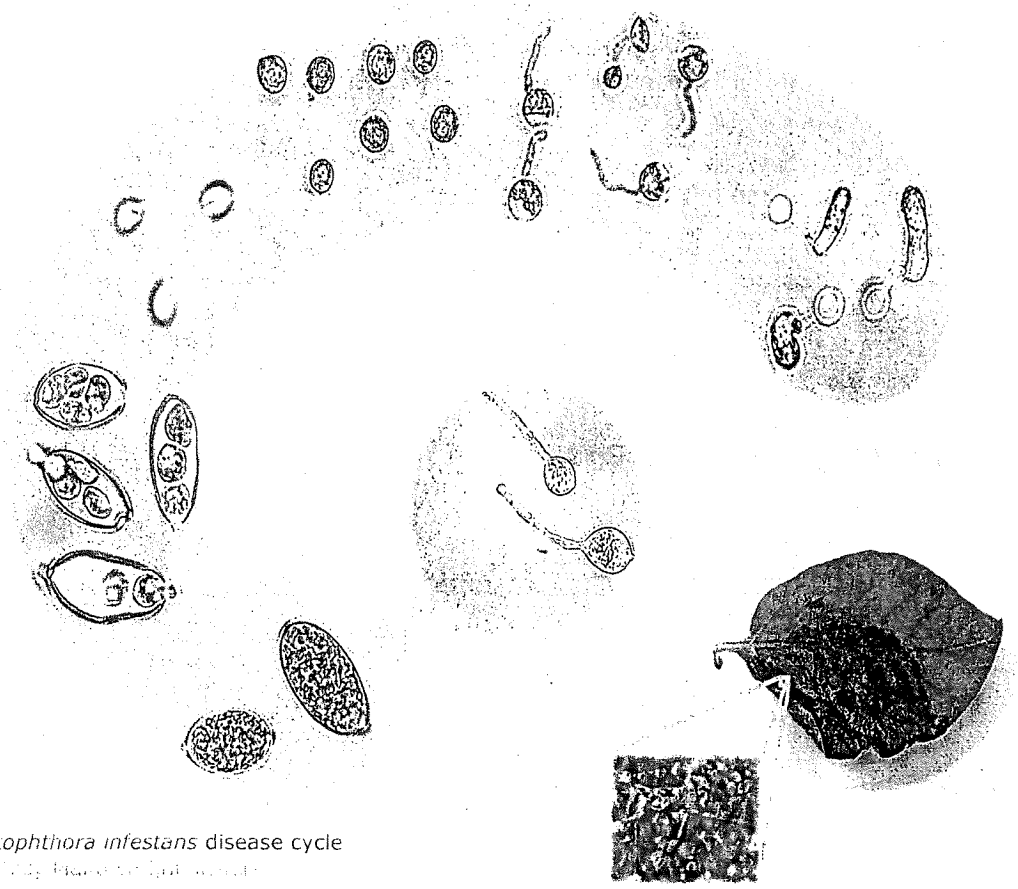
annual meeting

Phytophthora

Molecular Genetics Network



Phytophthora infestans disease cycle

The Ambassador Hotel, New Orleans, Louisiana
May 21-23, 2004

Supported by:
- NSF Research Coordination Networks in Biological Sciences
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PROGRAM

Phytophthora Molecular Genetics Network
The Ambassador Hotel, New Orleans, Louisiana
May 21-23, 2004

Friday May 21	arrival 4.00-7.00 PM; dinner 6.30 PM	
Saturday May 22	breakfast 7.00-8.00	
8.45 – 10.15 chair:	**Session 1** Francine Govers	**genomes, genomics.....**
50 min	Alan Collmer	Keynote lecture *Pseudomonas syringae* Functional Genomics: Searching for Patterns of Pathogenesis
20 min	Brett M. Tyler	Genome sequences of *Phytophthora sojae* and *Phytophthora ramorum*
20 min	Keith O'Neill	Sequencing the *Phytophthora infestans* genome: preliminary studies
coffee break		
10.40 – 12.00 chair:	**Session 2** Jim English	**...towards function**
20 min	Kurt H. Lamour	A Reverse Genetic Tool for *Phytophthora* Functional Genomics
20 min	Bill Fry	Transcriptional Regulatory Sequences in Oomycetes
20 min	Nicholas Champouret	Stable DNA Transformation of *Phytophthora infestans*: Towards Functional Analyses of Extracellular Proteases Inhibitors
20 min	Thorsten Nürnberger	Molecular analysis of *Phytophthora* derived molecular patterns
lunch 12.00 –1.30		
1.30-3.00 PM chair:	**Session 3** Paul Morris	**development and signaling**
20 min	Steve Whisson	Targeted pre-infection stage-specific discovery in *Phytophthora infestans*
20 min	Catherine Taylor	A Proteomic Approach to Identify Secreted Proteins from *Phytophthora infestans*
20 min	Harold Meijer	G-protein signaling and phosholipases in *Phytophthora infestans*
20 min	Howard S. Judelson	Gene expression and signal transduction during spore development in *Phytophthora infestans*
tea break		

3.30 – 4.30 PM chair:	**Session 4** Bill Fry	**population**
20 min	Carla Garzon	Moderate genetic differentiation in the plant pathogen *Pythium aphanidermatum*
20 min	Richard C. Hamelin	SNP genotyping in *Phytophthora ramorum,* the causal agent of sudden oak death
20 min	Luis Gomez & Jean B. Ristaino	Population history of *Phytophthora infestans* inferred from nuclear and mitochondrial DNA sequences
4.45 PM	Pre-dinner drinks and **poster session**	
dinner 6.00-7.30 PM		
8.00 – 9.00 PM chair:	**Session 5** Howard Judelson	**expression profiling**
20 min	Mark Gijzen	Microarray analysis of the soybean - *Phytophthora sojae* interaction
30 min	Sylvia Restrepo and/or Chris Smart	Expression profiling and molecular dissection of the potato – *Phytophthora infestans* interaction.
10 min	Sophien Kamoun	Oomycete Genome Initiative
mixer 9.00 PM	and poster session	

