Emerging technologies / Technologies naissantes

From sequence to phenotype: functional genomics of *Phytophthora*


Abstract: Oomycetes, such as *Phytophthora*, downy mildew causal agents, and *Pythium*, form a unique branch of eukaryotic-plant pathogens with an independent evolutionary history. Among the oomycetes, *Phytophthora* spp. cause some of the most destructive plant diseases in the world, and are arguably the most devastating pathogens of dicotyledonous plants. Large scale DNA sequencing (genomics) approaches promise to impact our understanding of the molecular basis of pathogenicity and host specificity in *Phytophthora* by facilitating the isolation of novel virulence and avirulence genes, as well as by helping to identify targets for chemical control. Structural genomic studies of *Phytophthora* are well under way. The challenge in the postgenome era is to link a sequence to a phenotype with as little experimental effort as possible, using computational tools for data mining and robust high throughput functional assays. In this article, we review our strategy of applying a sequence-to-phenotype (or functional genomics) paradigm to the discovery of novel virulence and avirulence genes, as well as the identification of novel fungicide targets in *Phytophthora*.

Key words: bioinformatics, data mining, gene silencing, gene knockout, complementation, virus expression systems, *Agrobacterium tumefaciens*.

Introduction

Oomycetes, such as *Phytophthora*, downy mildew causal agents, and *Pythium*, form a unique branch of eukaryotic-plant pathogens with an independent evolutionary history (Kamoun et al. 1999c; Kamoun 2000, 2001). Among the oomycetes, *Phytophthora* spp. cause some of the most destructive plant diseases in the world, and are arguably the most devastating pathogens of dicotyledonous plants (Erwin and Ribeiro 1996; Kamoun 2000). For example, *Phytophthora infestans* (Mont.) de Bary, the cause of the Irish potato famine, remains a destructive pathogen causing multibillion-dollar losses in potato and tomato production (Fry and Goodwin 1997a, 1997b). Other economically important diseases include root and stem rot caused by *Phytophthora sojae* (Kaufman & Gerdemann), which hampers soybean production in several continents, black pod of cocoa caused by *Phytophthora palmivora* (Butl.) Butl. and *Phytophthora megakarya* Bras. & Griff, a recurring threat...
to cocoa production, and sudden oak death caused by a recently discovered *Phytophthora* sp. that is decimating oak trees along the Pacific coast of the United States (Rizzo et al. 2001). Typically, these destructive diseases are difficult to manage, and sources of sustainable genetic resistance are limited.

Large scale DNA sequencing (genomics) approaches promise to impact our understanding of the molecular basis of pathogenicity and host specificity in *Phytophthora* by facilitating the isolation of novel virulence and avirulence genes, as well as by helping to identify targets for chemical control (Kamoun et al. 1999b; Qutob et al. 2000). Based on these premises, we and others in the *Phytophthora* research community have embarked upon cDNA and genomic sequencing projects that provided a first insight into gene diversity in *Phytophthora* (Kamoun et al. 1999b; Qutob et al. 2000; Waugh et al. 2000). However, once large sets of sequence data are available, the research focus needs to shift to functional analyses of the newly discovered genes. The challenge in the postgenome era is to link a sequence to a phenotype with as little experimental effort as possible, using computational tools for data mining and robust high throughput functional assays. To achieve this, we developed a sequence-to-phenotype (or functional genomics) paradigm for the discovery of novel virulence and avirulence genes, as well as for the identification of novel fungicide targets in *Phytophthora* (Fig. 1). In this article, we review our progress in applying this paradigm to plant pathogenic *Phytophthora* spp.

### Genomic databases for *Phytophthora*

Structural genomic studies of *Phytophthora* are well under way within the framework of the *Phytophthora* Genome Initiative (Waugh et al. 2000). These studies include expressed sequence tag (EST) projects from a variety of developmental and infection stages as well as targeted sequencing of contigs of bacterial artificial chromosome clones, representing selected regions of the genome. Completed pilot cDNA sequencing projects resulted in ca 2500 ESTs for *P. infestans* and 3000 ESTs for *P. sojae* (Kamoun et al. 1999b; Qutob et al. 2000) and were compiled in searchable databases available at the Web site of *Phytophthora* Genome Initiative (Waugh et al. 2000). Recently, funding for an additional 50 000 ESTs from a series of in vitro and potato infection stages has been secured (B. Tyler, personal communication). These sequences will be compiled at www.ncbi.nlm.nih.gov/ in an expanded and improved version of the *Phytophthora* Genome Initiative database, named *Phytophthora* Genome Consortium. In addition, an industry-funded genomics consortium has been initiated with academic collaborators and will complement public-sector efforts (Lam et al. 2001).

### Mining for candidate genes

We developed a series of specific criteria to select candidate genes for functional assays (Table 1). The rationale behind these criteria is that genes exhibiting the listed features are more likely to be involved in virulence or avirulence and could serve as optimal targets for fungicides. We designed specific algorithms and various data mining strategies to identify *Phytophthora* genes encoding degradative enzymes and extracellular proteins, as well as genes that are up-regulated during preinfection and infection stages and that are conserved among several pathogenic oomycetes. Such in-silico approaches yield lists of candidate genes that can be tested experimentally to confirm the predicted features. In addition, the selected candidate genes can then be functionally assayed using one or several of the assays described below.

