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Molecular Tools to Unravel the Role of Genes from *Phytophthora infestans*

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Voor mijn ouders
en Baukje

Stellingen

1 Kernen in een heterokaryotische cel van *Phytophthora infestans* zijn in staat met elkaar te communiceren.

dit proefschrift.

2 Het INF1 eiwit van *Phytophthora infestans* is een avirulentiefactor in de interactie tussen *Nicotiana benthamiana* en *P. infestans.*

dit proefschrift.

3 In tegenstelling tot wat Penington et al. (1989) beweren, zijn zoösporen van *Phytophthora* transcriptioneel actief.

Penington et al. (1989) Exp. Mycol. *13*, 158-168;
dit proefschrift.

4 Het gegeven dat RNA moleculen op een efficiënte wijze 'gene silencing' kunnen bewerkstelligen in nematoden ('RNA interference') en dat RNA moleculen waarschijnlijk een rol spelen in VIGS ('viral-induced gene silencing'), maakt het zeer aannemelijk dat de hypothetische silencingsfactoren in internucleare 'gene silencing' in *Phytophthora infestans* en in 'systemic aquired gene silencing' in planten, RNA moleculen zijn.

Fire et al. (1998) Nature *391*, 806-811;
Hamilton & Baulcombe (1999) Science *286*, 950-952.;
Palauqui et al. (1997) EMBO J. *16*, 4738-4745;
dit proefschrift.

5 Niet alle *ipi* genen van *Phytophthora infestans* blijken *in planta* geïnduceerde genen te zijn; derhalve moet de aanduiding *ipi* met een korreltje zout genomen worden.

Pieterse et al. (1993) Physiol. Mol. Plant Pathol. *43*, 69-79;
Pieterse et al. (1994) Mol. Gen. Genet. *244*, 269-277;
dit proefschrift.

6 Erfelijke eigenschappen worden niet alleen door DNA bepaald.

Jablonka et al. (1992) J. Theor. Biol. *158*, 245-268.

7 De acetylatiestatus van chromatine speelt een essentiële rol in de regulatie van genexpressie in eukaryotische organismen.

Ayer (1999) Trends Cell Biol. *9*, 193-198;
Privalsky (1998) Proc. Nat. Acad. Sci. USA *95*, 3335-3337.

8 In tegenstelling tot wat Money beargumenteert, verdient het geen aanbeveling om oomyceten schimmels te noemen; met een dergelijke redenatie worden vleermuizen ongetwijfeld vogels en dolfijnen wellicht vissen.

Money (1998) Mycol. Research *102*, 767-768.

9 Er zijn in Groot-Britannië vooralsnog meer mensen gestorven door de gekke-koeienziekte (Bovine Spongiform Encephalopathy) dan aan new variant-CJD (Creutzfeldt-Jakob Disease).

Farmers weekly, 1997-30 juli; 1999-13 sept.
www.cjd.ed.ac.uk/figures.htm.

10 If you believe in the theory of evolution, genetic modification is the 'highway to heaven'.

11 Het is opmerkelijk dat de natuur vaak de mooiste exemplaren twee X-chromosomen geeft.

12 Iedere species verdwijnt.

Stellingen behorend bij het proefschrift getiteld:

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Chapter 1

General Introduction

Potato Late Blight

In 1842, von Martius suggested, as one of the first, that a fungus could be the actual cause of a plant disease (von Martius, 1842). This new concept was welcomed by the British mycologist Berkeley (Figure 1), who translated the paper of von Martius in English. Twenty years before the germ theory of Pasteur, their views countered the widespread idea that fungi grew on organisms that previously became putrefied by "dampness" or similarly mysterious factors and many considered fungi and bacteria as the result but not the cause of disease (Bourke, 1991).

Shortly after von Martius' paper, the first epidemics of the late blight disease of potato occurred in Europe and in the north-eastern United States (Bourke, 1991; Peterson et al., 1992). Again there was great debate about the cause. Finally in 1876, Anton de Bary (Figure 1) determined conclusively that a micro-organism, which he named *Phytophthora* ("plant destroyer") *infestans*, was the cause of late blight (de Bary, 1876).