Sunday May 23	breakfast 7.00–8.00	
8.45 – 10.45 PM chair:	**Session 6** Mark Gijzen	**virulence, avirulence and resistance**
20 min	Miles Amstrong	Identification and functional characterisation of effector genes from *Phytophthora infestans.*
20 min	Francine Govers	Race-specific avirulence genes in *Phytophthora infestans*
20 min	Zhenyu Liu	Patterns of Diversifying Selection in the Phytotoxin-like *scr74* Gene Family of *Phytophthora infestans*
20 min	Dina Qutob	The *Phytophthora sojae* necrosis-inducing protein: recombinant protein production and Arabidopsis bioassays
20 min	Jim Beynon	*ATR13*: an avirulence gene from the *Peronospora parasitica* :Arabidopsis interaction.
20 min	John M. McDowell	Molecular Genetic Dissection of the *RPP7* Resistance Pathway
coffee break		
11.15-12.30 chair:	**Session 7** Brett Tyler	**Community activities and directions**
lunch 12.30		
14.00- 15.00	**Special news conference**	Release of the initial high quality assembly of the draft *P. sojae* and *P. ramorum* genome sequences, including access to the sequences via computer

ABSTRACTS ORAL PRESENTATIONS

Pseudomonas syringae Functional Genomics: Searching for Patterns of Pathogenesis

A. Collmer[1], J. R. Alfano[2], C. R. Buell[3], S. Cartinhour[4], and D. J. Schneider[4]
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Functional genomic analysis of *Pseudomonas syringae* is likely to yield insights into pathogenesis that are broadly useful because the host-specificity, symptoms, and plant defense responses associated with *P. syringae* diseases are similar to those accompanying disease caused by many important bacterial, Oomycete, and fungal pathogens. The Hrp type III secretion system (TTSS) of *P. syringae*, which injects virulence effector proteins into plant cells, plays a key role in the host-specific pathogenicity of the bacterium. More than 50 candidate TTSS effector genes have been identified in the complete genome sequence of *P. syringae* pv. *tomato* DC3000, a pathogen of tomato and Arabidopsis, based on the presence of characteristic promoter and N-proximal amino-acid patterns (Buell et al. 2003. Proc. Natl. Acad. Sci. USA 100:10181-10186). A draft sequence of the genome of *P. s. phaseolicola* 1448A has been obtained by The Institute for Genomic Research and is available through http://pseudomonas-syringae.org. The genome is currently being closed and then will be annotated and deposited into GenBank and the TIGR Comprehensive Microbial Resource. Refined experimental and computational tools for identifying TTSS effector genes are being applied to the 1448A genome. Comparison of the effector inventories, genomic islands, Hrp regulons, and mobile genetic elements of DC3000 and 1448A will provide a foundation for addressing many unanswered questions regarding the mechanisms and evolution of *P. syringae* pathogenicity. An emerging challenge that will be addressed in this talk involves the development of resources that promote ongoing functional genomics research in the *P. syringae* research community and comparisons of pathogenic processes by researchers working with other classes of pathogens.

GENOME SEQUENCES OF *PHYTOPHTHORA SOJAE* AND *PHYTOPHTHORA RAMORUM*

Brett M. Tyler

The genomes of the soybean pathogen *Phytophthora sojae* and the forest pathogen *P. ramorum* have now been sequenced to a depth of 9X. Assembly and machine annotation have begun and should be complete by May 2004. I will summarize the main findings of the analysis of these two genomes. The sequences are available at www.jgi.doe.gov and in the NCBI trace archive at http://www.ncbi.nlm.nih.gov/Traces/. Inspection of a preliminary version of the *P. sojae* assembly shows extensive proliferation and divergent evolution of gene families associated with the infection process such as *Avr1b*. In addition, the genome sequence together with the EST sequences are being used to construct Affymetrix GeneChips representing 15000 *P. sojae* and 32000 soybean genes. The GeneChips will be used to dissect the interplay between host and pathogen gene expression during infection of soybean cultivars carrying various levels of quantitative resistance.

Sequencing the *Phytophthora infestans* Genome: Preliminary Studies

[1]Keith O'Neill, [1]Michael C. Zody, [1]Elinor Karlsson, [2]Francine Govers, [2]Peter van de Vondervoort, [2]Rob Weide, [3]Stephen Whisson, [3]Paul Birch, [1]LiJun Ma, [1]Bruce Birren, [4]William Fry, [5]Howard Judelson, [6]Sophien Kamoun and [1]Chad Nusbaum

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Phytophthora infestans, the causal agent of Late Blight of potato, is arguably the most significant biotic threat to global food security. An annotated *P. infestans* genome sequence is a critically needed resource that will make a host of new research strategies available. To date, the relatively large size (237Mb), high repeat content and unknown amount of polymorphism have been barriers to the generation of a genome sequence. As sequencing costs drop and sequencing and assembly methods become more sophisticated, the *P. infestans* genome has become a realistic target. Accordingly, we have undertaken a one-year pilot project to explore the *P. infestans* genome and develop the optimal strategy, in terms of both cost and quality, for the generating a genome sequence. All work is being carried out in the T30-4 strain, which is an F1 of two aggressive strains isolated from potato in the Netherlands.

We have generated several new sequence data sets. We have sequenced paired ends of the entire *P. infestans* BAC library (27,648 clones) and completed a low-density survey of the genome through sequencing of a small-insert clone library (0.1X sequence coverage). Together these data represent more than 40Mb of *P. infestans* genome sequence. In addition, five pairs of BACs, representing overlapping regions from alternate haplotypes, were assembled with 10X sequence coverage and have been submitted to GenBank. All reads are available at the NCBI trace repository.

We have analyzed these data, along with the 486,177 read dataset generated by Syngenta, to characterize the *P. infestans* genome with respect to polymorphism and repeat content. We have assessed the polymorphism rate, and find a single nucleotide polymorphism (SNP) frequency of 1/2000 and a single base insertion/deletion frequency of less than 1/7700. Analysis of the nature and distribution of repeat elements is also underway and will be presented. Based on the results of this initial genome survey we have developed a strategy for complete genome sequencing of *P. infestans*, which will be discussed.

