Chapter 1

PHYTOPHTHORA

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Abstract: Members of the oomycete genus *Phytophthora* are among the most devastating plant pathogens. In this chapter, we summarize basic information on the biology of *Phytophthora* and review the main themes of molecular research on these pathogens, with special emphasis on recent advances in understanding virulence and pathogenicity, specificity in interaction with plants, chemotaxis, mating type determinants, genome structure, population genetics, and molecular phylogeny. In addition, we review the molecular tools used for genetic manipulation and study of *Phytophthora*. These include DNA transformation technology, reporter genes, gene silencing, genetic mapping, genomics, and functional genomics. A better understanding of the molecular genetics of these exceptional pathogens should lead to novel approaches to manage the numerous diseases they cause.

Ever since the potato late blight epidemics of the mid-nineteenth century, members of the oomycete genus *Phytophthora* have emerged as major pathogens of innumerable crops. Nowadays, with more than fifty species

recognized and with destructive diseases caused on thousands of plant species, *Phytophthora* remains an active subject of research and a nagging problem to farmers and growers. Renewed interest in *Phytophthora* diseases occurred in recent years with the reemergence of *Phytophthora infestans,* the Irish potato famine fungus. Late blight has turned into a global threat to potato and tomato production following a series of severe late blight epidemics that coincided with the migration to Europe and North America of aggressive A2 mating type strains (Fry and Goodwin, 1997a; Fry and Goodwin, 1997b). The International Potato Center estimated that worldwide losses in potato production caused by late blight have recently exceeded $3 billion annually, making *P. infestans* the single most important biotic constraint to global food production (Anonymous, 1996).

In parallel to the resurgence of *Phytophthora* in the field and in the news, oomycete research has entered an exciting phase. Recent technical developments, such as routine DNA transformation (Judelson, 1996a), use of reporter genes (Judelson, 1997b; Kamoun et al., 1998b; van West et al., 1998), genetic manipulation using gene silencing (van West et al., 1998; Kamoun et al., 1998c), and the development of detailed genetic maps (van der Lee et al., 1997; Whisson et al., 1995), will facilitate cloning and allow functional analyses of numerous candidate genes involved in interactions with the plant and other important phenomena. In addition, genomics approaches, or the wholesale study of *Phytophthora* genes, promises to bring research to yet another level (Kamoun et al., 1999b).

In this chapter, I summarize basic information on the biology of *Phytophthora* and review recent advances in molecular study and genetic manipulation of these pathogens. For additional detail, readers should refer to other recent reviews. Two review articles that discuss the genetics of the genus were published in the last three years (Judelson, 1996a; Judelson, 1997a). For more general but detailed information on *Phytophthora*, the monumental treatise by Erwin and Ribeiro (1996) describes many aspects of the biology of *Phytophthora*, provides detailed protocols and methods of study, separate and detailed descriptions of all known species, and an exhaustive list of references.

1. Oomycetes: A unique group of eukaryotic plant pathogens

Oomycetes include diverse organisms, some of which are pathogenic on fish, crayfish, and animals. Plant pathogenic oomycetes are usually classified in the Peronosporales and include in addition to *Phytophthora*, numerous genera of the biotrophic downy mildews (such as *Peronospora* and *Bremia*), the white rust pathogens *Albugo*, and more than 100 species of the genus *Pythium*. Altogether, these pathogens cause several billions of dollars of damages on crop, ornamental, and native plants. Some of the economically

important *Phytophthora* pathogens and their major host plants are listed in Table 1.

Table 1. Some economically important *Phytophthora* species

Phytophthora species	Major host plant(s)
Phytophthora capsici	Pepper
Phytophthora cinnamomi	Avocado and numerous forest species in the Australasian region
Phytophthora fragariae var. fragariae	Strawberry
Phytophthora infestans	Potato and tomato
Phytophthora palmivora	Cocoa
Phytophthora sojae	Soybean

Oomycetes exhibit filamentous growth habit, and are often inaccurately referred to as fungi. Modern biochemical analyses as well as phylogenetic analyses based on sequences of ribosomal and mitochondrial genes suggest that oomycetes share little taxonomic affinity to filamentous fungi, but are more closely related to golden-brown algae and heterokont algae in the Kingdom Protista (Kumar and Rzhetsky, 1996; Paquin et al., 1997; Van de Peer and De Wachter, 1997). Therefore, oomycetes include a unique group of eukaryotic plant pathogens, which evolved the ability to infect plants independently from true fungi. This suggests that oomycetes may have distinct genetic and biochemical mechanisms for interacting with plants. For example, in contrast to filamentous fungi (Osbourn, 1996a; Osbourn, 1996b), oomycetes contain little or no membrane sterols, the target of toxic saponins, and are therefore unaffected by these compounds which are abundantly present in plants.

2. Biology of *Phytophthora*

2.1. GENERAL CHARACTERSTICS

In the vegetative stage, *Phytophthora* exhibits filamentous mycelial growth (Erwin and Ribeiro, 1996). Mycelium is generally coenocytic with no or a few septa. The multiple diploid nuclei can differ genetically to form so called heterokaryotic strains. *Phytophthora* can produce both asexual and sexual spores. Asexual sporangia (or more accurately zoosporangia, singular zoosporangiospore) emerge directly from the hyphae through structures known as sporangiphores. Under optimal conditions of temperature and moisture, sporangia release swimming spores, the biflagellate zoospores. Sexual spores, or oospores, are formed when the male structure, antheridium, associates with the female, egg bearing oogonium. Some species of *Phytophthora* are self-fertile or homothallic, whereas others are self-sterile or heterothallic. Heterothallic species are divided into A1 and A2 mating types and crossing occurs when these two types of strains contact each other.