Rev. Miles Joseph Berkeley

Anton de Bary

Figure 1. Reverend Miles Joseph Berkeley (1803-1889) and Anton de Bary (1831-1888)

P. infestans (Mont.) de Bary is also known as the Irish potato famine fungus. It totally destroyed Ireland's potato crop during 1845 and 1846, which resulted in poverty and mass starvation. This led to great sociological and economic changes in that country and to the emigration of large numbers of people to the United States and elsewhere

(Figure 2) (Bourke, 1991). It is estimated that Ireland lost a quarter of its 8 million inhabitants to starvation and emigration. Probably no other single plant pathogen caused such widespread human suffering (Erwin & Ribeiro, 1996). World-wide losses in potato production caused by late blight, and control measures against the disease have been estimated to cost $3 billion annually (Duncan, 1999).

Figure 2. The Ejectment.
"There are no symptoms of life within their borders, no more than if they were situated in the midst of the Great Desert -- no more than if they were cursed by the Creator with the blight of barrenness. Those who laboured to bring those tracts to the condition in which they are -- capable of raising produce of any description -- are hunted like wolves, or they perish without a murmur. The tongue refuses to utter their most deplorable -- their unheard-of sufferings. The agonies endured by the 'mere Irish' in this day of their unparalleled affliction are far more poignant than the imagination could conceive, or the pencil of Rembrandt picture. We do not exaggerate; the state of things is absolutely fearful; a demon, with all the vindictive passions by which alone a demon could be influenced, is let loose and menaces destruction." (taken from: The Illustrated London News, Dec. 16, 1848).

The Disease Cycle of *Phytophthora infestans*

P. infestans is generally considered a specialised pathogen causing disease on leaves and fruits of potato and tomato crops, although natural infection of plants outside the genera *Solanum* and *Lycopersicon* have been reported (Erwin & Ribeiro, 1996). The disease cycle of *P. infestans* is well studied. Initially, pathogenesis by *P. infestans* involves asexual growth. Infections typically begin when sporangia land on the surface of a leaf (Figure 3). During wet conditions and at temperatures below 12 °C, zoospores are released from the sporangium, they encyst after a motile period and produce a

germ tube. Direct germination of the sporangia is also possible. Plant penetration occurs when the tip of the germ tube differentiates into an appressorium, which forms a penetration peg that pierces the cuticle and penetrates the underlying plant cell. An infection vesicle is produced in the epidermal cell and hyphae grow into the mesophyll cell layers both intra- and intercellularly. Occasionally, intracellular haustorial feeding structures are formed. After three to four days, *P. infestans* starts to grow saprophytically in the necrotised centre of the growing lesion. Hyphae emerge through the stomata and sporangiophores are formed which produce numerous new sporangia on the underside of the leaf. The sporangia do not desiccate and are relatively short-lived (Judelson, 1997a). Infected foliage first becomes yellow and then water-soaked and eventually turns black. Lesions may occur anywhere on the leaf, petiole, or even the stem of the potato plant. Tubers become infected later in the season. In the early stages, slightly brown or purple blotches appear on the skin. In damp soils the disease progresses rapidly, and the tubers decay either before or after harvest.

