Emerging technologies / Technologies naissantes

From sequence to phenotype: functional genomics of *Phytophthora*

S. Kamoun, S. Dong, W. Hamada, E. Huitema, D. Kinney, W.R. Morgan, A. Styer, A. Testa, and T.A. Torto

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*.

Résumé : Les oomycètes, tels que le *Phytophthora*, les agents du mildiou et le *Pythium*, forment un embranchement unique parmi les agents pathogènes des plantes eucaryotes, avec une évolution indépendante. Parmi les oomycètes, les *Phytophthora* spp. sont responsables de maladies parmi les plus destructrices des plantes et sont probablement les agents pathogènes les plus dévastateurs des dicotylédones. La venue du séquençage de l'ADN à grande échelle (la génomique) laisse présager une compréhension nouvelle des assises moléculaires de la pathogénicité et de la spécificité d'hôte du *Phytophthora* en simplifiant l'isolement de nouveaux gènes virulents et avirulents de même qu'en nous aidant à identifier des cibles pour la lutte chimique. Les études en génomique de structure du *Phytophthora* vont bon train. Le défi de l'ère postgénomique est de relier les séquences aux phénotypes, avec un minimum de nouvelles expériences, à l'aide d'outils informatiques capables d'exploiter les données et d'essais sur les fonctions à la fois robustes et à haut débit. Dans cet article, nous examinons notre stratégie consistant à utiliser un paradigme de séquence-à-génotype (ou génomique fonctionnelle) à la découverte de nouveaux gènes virulents et avirulents aussi bien qu'à l'identification de nouvelles cibles pour les fongicides dans le *Phytophthora*.

Mots clés : bioinformatique, exploitation de données, silençage génique, inactivation génique, complémentation, systèmes d'expression virale, *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

Accepted 28 November 2001.

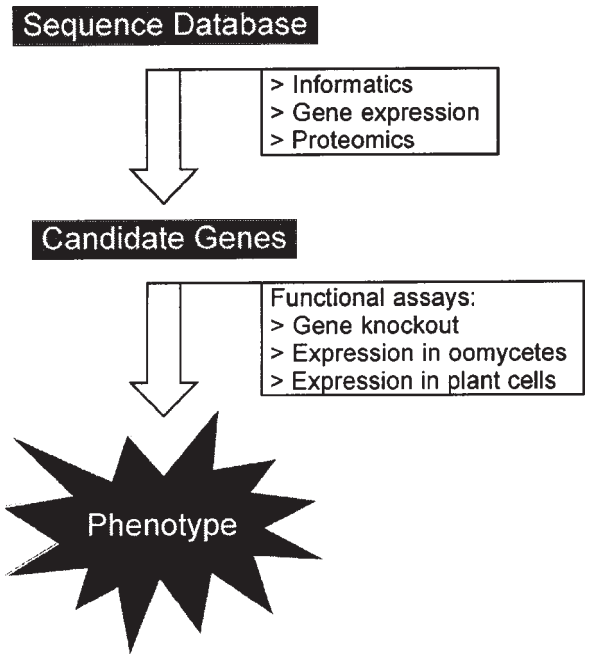
S. Kamoun,[1] S. Dong, W. Hamada, E. Huitema, D. Kinney, A. Styer, A. Testa, and T.A. Torto. Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH 44691, U.S.A.
W.R. Morgan. Department of Biology, The College of Wooster, Wooster, OH 44691, U.S.A.

[1]Corresponding author (e-mail: kamoun.1@osu.edu).

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.

Fig. 1. Flowchart illustrating the sequence-to-phenotype paradigm as applied to *Phytophthora*. The two-step process consists first of mining for candidate genes from sequence databases using informatics, gene expression proteomics, or a combination of these technologies. Then, functional assays are applied to the candidate genes to identify those that confer a desirable phenotype.



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.ncgr.org/pgc 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.

