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Pathogen profile

Phytophthora infestans enters the genomics era

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SUMMARY

Phytophthora infestans, cause of late-blight, is the most devastating disease of potato world-wide. Recent years have seen a dramatic intensification in molecular biological studies of *P. infestans*, including the development of novel tools for transformation and gene silencing and the resources for genetical, transcriptional and physical mapping of the genome. This review will focus on the increasing efforts to use these resources to discover the genetic bases of pathogenicity, avirulence and host-specificity.

Taxonomy: *Phytophthora infestans* (Mont.) de Bary—Kingdom Chromista, Phylum Oomycota, Order *Peronosporales*, Family *Peronosporaceae*, Genus *Phytophthora*, of which it is the type species.

Host range: Infects a wide range of solanaceous species. Economically important hosts are potato, tomato, eggplant and some other South American hosts (tree tomato and pear melon) on which it causes late blight.

Disease symptoms: Infected foliage is initially yellow, becomes water soaked and eventually blackens. Leaf symptoms comprise purple-black or brown-black lesions at the leaf tip, later spreading across the leaf to the stem. Whitish masses of sporangia develop on the underside of the leaf. Tubers become infected later in the season and, in the early stages, consist of slightly brown or purple blotches on the skin. In damp soils the tuber decays rapidly before harvest. Tuber infection is quickly followed by secondary fungal or bacterial infection known as 'wet rot'.

Useful web sites: http://www.ncgr.org/pgc/; http://www.oardc.ohio-state.edu/phytophthora/.

INTRODUCTION

In the mid-1840s, a devastating potato disease swept continental Europe, the British Isles and Ireland. It is estimated that Ireland, as a direct consequence of late blight, lost more than a quarter of its 8 million inhabitants to starvation and emigration, making this one of the most significant crop diseases in history. In the latter part of that decade, 20 years before the germ theory of Pasteur was widely accepted, a controversial debate raged through Europe and the USA as to whether the disease was caused by a fungus, excessive dampness, genetic deterioration in the cultivated potato, or by a 'poisonous miasma borne on the air', the offered sources of which included pollution, volcanic exhalations or 'some aerial taint originating in outer space' (reviewed in Bourke, 1991). However, it was not until 1876 that a micro-organism named *Phytophthora* (meaning 'plant destroyer') *infestans* was conclusively demonstrated to be responsible for potato late blight (de Bary, 1876). Today, although chemicals targeted against *P. infestans* provide some level of disease control, worldwide losses due to late blight and control measures are estimated to exceed $5 billion annually. *P. infestans* is thus regarded as a threat to global food security (Duncan, 1999).

P. infestans is a specialized pathogen, primarily causing disease on the foliage and fruits of a range of *Solanaceous* species (Erwin and Ribeiro, 1996). Infections may be initiated asexually when sporangia (Fig. 1) land on the surface of a leaf and, under damp conditions and temperatures below 12 °C, release motile zoospores that rapidly encyst and produce a germ tube. The tip of the germ tube develops into an appressorium and this produces an infection peg that penetrates the plant cell. Occasionally, intracellular haustorial feeding structures are formed. After 3 days, hyphae spread saprophytically throughout the growing lesion before emerging from stomata. Sporangiophores develop on the underside of the leaf and these release sporangia to propagate aerial spread of the pathogen (Fig. 1).

P. infestans is an heterothallic oomycete with two mating types, A1 and A2, which evolved in the Toluca valley, central Mexico. Until the 1980s the A2 mating type was confined to Mexico, previous spread of the pathogen being attributed to a single A1 isolate of the pathogen (Goodwin *et al.*, 1994). Since the 1980s the old A1 population has been gradually replaced by a new A1/A2 population. This has led to increased virulence and genetic variation worldwide, suggested to be the result of sexual reproduction (Fry *et al.*, 1993). The A1 and A2 mating types are

