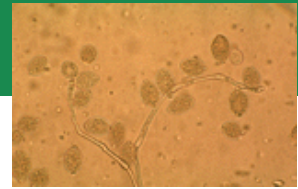


The Pathogen: Molecular Approaches



Research Investments in *Phytophthora infestans*: How to get to know the Pathogen?

Francine Govers¹

I would like to thank the organizers for giving me the opportunity to speak at this meeting.

I don't have to show this audience how disastrous the late blight disease can be. We all have the same long-term aim: to find ways to grow potatoes without continuous danger of crop loss due to the disease. There are various strategies one can follow to reach this goal or, in other words, there are many roads that lead to Rome.

If one describes a disease cycle —any disease cycle—the terminology used has much in common with that used in the eight o'clock news when the newsreader reports on yet another conflict anywhere in the world, whether it is in Kosovo, the Middle East, Africa or even in Ecuador, as I have seen last week. You hear "invasion", "attack", and "defense barriers" and, sadly enough, even "death and destruction".

In warfare, there are many different strategies to fight the enemy. One extreme is to build defense barriers that are strong enough to keep the enemy away from your territory, like the Berlin or Chinese Walls. Another extreme is to fight the enemy in his own territory or, in a very crude way, completely destroy the enemy and even destroy his territory. In this respect, one could think of the use of Agent Orange in the Vietnam War.

In a sense, one can consider breeding for late blight resistant potato lines —whether by classical breeding, molecular breeding or through marker-assisted breeding— as the one extreme: build defense barriers

and do not give the enemy an entry. The other extreme is to spray chemicals and completely eradicate the enemy. However, this involves all the negative side effects that chemicals may have on other, even beneficial, organisms.

Obviously, as in the real world, there is something in between. In the fight against late blight, I'm proposing a cold war strategy. I want to invade the territory of the enemy. In this allegory, the enemy is *Phytophthora infestans*.

I don't want to destroy the enemy in a very crude way. I respect the enemy. It is a very intriguing living creature, with all its peculiarities. It is also very challenging, from a biological point of view, to learn about this organism— its secrets, its biological niche, how it survives, propagates, spreads, attacks, invades and how it outsmarts us over and over again and gets around the defense barriers that we human beings try to create in the host plants.

So in my daily work, I consider *Phytophthora* a friend, not an enemy. And as you get to know a friend better and better, you will know his lifestyle, and you will also get to know what is vital for this friend to survive.

I would like to share with you the strategies that we Phytophthorologists exploit to get to know our friend. But before I do so I want you to get to know my friend a little bit better.

Phytophthora's have a fungal-like growth morphology, but strictly speaking, *Phytophthora* is not a fungus.

¹ Wageningen Agricultural University, The Netherlands

Oomycetes, the class to which the genus *Phytophthora* belongs, evolved independently from the higher fungi, such as the Ascomycetes and Basidiomycetes.

There is an extensive list of characteristics by which oomycetes distinguish themselves from higher fungi. I will not go into detail on all these differences, but the bottom line is that techniques that are available to study fungi at the biochemical, molecular and genetic level can not necessarily be applied to study oomycetes. These are the most important features of *Phytophthora infestans* that I want you to keep in mind: *P. infestans* is heterothallic with two known mating types, A1 and A2. The somatic cells, the hyphae, are diploid and can even be polyploid, and the genome size is extremely large—250 megabases, which is more than two times that of *Arabidopsis* and more than ten times the size of yeast. I want to stress the fact that this large genome is not a general feature of all *Phytophthora* species. Other species tested have sizes of around 50 – 60 megabases.

I would like to distinguish two levels of research. On the one hand there are studies at the population level and on the other hand studies at the cellular or molecular level.

The ability to identify isolates by means of DNA fingerprinting provides information on epidemiology, on population structure even on a worldwide scale, on the variability within populations, on survival in natural conditions, and on infection sources. RG57, the well-known DNA fingerprint probe derived from Bill Fry's lab, has been instrumental for studies at the population level. I will not go into detail, as you will hear more about these studies from the other speakers this afternoon and tomorrow. Instead, I would like to concentrate on studies at the cellular and molecular level.

Access to this level is obtained by isolating and cloning genes of interest. When dealing with a pathogen, genes of interest are those genes that play a role in the interaction with its host, for example, genes encoding pathogenicity factors. One can then study expression of these genes, find out how they are regulated and, in the end, get to know what their function is in the complicated network that defines a living creature. My lab and a few others have only in the last decade concentrated efforts on isolating genes of *Phytophthora infestans*.