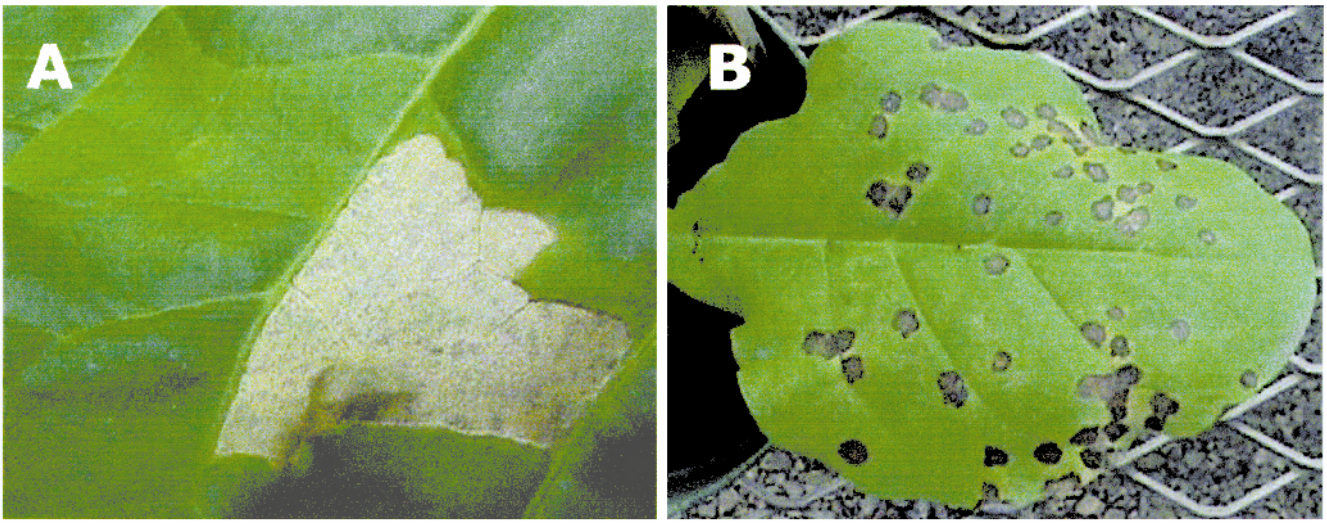
Table 1. Examples of features used for selecting candidate genes from sequence databases of *Phytophthora*.

Selection criteria	Rationale
Degradative enzymes	Putative virulence factors involved in host tissue penetration and degradation
Extracellular proteins	More likely to be involved in cross-talk with plants
Up-regulated during preinfection and infection stages	More likely to be involved in pathogenicity or virulence; putative fungicide targets
Conserved among pathogenic oomycetes	Putative fungicide targets

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-

Fig. 2. Heterologous expression of *Phytophthora* genes in plants. (A) Functional expression of a *P. infestans inf* elicitin gene in *Nicotiana* using agroinfiltration (Van der Hoorn et al. 2000) results in specific induction of the hypersensitive response (HR). Inactive (top left) and HR-inducing INF (bottom right) were infiltrated in adjacent leaf panels. (B) Functional expression of a *P. infestans inf* elicitin gene in *Nicotiana* using PVX results in induction of the HR and restriction of the virus to the HR lesions (Kamoun et al. 1999a). Normal symptoms induced by wild-type virus do not involve necrosis and are limited to mild mosaic (chlorotic) symptoms.



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 *inf1* gene could be used without modifications to transform *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 *Phytophthora*. Transposon mutagenesis, with either endogenous or ubiquitous transposons, is also a potentially useful technique. For both of these techniques, and considering that *Phytophthora* is a diploid at the vegetative stage, it will be more judicious to use homothallic species, such as *P. sojae*, instead of a heterothallic species such as *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 *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 microrganisms to identify genes encoding avirulence and virulence functions. In *Phytophthora*, Panabières et al. (1998) described functional expression of the gene from *Phytophthora cryptogea* Pethybr. & Lafferty encoding the basic elicitin cryptogein in *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 *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*.

Acknowledgements

We thank Melissa Barty and Alessandra Borello for technical assistance, and our collaborators in the oomycete research community for their help and assistance. In particular, we thank Pieter van West and Neil Gow (University of Aberdeen, Scotland, U.K.), Steve Whisson and Paul Birch (Scottish Crop Research Institute, Dundee, U.K.), Dinah Qutob and Mark Gijzen (Agriculture and Agri-food Canada, London, Ont., Canada), Vivianne Vleeshouwers and Francine Govers (Wageningen Agricultural University, The Netherlands), Elodie Gaulin, Martina Rickauer, and Marie-Thérèse Esquerre-Tugaye (Université Paul Sabatier, France), Brett Tyler (University of California, Davis, Calif., U.S.), Venkat Gopalan (The Ohio State University, Ohio, U.S.), and Isabelle Malcuit and David Baulcombe (Sainsbury Laboratory, Norwich, U.K.). This work was supported by the Research Enhancement Grant Program of the Ohio Agricultural Research and Development Center, the Ohio Soybean Council, and Syngenta Biotechnology Inc. The salaries and research support were provided by State and Federal Funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

References

Erwin, D.C., and Ribeiro, O.K. 1996. *Phytophthora* diseases worldwide. American Phytopathological Society Press, St. Paul, Minn.