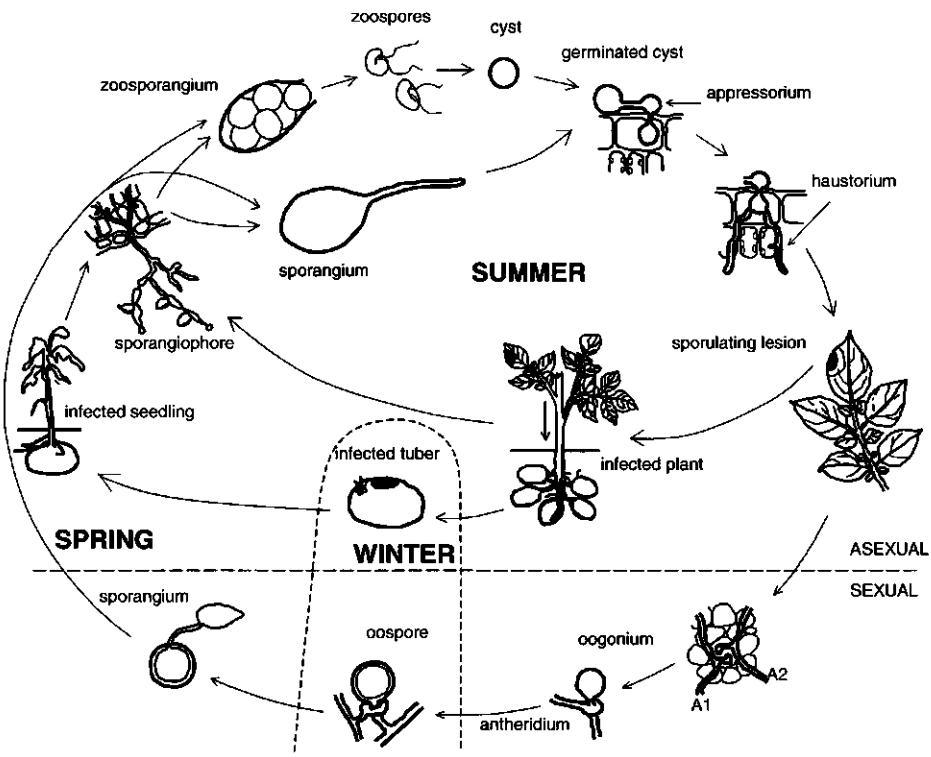

Figure 3. Life Cycle of *Phytophthora infestans*

P. infestans can also propagate via a sexual cycle (Figure 3). It is an heterothallic organism with two known mating types, A1 and A2. These represent compatibility types differing in hormone production and response, rather than dimorphic sexual forms (Judelson, 1997a). In response to hormones, male and female gametangia (antheridia and oogonia) are formed. Haploid nuclei are generated in the gametangia. An A1 and an A2 nucleus fuse and an oospore containing one diploid nucleus is generated. The oospore matures rapidly and a thick wall is formed, which enables the oospore to survive in the soil for many years. After germination of the oospore, new progeny, either A1 or A2, is able to infect newly planted tubers, or stems and leaves which come into contact with the soil (Drenth et al., 1995).

Phytophthora infestans Is Not a Fungus

The genus *Phytophthora* is closely related to the genus *Pythium*, and both genera are classified in the family Pythiaceae, which are water moulds belonging to the class oomycetes, order Peronosporales. Although oomycetes have many fungus-like characteristics, they are no longer classified in the Kingdom Fungi (Barr, 1983; Dick, 1995). Studies of cell wall composition (Bartnicki-Garcia & Wang, 1983), metabolism (Vogel et al., 1964; Pfyffer et al., 1990), and rRNA sequence (Förster et al., 1990; Illingworth et al., 1991) indicate that oomycetes are better classified with chrysophytes, diatoms, and golden-brown algae in the Kingdom Protista. Among the eukaryotic plant pathogens, oomycetes are a unique group which must have gained the ability to infect plants independently from "true" fungi. This suggests that oomycetes may have distinct genetic and biochemical mechanisms to interact with plants. Interestingly, some biological characteristics of oomycetes are relatively uncommon in "true" fungi (Zentmyer, 1983; Griffith et al., 1992). Oomycetes are for the major part of their life cycle diploid, whereas the true fungi are haploid. *Phytophthora* species have coenocytic mycelium with no, or few septa. The cell wall of *Phytophthora* is composed of cellulose and β-glucans but no chitin, which is the common cell wall component of true fungi (Bartnicki-Garcia & Wang, 1983). The zoospores are biflagellate with one whiplash and one tinsel flagellum (Hemmes, 1983). The rootlet morphology of the flagella is similar to that of the heterokont algae (Barr, 1981). *Phytophthora* species require an exogenous source of β-hydroxy sterols for sporulation as they do not synthesise sterols themselves (Hendrix, 1970; Elliott, 1983). Furthermore, members of the Pythiaceae are uniquely resistant to polyene antibiotics such as pimaricin and nystatin (Eckert & Tsao, 1962).

