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Towards the identification of avirulence genes from *Plasmopara viticola*, the causal agent of grapevine downy mildew

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Grapevine Downy Mildew, caused by the biotrophic Oomycete *Plasmopara viticola,* is one of the most important diseases affecting vineyards. The pathogen attacks grapevines worldwide causing important economical losses. The current strategy to control the disease relies totally on the use of chemical fungicides. This practice not only is expensive (in France alone its cost is estimated to be around 150 million euro per year) but also causes a slow and progressive damage to the environment. On top of this, the arising of pathogen strains resistant to fungicides diminishes the efficiency of this practice.

An alternative to the systematic use of fungicides is the use of varieties showing resistance to the pathogen, which is cost-effective and environment friendly. However, since all cultivated European grapevine varieties are susceptible to *Plasmopara viticola*, the resistance needs to be introduced from other *Vitaceae* through breeding programmes that ensure that the agronomically and technologically important characteristics of the varieties are maintained. Breeding programmes have resulted in the creation of downy mildew resistant varieties that are currently grown on limited acreages, such as Regent and Solaris. At INRA Colmar we are developing a breeding programme for resistance to grapevine downy mildew that exploits mainly the resistance to *P. viticola* found in *Muscadinia rotundifolia*, whereas other sources of resistance are being characterised.

Efficient breeding for disease resistance requires a solid understanding of the pathosystem. In order to ensure that the resistance will be efficient and durable we need, on one hand, to understand the genetic and molecular basis of the different sources of resistance, and, on the other hand, to evaluate the genetic diversity and evolutionary potential of the pathogen populations. The last few years have witnessed progress in our knowledge about the genetic diversity of *P. viticola* populations as well as in the characterisation of the genetic basis of the resistance derived from *M. rotundifolia, Vitis riparia* and the varieties Regent and Bianca.

Despite the advances towards the characterisation of genetic basis of the resistance to grapevine downy mildew nothing is known about the nature of the corresponding avirulence genes from the pathogen. Plant disease resistance proteins recognise pathogen-encoded factors, so called avirulence genes, and trigger defense responses leading to pathogen arrest. The identification of avirulence genes is a necessary step not only to understand the biology of the interaction but also to design appropriate strategies to fight the pathogen. The analysis of variability for avirulence genes in pathogen populations is an important tool for the prediction of the evolutionary potential of the pathogen, which in turn is useful to design adequate strategies for the efficient deployment of durable resistances. Moreover, the isolation and characterisation of the avirulence genes corresponding to known resistance genes allows to estimate the durability of the resistance based on the penalty imposed upon the pathogen by the resistance gene.

Avirulence genes identified so far from Oomycetes are small secreted proteins, carrying a signal peptide and a particular RXLR motif, whose expression is induced upon infection and whose ectopic expression cause an hypersensitive response in the presence of the corresponding resistance gene. Thus, based on knowledge from other Oomycetes, *P. viticola* effectors can be identified using a candidate gene strategy based on data mining of genomic resources. Public genomic resources of *P. viticola* are very limited, not to say inexistent. A recent search at NCBI/EMBL databases produced 79 *P. viticola* entries (10 ESTs and 69 core nucleotides), the majority sequences of mitochondrial or ribosomal origin. In consequence, the identification of avirulence genes by means of a candidate gene strategy requires developing genomic resources for *P. viticola.*

Since the expression of avirulence genes is induced upon infection, we first attempted to obtain a cDNA library enriched for sequences induced upon infection using suppression subtractive hybridization (SSH): cDNAs from in-vitro germinated cysts and from chemically induced plant leaves were subtracted from cDNA from infected leaves. Using this procedure we expected to minimize the amount of pathogen-induced grapevine genes included in the library as well as the pathogen housekeeping genes. The results obtained with this strategy were disappointing: there were a very low proportion of pathogen sequences and the sequences were truncated, which makes them not suitable for a candidate-gene approach.

As an obligate biotrophe, *P. viticola* can only grow on living tissues and the pathogen biomass in the invasive stages of infection is quite low compared to the plant biomass. Nevertheless, zoospores are easily obtained by washing off sporangia from leaves at the late stages of infection. Interestingly, the first stages of pathogen development (growth of germinative tubes and vesicle formation) can be reproduced in vitro just by adding sodium chloride to an aqueous suspension of spores (Figure 1).

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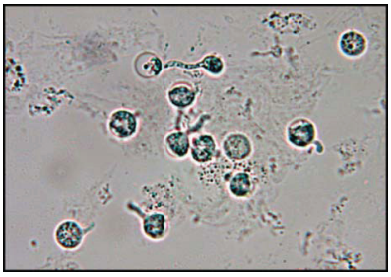

Figure 1*: In vitro* germinated spores of *Plasmopara viticola*

We obtained two cDNA libraries, one from grapevine leaves in the invasive phase of *P. viticola* infection (non-sporulating) and a second one from *in vitro* germinated zoospores. The availability of the grapevine genome sequence and the genome sequences of several Oomycetes allows us to distinguish between sequences from plant and pathogen origin. Sequencing an aliquot of clones from each library showed that 15-20% of sequences of the cDNA from infected tissue are expected to be from pathogen origin, while around 5% of sequences from the cDNA from germinated spores are from plant origin, due to contamination in the purification procedure. Sequencing 1920 clones from the library obtained from germinated zoospores resulted in the identification of 1063 nuclear ESTs (Expressed Sequence Tags) from *P. viticola*, the rest belonging to sequences from ribosomal, mitochondrial and plant origin. Sequence analysis revealed the presence of 58 ESTs from genes putatively involved on pathogenicity (secreted proteins, glucanase-inhibiting proteins, RXLR proteins, protease inhibitors, etc) and thus candidates to behave as avirulence genes.

In summary, in vitro germinated zoospores proved to be a suitable material for the identification of avirulence genes. The availability of next-generation sequencing technologies allows obtaining huge amounts of sequence data at a reasonable cost. Sequencing cDNA from germinated spores using such technologies should provide us with the genomic resources necessary to identify avirulence genes using the above-described candidate-gene based approach.

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