### Functional assays in *Phytophthora*

Currently, gene silencing is the only proven technology to generate *Phytophthora* strains deficient in particular gene products (Kamoun et al. 1998; van West et al. 1999). How-

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**Table 1.** Examples of features used for selecting candidate genes from sequence databases of *Phytophthora*.

<table>
<thead>
<tr>
<th>Selection criteria</th>
<th>Rationale</th>
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<tr>
<td>Degradative enzymes</td>
<td>Putative virulence factors involved in host tissue penetration and degradation</td>
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<tr>
<td>Extracellular proteins</td>
<td>More likely to be involved in cross-talk with plants</td>
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<tr>
<td>Up-regulated during preinfection and infection stages</td>
<td>More likely to be involved in pathogenicity or virulence: putative fungicide targets</td>
</tr>
<tr>
<td>Conserved among pathogenic oomycetes</td>
<td>Putative fungicide targets</td>
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ever, this approach has only been proven for a handful of genes so far, and it remains to be determined whether it can be systematically applied. Gene silencing can also be associated with a lack of specificity if a family of closely related genes is targeted. There are, however, a number of advantages in using gene silencing to generate strains with altered phenotype. For example, no specific plasmid needs to be constructed for the transformation experiment since van West et al. (1999) showed that a promoterless full-length cDNA clone of the \textit{inf1} gene could be used without modifications to transform \textit{P. infestans} and generate silenced strains. Therefore, strategies might be devised to assay pools of cDNA clones simultaneously. Another exciting prospect of gene silencing for functional genomic analyses is the observed spread of the silenced state from silenced transgenic nuclei to nontransformed nuclei, when mixed together in a heterokaryotic strain (van West et al. 1999). This suggests that it might be sufficient to engineer silencing in a limited number of nuclei of a hyphae to ultimately silence the entire mycelium.

Other methods for targeted gene knockout are still being developed (Kamoun 2000). A strategy for ribozyme-mediated disruption of gene expression is being developed by the laboratory of Venkat Gopalan (Department of Biochemistry, Ohio State University) in collaboration with our group. Gene disruption through homologous recombination has only been examined superficially in \textit{Phytophthora}. Transposon mutagenesis, with either endogenous or ubiquitous transposons, is also a potentially useful technique. For both of these techniques, and considering that \textit{Phytophthora} is a diploid at the vegetative stage, it will be more judicious to use homothallic species, such as \textit{P. sojae}, instead of a heterothallic species such as \textit{P. infestans}. This will allow facile selfing of the transformed strains to recover a progeny that is homozygous at the mutated locus.

Functional assays in heterologous oomycetes

In addition to loss of function assays, gain of function or complementation assays can be set up using heterologous \textit{Phytophthora} spp. or even heterologous pathogenic or saprophytic oomycetes. Candidate genes of interest can be used to transform heterologous species that are known to lack a functional copy of the gene or carry a mutated ortholog. Traditionally, gain of function experiments have been extensively used in studies on plant pathogenic microorganisms to identify genes encoding avirulence and virulence functions. In \textit{Phytophthora}, Panabières et al. (1998) described functional expression of the gene from \textit{Phytophthora cryptogea} Pethybr. & Lafferty encoding the basic eliciten cryptogene in \textit{P. infestans}. A gain of function approach should prove appropriate for many classes of genes. In addition, complementation assays are technically easier to perform than gene knockout experiments.

Functional assays in plants

Plant pathogenic microbes produce an extraordinary diversity of signal molecules, encoded by virulence genes, that can manipulate molecular and cellular processes in plants by inducing symptom-like responses and altering defense responses. Ectopic expression of single pathogen genes in plant cells often leads to phenotypic effects. Typically, expression of bacterial, fungal, or oomycete avirulence genes in plant cells that contain the matching resistance gene results in cell death typical of the hypersensitive response (Kamoun et al. 1999a; Kjemtrup et al. 2000; Lauge and De Wit 1998). Therefore, plant gene expression systems can be adapted to systematically screen large numbers of \textit{Phytophthora} genes for functional response in plants.
In our laboratory, we routinely perform high throughput functional expression screens of *P. infestans* cDNAs in tomato and tobacco using vectors based on *Potato virus X* (PVX) and *Agrobacterium tumefaciens* (Smith & Townsend) Conn (Kamoun et al. 1999a; Van der Hoorn et al. 2000). This approach allowed us to identify cDNAs that induce resistance responses and disease-like symptoms (Testa and Kamoun 2001; Torto et al. 2001). Examples of functional expression of *Phytophthora* genes in plants are shown in Fig. 2.

**Future directions**

With the advent of genomic technology, the pace of discovery and functional analyses of *Phytophthora* genes has greatly accelerated. Here, we outlined our strategy for systematic mining of the genome sequence data, selection of candidate genes, and high throughput functional screening of selected genes. In the future, this approach will be enhanced by additional improvements in computational methods and algorithms for data mining and increased sequence data for oomycetes and other organisms. In addition, even though ectopic expression of pathogen genes in plant cells can now be performed at a remarkable high throughput rate, there is still a need for improvement and adaptation to large-scale analyses of the gene knockout and complementation assays currently available for *Phytophthora*.

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**References**