To give an idea of how fast (or I should say how slow) progress is in this field, I refer to a table taken from the Ph.D. thesis of Corne Pieterse in 1993. The table lists

13 sequences but, in fact, these represent only eight genes or gene families of the estimated 15000 genes of *P. infestans*

Recently I screened the database and found out that it contains 48 *P. infestans* accessions, of which more than half are sequences that do not encode proteins, but represent repetitive non-coding sequences, including ITSs of ribosomal RNA genes. If one compares carefully, only three new genes or gene families have been added in six years time. That is not much.

However, that doesn't mean that we were just sitting there waiting for something to happen. On the contrary, besides isolating and characterizing genes, we have been working on tools that are crucial, or at least very desirable, to have when one wants to study gene function in an organism. And since oomycetes are so different from other organisms, we had to develop those tools and adapt the protocols to make them work in *P. infestans*.

It is very crucial to have the ability to reintroduce isolated genes into their original background, either after modifying them or after fusing the coding sequence to a constitutive promoter. DNA transformation of *P. infestans* was first accomplished in the early nineties by Howard Judelson, at that time at UC Davis. That was certainly a milestone in *Phytophthora* research. The selectable antibiotic resistance genes that are used are hygromycin and geneticin, but in all cases expression of these genes has to be regulated by an oomycete promoter. Gene promoters from higher fungi or plants do not function in *Phytophthora*.

DNA transformation allows you to study gene expression by using reporter genes. The reporter gene we have been using is the GUS gene, which is also used extensively in plants. I'm going to give you one example: Pieter van West, a PhD student in my lab, has been studying a gene that we have called *ipiO* that belongs to the category of *in planta* induced genes. By making use of a gene promoter-GUS fusion we demonstrated that *ipiO* is highly expressed in the hyphal tips at the edge of the expanding lesion where the pathogen is invading healthy plant cells. This suggests that *IPI-O* is localized at the interface between the invading hyphae and the plant cells, and could play a role in pathogenicity.

Another crucial technique to study gene function is inactivating a particular gene. Since oomycetes are diploid, it is not possible to simply inactivate a gene by homologous recombination, as is done in yeast or *Aspergillus*. In plants, also diploids or polyploids, homology-dependent gene silencing is a frequently used

approach to inactivate genes. So we choose to apply a similar approach to inactivate genes in *Phytophthora infestans*. As a target gene, we took the elicitor gene *inf1*. One reason to take this single copy gene as a target is that the protein derived from the gene, the elicitor INF1, is very easily detected. It is secreted in the culture filtrate in huge amounts. Therefore, inactivation of the gene can be easily monitored. We transform the constructs to *P. infestans*. We have done some studies on the mechanism of inactivation, but I will not go into detail on that now. The bottom line is that we have shown we can perform targeted mutagenesis in *P. infestans* for this particular gene *inf1*. The next step will be to demonstrate that we can inactivate other genes by a similar approach.

As I mentioned earlier, we chose *inf1* because we can easily monitor inactivation, but of course, we also were very much interested in the phenotype of the INF1 deficient mutants. INF1 is an elicitor that has elicitor activity on tobacco. Injection of purified elicitor in tobacco causes a hypersensitive response and, based on biochemical data, it was suggested that elicitors might function as species-specific avirulence factors.

To test this hypothesis, we needed *Phytophthora* strains that do not produce elicitor. As I mentioned, we obtained those strains by transformation of *P. infestans* with sense and antisense *inf1* constructs. When we tested those transformants on various plant species, we found that INF1 deficient mutants have an extended host range. They can still infect potato and tomato but, in addition, they are able to infect *Nicotiana benthamiana*. Based on those results, we concluded that resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of elicitor protein INF1.

To conclude, I have shown you the DNA transformation, the reporter gene and the targeted mutagenesis and, as examples, I talked about just two genes that are very interesting in terms of the interaction. One (*ipiO*) that has such a specific expression pattern that it makes one wonder what its function is during the interaction with the host; and the other gene (*inf1*) which encodes an elicitor and thus plays a role in eliciting defense responses, in this case, in non-host plants. Just two genes, but we expect *P. infestans* to have around 10000-15000 genes, so how do we select the genes of interest?