2.2. LIFE AND DISEASE HISTORY OF A FOLIAR *PHYTOPHTHORA*

A typical aerial species is the late blight pathogen *P. infestans*. *P. infestans* is generally considered a specialized pathogen causing disease on leaves and fruits of potato and tomato crops. In potato, infection of tubers may also occur. Only sporadic reports of natural infection of plants outside the genera *Solanum* and *Lycopersicon* have been reported (Erwin and Ribeiro, 1996). The life cycle and infection process of *P. infestans* is well known (Coffey and Wilson, 1983; Hohl and Suter, 1976; Judelson, 1997a; Kamoun et al., 1999c; Pristou and Gallegly, 1954). Infection generally starts when motile zoospores, that swim on the leaf surface, encyst and germinate. Germ tubes form an appressorium and a penetration peg, which pierces the cuticle and penetrates an epidermal cell to form an infection vesicle. Branching hyphae with narrow, digit-like haustoria expand from the site of penetration to neighboring cells through the intercellular space. Later on, infected tissue necrotizes and the mycelium develops sporangiophores that emerge through the stomata to produce numerous asexual spores called sporangia. Penetration of an epidermal cell by *P. infestans* was noted in all examined interactions including those with plant species unrelated to the solanaceous hosts (Gross et al., 1993; Kamoun et al., 1999c; Kamoun et al., 1998c; Naton et al., 1996; Schmelzer et al., 1995). Fully resistant plants, such as some of the potato lines bearing *R* genes or the nonhosts *Solanum nigrum*, parsley, and *Nicotiana* spp. display a typical localized hypersensitive response (HR), a programmed cell death of plants, at all infection sites (Colon et al., 1992; Freytag et al., 1994; Gees and Hohl, 1988; Gross et al., 1993; Kamoun et al., 1999c; Kamoun et al., 1998c; Naton et al., 1996; Schmelzer et al., 1995). The HR can be highly localized to a single epidermal cell or can affect a group of cells surrounding the penetrating hyphae (Kamoun et al., 1999c; Kamoun et al., 1998c). It appears that the HR is associated with all known forms of genetic resistance to *P. infestans* and may be involved in nonhost resistance and partial resistance phenotypes (Kamoun et al., 1999c).

2.3. LIFE AND DISEASE HISTORY OF A SOIL *PHYTOPHTHORA*

A typical soil species is the root and stem rot pathogen *Phytophthora sojae* (formerly known as *Phytophthora megasperma* f. sp. *glycinea*). *P. sojae* is a specialized pathogen causing disease on soybean and some lupine species (Erwin and Ribeiro, 1996; Schmitthenner, 1989). This pathogen generally infects roots and sometimes spreads to the stem of soybean plants. However, leaves may also be infected if they get in contact with soil particles containing the pathogen (Schmitthenner, 1985; Schmitthenner, 1989). The life history of *P. sojae* is well known, but contradictory reports on the cytology of infection have been published (Enkerli et al., 1997; Ward et al., 1989). In the field, infection generally starts when motile zoospores,

swimming in the water interface in the soil, are attracted to host plant root exudates. Once on the root surface, zoospores encyst and germinate. In a study that mimicked natural infection of soybean roots, Enkerli et al. (1997) examined the ultrastructure of early infection events on both resistant and susceptible plants. On all plant genotypes, penetration by germ tubes occurred directly between two epidermal cells without the formation of an appressorium. On susceptible soybean roots, *P. sojae* established a short biotrophic phase by growing in the intercellular space, forming numerous haustoria and inducing little response from the plant. Later on, starting at about 15 hours after inoculation, the root turned necrotic as the pathogen reached the vascular tissue and started growing intracellularly. In roots of resistant soybean plants containing the root rot resistance genes *Rps1a* or *Rps1b*, the timing of events was different. Rapid host cell necrosis occurred and the pathogen rarely established haustoria. Penetration of root tissue was limited as *P. sojae* rarely reached the vascular tissue.

Even though *P. sojae* infects roots, many researchers use hypocotyl infection assays for convenience and reproducibility. Interestingly, cytological examination of hypocotyl infection revealed the formation of appressoria by *P. sojae* germinating cysts prior to penetration unlike the direct intercellular penetration observed on roots (Ward et al., 1989). It remains unclear at this stage whether this discrepancy truly reflects different modes of infection of soybean tissues by the pathogen or simply the different experimental procedures used.

2.4. *P. INFESTANS* VS. *P. SOJAE*: TWO CONTRASTING PATHOGENS

The genus *Phytophthora* is diverse. Table 2 illustrates contrasting features in the biology and genetics of two economically important *Phytophthora* pathogens, *P. infestans* and *P. sojae*. The noted differences in such basic aspects as mode of sexual reproduction or genome size suggest that genetic studies on *Phytophthora* should consider this diversity of the genus and must incorporate comparative analyses. This will prove essential in order to achieve a thorough understanding of these organisms.

Table 2. General characteristics of *Phytophthora infestans* and *Phytophthora sojae*

Feature	*P. infestans*	*P. sojae*
Host plants	Potato, tomato, and related species	Soybean and lupines
Infected tissue	Leaves, stems, and tubers	Roots and stems, occasionally leaves
Penetration of plant tissue	Intracellular penetration of an epidermal cell through appressoria formation	Intercellular penetration between two root epidermal cells, no appressoria formation
Infection process	Early biotrophic phase with	Early biotrophic phase with

Feature	*P. infestans*	*P. sojae*
	formation of haustoria followed by saprophytic growth	formation of haustoria followed by saprophytic growth
Sporangia	Deciduous, dislodge easily from the sporangiophores	Persistent, remain attached to the sporangiophores
Main habitat	Aerial plant parts	Soil
Sexual behavior	Self-sterile or heterothallic	Self-fertile or homothallic
Genome size	250 Mb	62 Mb
Haploid chromosome count	8-10	10-13

3. Main themes of molecular research

3.1. VIRULENCE AND PATHOGENICITY

Due to the lack of random mutagenesis systems in *Phytophthora*, indirect approaches have been taken to identify genes that may play a role in virulence and pathogenicity. Pieterse et al. (1993a) showed that a number of *P. infestans* genes are activated during interaction with potato. Using a differential hybridization approach, several in planta-induced genes (so called *ipi* genes) were isolated (Pieterse et al., 1994a; Pieterse et al., 1993a; Pieterse et al., 1991; Pieterse et al., 1993b). Based on their typical pattern of expression, it was proposed that these genes might play a role in pathogenicity. Two such *in planta*-induced genes are the closely related *ipiO1* and *ipiO2* (Pieterse et al., 1994a; Pieterse et al., 1994b). The putative 152 amino-acid IPIO1 protein bears a signal peptide at the *N*-terminus and is presumably extracellular. A careful analysis of the expression of *ipiO in vitro* and during various stages of infection of potato using RNA blot hybridizations and histochemical localization of GUS activity from a *P. infestans* transformant carrying a fusion between the *ipiO1* promoter and the β-glucuronidase reporter gene, was recently reported by van West et al. (1998). These experiments showed that *ipiO* expression is limited to particular stages of the life cycle of *P. infestans*. *IpiO* mRNA was detected in zoospores, cysts, germinating cysts, and younger mycelium, but not in sporangia and mature mycelium. In diseased potato leaves, *ipiO* expression was limited to the outer zones in invading biotrophic hyphae, but did not occur in the necrotic areas of lesions. This suggests that the IPIO protein could be located at the interface between the invading hyphae and plant cells (van West et al., 1998). Other *ipi* genes identified through the differential hybridization procedure of Pieterse et al. (1993a) encode homologs of ubiquitin (encoded by *ubi3R*) and calmodulin (*calA*), and a novel glycine-rich protein family (*ipiB1*, *ipiB2*, and *ipiB3*) (Pieterse et al., 1991; Pieterse et al., 1994b; Pieterse et al., 1993b). However, future functional genetic analyses are required to unequivocally determine whether these *ipi* genes play a role in pathogenicity.