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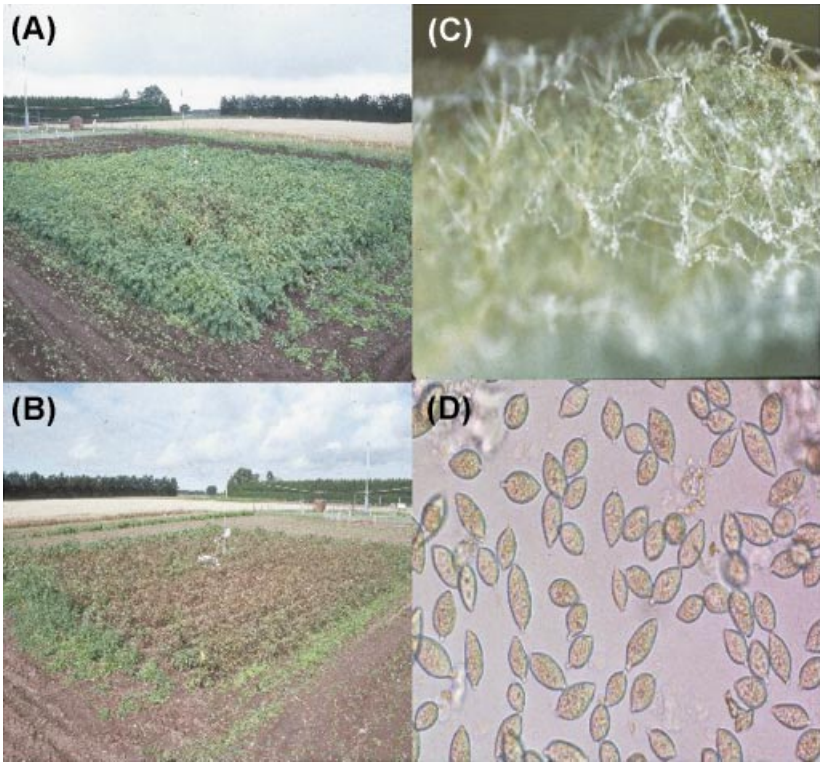

Fig. 1 Natural infection by *P. infestans* of a plot containing susceptible potato cultivar Bintje (A and B). Early symptoms of infection can be seen in the centre of the plot (A), followed by massive devastation of the plant foliage 10 days later (B). Aerial hyphae growing from the underside of a susceptible leaf (C) develop sporangiophores which produce sporangia (D) in the later stages of infection.

believed to represent compatibility types that differ in hormone production and response (Judelson, 1997). The development of male and female gametangia (antheridia and oogonia) is stimulated by the hormones within a mating zone, where normal vegetative growth and asexual sporulation are inhibited. The haploid A1 and A2 nuclei fuse to form a diploid nucleus within a structure called the oospore. The oospore develops a thick wall, allowing survival for many years in soil. Germination of the oospore releases progeny of either A1 or A2 mating type that are able to infect newly planted tubers, or stems and leaves that come into contact with the soil (Drenth *et al*., 1995).

The oomycetes comprise a large number of economically important and highly destructive plant pathogens, including the downy mildews and over 60 species of the genus *Phytophthora*. Because of their filamentous growth, taxonomists have historically grouped them with fungi, such as the ascomycetes and basidiomycetes. However, contemporary studies of metabolism (Pfyffer *et al*., 1990) and rRNA sequence (Cooke *et al*., 2000; Förster *et al*., 1990) have shown that the oomycetes are taxonomically distinct from the fungi and are more closely associated to diatoms and brown algae. Until recently, *P. infestans* has thus been relatively poorly studied in comparison to fungi. Nevertheless, the last 10 years has seen an increasing effort to understand the genetics of *P. infestans* and to develop the molecular tools for investigating gene function (Judelson, 1997). This review will serve as an up-date of the advances in *P. infestans* molecular biology and will focus specifically on genomic studies that are helping to unravel the mysteries of pathogenicity and host-specificity.

THE *P. INFESTANS*-POTATO GENE-FOR-GENE INTERACTION

Genetic resistance to *P. infestans* in both wild and cultivated potato species may be of two forms, either race specific or race nonspecific (field or partial resistance) (reviewed in Wastie, 1991). Race specific resistance is characterized by interactions between the products of dominant resistance (*R*) gene alleles in the host and corresponding avirulence (*Avr*) gene alleles in the pathogen, the so-called gene-for-gene hypothesis (Flor, 1971). The result is a form of localized programmed cell death called the hypersensitive response (HR) that prevents a further spread of the pathogen. Race nonspecific resistance is poorly understood, although recent evidence implies a central role for the HR in all forms of resistance to oomycetes (Kamoun *et al*., 1999a; Vleeshouwers *et al*., 2000).