I have listed various approaches to select genes. Some of these have been used or are being used to isolate *P. infestans* genes. From the genes that have been isolated from *P. infestans*, some are housekeeping genes that have been isolated based on homology with similar genes

from other organisms. One can do differential screening and select for genes that are expressed in certain developmental stages. I already mentioned the *in planta* induced genes and these have been selected by a black box approach. One doesn't know beforehand what kind of genes one will isolate, as in the case of *ipiO* with no homology to any known gene. Reverse genetics have been applied to isolate elicitor genes. Abundant proteins which can be easily purified and based on the amino acid sequence PCR primers are designed and the gene is isolated. Positional cloning is another approach, a very labor-intensive approach, I would say, but for some genes it is the only approach that one can apply. Howard Judelson, now at UC Riverside, is hunting for the mating type locus and the metalaxyl resistance gene. He has linked DNA markers and BAC libraries, so that should lead to something sooner or later. In my lab, Theo van der Lee is using the positional cloning approach to obtain avirulence genes, the pathogen's counterparts in the R gene-for-avr-gene interaction. Identification of these genes will teach us how *P. infestans* is able to avoid recognition by major R genes and how the race specificity changes so easily in a population when there is pressure from a major R gene on the population. Attempts to isolate the race-specific elicitors by a biochemical approach were unsuccessful, so the positional cloning approach is the only method we can use to isolate these genes. Random mutagenesis or transposon tagging might be additional approaches but, as they have not been explored for *P. infestans*, we have to go with positional cloning.

Just as an update, we have constructed the first molecular genetic linkage map of *P. infestans* using AFLP markers. The map contains 10 major linkage groups, possibly representing 10 chromosomes, and a few minor groups which will probably be integrated in the major groups when we analyze more markers. On this map we positioned the mating type locus and four avirulence genes, one cluster of three containing *Avr3*, *Avr10*, *Avr11* and *Avr4*. They are all dominant. More markers linked to this avr cluster were selected by bulked segregant analysis. We pooled DNA of virulent and avirulent progeny and screened a high numbers of markers for linkage. We now have a high-density linkage map that should form the basis for cloning these avirulence genes.

Last, but not least, advances in technology related to genomics now allow us to sequence complete genomes of model organisms within a limited time frame. Information that is gained from random sequencing projects is extremely valuable and it is certainly the fastest way to obtain genes of interest from any organism, even *Phytophthora*. With that in mind, we started in

1997 with a pilot-scale EST project. This project is a collaboration between three labs and a bioinformatics institute, NCGR. We preferred an EST approach to genomic sequencing. The genome size of *P. infestans* is simply too large to accomplish complete sequencing of the genome when taking into account the limited input of manpower and resources. Two weeks ago, we submitted a manuscript with the first data of this EST pilot study— the first 1000 Expressed Sequence Tags derived from genes expressed in mycelium of *P. infestans*.

The power of EST sequencing has tremendously increased the number of accessions of expressed *P. infestans* sequences. Among the genes that we have sequenced there are several candidates that merit further investigation. We have classified the ESTs in functional categories. Several genes encode housekeeping enzymes; others can be expected to have functions in signaling, in cell cycle control, and in cell division. We found some genes with high similarity to unique plant genes and we found a whole new group of genes that encode elicitor-like proteins such as members of the family to which INF1 belongs. Those genes were not isolated by other approaches that we applied (such as PCR or heterologous hybridization) to get members of this family. This shows how powerful random sequencing is. All these genes are expressed in mycelial stages. It will be extremely informative to get similar information about genes expressed in other developmental stages, in particular in the pre-infection stages and in the *in planta* growth stages. Based on the sequence data and the expression patterns, we will be able to select genes for further study. In my laboratory, we are particularly interested in genes involved in the various signal transduction pathways because we

expect these pathways to be potential targets for control. Also, we will continue to study the elicitors and the avirulence and pathogenicity factors. This will, we hope, teach us how our friend *Phytophthora* is able to invade and destroy the host plant, and what type of signals elicit defense response.

I want to conclude with a final statement. Research investments in *Phytophthora infestans* are worth it. The result is that we will get to know the pathogen and, within a few years, we will know the vital links in the lifecycle and how we can use this knowledge for control of late blight. We now have fast methods to identify genes and to determine the function of genes. We have an enthusiastic group of *Phytophthora* researchers in various labs worldwide who are prepared to go for it. We could, however, use a bit more financial support. I hope I have convinced you that the cold war strategy—getting to know the enemy— is worth exploring in the fight against late blight, and that this knowledge will be very useful, maybe even essential, in designing molecular breeding strategies and in developing new control agents.

I would like to thank all the people who were and/or still are members of the *Phytophthora* research group in Wageningen over the last nine years and the various collaborators from other labs. In my presentation I mentioned the work of Pieter van West, Theo van der Lee and Sophien Kamoun. The EST project is a collaboration with Donald Nuss at the University of Maryland; Sophien Kamoun, who is now at Ohio State University; and the bioinformatics specialists Bruno Sobral and Peter Hrabec at NCGR in Santa Fe.