamiana*. These results demonstrate that the recognition of INF1 elicitin is a major component of the resistance of *N. benthamiana* to *P. infestans* and that INF1 functions as an avirulence factor in this interaction.

1. Kamoun S, Young M, Glascock C, Tyler, BM. 1993. Mol. Plant-Microbe Interact. 6, 15-25.
2. Yu L. 1995. Proc. Nat. Acad. Sci. 92, 4088-94.
3. Kamoun S, van West P, de Jong AJ, de Groot K, Vleeshouwers V, Govers F. 1997. Molec. Plant-Microbe Interact. 10, 13-20.

Session 3

Evolutionary and Ecological Genetics

WHITHER *PHYTOPHTHORA*? A REVISED CLASSIFICATION OF *PHYTOPHTHORA* AND OTHER OOMYCETES ON THE BASIS OF ITS ANALYSIS.

David Cooke

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Despite the importance of *Phytophthora* species as plant pathogens, our understanding of their population biology, evolutionary history and ongoing speciation processes is scant. We even lack consistent descriptions of some species which remain as poorly defined assemblages grouped on the basis of inconsistent morphological characters.

A re-examination of the genus on the basis of molecular data is clearly needed. The Internal Transcribed Spacer (ITS) regions of rDNA are ideal for such a study since they resolve at the appropriate taxonomic level yet concerted evolution generally ensures intraspecific uniformity, they have well conserved flanking regions ideal for PCR amplification and their arrangement in tandem repeats increases the sensitivity of PCR-based detection systems. Also, data from different research groups can easily be integrated. However there are also limitations which will be discussed in this presentation.

We have now established a comprehensive collection of *Phytophthora* isolates and sequenced the ITS regions of over 180, representing 50 known species, in order to resolve taxonomic difficulties and examine the evolutionary biology of the genus. We have also established a database of restriction digest patterns of over 200 isolates. There is sufficient ITS polymorphism to differentiate most species and a coherent phylogenetic tree indicates the genus is monophyletic. In some species an examination of the interface between population genetics and phylogeny (i.e. the speciation process) has been possible. The challenge of relating this molecular phylogeny to factors such as ecology, morphology, physiology, pathology and geographic distribution will be discussed.

Widening the study to other oomycota indicates considerably more diversity within *Pythium* compared to *Phytophthora*. All *Peronospora* species studied to date have shown an affinity with *Phytophthora* suggestive of their evolution as biotrophic Phytophthoras. A revisiting of Gaumann's hypothesis is now possible.

THE EVOLUTION OF *PHYTOPHTHORA* SPECIES

André Drenth

Cooperative Research Centre for Tropical Plant Pathology, The University of Queensland, Brisbane 4072, Australia.

An area of research currently attracting much interest concerns the evolution and origin of different *Phytophthora* species. The ability to rapidly obtain DNA sequence information from almost any species provides ample opportunities to investigate the evolution of species, including those of the genus *Phytophthora*. With the recent preparation and analysis of a database containing ribosomal DNA sequences of over 50 species of *Phytophthora,* and a number of *Pythium* and downy mildew species, it is possible to test hypotheses concerning the origins and relationships between the different species. The first issue to be resolved concerns the definition of biological species which are considered to be the operational units of evolution. Genetic diversity and DNA sequence data, combined with existing morphological and pathogenicity data, will provide ways in which to refine current species concepts.

The second issue is to try and identify the major evolutionary forces contributing to speciation in plant pathogenic fungi such as: *(i)* the level of genetic diversity in the original population, *(ii)* natural selection through changes in the host species leading to host specificity or even resistance gene specificity (e.g. gene-for-gene coevolution), *(iii)* reproductive isolation which can either be geographically based (allopatric speciation) or genetically based, (either pre- or post- zygotic) typically leading to sympatric speciation, and *(iv)* the genetic system, as reproduction in fungi can either be heterothallic, homothallic, asexual or combinations of these.