At least 11 single *R* genes for resistance to *P. infestans* (termed *R1–R11*) have been introgressed into the cultivated potato from *Solanum demissum* (Wastie, 1991). A number of these genes have been mapped, including *R1* and *R3* (El-Kharbotly *et al*., 1994; Leonards-Schippers *et al*., 1992), *R2* (Li *et al*., 1998), *R6* and *R7*

(El-Kharbotly *et al*., 1996). However, none of them has yet been isolated. Genetic studies on *P. infestans* have been carried out to reveal the genetic basis of avirulence (Al-Kherb *et al*., 1995; Spielman *et al*., 1989, 1990). These studies have revealed that specificity towards *R* genes in potato is conditioned by single dominant *Avr* genes for most interactions. Nevertheless, an investigation of different isolates of the pathogen has revealed contradictory results, in that *Avr2* and *Avr4* were dominant in some isolates (Al-Kherb *et al*., 1995) and recessive in others (Speilman *et al*., 1989). The differences between these strains may be explained either by independent loci determining avirulence in each, or by the occurrence of epistatic inhibitor loci. Evidence for the latter was presented by Al-Kherb *et al*., 1995 in the case of *Avr10*.

To facilitate the positional cloning of avirulence genes from *P. infestans* a genetic linkage map has been constructed using 183 AFLP and 7 RFLP markers (van der Lee *et al*., 1997). The map contains 10 major linkage groups and represents 1200 cM. Recently, using bulked segregant analysis, the positions of six dominant *Avr* loci have been placed on the map (van der Lee *et al*., 2001). *Avr4* was positioned on linkage group (LG) A2-a, *Avr2* on LG VI, *Avr1* on LG IV and *Avr3*, *Avr10* and *Avr11* were shown to be tightly linked on LG VIII. The clustering of avirulence loci, whilst not observed in the true fungi, has been demonstrated in another oomycete, *P. sojae* (Whisson *et al*., 1995).

The genome size of *P. infestans* is 250 Mb, unusually large for an oomycete (Tooley and Therrien, 1987). A DNA library with large insert sizes and several-fold genome redundancy may thus prove essential for positional cloning. Recently, a Bacterial Artificial Chromosome (BAC) library was constructed from an F_1 individual of the mapping population analysed by van der Lee *et al*. (1997, 2001) possessing all six *Avr* alleles and the AFLP markers linked to them (Whisson *et al*., 2001). The library comprises clones representing 10 genome equivalents, with an average insert size of 98 kb. A three-dimensional pooling strategy was developed for screening the BAC library with AFLP markers and was used to construct a contig of 11 BAC clones spanning the *Avr11* locus (Whisson *et al*., 2001). Work is under way to generate contigs across all of the *Avr* loci, and to seek the genes responsible for the avirulent phenotype.

THE *P. INFESTANS*–PLANT INTERACTION TRANSCRIPTOME

A number of developmental processes are required for *P. infestans* to successfully invade its plant host, including the formation of zoospores, their encystment, the production of a germ tube, and the development of appressoria, hyphae, haustoria and, finally, sporangiophores. Successful infection and the development of disease symptoms are often termed a compatible interaction. If the plant, through mounting a series of defences, is able to interrupt or inhibit any of these processes, colonization of the pathogen, and its further spread to other plants, will be prevented. This is often called an incompatible interaction. The perception of *P. infestans* by its host, and the ability of the pathogen to avoid or overcome the host's defences, implies a complex, dynamic communication network between the interacting organisms. The induction of biochemical response pathways, or the development of cell types specific to the interaction, requires the up- or down-regulation of countless genes. We have recently coined the term 'interaction transcriptome' to mean the sum of the transcripts, from both host and pathogen, that are produced during their association (Birch and Kamoun, 2000). A key initial step in understanding the mechanisms and processes involved in the *P. infestans*–plant interaction involves determining the interaction transcriptome.