Finally, we are building a dense genetic map of *P. infestans* to anchor the genome sequence and to provide a fully integrated mapping resource to the community. We are in the process of large-scale SNP detection, by mining SNPs from new reduced-representation shotgun sequences as well as the Syngenta data. SNPs will be genotyped on the progeny of Cross 71, the cross that generated T30-4. The new genetic map will be integrated with the existing AFLP map.

A Reverse Genetic Tool for *Phytophthora* Functional Genomics

Lamour, K. H.

The completion of genomic sequences for *Phytophthora* encourages the development of general tools for studying gene function. Reverse genetics provides a powerful mechanism for both knocking-out and modifying specific genes. A general reverse genetic strategy utilizing *Phytophthora sojae* as a model is being developed with the goal of providing gene-specific mutants to the Phytophthora Molecular Genetic community. A library of 3000 EMS mutagenized *P. sojae* individuals has been constructed and screening for point mutations within specific gene-targets will begin late 2003. A key component of this project has been the miniaturization of high-throughput techniques for growing, transferring, storing, and extracting DNA from the isolates. Initial targets include genes involved in uracil biosynthesis with the goal of developing stable auxotrophs. Further targets have been identified by the community at large and include genes involved with spore production, motility, encystment, virulence, and drug resistance. The identification of targets and access to information and mutants will be discussed.

Developing an Enhanced Transformation System for the Identification of Transcriptional Regulatory Sequences in Oomycetes

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A basic understanding of transcriptional regulatory sequences will provide critical information necessary to understand oomycete gene expression. We have identified the core promoter region and transcriptional start sites for several genes in *Phytophthora infestans*. The goal of the current study is to identify and characterize additional transcriptional regulatory sequences in oomycetes. The lack of a rapid and efficient transformation system has hampered our investigations. Therefore, we have developed a new polyethylene glycol protoplast transformation method. Subsequently, twenty constructs containing several oomycete promoter and terminator sequences fused to the ß-glucuronidase (GUS) gene were tested for transcriptional activity in transient assays in *P. infestans* and *Pythium aphanidermatum*. Three of the constructs had no activity in the transient assays in both *P. infestans* and *P. aphanidermatum*. Construct DBpham35G, containing a fusion of the *ham34* promoter from *B. lactucae* and the *Piexo1* promoter from *P. infestans*, drove the highest GUS expression levels in *P. infestans*. The DBpham35G construct also yielded the highest GUS expression levels in *P. aphanidermatum*. The transformation method was further developed into a stable transformation method for *P. aphanidermatum* and *Phytophthora sojae*. Currently, five of the promoter-GUS constructs are being evaluated for their ability to drive GUS expression in stable *P. aphanidermatum* and *P. sojae* transformants. The selectable marker gene for geneticin resistance was incorporated into these vectors, and they are currently being tested for their ability to select for stable transformants in *P. aphanidermatum* and *P. sojae.*

Stable DNA Transformation of *Phytophthora infestans*: Towards Functional Analyses of Extracellular Protease Inhibitors

N. Champouret[1, 2, *], M. Tian [1], F. Mauch [2] and S. Kamoun [1]
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Transformation of microbial plant pathogens is one of the key molecular tools to study genes relevant to disease. The oomycete *Phytophthora infestans* is an economically important plant pathogen, which causes disease on solanaceous crop plants, such as potato and tomato. The plant intercellular space is the battlefront where interactions between proteins from plant and pathogen take place. Data mining of *Phytophthora* sequence databases for secreted proteins identified a number of genes with significant similarity to the Kazal family of serine protease inhibitors (named extracellular protease inhibitors or EPIs). To complement biochemical studies, we carried out stable DNA transformation of *P. infestans* using sense and antisense constructs of the *epi1* and *epi2* genes. We used the GATEWAY conversion technology to generate transformation vectors in which the gene of interest is bordered by the oomycete *ham34* promoter and terminator, followed by the bacterial resistance gene G418 (Geneticin) fused to *hsp70* promoter and terminator. We introduced vector DNA into protoplasts as a complex with cationic liposomes using polyethylene glycol and $CaCl_2$–mediated transformation, and optimized the conditions over several rounds of transformation. A total of 96 stable transformants were confirmed using PCR, resulting in 12 transformants with the antisense construct for each *epi* gene, 32 with the *epi1* sense and 40 with the *epi2* sense construct. The expression of *epi1* and *epi2* in the various transformants are being quantified by western-blot analyses of proteins in the extracellular medium and RT-PCR to identify transformants altered in *epi* expression. These transformants will be used to inoculate host plants to assess virulence phenotype. These studies will lead to a better understanding of the role of these effectors proteins in plant-*Phytophthora* interactions. Improvements in the classical chemical transformation protocol of *P. infestans* will be presented.

MOLECULAR ANALYSIS OF PHYTOPHTHORA-DERIVED MOLECULAR PATTERNS

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Immunity of an entire plant species to microbial infection (non-host resistance) is determined by intertwined layers of defense including both constitutive barriers and inducible reactions. Activation of non plant cultivar-specific inducible responses is likely based upon recognition of pathogen-associated molecular patterns, which bind to plant receptors. We have identified a cell wall transglutaminase (TGase) from phytopathogenic *Phytophthora* spp. that triggers defense responses in parsley and potato. Pep-13, constitutes a surface-exposed fragment within this TGase, which is both necessary and sufficient for defense activation. TGase transcripts and TGase activity are detectable in all *Phytophthora* species analyzed, with the Pep-13 sequence being fully conserved in all enzymes. Mutational analysis within Pep-13 identified the same amino acids indispensable for both TGase and defense-eliciting activity. Altogether, Pep-13 possesses characteristics of PAMPs known to mediate innate immunity in animals, and elicitors of