Another interesting feature of *Phytophthora* is that it has multinucleated sporangia and hyphal cells, but single nucleated zoospores (Erwin & Ribeiro, 1996). *Phytophthora* strains that have two or more different nuclei per cell, so called heterokaryons, can be formed naturally when hyphae of two different genotypes fuse and the nuclei are mixed (Layton & Kuhn, 1990). Whether such a process, often referred to as parasexuality,

plays a role in generating new pathogenic races of *P. infestans* is at present unknown. Attempts to obtain hyphal fusion during growth of *P. infestans in planta* were so far unsuccessful (Judelson, 1997a; Judelson & Yang, 1998). However, heterokaryotic and polyploid strains could be obtained *in vitro*, by mixing zoospores of two different strains (Judelson & Yang, 1998). Also in this thesis (Chapter 5), we describe *in vitro* protoplast fusion of strains bearing different antibiotic resistance genes, that resulted in new heterokaryotic strains with a novel phenotypic trait. These findings demonstrate that the parasexual cycle is a powerful process for generating genetic diversity in *P. infestans* and possibly this also occurs in nature.

The *Phytophthora*-Plant Interaction: Who Is the Champion?

A successful infection of its hosts by *P. infestans* requires many developmental events, involving formation and release of zoospores from a sporangium, encystment of the zoospores, germination of the cyst, differentiation of the germtube into an appressorium, penetration of the plant leaf surface by an appressorium via a penetration peg, differentiation of the penetration peg into hyphae, formation of haustoria, production of aerial sporangiophores, and finally the production of sporangia. If only one of these processes is interrupted, for example due to active plant defences, *P. infestans* fails to colonise successfully, and further spread of the disease is prevented. Therefore, it is absolutely essential for the propagation of the pathogen that "everything goes right". This requires a lot of "cat and mouse" tactics between *P. infestans* and its host plant. When *P. infestans* is able to colonise and propagate on a host plant, a compatible interaction develops, whereas in an incompatible interaction the plant can arrest colonisation, and *P. infestans* is unable to propagate successfully. To investigate the key processes that determine the outcome of the interaction, genes encoding pathogenicity and avirulence factors have to be isolated and their functions have to be determined.

Pathogenicity

The interactions between *P. infestans* and its hosts and non-hosts involve complex biological processes. Only very little is known about the molecular basis underlying pathogenicity of *P. infestans* (Govers et al., 1997). This is mainly due to a lack of random mutagenesis systems. Pathogenicity, in the broadest sense, comprises all the cellular and molecular processes that are necessary for the pathogen to establish and maintain a successful colonisation. This definition implies that genes which are generally considered as "house-keeping genes" do not encode pathogenicity factors. However, proteins and other macromolecules involved in overcoming physical and chemical barriers (such as cutinases or plant cell wall degrading enzymes), detoxification of plant substances (such as phytoalexins), production of toxins, and

formation of infection structures, are considered to be potential pathogenicity determinants (reviewed by Oliver & Osbourn, 1995).

In the potato-*P. infestans* interaction, only a few potential pathogenicity factors have been studied. These include mainly enzymes that degrade cell walls, such as endocellulases, 1,3-β-glucanases, β-glucosidases, pectin-esterases, galactanases and polygalacturonases (Friend, 1991), and phytotoxins (Seidel, 1961). These enzymes and toxins were found in culture fluids of axenically-grown *P. infestans* and therefore, it is unknown to what extend these factors contribute to disease development in plants. Furthermore, the biotrophic nature of *P. infestans*, and its own hyphal cell wall composition (consisting of β-glucans and celluloses), suggest that the cell wall degrading enzyme system of the pathogen has to be under tight control. Cell wall degrading enzymes may be secreted *in planta* to some extend to facilitate hyphal growth through the tissue, but the host cells are not immediately destroyed.

It seems likely that in the potato-*P. infestans* interaction, successful colonisation requires the up, or down regulation of expression of particular genes of *P. infestans*. Genes of which the expression is induced during colonisation, so called *in planta*-induced or *ipi*-genes, may encode essential pathogenicity factors. In our laboratory, Pieterse et al. (1993a) took an approach to isolate *ipi*-genes, using a differential hybridisation screening protocol. Two of the genes isolated encode homologues of ubiquitin (encoded by *ubi3R*) and calmodulin (*calA*), respectively (Pieterse et al., 1991; 1993b). In this thesis the characterisation of two other *ipi*-genes, *ipiO* and *ipiB*, is described.