The development of low-cost, high throughput DNA sequencing has allowed plant pathology to enter the 'genomics era'. In particular, projects involving large scale sequencing of cDNAs (Expressed Sequence Tags or ESTs) are on-going for a wide variety of crop plants. Recently, EST information has also emerged from *P. infestans* (Kamoun *et al*., 1999b) and *P. sojae* (Qutob *et al*., 2000). Approximately 2000–3000 ESTs from each species are currently housed in the *Phytophthora* Genome Initiative (PGI) database (Waugh *et al*., 2000; http://www.ncgr.org/pgi/index.html). The PGI was set up by researchers from around the world to coordinate genome-scale studies of *P. infestans* and *P. sojae*. Last year, the US Department of Agriculture funded a project to sequence a further 41 000 *P. sojae* and 14 000 *P. infestans* ESTs (http://www.ncgr.org/pgc).

ESTs generated from cDNA libraries constructed from *Phytophthora*-infected plant tissue could be of either pathogen or host origin. Plant and *Phytophthora* EST populations can nevertheless be easily distinguished using bioinformatic analyses. Qutob *et al*. (2000) showed that plant and *Phytophthora* ESTs have a markedly different GC content, and thus most ESTs can be easily distinguished on this basis. Percentage GC content was assessed for sequences from cDNA libraries derived solely from either *P. sojae* or soybean. Both sets of sequences produced distinct, slightly overlapping, normal distribution curves, with the pathogen ESTs averaging 58% GC content and the soybean ESTs 46% GC content (Qutob *et al*., 2000). Similar analysis of sequences from a cDNA library constructed after infection of soybean with *P. sojae* revealed ESTs to be clustered around two peaks corresponding to 46% and 58% GC content. Two-thirds of the ESTs from this library fell into the latter category and were thus predicted to be from the pathogen. Many showed a strong identity to *P. sojae* ESTs from non-interaction cDNA libraries. Additional cDNAs were confirmed to originate from the pathogen by Southern hybridization. A similar difference in GC content has been shown between potato and *P. infestans* ESTs (S. Kamoun; personal communication). Thus, *in silico* analyses can prove powerful in distinguishing candidate plant and pathogen genes in the *Phytophthora*–plant

interaction transcriptome, although it remains to be seen whether clear differences in GC content between host and pathogen cDNAs will be observed in other pathosystems.

Many proteins that play a role in pathogenicity, or elicit a defence response in the plant, are likely to be surface components of the pathogen. Torto *et al.* (2001) have been exploiting the increasing EST data to search for genes encoding potential extracellular proteins in *P. infestans*. They have developed an algorithm called PexFinder V1.0 (where Pex represents *Phytophthora* extracellular protein) to rapidly identify putative secreted or membrane-associated proteins encoded by the ESTs, with the expectation that candidate genes with an essential survival or virulence role would be targeted for downstream functional analyses. To date, analysis of more than 2000 ESTs has revealed 145 independent *Pex* genes, of which 85 show no similarity to sequences in international databases. High throughput functional analyses, using virus and *Agrobacterium* vectors, is currently under way to determine whether they are involved in virulence or avirulence, both in interactions with the host, potato, and in non-host interactions (see below) with a variety of *Nicotiana* spp.

Many of the genes with a key role in either host defence or pathogenicity will be up-regulated during the *P. infestans*–plant association. A number of methods exist for isolating such differentially expressed genes. Pieterse *et al.* (1991, 1993, 1994a,b) isolated a number of *in planta* induced (*ipi*) genes by differential screening of a *P. infestans* genomic DNA library with cDNA derived from mRNA prepared after infection of potato leaves with *P. infestans* and from mRNA prepared after growth of *P. infestans* on a basic medium in culture. Amongst the up-regulated *P. infestans* sequences were the *ipiO* and *ipiB* genes, each of which is a member of a cluster of related sequences (Pieterse *et al.*, 1994a,b). The *ipiO* gene has since been shown to be expressed in invading hyphae during the early stages of infection (van West *et al.*, 1998).