non-cultivar-specific plant defense may thus be considered physiological equivalents of PAMPs.
Pep-13 binds specifically to a 100-kD monomeric parsley plasma membrane protein. Bulk preparations of this low-abundant receptor protein are currently completed. Signaling of the elicitor stimulus involves immediate Ca^{2+} influx, sustained increases in the cytoplasmic Ca^{2+} concentration and requires reactive oxygen species and MAP kinase activity, all of which are necessary for Pep-13-mediated PR gene expression and phytoalexin production. cDNA-AFLP analyses have provided approximately 250 genes of which transcripts accumulate within minutes upon addition of Pep-13. RNAi-based inactivation of these genes is under way to establish their involvement in elicitor signaling.
NPP1, homologs of which are found in various phytopathogenic oomycetes, fungi and bacteria, was shown to trigger a similar cascade of events through a receptor system distinct of the Pep-13 receptor. In addition, NPP1 triggers a complex pattern of defense responses in *Arabidopsis* Col-0 plants. Plants infiltrated with NPP1 develop lesions reminiscent of the hypersensitive response, apposition of callose and transcriptional activation of several PR genes. Currently, a conditional lethal screen for *Arabidopsis* mutants resistant to NPP1 (NPP1 gene fused to a dexamethasone-inducible promoter allows initiation of conditional systemic cell death in NPP1-susceptible lines) is being performed, which may provide access to components involved in NPP1 perception and/or signal transmission. Moreover, microarray analyses performed with RNA from NPP1-treated *Arabidopsis* plants revealed numerous genes, whose expression was altered upon elicitor treatment. Plants carrying T-DNA insertions in selected genes are currently tested for their effects on non-host resistance as well as basal resistance.

Targeted pre-infection stage-specific discovery in *Phytophthora infestans*

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Phytophthora infestans, the causal agent of potato and tomato late blight, produces several different cell types prior to penetration of the host plant and during the early stages of infection. Cell types including sporangia, zoospores, germinating cysts, and appressoria can be generated in the absence of the host plant and so form the basis for stage-specific gene discovery. Amplified fragment length polymorphism based mRNA fingerprinting (cDNA-AFLP) and suppression subtractive hybridisation (SSH) are being used to target transcripts specifically up-regulated during cyst germination and appressoria formation; structures formed just prior to infection of potato. These structures are likely to contain many transcripts involved in successful penetration of the host, and establishment of a compatible interaction. Transcripts identified encode proteins potentially involved in adhesion, cell wall degradation, signalling, virulence, amino acid and protein biosynthesis, stress response, and detoxification. Expression of genes identified using SSH and cDNA-AFLP is being quantified by real-time RT-PCR in cultured mycelium, sporangia, zoospores, germinating cysts, germinating cysts with appressoria and at several time points post-inoculation of susceptible potato cultivar Bintje. Based on the expression profile of the identified transcripts, they have been prioritised for functional analysis to determine their role in the pathogen lifecycle and interaction with potato. Progress towards determining gene function using homology dependent gene silencing will be presented.

A Proteomic Approach to Identify Secreted Proteins from *Phytophthora infestans*

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A thorough understanding of the molecular events taking place during early interactions between *P. infestans* and host and non-host plants is crucial for developing new control strategies. At the plant-pathogen interface, an exchange of molecular signals is thought to determine the outcome of the interaction. We anticipate that secreted and cell wall proteins from pre-infection stages, namely zoospores, germinating cysts and appressoria will be rich in important signalling molecules involved in disease resistance or establishing successful infection. A proteomic approach is employed to identify novel extracellular and cell wall proteins from pre-infection stages. Here we present our latest results.

G-protein signaling and phosholipases in *Phytophthora infestans*

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Transduction of environmental signals involves perception and transmission via signaling cascades thereby activating appropriate responses. Extracellular signals can be perceived by receptors at the plasma membrane and upon sensing the signal the receptor can undergo a conformational change resulting in activation of a downstream pathway. In the case of G-protein signaling, the receptor binds to heterotrimeric G-proteins that consist of an α, β and γ-subunit. Upon binding, the Gα-bound GDP is replaced by GTP. Subsequently the G-protein dissociates into the α subunit and the $\beta\gamma$ subunits that activate or inactivate downstream targets. In *Phytophthora infestans* one Gα and one Gβ-subunit gene, have been identified. Mutants in which the Gβ-subunit gene *Pigpb1* was silenced formed an abnormally dense mat of aerial mycelium and produced very little or no sporangia (Latijnhouwers and Govers, 2003, Eukaryotic Cell 2, 971-977). Gα mutants with a silenced *Pigpa1* gene, grew normally and produced sporangia but the zoosporogenesis and zoospore release were much less efficient. Moreover, the zoospores had lost the ability to aggregate and the virulence of the PiGPA1-knock-down mutants on potato leaves was strongly reduced (Latijnhouwers *et al.*, 2004, Mol. Microbiol. 51, 925-936). The elucidation of the signaling cascades in *P. infestans* under control of PiGPA1 and PiGPB1 is now well under way. In animal and plant cells G-proteins activate phospholipid based signaling. Activation of phospholipases, results in the formation of second messengers that also activate cascades, either in parallel or integrated with other G-protein activated pathways. Several aspects of the recent work will be discussed.

Gene expression and signal transduction during spore development in *Phytophthora infestans*

Howard S. Judelson*, Audrey Ah Fong, Flavio Blanco, Kyoung Su Kim, and Shuji Tani. Department of Plant Pathology, University of California, Riverside, CA 92521 USA

Spores represent the main infectious propagules of the late blight diseases. To understand the molecular and cellular events involved in their formation and germination, an integrated genetic, biochemical, and pharmacological approach is being used. Expression profiling experiments using cDNA arrays identified >150 genes that exhibit major changes in mRNA abundance during the formation and/or germination of spores. Distinct classes in their pattern of expression was revealed by studying the effects of chemical inhibitors and mutations, and by performing timecourses of development. Genes with potential regulatory roles, such as kinases and transcription factors, have been targeted for detailed analyses. To better understand their functions, transformants silenced in their expression have been generated, interacting proteins identified using the yeast two-hybrid approach, and cellular localizations determined using GFP fusions. Silencing of some genes, for example, has resulted in deficiencies in sporulation or zoospore behavior. In addition, transformants expressing promoter-GUS fusions have been generated, resulting in the identification of sporulation and zoosporogenesis-specific promoters. Deletion analyses of these promoters is identifying potential binding sites for transcription factors, and the study of these factors will help identify early events required for the formation or germination of the spores.