Penetration of plant cells by *P. infestans* germinating cysts requires the formation of specialized infection structures, such as appressoria. Kraemer et al. (1997) used a biochemical approach to identify polypeptides associated with these infection structures. Two-dimensional SDS-polyacrylamide gel electrophoresis of total and newly synthesized proteins in hyphae, cysts, germinating cysts and appressoria induced *in vitro* on artificial membranes were compared. Several polypeptides that showed specific developmental changes were identified but their identity remains unknown.

Degradation of plant cell wall is likely to be a component of virulence of *Phytophthora*, particularly during penetration of plant epidermis and establishment of haustoria. However, little is known about genes encoding degrading enzymes. Munoz and Bailey (1998) described a *Phytophthora capsici* gene encoding a 218 amino-acid cutinase that may be essential for infection of pepper. Following a pilot sequencing project of *P. infestans* cDNAs, a number of sequences encoding homologs of degradative enzymes, such as polygalacturonases, were identified (Kamoun et al., 1999b). Future functional assays should help determine whether these degradative enzymes are important virulence factors for *Phytophthora*.

3.1. SPECIFICITY IN INTERACTION WITH PLANTS

3.1.1. Race-specific avirulence genes

The molecular basis of host-specificity at the plant cultivar-pathogen race level is well understood. Perception by the plant of signal molecules, elicitors, produced by the avirulent pathogen, leads to the induction of effective defense responses including a programmed cell death response termed hypersensitive response (HR) (Baker et al., 1997; Dangl et al., 1996; Lamb et al., 1989). This model has been genetically defined by Flor's gene-for-gene hypothesis (Flor, 1956; Flor, 1971). According to this hypothesis, a resistance reaction is determined by the simultaneous expression of a pathogen avirulence or *Avr* gene with the corresponding plant resistance or *R* gene (Staskawicz et al., 1995). In recent years, the gene-for-gene hypothesis has received tremendous experimental support through the identification and functional characterization of both *Avr* and *R* genes. However, to date, no plant *R* gene targeted against *Phytophthora* or race-specific *Avr* gene of *Phytophthora* has been described (Judelson, 1996a; Kamoun et al., 1999c). Subsequently, the molecular mechanisms underlying the evolution of new virulence traits in *Phytophthora* remain unknown even though races of *P. infestans* virulent on a wider range of plant genotypes have been known for fifty years (Black and Gallegy, 1957). However, preliminary genetic analyses of *P. infestans* and *P. sojae* indicate that in many cases race/cultivar specificity follows Flor's gene-for-gene model.

Through classical breeding, a total of eleven dominant late blight *R* genes have been introgressed into potato from the Mexican wild species *Solanum*

demissum. The genetics of *P. infestans* virulence/avirulence on these potato lines has been examined. In a first study describing genetic crosses between *P. infestans* isolates with different virulence patterns, it was shown that avirulence on potato plants carrying the *R-2* and *R-4* resistance genes is dominant (Al-Kherb et al., 1995). In a second study using a different cross, van der Lee et al. (1998) reported that avirulence on potato plants carrying one of the *R-3*, *R-4*, *R-10*, and *R-11* resistance genes is dominant. Interestingly, *Avr3*, *Avr10*, and *Avr11* appeared closely linked in this cross and formed a tight cluster on linkage group VIII of an AFLP linkage map generated with the same progeny (van der Lee et al., 1997). van der Lee et al. (1998) then screened pooled DNA from virulent and avirulent progenies using the bulked segregant analysis (BSA) approach. This led to the identification of a total of 18 AFLP markers linked to the *Avr* cluster. These tightly linked markers should greatly facilitate the molecular cloning of this *P. infestans Avr* gene cluster.

Genetically resistant soybean cultivars containing one or combinations of thirteen known *Rps* resistance genes introduced from related *Glycine* germplasm have resulted in significant protection in the field against *P. sojae*. However, new races of *P. sojae* that can infect *Rps* cultivars are rapidly evolving (Forster et al., 1994). Populations of *P. sojae* that can defeat the most commonly displayed *Rps* genes, including the successful *Rps1-k*, have recently increased in number and distribution (Schmitthenner et al., 1994; A.F. Schmitthenner and A. Dorrance, pers. comm.). More than forty races of the pathogen have been described and dozens of new races are being characterized countrywide. Genetic analyses of crosses between *P. sojae* strains differing in virulence patterns have shown that avirulence to *Rps1b*, *Rps1d*, *Rps1k*, *Rps3b*, *Rps4*, and *Rps6* is dominant (Gijzen et al., 1996; Tyler et al., 1995; Whisson et al., 1994; Whisson et al., 1995). Interestingly, a number of *P. sojae* avirulence genes, such as *Avr1b* and *Avr1k*, and *Avr4* and *Avr6*, appear very tightly linked (Gijzen et al., 1996; Whisson et al., 1995). Progress has been made toward cloning these two *Avr* clusters. A Bacterial Artificial Chromosome (BAC) clone that contains both the *Avr1b* and *Avr1k* genes was identified and fine mapping of the two genes was performed (Arredondo et al., 1998). In addition, three overlapping cosmids containing *Avr4* and *Avr6* and covering 67.3 kb and 10.1 cM were identified (Whisson et al., 1998). Currently, both of these *Avr* clusters are being characterized using subcloning and complementation experiments.

A summary of all known clusters of *Avr* genes in *Phytophthora* is shown in Table 3.

It should be noted that in both *P. infestans* and *P. sojae*, avirulence did not always segregate as a dominant trait (Al-Kherb et al., 1995; Whisson et al., 1998). In some cases, avirulence was recessive in some but not all

crosses, and in other cases aberrant segregation ratios were observed. It remains unclear at this stage whether this departure from Flor's hypothesis reflects a genetic basis of cultivar/race specificity in *Phytophthora* that sometimes does not fit the gene-for-gene model or whether these genes are subject to aberrant segregation in some crosses.