Using standard subtractive hybridization techniques, Görnhaldt *et al.* (2000) isolated a family of mucin-like genes, termed *car* genes, that are up-regulated in germinating cysts shortly before the onset of infection. The *car* genes were shown to be clustered in the genome. The authors postulated that the *car* gene products may serve to provide a mucous cover to protect the germinating cysts from desiccation, physical damage and plant defences.

More recently, a PCR-based method for isolating differentially expressed genes, suppression subtractive hybridization (SSH), has been used to study potato–*P. infestans* interactions. This technique can be readily combined with large-scale sequencing approaches and allows the detection of low-abundance differentially expressed transcripts. This is a major advantage in analysing plant–micro-organism associations, where often only small amounts of biological material are available. SSH has been used to isolate potato genes that are up-regulated in the incompatible (Avrova *et al.*, 1999; Birch *et al.*, 1999) and compatible (Dellagi *et al.*, 2000) interactions with *P. infestans*. Moreover, SSH-derived cDNA populations enriched for sequences expressed specifically at early (15 h post-inoculation) and late (72 h post-inoculation) stages of infection have been generated and used as probes to screen the *P. infestans* BAC library constructed by Whisson *et al.* (2001). Each probe hybridized to a number of BACs, but neither hybridized to the same clones. Quantitative RT-PCR has been used to demonstrate that these BACs contain sequences that are up-regulated specifically at the early or late stages of infection (Avrova, Whisson, de Luca & Birch, unpublished results) and are thus candidates for a role in pathogenicity.

THE MOLECULAR BASIS OF NON-HOST RESISTANCE

Most plant pathogens exhibit specialization and can only infect a limited number of plant species. *P. infestans* is no exception, primarily infecting the leaves of a variety of *Solanaceae*. This implies that the majority of non-host plants possess a series of either pre-formed or inducible mechanisms to successfully prevent infection by this pathogen. It has recently been suggested that non-host resistance to *P. infestans*, and indeed to oomycetes in general, involves the HR, presumably activated by the perception of elicitors generated by the pathogen (Kamoun *et al.*, 1999a; Vleeshouwers *et al.*, 2000). As with race-specific resistance of potato to *P. infestans*, non-host resistance may thus involve a gene-for-gene interaction. This is exemplified by the well-characterized interactions between *Phytophthora* spp. and tobacco.

Most *Phytophthora* spp. studied secrete elicitins, 10 kDa holoproteins that elicit an HR-like response and systemic acquired resistance (SAR) specifically in *Nicotiana* spp. within the *Solanaceae* family (Kamoun *et al.*, 1993; Ricci *et al.*, 1989). Most isolates of *P. nicotiana* which are highly virulent to tobacco, causing the disease black shank, do not secrete these proteins (Kamoun *et al.*, 1993; Ricci *et al.*, 1989). Elicitins are thus believed to act as avirulence factors in tobacco–*Phytophthora* interactions.

Similar to many other *Phytophthora* spp. that have been studied, *P. infestans* contains a family of elicitin-like genes (Kamoun *et al.*, 1997a, 1999b). The gene *inf1*, encoding the major elicitin secreted by *P. infestans*, is highly expressed in mycelium grown in various culture media, but is not expressed in sporangia, zoospores, cysts or germinating cysts. During infection, the gene is down-regulated in the early, biotrophic stages of the interaction, but is highly expressed in the later stages of infection, when profuse sporulation and necrosis occur (Kamoun *et al.*, 1997b). A gene-silencing strategy has been adopted to inhibit INF1 production. INF1-deficient strains are still pathogenic on potato, but also induce disease lesions when inoculated on *Nicotiana benthamiana*. In contrast, wild-type *P. infestans* elicits a typical, localized necrosis on this non-host plant, indicating that INF1 functions as an avirulence factor in the *P. infestans–N. benthamiana* interaction (Kamoun *et al.*, 1998a).