Moderate genetic differentiation in the plant pathogen *Pythium aphanidermatum*

C. D. Garzon, D. M. Geiser and G. W. Moorman

Pythium aphanidermatum is one of the most devastating plant pathogenic Oomycetes, reducing vigor, quality and yield, and often killing a large percentage of plants affected. Pythium diseases caused by this species are particularly severe in greenhouse crops and turf growing at warm temperatures (25 C to 35 C). Despite the economic relevance of this pathogen little is known about its genetics. The objectives of this investigation were to evaluate the genetic diversity of *P. aphanidermatum*, to determine a population structure within the species, and to identify the factors associated with it. The ITS region and COX II were sequenced in a diverse sample of 34 and 21 isolates, respectively. No variation was found in the sequences of these genes. AFLP analysis using 2 selective primer combinations provided 111 polymorphic markers that were used in population analysis. Population structure was analyzed using the fixation index (F_{ST}) on populations defined by host, geographic origin, and sensitivity to mefenoxam. The F_{ST} analysis indicated that 9.5% of the genetic diversity of the sample was associated with host and 9.3% with sensitivity to mefenoxam. The contribution of geographic origin to population structure was low (4%). Sexual reproduction and long distance movement may be factors contributing to the little geographical structure of the sample. One marker associated with resistance to mefenoxam was identified.

SNP genotyping in *Phytophthora ramorum,* the causal agent of sudden oak death.

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In June 2003, *Phytophthora ramorum,* the organism responsible for Sudden oak death, was found in British Columbia (B.C.), Canada, for the first time. Molecular monitoring and diagnostic tools are important to prevent spread of this pathogen and enforce its eradication. The internal transcribed spacer (ITS), elicitin, ß-tubulin and CBEL (cellulose binding elicitor lectin) genes were sequenced and compared among *Phytophthoras* and within *P. ramorum*. We discovered single nucleotide polymorphisms (SNP) within *P. ramorum* for CBEL and ß-tubulin that were linked to geographic origin. SNP genotyping assays using primer extension were developed that can rapidly genotype these SNP in a multi-plate format. A worldwide collection of *P. ramorum* was genotyped using these assays. All European isolates genotyped were heterozygous at ß-tubulin for SNP ß-tub858 (A/G) but homozygous for ß-tub279 (G/G). By contrast, all California isolates were homozygous for SNP ß-tub858 (A/A), but heterozygous for ß-tub279 (T/G). For CBEL, all European isolates were heterozygous for CBEL245 (G/C) and CBEL412 (G/A), but California samples were homozygous at both SNP loci CBEL245 (G/G); CBEL412 (G/G). Isolates from Oregon and B.C. had SNP profiles identical to those from Europe. These results suggest that the newly discovered *P. ramorum* in western North America may represent a novel introduction from Europe. The likelihood that migration took place from California is low. This has important implications for our understanding of the epidemiology of this disease.

Population history of *Phytophthora infestans* inferred from nuclear and mitochondrial DNA sequences

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The center of origin of *P. infestans* as well as the source of inoculum for the epidemic that caused the Irish potato famine in 1845 is an intriguing subject. Nuclear and mitochondrial DNA variability is being used to examine the population history of *P. infestans* in an attempt to answer two questions: 1) What is the phylogeographic pattern of molecular variation in *P. infestans*? 2) Does mitochondrial and nuclear DNA sequence evidence justify the specific hypothesis that a common ancestor of *P. infestans* originated in South American or Mexican populations? DNA sequence data from three nuclear regions and one mitochondrial region were collected from fifty-two isolates obtained from various locations including Brazil, Bolivia, Ecuador, Peru, Costa Rica, Mexico, USA, and Ireland. Gene genealogies were constructed for the Ras, Intron Ras, and ß-tubulin nuclear regions as well as for the mitochondrial P4 region (Cox I). Ras and Intron-Ras genealogies consisted of three haplotypes, and ß-tubulin of two haplotypes. For the P4 region two main haplotypes were resolved corresponding to the I and II mtDNA haplotypes described elsewhere. Peruvian, USA and Irish populations shared the same haplotypes across the regions suggesting a common ancestry among these populations.

Microarray analysis of the soybean - *Phytophthora sojae* interaction

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To investigate patterns of gene expression in soybean and *Phytophthora sojae* during infection, we constructed a 4896 gene microarray of host (83%) and pathogen (17%) cDNA transcripts. Samples of mRNA were purified from infection sites at 3, 6, 12, 24 and 48 hours after inoculation of soybean plants with *Phytophthora sojae* zoospores. Control plants were water-inoculated. A total of 50 hybridizations were conducted using mRNA isolated from three independent experiments. Soybean genes that were strongly up-regulated during infection included those encoding enzymes of phytoalexin biosynthesis and defense and pathogenesis-related proteins. Expression of these genes generally peaked at 24 hours after infection but individual gene families displayed varied patterns of expression during the time course. Surprisingly, expression levels of many genes normally associated with defense responses declined during infection. Selected lipoxygenases and peroxidases were among the most strongly down-regulated genes on the array. Other host genes that declined in expression during infection included photosynthetic components, beta-amylases, metallothioneins, and genes encoding key steps in methionine and cellulose biosynthesis. The number of pathogen genes expressed during infection reached a maximum at 24 hours, during the period of transition from biotrophy to necrotrophy. Pathogen transcripts encoding proteinases, glucanases, cutinases, and necrosis and elicitor proteins were detected and showed distinct expression profiles over the time course of infection.

Identification of genes differentially expressed during potato – *Phytophthora infestans* compatible interactions.

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Potato cDNA microarrays were exploited in a search for genes that are differentially expressed during a compatible interaction with *Phytophthora infestans*. The cDNA microarray used was constructed at TIGR and included 5000 cDNAs. It was derived from ESTs identified from stolon, leaf, and pathogen-challenged libraries. In this study, leaves of the potato cultivar Kennebec were harvested for RNA extraction 0, 6, 12, 24, 48 and 72h after inoculation with *P. infestans* (US-8), and the entire experiment was repeated three times. RNA was extracted, labeled and hybridized to two arrays for each time point of each experiment for a total of 36 arrays. Data were subjected to rigorous statistical analyses. A significant differential expression was observed for 13% of the genes. The transcripts for many proteins involved in photosynthesis were repressed at all time points after the inoculation. Genes involved in the biosynthesis of ethylene were strongly down-regulated 48h after the inoculation following an initial induction at 6h. In addition, an early and transient induction was observed for a group of genes encoding DNA binding proteins and proteinase inhibitors. Interestingly, a salicylic acid – binding protein, carbonic anhydrase was repressed during the time course of the experiment. The technical and biological validations of these results are in progress.