Table 3. Clusters of race-specific *Avr* genes in *Phytophthora*

Phytophthora spp.	*Avr* cluster	Plant spp./*R* gene	Reference
P. infestans	*Avr3*/*Avr10*/*Avr11*	*Solanum demissum* *R3*/*R10*/*R11*	Van der Lee et al. (1998)
P. sojae	*Avr1b*/*Avr1k*	*Glycine* *Rps1b*/*Rps1k*	Whisson et al. (1995), Arredondo et al. (1998)
P. sojae	*Avr4*/*Avr6*	*Glycine Rps4*/*Rps6*	Whisson et al. (1995), Gijzen et al. (1996)

3.1.2. Elicitins

A family of extracellular protein elicitors, termed elicitins, has been identified in *P. infestans* and other *Phytophthora* species and evidence has accumulated for a role of these molecules in delimiting the host-range of *Phytophthora* (Grant et al., 1996; Kamoun et al., 1998c; Yu, 1995). Elicitins are highly conserved 10-kDa proteins that are secreted by all *Phytophthora* species and several *Pythium* species (Huet et al., 1995; Kamoun et al., 1993b; Pernollet et al., 1993). A list of all known elicitins and elicitin-like proteins from *Phytophthora* is shown in Table 4. Elicitins induce defense responses, including the HR, on a restricted number of plants, specifically *Nicotiana* species within the Solanaceae family, and radish and turnips within the Cruciferae family (Bonnet et al., 1996; Kamoun et al., 1994). Recognition by plant cells is thought to be determined by the interaction of elicitins with a high-affinity binding site in the tobacco plasma membrane (Wendehenne et al., 1995; Yu, 1995).

Table 4. The elicitin family of *Phytophthora*

Phytophthora spp.	Elicitin	Class	Predicted cellular localization
P. cactorum	CAC-A	I-A (acidic)	Secreted
P. capsici	CAP-A (capsicein)	I-A (acidic)	Secreted
P. cinnamomi	CIN-B	I-B (basic)	Secreted
P. cryptogea	CRY-A1	I-A (basic)	Secreted
P. cryptogea	CRY-B (cryptogein)	I-B (basic)	Secreted
P. cryptogea	CRY-HAE20, CRY-HAE26	II (highly acidic)	Secreted
P. drechsleri	DRE-A, DRE-B	I-B (basic)	Secreted
P. infestans	INF1	I-A (acidic)	Secreted
P. infestans	INF2A, INF2B	III	Surface protein

Phytophthora spp.	Elicitin	Class	Predicted cellular localization
P. infestans	INF4	Not yet classified	Secreted
P. infestans	INF5, INF6	Not yet classified	Surface protein
P. infestans	INF7	Not yet classified	Surface protein
P. megasperma	MGM-A	I-A (acidic)	Secreted
P. megasperma	MGM-B	I-B (basic)	Secreted
P. parasitica	PARA1 (parasiticein)	I-A (acidic)	Secreted
P. parasitica	PARA2	II (highly acidic)	Secreted
P. parasitica	PARA3	Not yet classified	Surface protein
P. sojae	SOJ-1, SOJ-2, SOJ-3, SOJ-4 (sojein)	I-A (acidic)	Secreted

The three-dimensional structure of cryptogein, the major basic elicitin of *Phytophthora cryptogea*, was determined using crystallography and nuclear magnetic resonance spectroscopy (Boissy et al., 1996; Fefeu et al., 1997; Gooley et al., 1998). Cryptogein displays on one side five loosely conserved alpha-helices and on the other side, a highly conserved beak structure formed by two antiparallel beta sheets and a Ω-loop. Based on the high amino-acid sequence similarity between different members of the elicitin family, this overall structure is likely to be conserved.

There are a number of experiments that suggest that elicitins function as avirulence factors in *Phytophthora*-plant interactions (Table 5). Treatment of tobacco plants with purified elicitins or expression of an elicitin gene in transgenic tobacco induces resistance to *Phytophthora parasitica* var. *nicotiana* (Ricci et al., 19989; Kamoun et al., 1993b; Keller et al., 1999; Tepfer et al., 1998). In *P. parasitica*, the absence of elicitin production correlates with virulence on tobacco, a plant species that strongly responds to elicitins (Bonnet et al., 1994; Kamoun et al., 1994; Kamoun et al., 1993b; Ricci et al., 1989). Moreover, in a sexual progeny of *P. parasitica*, elicitin production segregated with low virulence (Kamoun et al., 1994), suggesting that elicitins function as avirulence factors in *P. parasitica*-tobacco interactions (Kamoun et al., 1993a; Yu, 1995). Elicitin recognition has also been proposed to be a component of nonhost resistance of *Nicotiana* species to *P. infestans* (Kamoun et al., 1997b; Kamoun et al., 1998c). Conclusive direct evidence of the role of elicitins as avirulence factors came from functional analyses of the *P. infestans* elicitin gene *inf1*. Using a single step transformation procedure with an antisense construct of the *inf1* elicitin gene, stable *P. infestans* strains deficient in the production of INF1 elicitin were engineered (Kamoun et al., 1998c). Two of these strains showed increased virulence on the plant species *N. benthamiana*, indicating that the recognition of INF1 is a major determinant of the resistance response of *N. benthamiana* to *P. infestans*.

Elicitins can also induce resistance to pathogens other than *Phytophthora* (Table 5). Keller et al. (1999) generated transgenic tobacco plants containing a fusion between the pathogen inducible tobacco promoter hsr203J and the *P. cryptogea* cryptogein (*cry-B*) gene. These transgenic plants exhibited the HR under inducing conditions and showed enhanced resistance to the fungal pathogens *Thielaviopsis basicola*, *Erysiphe cichoracearum*, and *Botrytis cinerea*. Kamoun et al. (1993b) showed that coinfiltration of radish leaves with purified elicitin solutions and the bacterial pathogen *Xanthomonas campestris* pv. *armoraciae* resulted in reduced leaf spot symptoms and decreased growth of the pathogen. Functional expression of the *inf1* avirulence gene from engineered potato virus X (PVX) genome resulted in localized HR lesions on tobacco plants and inhibited spread of the engineered virus (Kamoun et al., 1999a). In contrast, a PVX construct producing an INF1 mutant form with reduced elicitor activity caused systemic necrotic symptoms, and was unable to inhibit PVX spread (Kamoun et al., 1999a). Taken together, these results demonstrate that the HR induced by elicitins is a highly versatile defense mechanism active against a number of unrelated pathogens.