TOOLS FOR THE STUDY OF GENE FUNCTION IN *P. INFESTANS*

For any aspect of gene function to be assessed in *P. infestans*, it is essential that a DNA-mediated transformation system is available. The first reported transformation of *P. infestans* was by Judelson *et al.* (1991), albeit at low efficiency, using protoplasts. Nevertheless, it has been used successfully for anti-sense inhibition of a transgene (Judelson *et al.*, 1993) and for co-transformation using intermolecular ligation (Judelson, 1993). It has also been used to develop an *in planta* reporter system (e.g. Kamoun *et al.*, 1998b) and for heterologous expression in *P. infestans* of a gene from another *Phytophthora* spp. (Panabières *et al.*, 1998). However, the transformation procedure used in these reports required the digestion of the cell wall using Novozym 234, an enzyme mixture that is no longer produced. Therefore, different methods for transformation must be adopted. One such method, utilizing microprojectile bombardment to transform *P. infestans*, has recently been reported (Cvitanich and Judelson, 2001) and overcomes the requirement for protoplast formation with Novozym.

For map-based cloning of genes and studies of genome structure and organization, two BAC libraries have been constructed from *P. infestans*. Randall and Judelson (1999) reported the generation of a library comprising fourfold genome coverage and an average insert size of 75 kb, and Whisson *et al.* (2001) have generated a BAC library comprising 10-fold genome coverage and an average insert size of 98 kb. Randall and Judelson (1999) reported the transformation of entire BAC clones into *P. infestans*, making it possible to determine the presence of a gene, such as an avirulence gene, within large regions of the genome.

P. infestans is diploid, making it difficult to perform gene knockouts, as is often done in the fungi that have a haploid stage in their life cycle. The occurrence of post-transcriptional gene silencing in *P. infestans* (van West *et al.*, 1999) is therefore a promising development in the area of *P. infestans* functional genomics. However, at this time gene silencing in *P. infestans* is not yet routine and its molecular basis is poorly understood. The utility of gene silencing as a functional tool in *P. infestans* has been shown by the development of transgenic strains silenced for transcription of the elicitin-encoding *inf*1 gene (see above) (Kamoun *et al.*, 1998a).

Functional analysis of cloned *P. infestans* genes may also be performed without transformation or silencing in *P. infestans*. Evidence that the cloned *Piypt* gene from *P. infestans* was involved in vesicle transport and secretion was provided by functional complementation of a *Saccharomyces cerevisiae* mutation in the equivalent gene (Chen and Roxby, 1996). This approach could be extended to other genes in *P. infestans* that are highly conserved in yeast, and for which yeast mutants exist.

The construction of a binary Potato Virus X (PVX)-*Agrobacterium* vector (Takken *et al.*, 2000) for expression of pathogen genes *in planta* will allow investigation of the functions of *P. infestans* genes products which interact directly with host components. High throughput analysis of such genes, which might encode potential non-host elicitors or avirulence genes, is currently under way (Torto *et al.*, 2001).

CONCLUSIONS AND FUTURE PROSPECTS

A wealth of EST information is being generated from *P. infestans* and from the host plants with which it interacts, and new bioinformatic tools are being developed to interpret this explosion of data. BAC libraries are facilitating not only map-based cloning of genes involved in development and avirulence, but also fundamental studies of genome structure and organization in this pathogen, and plans are afoot to integrate transcriptional, physical and genetic maps. Proteomic studies of *P. infestans* have recently been initiated to identify developmental stage-specific and extracellular proteins (van West *et al.*, 2001) and these studies will complement on-going, targeted gene discovery using techniques such as SSH. Novel methods for DNA-mediated transformation and gene silencing in *P. infestans* have been developed, although these are not yet sufficiently high throughput to analyse the functions of hundreds of candidate virulence and avirulence genes that are being identified. Nevertheless, a role for some of these genes may be determined by direct expression in host or non-host plants using virus and *Agrobacterium* vectors. *P. infestans*, along with its oomycete relatives *P. sojae*, a soybean pathogen, and *Peronospora parasitica*, pathogen of the model plant *Arabidopsis thaliana*, are starting to embrace the genomics era. Comparative genomics between these developing model oomycetes, all very different in their life styles and modes of infection, will inform us about the requirements for survival and pathogenicity in this hugely important and poorly understood group of organisms.

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