Molecular dissection of the potato – *Phytophthora infestans* interaction

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Microarray analyses were utilized to identify genes that exhibit differential expression during a compatible interaction between potato and *Phytophthora infestans.* Clusters of potato genes that had a similar pattern of expression during the interaction between potato and *P. infestans* were identified. To determine the accuracy of these expression patterns, we are utilizing real-time PCR. We have chosen one or more genes within different clusters, and the pattern of expression is being followed over time in both compatible and incompatible interactions. The data generated using real-time PCR are being compared to data generated using microarrays, which allows us to gain an accurate idea of which potato genes are modified upon inoculation with *P. infestans.* Additionally, candidate genes identified in the microarray experiment are being subjected to functional analysis. We are currently in the process of silencing host genes that may play a role in pathogenicity. In these experiments we silence genes in the model plant *Nicotiana benthamiana* via virus-induced gene silencing (VIGS). Following gene silencing, plants are inoculated with a *P. infestans* strain that is compatible with *N. benthamiana*, and the interaction is followed at the molecular (real-time PCR), microscopic (cytology of infection) and macroscopic (symptom development) levels.

Identification and functional characterisation of effector genes from *Phytophthora infestans.*

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Phytophthora infestans is a hemi-biotrophic pathogen that exhibits gene-for-gene interactions with its host potato. Compared to bacterial pathogens, relatively few fungal effector genes have been cloned and even fewer from the oomycetes. We aim to clone and characterise effector genes from the oomycete pathogen *P. infestans* as a first step in studying the molecular basis of host/oomycete recognition and specificity. Screening secreted genes for SNPs that associate with avirulence phenotypes has been used to augment traditional map-based cloning to identify candidate avirulence effectors. SNP discovery has also allowed the identification of proteins under strong diversifying selection and has been used to prioritise candidate genes for functional analysis. We have used a variety of functional screens to evaluate putative effectors including: heterologous expression of *P. infestans* proteins from a PVX vector as a high throughput screen, a co-bombardment strategy involving transient expression of GFP to examine elicitation of localised cell death, transformation, and homology dependant gene silencing. Progress in these areas will be described.

- data mining
- from SSH

Race-specific avirulence genes in *Phytophthora infestans*

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Phytophthora infestans is a destructive oomycete pathogen causing potato late blight worldwide. Genetic analyses of potato and *P. infestans* have demonstrated that in this pathosystem, monogenic resistance mediated by resistance (*R*) genes, is based on a gene-for-gene interaction. Our aim is to clone and characterise avirulence (*Avr*) genes in *P. infestans*. We constructed high density linkage maps of two regions carrying *Avr* genes (van der Lee *et al.* 2001 Genetics 157: 949-956) and performed cDNA-AFLP analysis on F1 progeny of the mapping population. The cDNA-AFLP analysis resulted in a number of Transcript Derived Fragments (TDFs) and RT-PCR showed that some of the TDFs represent genes differentially expressed in the different races. By marker landing and genome walking using the cloned TDFs we could identify a candidate avirulence gene in the *Avr3-Avr10-Avr11* cluster. An update of recent work will be presented.

Patterns of Diversifying Selection in the Phytotoxin-like *scr74* Gene Family of *Phytophthora infestans*

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ω = dN/dS ratio
ω > 1 diversifying selection
ω = 1 → neutral
ω < 1 purifying selection
scr74 candidate gene

Phytophthora infestans, the Irish famine organism causes late blight, a reemerging disease of potato and tomato. Little is known about the molecular evolution of *P. infestans* genes and gene families. Diversifying selection acting on genes involved in the infection process is likely to reflect an "arms race" coevolution between the host and the pathogen. Thus, we hypothesized that diversifying selection could be exploited as a criterion for selection of candidate effector genes (virulence and avirulence genes) from *P. infestans*. We used an *in silico* approach aimed at identifying *P. infestans* genes under diversifying selection. At the molecular level, the most reliable indicator of diversifying selection is a higher nonsynonymous nucleotide substitution rate (d_N) than synonymous nucleotide substitution rate (d_S) between two protein-coding DNA sequences, or their rate ratio d_N / d_S (omega) > 1. We mined expressed sequence tag (EST) data from a variety of infection and developmental stages of *P. infestans* for secreted and potentially polymorphic genes. This led to the identification of the highly polymorphic *scr74* gene family, which encodes secreted small cysteine-rich proteins with similarity to the *Phytophthora cactorum* phytotoxin PcF. We investigated the molecular evolution of *scr74* genes using the approximate method of Nei and Gojobori and Maximum-Likelihood method (ML). Our results indicated that the extensive polymorphism observed within the *scr74* gene family is likely to be caused by diversifying selection. Pairwise comparisons of 17 *scr74* sequences revealed elevated ratios of nonsynonymous to synonymous nucleotide substitution rates, particularly in the mature proteins. Using ML, all 21 polymorphic amino acid sites were identified to be under diversifying selection. Nineteen of these 21 amino acids are located in the mature protein region suggesting that selection may have targeted the functional portion of the proteins. We propose an evolutionary model that involves duplications followed by functional divergence of *scr74* genes.