Table 5. Experimental evidence for a role of elicitins as avirulence factors

elicitin	Plant tested	Experiment	Reference
CAP-A, cryptogein (CRY-B), PARA1	Tobacco	Application of purified elicitins to tobacco plants induced resistance to *Phytophthora parasitica* var. *nicotiana*	Ricci et al. (1989), Kamoun et al. (1993b)
CRY-B	Tobacco	Transgenic tobacco plants producing cryptogein displayed enhanced resistance to *P. p.* var. *nicotiana*, as well as to the fungal pathogens *Thielaviopsis basicola*, *Erysiphe cichoracearum*, and *Botrytis cinerea*.	Keller et al. (1999), Tepfer et al. (1998)
CRY-B, PARA1	Radish	Application of purified elicitins to radish plants induced resistance to the bacterial pathogen *Xanthomonas campestris* pv.	Kamoun et al. (1993b)

elicitin	Plant tested	Experiment	Reference
		armoraciae	
INF1	*Nicotiana benthamiana*	*P. infestans* strains silenced for the *inf1* gene were able to infect *N. benthamiana*	Kamoun et al. (1998c)
INF1	Tobacco	A recombinant potato virus X (PVX) expressing the *inf1* gene became avirulent on tobacco	Kamoun et al. (1999a)

Elicitin-like genes were isolated from *P. infestans* using PCR amplification with degenerate primers, low stringency hybridization techniques, and random sequencing of cDNAs (Kamoun et al., 1997a; Kamoun et al., 1999b; unpublished data). In total, seven elicitin and elicitin-like genes have now been identified in *P. infestans* (Table 4). All these genes encode putative extracellular proteins that share the 98 amino-acid elicitin domain that corresponds to the mature INF1. Major structural features of the elicitin domain (six cysteins, predicted secondary structure etc...) are conserved in the *inf* gene family. Five *inf* genes (*inf2A*, *inf2B*, *inf5*, *inf6*, and *inf7*) encode predicted proteins with a C-terminal domain in addition to the N-terminal elicitin domain. Sequence analysis of these C-terminal domains shows a high frequency of serine, threonine, alanine, and proline. The amino-acid composition and the distribution of these four residues suggest the presence of clusters of *O*-linked glycosylation sites (Kamoun et al., 1997a; Wilson et al., 1991). Interestingly, numerous surface and cell wall associated proteins consist of a signal peptide and a functional extracellular domain followed by a serine-threonine rich *O*-glycosylated domain (Jentoft, 1990). Such proteins were shown to have a 'lollipop on a stick' structure in which the *O*-glycosylated domain forms an extended rod that anchors the protein to the cell wall leaving the extracellular N-terminal domain exposed on the cell surface (Jentoft, 1990). Therefore, these atypical INF proteins may be surface or cell wall associated glycoproteins that interact with plant cells during infection. Whether these elicitin-like genes encode active elicitor proteins and function as avirulence determinants remains to be determined.

The *P. infestans-Nicotiana* system is proving useful as a model system for dissecting the molecular components that determine nonhost resistance. In addition to the use of gene silencing technology for functional analysis of elicitin genes in *Phytophthora* (Kamoun et al., 1998c), the use of viral vectors, such as potato virus X (PVX), for expression of elicitin genes in *Nicotiana* (Kamoun et al., 1999a) should help in determining patterns of

elicitor activity and specificity of the individual members of this diverse gene family and will allow the dissection of *Nicotiana* resistance into specific components (Kamoun, 1998).

The intrinsic biological function of elicitins in *Phytophthora* has long remained a mystery. Due to their high expression level in sporulating mycelium and abundant secretion, elicitins were thought to be structural proteins (Yu 1995; Kamoun et al., 1997b). However, more conclusive evidence came recently with the demonstration that these proteins can bind a number of sterols, such as dehydroergosterol, and may function as sterol-carrier proteins (Mikes et al., 1998; Mikes et al., 1997), a biological function of essential importance to *Phytophthora* spp. since they cannot synthesize sterols and must assimilate them from external sources (Hendrix, 1970). Functional genetic analyses and *in vivo* studies should help determine whether elicitins function indeed as sterol carriers in *Phytophthora*. In addition, it would be interesting to determine by structure-function mutagenesis studies whether or not the residues involved in sterol binding are distinct from those involved in interactions with tobacco receptor(s).

3.1.3. Other elicitors

Parsley is a nonhost of *P. sojae* and *P. infestans*. Following inoculation with *Phytophthora*, parsley cells exhibit a complex and coordinated series of morphological and biochemical defense responses that culminate into HR cell death (Hahlbrock et al., 1995; Naton et al., 1996; Somssich and Hahlbrock, 1998). An extracellular 42 kD glycoprotein elicitor from *P. sojae* or a 13 amino-acid oligopeptide (Pep-13) derived from this protein are sufficient to induce changes in plasma membrane permeability, an oxidative burst, activation of defense genes, and accumulation of defense compounds (Nurnberger et al., 1994). In addition to molecular signals, local mechanical stimulations, perhaps similar to those caused by the invading pathogen, induce some of the early morphological reactions and potentiate the response to the elicitor (Gus-Mayer et al., 1998). However, the signal(s) that lead to the HR in the parsley system remain unknown.

A 34-kDa glycoprotein elicitor (known as CBEL) was identified from *Phytophthora parasitica* var. *nicotianae*, a pathogen of tobacco (Sejalon-Delmas et al., 1997; Villalba Mateos et al., 1997). CBEL induced lipoxygenase (LOX) activity and accumulation of hydroxyproline-rich glycoproteins in tobacco (Sejalon-Delmas et al., 1997). A cDNA encoding CBEL was described (Villalba Mateos et al., 1997). The deduced amino acid sequence contained two direct repeats of a cysteine-rich domain, joined by a threonine/proline-rich region reminiscent of the cellulose-binding domain of fungal glycanases. However, CBEL did not show hydrolytic activity on a variety of glycans. Instead, CBEL showed lectin-like activity and bound

purified fibrous cellulose as well as plant cell wall extracts suggesting a dual function for this protein.

3.1.4. P. mirabilis vs. P. infestans

Phytophthora mirabilis, a host-specific species closely related to *P. infestans*, infects *Mirabilis jalapa* (four-o'clock) but is unable to infect potato and tomato. Interspecific hybrids between these two *Phytophthora* species were essentially unable to infect the original host plants suggesting that avirulence on the nonhosts is dominant (Goodwin and Fry, 1994). In addition, in contrast to the parental strains, large HR-like necrotic lesions were induced by several of the hybrids on tomato indicating an alteration of the extent of the HR. Future genetic work could help identify the components of host-specificity in these interactions. Since both *P. infestans* and *P. mirabilis* naturally occur in central Mexico on different hosts and are reproductively isolated, Goodwin (1998) proposed that this is an example of sympatric speciation in *Phytophthora*.

3.2. CHEMOTAXIS

Zoospores of *Phytophthora* exhibit a number of tactic responses that allow them to reach infection sites in roots of host plants (Erwin et al., 1983). Electrotactic swimming of zoospores of *Phytophthora palmivora* has been reported and is thought to reflect attraction to weak electrical fields generated by roots (Morris and Gow, 1993). In addition, positive chemotaxis of zoospores towards plant derived compounds is well known. For example, zoospores of *P. sojae* are attracted to the isoflavones daidzein and genistein, which are exuded from the roots of soybean plants into the rhizosphere (Morris and Ward, 1992; Tyler et al., 1996). Morris and Ward (1992) observed that zoospores of other *Phytophthora* species are not attracted to soybean isoflavones suggesting that selective chemotaxis might play a role in host-range determination. Tyler et al. (1996) identified differences in the ability of a series of *P. sojae* strains to sense various compounds with structural similarity to soybean isoflavones. This led to the prospect of genetic analysis of chemotactic response in *P. sojae*.