To stimulate discussion and to put evolutionary studies into perspective the following six questions can be raised:

1. have hemi-biotrophic *Phytophthora* spp. evolved from necrotrophic species? ??
2. have host specific *Phytophthora* spp. evolved from the broad host range species? yes
3. have the host specific *Phytophthora* spp. coevolved with their host plants or has sympatric speciation occurred at a much later stage in the evolution of the pathogen? NO generalization possible
4. has specificity in *Phytophthora* towards plant roots and foliage evolved from each other or independently? many foliar disease fall within three related clades
5. have homothallic *Phytophthora* spp. recently evolved from genetically diverse heterothallic species? yes, occurred independently in different clades
6. has hybridisation between different *Phytophthora* spp. led to new species? yes

- allopatric
- sympatric.

mating: takes place within the host
new host: reproductive isolation
annual plant: strong selection for homothallism

Peronosporaceae → more advanced

PROBABLE SYMPATRIC SPECIATION IN *PHYTOPHTHORA* MEDIATED BY CHANGES IN HOST SPECIFICITY

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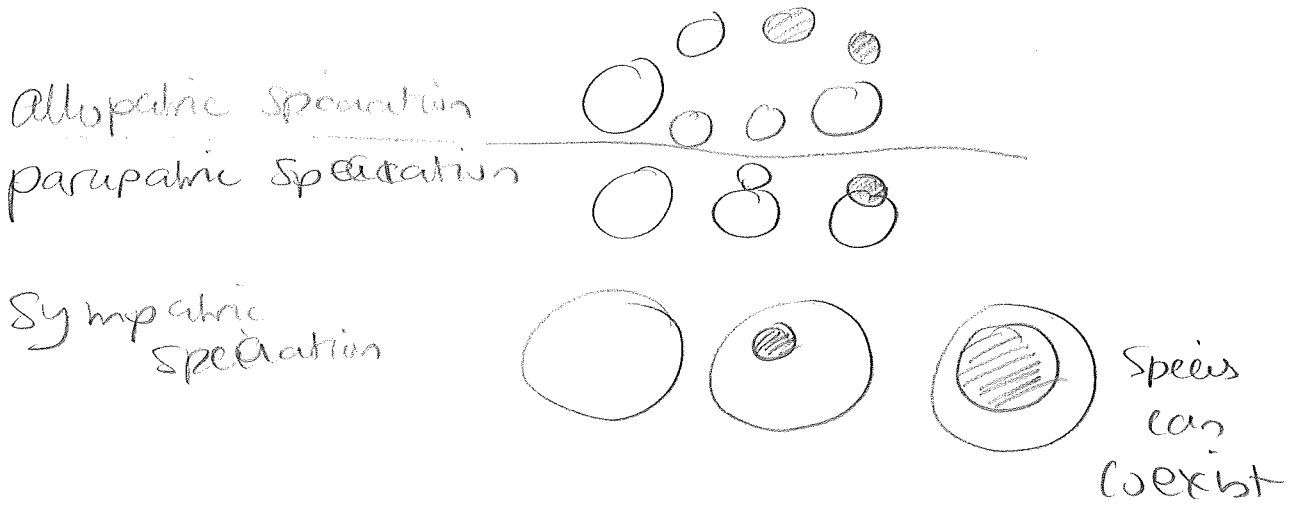
Sympatric speciation occurs when species form without geographical separation. Although the theory of sympatric speciation has been well developed for more than 30 years, there are few good examples in the literature, and whether it occurs at all is still highly controversial. The best-known example concerns host shifts in *Rhagoletes* flies, but host specificity is not absolute in that genus, and whether it represents true sympatric speciation has been questioned. A change in host specificity could provide an instant reproductive isolating mechanism for pathogens that complete their life cycles on their hosts. Such reproductive isolation could result in rapid, frequent sympatric speciation. Many plant-pathogenic fungi are host specific, complete their life cycles on their hosts, and thus might have arisen by sympatric speciation. An analysis of host specificities, degree of reproductive isolation, and genetic incompatibilities in F_1 interspecific hybrids, revealed evidence for probable recent sympatric speciation between *Phytophthora infestans* and *P. mirabilis* in central Mexico. The two species are host specific, sympatric, and reproductively isolated. Differences between *P. infestans* and *P. mirabilis* were found for mitochondrial DNA, isozymes, DNA fingerprints and RAPDs. Cluster analyses using a large battery of molecular markers provided clear separation between all six species in *Phytophthora* Group IV. Gene flow analyses using data from 130 presumed genetic loci revealed that *P. infestans* and *P. mirabilis* are as reproductively isolated from each other as they are from the other species in *Phytophthora* Group IV. Extensive efforts to develop F_2 progenies from F_1 interspecific hybrids failed. They clearly are different species. However, they are so closely related that the sequences of their ribosomal DNA Internal Transcribed Spacer II regions are identical. Because their hosts are very different (one is in the Nyctaginaceae, the other in the Solanaceae), gradual divergence during allopatric speciation seems highly unlikely. The only likely explanation for the data is that these two species arose by recent, sympatric speciation that was driven by a change in host specificity.