The *Phytophthora sojae* necrosis-inducing protein: recombinant protein production and Arabidopsis bioassays

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The 24 kD necrosis-inducing protein from *Phytophthora sojae*, PsojNIP, causes plant cell death and necrosis. Our hypothesis is that PsojNIP assists pathogen colonization of soybean tissues during the necrotrophic phase of disease development. To further study the mode of action of PsojNIP, methods were developed for recombinant protein expression (rPsojNIP) in *E. coli* for use in bioassays with Arabidopsis seedlings. Large amounts of the protein were produced in *E. coli* in the form of inclusion bodies. The inclusion bodies could be solubilized with 6 M guanidine-HCl or 1% SDS. Biologically active protein, causing necrosis in a tobacco leaf infiltration assay, was recovered by step-wise dialysis in decreasing concentrations of denaturing agent, concluding with final reconstitution in 10 mM Tris, pH 8.0. Analysis by gel filtration chromatography indicated that the refolded rPsojNIP was present in solution as aggregates of >600 kD. Nonetheless, germination of Arabidopsis seeds on MS-agar plates supplemented with rPsojNIP dramatically reduced lateral and primary root growth and overall seedling vigor. Identical solutions of heat-denatured rPsojNIP did not have any effect. The activity of rPsojNIP on Arabidopsis seedlings was concentration dependent. Supplements of 1 μg ml^{-1} of rPsojNIP were active in reducing root growth, but higher concentrations led to greater reductions of root length and the appearance of necrotic symptoms. A screen of 20 different Arabidopsis lines and ecotypes suggested that there may be natural variation in sensitivity to rPsojNIP.

***ATR13*: an avirulence gene from the *Peronospora parasitica* :Arabidopsis interaction.**

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The resistance gene *RPP13* is a classic resistance gene of the coiled-coil: nucleotide binding site: leucine rich repeat class. The gene is present as a functional allelic series where different alleles recognise different pathogen isolates. It is the most variable plant gene cloned to date and exhibits extreme variation within the leucine rich repeat domain. This suggests it is under positive environmental selection for change. A key candidate that could be driving this change is the complementary avirulence gene *ATR13*. Hence, we have applied map-based cloning techniques to define a mapping interval for *ATR13*. We used the SSH technique to identify *in planta* expressed genes and one of these co-segregated with *ATR13*. *In planta* assays were used to confirm that this gene caused an *RPP13* specific response. We will report the structure of *ATR13* and the relationship between *RPP13*alleles and recognition of *ATR13* alleles.

Molecular Genetic Dissection of the *RPP7* Resistance Pathway

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The *RPP7* gene activates race-specific resistance to the downy mildew pathogen *Peronospora parasitica*. Previously published genetic epistasis tests have established that *RPP7* activates defense responses through a signaling mechanism that does not require accumulation of salicylic acid (SA) or components of the ethylene and jasmonic acid response pathways encoded by *EIN2*, *JAR1*, or *COI1*. Furthermore, *RPP7* is not suppressed by mutations in a variety of putative signal transducers that are required by various NBS-LRR resistance genes (e.g. *pad4-1*, *ndr1-1*, *npr1-1*, *pbs2-1*). In an effort to better understand the genetic requirements for signal transduction from *RPP7*, we have constructed a series of double mutants to test for additive or functionally redundant contributions by known defense signaling components. Most of these combinations display a slightly enhanced level of asexual sporulation, with the *ndr1/pad4* combination having the strongest effect. All of the double mutants are capable of inducing the ROI production and the HR, but this response is delayed to varying degrees. These results reveal "cryptic" roles for *PAD4*, *PBS2*, *NDR1*, and *NPR1* in *RPP7* signaling, and suggest that *RPP7* activates resistance through multiple signaling pathways that collectively regulate the kinetics of the HR. We have also cloned the *RPP7* gene, using map-based methods. This gene belongs to a cluster of eight highly related CC-NBS-LRR genes on Chr.1, which share significant similarity with *RPP8*. The *RPP7* gene is 18.6 Kb long. Most of the gene is comprised of long introns within short 5' and 3' UTRs. The RPP7 transcript is alternatively spliced.
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POSTER ABSTRACTS

A Population Genomics Approach to the Study of Molecular Evolution in *Phytophthora infestans*.

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The mitochondrial genome of extant haplotypes of *Phytophthora infestans*, the late blight pathogen of potato and tomato will be sequenced. The specific objectives are to: 1) Sequence the entire genome of three isolates representing the Ia, IIa, and IIb haplotypes; 2) Use whole mitochondrial genome analysis to develop novel polymorphic markers; 3) Determine whether the mitochondrial genome of *P. infestans* undergoes recombination, and 4) Study the rate of evolution of the pathogen. The three haplotypes employed in this study originated from the Netherlands (Ia haplotype, isolate 80029), Ireland (IIa haplotype, isolate 15/99), and the US (IIb haplotype, isolate 94-52). Mycelia, derived from either single zoospore or single sporangia, was grown in pea-broth media at 18 °C without shaking. Total DNA was extracted by a modified cetyltrimethylammonium bromide method, and mitochondrial DNA (mtDNA) was separated from nuclear DNA by a CsCl equilibrium density gradient in the presence of bisbezimide. The Institute for Genomic Research (TIGR) is sequencing the isolates

using a shotgun sequencing procedure. Shotgun clones are being sequenced to generate approximately 8-10-fold coverage of each mitochondrial genome. The results of the mitochondrial genome sequencing project will be discussed.

The glucanase inhibitor protein (*GIP*) gene family from *Phytophthora infestans*.

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Glucanase inhibitor proteins (GIPs) are a class of proteins that bind and inhibit the activity of plant extracellular endo-β-1,3-glucanases (EGases), blocking release of glucan elicitors and their triggered defense responses. GIPs are produced by some *Phytophthora* species and secreted into the apoplast during infection. They were first identified in *P. sojae* and appear to have a high specificity for particular EGase isoforms. GIP1 from *P. sojae* (PsGIP1) was shown specifically to bind EGaseA from soybean but not to isoform EGaseB. Despite their potential importance as suppressors of EGase-mediated defense responses, the molecular basis of GIP action and specificity are not well understood. We have recently identified four GIP homologs (*PiGIP1*-4) in *P. infestans* by genomic library screening and EST and BAC databases searches. Genomic Southern blot analysis further indicated the existence of a four-member GIP family and Western analysis identified GIP isoforms that are secreted in *P. infestans* growth media and into the apoplast of infected tomato leaves. We also used structural molecular modeling to identify putative docking sites on the surfaces of EGases and GIPs that may be involved in the high affinity binding between these proteins. Codon evolution model analysis of some of these amino acid residues indicated that they are evolving under positive selection, suggesting the existence of a 'molecular arms race' between GIPs and EGases. We are currently trying to identify the GIP-EGase complexes from tomato apoplastic fluid using co-immunoprecipitation assays and further results will be presented.