In addition to zoospore chemotaxis, chemotropic and contact-induced responses have been noted for germinating cysts *of P. sojae* (Morris et al., 1998). The hyphal tips of germinating cysts detected and penetrated pores in artificial membranes and produced multiple appressoria on smooth, impenetrable surfaces (Morris et al., 1998). These processes may help the hyphae identify appropriate penetration sites on the root surface.

3.3. MATING TYPE DETERMINANTS

Heterothallic species of *Phytophthora* display two types of strains with either the A1 or A2 mating type. A genetic model of mating type inheritance was developed based on genetic analyses of mating type and molecular markers linked to the mating type locus of *P. infestans* and *P. parasitica*

(Fabritius and Judelson, 1997; Judelson, 1996b; Judelson, 1996c). Apparently, heterozygosity (A/a) determines the A1 type and homozygosity (a/a) the A2 type. A number of abnormal genetic events, such as distorted segregation, nonrandom assortment of alleles, translocations, and duplications, occur at the mating type locus of several *P. infestans* strains (Judelson, 1996b; Judelson, 1996c; Judelson et al., 1995). Cosmid and BAC contigs containing the mating type locus were assembled (Judelson et al., 1998). Fine genetic mapping, complementation assays, and identification of expressed sequences in this region are under way and will help characterize the mating type locus.

3.4. GENOME STRUCTURE

The genome size of different *Phytophthora* species shows great divergence. For example, whereas the *P. sojae* genome was estimated at 62 Mb (Mao and Tyler, 1991), the *P. infestans* genome is thought to be 250 Mb (Tooley and Therrien, 1987). The haploid chromosome count of many *Phytophthora* species is not exactly known due to the occurrence of several small chromosomes that are difficult to resolve under light microscopy. The haploid chromosome number of *P. infestans* was estimated at n=8-10 by microscopy (Sansome and Brasier, 1973), and that of *P. sojae* at n=10-13 (Sansome and Brasier, 1974). Using pulse field gel electrophoresis, a total of eight chromosome-sized DNA bands were identified (Judelson et al., 1992b). However, based on intense ethidium bromide staining, at least two of these bands were suspected to be doublets or triplets (Judelson et al., 1992b).

One frequently ascribed feature to *Phytophthora* species is a high abundance of repetitive sequences. In *P. sojae*, five families of tandemly repeated sequences were identified following genomic substraction of chromosomal DNA from different isolates of the pathogen (Mao and Tyler, 1996). These sequences varied in copy number between the isolates and were all localized on single chromosomes of *P. sojae*. In *P. infestans*, repetitive DNA was estimated to cover at least 50% of the genome (Judelson and Randall, 1998). Following screening of a library of *P. infestans* for repetitive sequences, a total of 33 distinct families of repetitive DNA were discovered (Judelson and Randall, 1998). These elements were either tandemly repeated or dispersed throughout the *P. infestans* genome. Copy numbers varied from 70 to 8,400 per haploid genome. A number of these repeated sequences occurred in other distantly related *Phytophthora* species. However, some elements were specific to *P. infestans* and the closely related *P. mirabilis*.

Based on sequencing of a 60 kb BAC clone of *P. sojae*, a high density of genes was uncovered (Arredondo et al., 1998). Several pairs of genes are less than 300 bp apart. This led to the suggestion that in *P. sojae*, functional

genes are located in high-density gene islands separated by clusters of repetitive sequences, which may constitute around 50% of the genome (Arredondo et al., 1998).

3.5. POPULATION GENETICS

Modern analyses of population genetics of *Phytophthora* using variation in isozymes and RFLP markers resulted in great insight into the population structures and macroevolution of several species (Fry et al., 1993; Goodwin et al., 1994; Drenth et al., 1994; Drenth et al., 1996; Goodwin, 1997). For example, based on DNA and isozyme fingerprint analyses, it was suggested that before the 1980s a single clonal lineage of the A1 mating type, termed US-1, dominated most populations of *P. infestans* worldwide (Goodwin et al., 1994). US-1 isolates are thought to have propagated by asexual reproduction from *P. infestans* strains introduced in the 1840s from Mexico to North America and later to Europe and the rest of the world (Fry et al., 1993; Goodwin et al., 1994; Fry and Goodwin, 1995; Fry and Goodwin, 1997a; Fry and Goodwin, 1997b; Goodwin, 1997). However, recent migration events, probably from Mexico, to Europe and North America of populations of *P. infestans* that include aggressive and A2 mating type strains led to the displacement of US-1 populations and the establishment of a sexual cycle in some localities (Fry et al., 1993; Drenth et al., 1994; Fry and Goodwin, 1995; Fry and Goodwin, 1997a; Fry and Goodwin, 1997b). Particularly devastating epidemics caused by the migrant strains were recently observed worldwide as late blight re-emerged as a serious threat to potato production worldwide (Anonymous, 1996; Fry and Goodwin, 1997a; Fry and Goodwin, 1997b).

Similar to *P. infestans*, the use of RFLP markers helped dissect the distribution of genetic variation in *P. sojae*. *P. sojae* appears to show a moderate degree of diversity (Forster et al., 1994; Drenth et al., 1996). A number of isolates with different virulence patterns on soybean plants containing *Rps* genes have identical or near identical RFLP patterns, suggesting that some race types may have arisen in clonal lineages (Forster et al., 1994). In addition, and based on the distribution of alleles among isolates within each group, some races of *P. sojae* may also have arisen by rare outcrosses in this homothallic species (Forster et al., 1994). Drenth et al. (1996) compared US and Australian populations of *P. sojae* using RFLP markers. Genotypic diversity was much lower in Australian populations of *P. sojae* (ranging from 2.5 to 14.3%) compared to US populations (60%), suggesting that *P. sojae* may have been established in Australia following a single introduction of the pathogen. In addition, all five races that occur in Australia appear to have emerged in clonal lineages.

The levels of analysis obtained using RFLP and isozyme markers remain limited particularly in populations showing little polymorphism, such as

clonal lineages of *P. infestans* and *P. sojae*. The AFLP DNA fingerprinting technique, which was used to generate a high-resolution genetic map of *P. infestans* (van der Lee et al., 1997), could also be useful in achieving fine levels of genetic analysis of *Phytophthora* populations. For example, Kamoun et al. (1998a) identified a total of 49 polymorphic AFLP bands in a set of 18 US-1 isolates of *P. infestans* with no known isozyme or RFLP polymorphisms.