NEW TOOLS FOR AN OLD PROBLEM: ADVANCES IN THE STUDY OF THE UK *P. INFESTANS* POPULATION STRUCTURE.

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In the UK over the last 15 years, there has been a replacement of the old clonal A1 strain of *P. infestans* with new populations of diverse genotypes and of both mating types. This was probably due to importation of blighted ware tubers from Mexico to Europe. To determine the level of genetic diversity in UK populations, phenotypes were determined using a selection of molecular markers; including the multi-locus fingerprinting probe, RG57, a telomeric DNA probe from *Arabidopsis thaliana*, mtDNA haplotype, AFLPs and microsatellite markers which are multi-allelic and have the advantage of being co-dominant, allowing allele frequencies to be determined.

Sampling of 5-10 isolates per site from 15 commercial sites throughout the UK in 1996 revealed 100% A1 mating type and the presence of 3 dominant RG57 fingerprints suggesting clonal propagation at these sites. Intensive sampling of 50-100 isolates per site from 5 garden and small-scale commercial sites revealed greater genetic diversity. Between 6-15 RG57 fingerprints were identified per site. Larger sample sizes resulted in the detection of rare fingerprints, which occurred once only; half of the RG57 fingerprints occurred as single isolates. Sites showing high diversity often included the A2 mating type (maximum 46%) as well as A1, but at the most variable site (15 fingerprints) A2 was present at only 2%.

AFLP analysis produced trees basically similar to trees generated from RG57 fingerprints, however using 2 primer pairs, the frequently occurring RG57 fingerprints could be further separated. A primary branch of both trees consisted of isolates that showed the type IIa mtDNA haplotype, additionally a branch of the AFLP tree consisted of A2 mating types, suggesting that populations were not sexually recombining. Analysis of 457 individuals from garden populations using microsatellite markers revealed relative uniformity at 3 microsatellite loci compared to populations from Mexico were sexual recombination is considered to be frequent.

Greater genetic diversity at garden sites containing high levels of the A2 mating type suggests sexually-generated recombination, but may be explained by migration, the variety of potato and tomato grown, diverse origin of seed, frequent occurrence of ground keepers, cull piles and compost heaps which encourage overwintering within tubers.

SPECIATION PROCESSES AND NEWLY EMERGING SPECIES HYBRIDS IN *PHYTOPHTHORA*

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Over the millennia, the main ingredients of speciation in *Phytophthora* are likely to have been classic geographic isolation, local sympatric radiation of ancestral *Phytophthora* taxa and the selective influences of climate, host plant and other organisms. Over the past century or so, however, increasing world trade in plants and the rapid redistribution of *Phytophthora* taxa beyond their original habitats by man has probably lead to radical new evolutionary opportunities for Phytophthoras (i) in terms of adaptation of existing taxa to new environments; (ii) in terms of novel interspecific gene flow between previously geographically isolated species, ranging from limited genetic exchange to full interspecific hybridisation. Ecological and genetical processes involved in such hybridisation events will be discussed. Molecular, cytogenetic and phenotypic evidence for the recent emergence of several new *Phytophthora* species hybrids will be presented.

P. meadii ♀ - Ploidy — Comparative oogonium — Oospore fr

Various ♂ Strains

4n — large — normal

2n + 4n — Small to large — failure

2n — Small — failure

2n — Small — failure

Genome, Brasier, Hansen

fusion

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