Protein scaffold design for delivery of peptides that induce encystment of *Phytophthora capsici* zoospores.

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The ability of *Phytophthora* to locate a host, infect, and cause disease depends on its responsiveness to environmental signals that trigger critical developmental steps. In the past, we used combinatorial, phage-display technology to identify peptides that induce encystment (encystment agonists) of *P. capsici* zoospores. We are currently investigating the potential for delivery of these peptides as defense molecules via display on carrier proteins. We have identified several exportable plant proteins as candidate peptide carriers. In initial constructs, we incorporated agonist or non-agonist peptides in a monomeric format at the exposed C-terminus of a selected exportable protein. By in vitro assay agonists peptides, known to induce encystment in phage display or synthesized formats, also induced encystment as part of the protein scaffold. Non-agonist peptides, known to bind to zoospores but not to induce encystment in phage display format, did not induce encystment when displayed on the carrier protein. In ongoing experiments, we are comparing agonist and non-agonist peptides in concatameric formats and in alternative scaffold designs in selected carrier proteins.

Identifying determinants of spore-specific transcription using the *PiCdc14* promoter from *Phytophthora infestans*

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Phytophthora infestans is the causative agent of the tomato and potato late blight diseases. The asexual spores are an important part of the life cycle and are the main propagules for the spread of infection. Several genes, e.g. the cell cycle regulator *PiCdc14*, were found to be involved in asexual spore development. To understand the mechanism controlling spore-specific expression, a promoter deletion analysis of the *PiCdc14* gene was conducted. A 945 bp upstream regulatory sequence obtained from a genomic clone and various deletion sequences were fused with the *uidA* gene and used to transform *P. infestans*. With the full-length promoter, GUS activity was detected in sporangiophore initials, in sporangiophores bearing immature sporangia, and later to mature sporangia only. A 415 bp fragment upstream of the ATG still resulted in spore-specific expression. However, the 110 bp promoter exhibited constitutive GUS expression which suggests that the –415 bp to –110 bp region contains negative regulatory element(s). A more comprehensive deletion analysis of the region, site directed mutagenesis, and electrophoretic mobility shift assays will confirm the presence of *cis*-regulatory elements involved in spore-specific transcription. Parallel studies of other spore-specific promoters will help to identify conserved motif(s) responsible for driving spore-specific expression.

Population history of *Phytophthora infestans* inferred from nuclear and mitochondrial DNA sequences

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The center of origin of *P. infestans* as well as the source of inoculum for the epidemic that caused the Irish potato famine in 1845 is an intriguing subject. Nuclear and mitochondrial DNA variability is being used to examine the population history of *P. infestans* in an attempt to answer two questions: 1) What is the phylogeographic pattern of molecular variation in *P. infestans*? 2) Does mitochondrial and nuclear DNA sequence evidence justify the specific hypothesis that a common ancestor of *P. infestans* originated in South American or Mexican populations? DNA sequence data from three nuclear regions and one mitochondrial region were collected from fifty-two isolates obtained from various locations including Brazil, Bolivia, Ecuador, Peru, Costa Rica, Mexico, USA, and Ireland. Gene genealogies were constructed for the Ras, Intron Ras, and _-tubulin nuclear regions as well as for the mitochondrial P4 region (Cox I). Ras and Intron-Ras genealogies consisted of three haplotypes, and _-tubulin of two haplotypes. For the P4 region two main haplotypes were resolved corresponding to the I and II mtDNA haplotypes described elsewhere. Peruvian, USA and Irish populations shared the same haplotypes across the regions suggesting a common ancestry among these populations. A combined analysis as well as the reconstruction of the evolutionary history of the haplotyes is in progress using a combination of parsimony, maximum likelihood and coalescent methods

Heterologous expression of a pleotropic drug resistance transporter from the soybean pathogen *Phytophthora sojae* in yeast transporter mutants.

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A system for the expression and functional characterization of an ATP Binding Cassette (ABC) transporter from the soybean pathogen Phytophhtora sojae in yeast transporter mutants is described. The gene for PsPDR1 has a single open reading frame coding for a 1310 amino acid protein with homology to the Pleiotoptic Drug Resistance family of ABC transporters. PsPDR1 was amplified by PCR from a BAC clone using sequence specific primers and directionally cloned in the yeast expression vector pYES2. Purified plasmid containing PsPDR1 under the control of the GAL1 promoter was transformed by lithium acetate transformation into the URA- yeast strain YYM3 which is deficient in the ABC transportersPDR5 and SNQ2. Transformants were identified by uracil selection on minimal media plates. Expression of PsPDR1 conferred a drug resistance phenotype against 8-hydroxyquinoline, cyclohexamide and Rhodamine 123 in drug susceptibility disk assays. We believe this to be the first demonstration that Phytophthora sojae genes can be functionally expressed and characterized in yeast cells. Thus controlled expression of ABC transporter genes in yeast transporter mutants can be used to efficiently assess the potential drug substrates of the other members of this family of ABC transporters in P. sojae.. In future work, substrate specificity of this transporter will be assessed using yeast strains deficient in seven ABC genes.

Molecular Detection of *Phytophthora capsici* from soil using polymerase chain reaction (PCR).

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Phytophthora blight, caused by the oomycete pathogen *Phytophthora capsici* is a devastating disease on bell pepper and cucurbits in the United States and worldwide. A rapid and accurate method for detection of *P. capsici* in soil is critical for the identification of infested fields and studies of the spread of Phytophthora blight. Sporangia were added to soil at levels of 12 to 120,000 sporangia per half gram of soil. DNA was extracted from soil using a MO BIO kit. The PCR primers PCAP and ITS1 were used to amplify the ribosomal DNA (rDNA). *P. capsici* was readily detected with as few as 12 sporangia per half gram of soil. Our PCR data suggest that direct detection of *P. capsici* from soil with PCAP and ITS1 primer pairs will provide a valuable tool for quick detection and monitor of *P. capsici*.

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