Fry, W.E., and Goodwin, S.B. 1997a. Re-emergence of potato and tomato late blight in the United States. Plant Dis. 81: 1349–1357.

Fry, W.E., and Goodwin, S.B. 1997b. Resurgence of the Irish potato famine fungus. Bioscience, 47: 363–371.

Kamoun, S. 2000. *Phytophthora*. *In* Fungal pathology. *Edited by* J. Kronstad. Kluwer Academic Publishers, Dordrecht. pp. 237–265.

Kamoun, S. 2001. Nonhost resistance to *Phytophthora*: novel prospects for a classical problem. Curr. Opin. Plant Biol. 4: 295–300.

Kamoun, S., van West, P., Vleeshouwers, V.G., de Groot, K.E., and Govers, F. 1998. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. Plant Cell, 10: 1413–1426.

Kamoun, S., Honee, G., Weide, R., Lauge, R., Kooman-Gersmann, M., de Groot, K., Govers, F., and De Wit, P.J.G.M. 1999a. The fungal gene *Avr9* and the oomycete gene *inf1* confer avirulence to potato virus X on tobacco. Mol. Plant–Microbe Interact. 12: 459–462.

Kamoun, S., Hraber, P., Sobral, B., Nuss, D., and Govers, F. 1999b. Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences. Fungal Genet. Biol. 28: 94–106.

Kamoun, S., Huitema, E., and Vleeshouwers, V.G.A.A. 1999c. Resistance to oomycetes: a general role for the hypersensitive response? Trends Plant Sci. 4: 196–200.

Kjemtrup, S., Nimchuk, Z., and Dangl, J.L. 2000. Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. Curr. Opin. Microbiol. 3: 73–78.

Lam, S.T. 2001. *Phytophthora* genomics consortium. Phytopathology, 91(Suppl.): S158 (Abstr.).

Lauge, R., and De Wit, P.J. 1998. Fungal avirulence genes: structure and possible functions. Fungal Genet. Biol. 24: 285–297.

Panabières, F., Birch, P.R., Unkles, S.E., Ponchet, M., Lacourt, I., Venard, P., Keller, H., Allasia, V., Ricci, P., and Duncan, J.M. 1998. Heterologous expression of a basic elicitin from *Phytophthora cryptogea* in *Phytophthora infestans* increases its ability to cause leaf necrosis in tobacco. Microbiology (Reading, U.K.), 144: 3343–3349.

Qutob, D., Hraber, P.T., Sobral, B.W., and Gijzen, M. 2000. Comparative analysis of expressed sequences in *Phytophthora sojae*. Plant Physiol. 123: 243–254.

Rizzo, D.M., Garbelotto, M., Davidson, J.M., Slaughter, G.W., and Koike, S.T. 2001. A new *Phytophthora* canker disease as the probable cause of sudden oak death in California. Phytopathology, 91(Suppl.): S76 (Abstr.).

Testa, A., and Kamoun, S. 2001. Necrosis-inducing cDNAs from *Phytophthora infestans* identified using high throughput functional expression assays. 10th International Congress of Molecular Plant–Microbe Interactions, 10–14 July 2001, University of Wisconsin, Madison, Wisc. (Abstr.)

Torto, G.A., Styer, A., and Kamoun, S. 2001. EST data mining: novel extracellular proteins from the oomycete plant pathogen *Phytophthora infestans*. Fungal Genet. Newsl. 48(Suppl.): 111 (Abstr.).

Van der Hoorn, R.A., Laurent, F., Roth, R., De Wit, P.J. 2000. Agroinfiltration is a versatile tool that facilitates comparative analyses of *Avr9*/*Cf-9*-induced and *Avr4*/*Cf-4*-induced necrosis. Mol. Plant–Microbe Interact. 13: 439–446.

van West, P., Kamoun, S., van't Klooster, J.W., and Govers, F. 1999. Internuclear gene silencing in *Phytophthora*. Mol. Cell, 3: 339–348.

Waugh, M., Hraber, P., Weller, J., Wu, Y., Chen, G., Inman, J., Kiphart, D., and Sobral, B. 2000. The *Phytophthora* genome initiative database: informatics and analysis for distributed pathogenomic research. Nucleic Acids Res. 28: 87–90.