3.6. MOLECULAR PHYLOGENY

Classical taxonomy of the genus *Phytophthora* has been based on often-inconsistent morphological markers. This classification has been confusing and unsatisfactory. Recent studies re-examined the evolution of the genus using molecular markers, such as the Internal Transcribed Spacer (ITS) regions of rDNA. Cooke (1998) reported sequencing the ITS region of over 180 isolates representing 50 *Phytophthora* species. There was sufficient polymorphism in the ITS sequences to differentiate most species and to generate a coherent phylogenetic tree. The genus appeared monophyletic and showed less overall diversity than *Pythium*. However, the downy-mildews *Peronospora* showed strong affinity with *Phytophthora* raising the possibility that this group evolved from a biotrophic stock of *Phytophthora*. A good understanding of the phylogenetic relationships between *Phytophthora* species is essential for comparative biological studies and for designing sound disease management approaches.

A more detailed level of phylogenetic analysis can also be achieved in asexual populations of *Phytophthora*. For example, since *P. infestans* isolates of the US-1 genotype have propagated clonally by asexual reproduction, it is possible to reconstruct the phylogeny of these isolates given enough polymorphic molecular markers. Kamoun et al. (1998a) conducted a phylogenetic analysis on a set of 18 European US-1 isolates using markers generated by AFLP DNA fingerprinting. A phylogenetic tree was generated using parsimony analysis and 14 of the tested isolates were found to belong to three statistically significant branches within the US-1 lineage. Since five of the examined isolates were deficient in the production of the extracellular protein INF1 elicitin, this analysis helped determine whether this phenotype arose from one common mutant ancestor or whether loss of INF1 production occurred repeatedly in *P. infestans* (Kamoun et al., 1998a).

4. Molecular tools for genetic manipulation

4.1. DNA TRANSFORMATION

Stable DNA transformation is a prerequisite for genetic manipulation and functional analysis. Protocols and plasmids for transformation of oomycetes had to be developed from scratch as vectors available for transformation of filamentous fungi did not work in *Phytophthora* probably due to different

sequence requirements for the transcriptional machinery (Judelson and Michelmore, 1991; Judelson et al., 1992a). A number of promoters, such as *ham34* and *hsp70*, from the oomycete downy-mildew *Bremia lactucae*, showed strong activity in transient transformation assays of *P. infestans* protoplasts (Judelson and Michelmore, 1991). These were then linked to genes encoding antibiotic resistance (*nptII* and hygromycin resistance genes) and successfully used as selection vectors for stable DNA transformation of various *Phytophthora* species. The standard transformation protocol is based on liposome-PEG mediated transformation of protoplasts, followed by regeneration of the protoplasts and antibiotic selection on agar medium (Judelson et al., 1991; Judelson et al., 1992b). High frequency rates of co-transformation (up to 50%) were observed especially if the two plasmids are linearized with restriction enzymes with compatible ends (Judelson, 1993). This finding turned out to be quite useful as the gene of interest can be rapidly cloned in convenient expression cassettes and co-transformed with the selection plasmid. So far, homologous recombination has not been detected and integration of the introduced DNA into the genome is thought to be through heterologous recombination. High rates of tandemly integrated plasmids are often observed. Overall, the rates of transformation remain limited for some applications, however, a single transformation experiment with *P. infestans* can now lead up to 200 independent transformants.

Several species, such as *P. infestans*, *P. sojae*, and *P. palmivora*, have been transformed using the PEG/liposome protoplast protocol (Judelson et al., 1991; Judelson et al., 1992b; P. van West, pers. comm.). The limiting step in transformation appears to be in the heterologous integration of the introduced plasmids and the low regeneration rate of the protoplasts. Recently, transformation of *P. sojae* via electroporation of zoospores, which naturally lack a cell wall, has been achieved (B. Tyler, pers. comm.). This could help in avoiding problems with low frequencies of protoplast regeneration and may ultimately result in improved frequencies of transformation.

4.2. REPORTER GENES

Several reporter genes including those encoding β-glucuronidase (GUS), luciferase, and the green fluorescent protein (GFP) have been used successfully in *Phytophthora* (Judelson, 1997b; van West et al., 1998; P. van West, pers. comm.). *Phytophthora* transformants expressing the GUS reporter gene have been used to monitor disease progression *in planta*, to evaluate disease resistance, to study promoter expression and to visualize morphological structures during mating (Judelson, 1997b; Kamoun et al., 1998b; van West et al., 1998). A transgenic *P. infestans* strain containing a transcriptional fusion between the promoter of the plant-induced *ipiO* gene and GUS proved useful in determining spatial patterns of expression of the

ipiO promoter during infection of potato (van West et al., 1998). GUS staining was limited to the biotrophic stage of infection, particularly the edge of the invading hyphae suggesting that *ipiO* is highly expressed at that stage. Kamoun et al. (1998b) described the use of a *P. infestans* strain constitutively expressing high levels of GUS to measure fungal biomass and to estimate levels of general resistance of potato. However, due to the cost of GUS assays and the biosafety requirements for a transgenic strain, it is unclear whether using such GUS-tagged strains would confer significant advantages in routine evaluation of disease resistance in *P. infestans*-potato studies over using traditional methods for disease evaluation.

P. infestans strains expressing GUS were also used in studies on the mating process. By pairing strains containing a GUS transgene with nontransformed strains, Judelson (1997b) easily determined whether oospores resulted from hybridization or from self-interactions. Depending on the cross examined, 5 to 99% of the total oospores formed resulted from outcrossing. The use of the GUS marker also allowed determining levels of sexual preference, whether a strain is likely to act as a male or a female in a particular cross. A1 and A2 isolates behaved mainly as females and males, respectively. However, the sexual preference of a particular strain varied depending on its mating counterpart.

4.3. GENE SILENCING

Since no homologous recombination was detected following transformation of *Phytophthora*, a classical gene disruption approach was considered to have a low chance of success in this diploid organism. Therefore, attempts at targeted gene knockout followed the gene silencing approach that proved successful in plants. Following transformation of *P. infestans* with sense, antisense, and promoterless constructs of the endogenous single locus *inf1* gene, silencing of *inf1* was observed in up to 20% of the transformants (van West et al., 1999). Silencing was accompanied by the complete absence of *inf1* mRNA and INF1 protein and proved stable over repeated vegetative culture of the pathogen both *in vitro* and *in planta* (Kamoun et al., 1998c; van West et al., 1999). Nuclear run-on assays indicated that silencing is regulated at the transcriptional level (van West et al., 1999). No hypermethylation was observed in both transgenic and endogenous sequences of *inf1*. Due to the stability and total efficacy of this phenomenon, functional analyses could be performed with the silenced strains.

Efficient silencing of the *inf1* gene was also manifested in heterokaryotic mycelia, obtained after protoplast fusion of a transgenic-silenced strain and a non-silenced strain (van West et al., 1999). This observation suggests the involvement of a *trans*-nuclear silencing factor and rules out DNA-DNA interactions as the basis for gene silencing. Furthermore, homokaryotic wild-

type strains, obtained following nuclear separation of silenced heterokaryotic strains, displayed stable gene silencing indicating that the presence of nuclear transgenic sequences was not essential to ensure and maintain silencing of the endogenous *inf1* gene. Apparently an inter-nuclear process, perhaps based on a *trans*-acting silencing factor, is responsible for the gene silencing phenomenon in the heterokaryotic *P. infestans* strains (van West et al., 1999). The facile transfer of gene silencing from one genetic background to another by passage through a heterokaryon should prove a very useful technology for constructing strains silenced for multiple genes.

Spontaneous silencing of transgenic GUS sequences was described in *P. infestans* (Judelson and Whittaker, 1995) and could be mechanistically related to the transcriptional silencing of the *inf1* gene. Following culturing, *P. infestans* strains transformed with GUS spontaneously lost all detectable GUS mRNA and activity. No correlation with changes in transgene structure or hypermethylation was detected. There is a tremendous variation in incidence of this spontaneous gene silencing phenomenon in *P. infestans*. For example an independent set of transgenic *P. infestans* strains containing the GUS gene failed to display spontaneous gene silencing (S. Kamoun, unpublished data). Thus it remains unclear whether spontaneous gene silencing is a major problem that can hamper stable expression of genes in *Phytophthora*.

4.4. GENETIC MAPPING

The development of comprehensive genetic and physical maps should greatly facilitate gene isolation from *Phytophthora* species. A detailed genetic map of *P. infestans* based on AFLP markers has been published (van der Lee et al., 1997). The data was generated from 73 F1 progeny from a cross between two homokaryotic and diploid isolates of *P. infestans*. A total of 183 AFLP markers, 7 RFLP markers and the mating type locus were mapped into 10 major and 7 minor linkage groups covering a total of 827 cM. More recently, a tight cluster of three avirulence genes, *Avr3*, *Avr10*, and *Avr11*, were placed on linkage group VIII of the AFLP map (van der Lee et al., 1998). A linkage map of *P. sojae* was constructed using 106 F2 individuals from two crosses (Whisson et al., 1995). The map was based on 22 RFLP markers, 228 RAPD, and 7 avirulence genes and covered 10 major and 12 minor linkage groups for a total of 830.5 cM. Progress toward a full physical map is under way. Arredondo et al. (1998) reported progress toward the construction of a BAC contig covering the entire 62 Mb genome of *P. sojae*.

4.5. GENOMICS

It is obvious that cDNA and genomic sequencing approaches can accelerate the genetic characterization of *Phytophthora* and other oomycetes. Understanding the genetic make-up of economically important species, such

as *P. infestans* and *P. sojae*, promises to lead to novel approaches for disease control and management. Based on these premises, a number of *Phytophthora* geneticists initiated a collaborative genomics effort, known as the *Phytophthora* Genome Initiative (PGI; http://www.ncgr.org/pgi/index.html). Within, the framework of PGI, a pilot cDNA sequencing project was performed (Kamoun et al., 1999b). A total of 1,000 Expressed Sequence Tags (ESTs) corresponding to 760 unique sets of sequences were identified using random sequencing of clones from a cDNA library constructed from mycelial RNA of *P. infestans*. A number of software programs, represented by a relational database and an analysis pipeline, were developed for the automated analysis and storage of the EST sequence data. A set of 419 non-redundant sequences, which correspond to a total of 632 ESTs (63.2%), were identified as showing significant matches to sequences deposited in public databases. A putative cellular identity and role was assigned to all 419 sequence sets. All major functional categories were represented by at least several ESTs. Four novel cDNAs containing sequences related to elicitins were among the most notable genes identified. Two of these elicitin-like cDNAs were among the most abundant cDNAs examined (1.7% of all cDNAs).

A number of high throughput genomics projects are being initiated and include large scale EST sequencing of particular developmental and infection stages, BAC-end sequencing for the construction of BAC contigs, and targeted sequencing of BAC contigs of particular interest. There is no doubt that as with other organisms, the genetic study of *Phytophthora* will be totally transformed by the anticipated overflow of DNA sequence data.

4.6. FUNCTIONAL GENOMICS

Once a sizable amount of DNA sequence data is available for *Phytophthora*, there will be an essential need for robust assays to perform functional genetic analyses. Currently, gene silencing is the only proven technology to generate *Phytophthora* strains deficient in particular gene products (Kamoun et al., 1998c; van West et al., 1999). However, this approach has only been proven for a single gene so far, and it remains to be determined whether it will prove successful for a reasonable number of genes to allow its systematic application. Another disadvantage of gene silencing is a potential lack of specificity if a family of closely related genes is involved. In addition, depending on the gene examined, screening for silenced transformants can prove tedious and should be done preferably at the protein level, in addition to the RNA level. There is however a number of advantages for using gene silencing to generate strains with altered phenotype. For example, no specific plasmid needs to be constructed for the transformation experiment. 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. However, the most exciting prospect of gene silencing for functional genomic analyses is the observed spread of the silenced state from silenced transgenic nuclei to non-transformed 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 hyphae. One can easily devise rapid or transient transformation assays to test such an approach. Similar approaches are being contemplated in *Caenorhabditis elegans*, since it was observed that localized treatments of the worms with double-stranded RNA resulted in systemic gene silencing and resulted in a progeny silenced for the target gene (Fire et al., 1998).

Other methods for targeted gene knockout need also to be investigated for development of functional analysis tools for *Phytophthora*. Gene disruption through homologous recombination has only been examined superficially in *Phytophthora* and needs to be revisited with perhaps constructs containing large pieces of flanking DNA. Transposon mutagenesis, with either endogenous or ubiquitous transposons, is also a potentially useful technique and may also result in increased transformation frequencies, particularly if it turns out that frequency of transposition is higher than the low frequency of heterologous recombination. 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 progeny that are homozygous at the mutated locus.

5. Conclusion

Research on *Phytophthora* has entered an exciting phase, with a noticeable increased discovery pace in recent years. In the coming years, continuous technological improvements as well as the expected impact of genomics should strengthen this trend and hopefully attract fresh talent to the field. Even though the classical biology of many *Phytophthora* species is well known (Erwin et al., 1983; Erwin and Ribeiro, 1996), there remains an urgent need for additional molecular and genetic studies. Hopefully, this will lead to a better understanding of the molecular genetics of these exceptional organisms and to novel approaches for management of the devastating diseases they cause.

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