In order to establish conclusively whether genes encoding cell wall degrading enzymes or *ipi*-genes play essential roles in pathogenicity, *P. infestans* mutants have to be generated in which expression of those genes is altered or abolished.

Race Specific and Host Specific Avirulence

The molecular basis of host-specificity at the plant cultivar-pathogen race level is fully recognised. In general it is assumed that recognition by the plant of signal molecules (elicitors) produced by the avirulent race/strain leads to the induction of effective defence responses, including a programmed cell death response, also termed hypersensitive response (HR) (Dangl et al., 1996; Kamoun et al., 1999). This model was first proposed by Flor in his gene-for-gene hypothesis in the rust-flax interaction (Flor, 1956; 1971). According to this hypothesis, a resistance reaction is determined by the simultaneous expression of an avirulence or *Avr*-gene of the pathogen and the corresponding resistance or *R*-gene from the plant. In the last decade many *R*-genes have been isolated (Lawrence et al., 1995; Anderson et al., 1997; Parker et al., 1997; Ori et al., 1997). Also many *Avr*-genes, mainly bacterial and a few fungal ones, have been cloned (reviewed by Laugé & de Wit, 1998). However, to date, neither plant *R*-genes targeted against *P. infestans* nor race-specific *Avr*-genes of *P. infestans* have been isolated. Isolation of *Avr*-genes from *P. infestans* by positional cloning is in

progress. A high density linkage map of a chromosomal region of *P. infestans* containing an *Avr*-gene cluster, *Avr3*, *Avr10*, and *Avr11*, has been established (van der Lee et al., 1997; 1999).

Interestingly, *P. infestans* is also able to penetrate non-host plants such as *Nicotiana* species. These plants remain resistant because a HR prevents further spread of *P. infestans*. In many non-host interactions the HR remains limited to one or a few cells, whereas in incompatible interactions of *P. infestans* with potato cultivars containing *R*-genes, often a group of cells displays the HR and infection is abolished at later stages (Kamoun et al., 1999). Previously, it has been shown that *P. infestans* and other *Phytophthora* species produce 10 kDa extracellular proteins, termed elicitins, which induce a HR in several *Nicotiana* species (Ricci et al., 1989; Kamoun et al., 1993). It was hypothesised that these elicitins could play a role in the resistance response of *Nicotiana* against *Phytophthora*. In this thesis, we present the characterisation and functional analysis of a gene encoding the predominantly secreted elicitin, INF1, of *P. infestans*. The *inf1* gene belongs to a family of genes of which currently seven members have been identified in *P. infestans* (Kamoun et al., 1999). It was found that purified elicitins are capable of binding sterols *in vitro* and hence it has been suggested that they might function as sterol carrier proteins *in vivo* (Mikes et al., 1997; 1998). Since *Phytophthora* species can not synthesise sterols (Hendrix, 1970; Elliott, 1983), it is possible that they obtain sterols from the host plant and that elicitins may play a role in this process.

Molecular Tools to Study Gene Function in *Phytophthora infestans*

P. infestans can easily be grown in the laboratory and has been an amenable organism for many scientific studies. Biochemical, cytological, and physiological studies on *in vitro*-grown mycelia and *in planta* interactions have been performed successfully. Unfortunately, the development of efficient genetic and molecular biological tools has been difficult. Only in 1991, a $CaCl_2$ and polyethylene-glycol-based DNA transformation protocol was developed by Judelson et al. (1991). Stable integrative transformants can be obtained by integration of transformation vectors harbouring chimeric genes composed of oomycete promoters and terminators and coding sequences of bacterial genes conferring resistance to the antibiotics geneticin (G418) and hygromycin. Co-transformation of a vector carrying a selection marker and a plasmid carrying the gene of interest is performed to generate transgenic isolates. In experiments described in this thesis the transformation technique was used to engineer mutants deficient for INF1, and to obtain transformants expressing the β-glucuronidase reporter gene under control of the *ipiO* gene promoter.

Reporter Gene Studies

Up till now *P. infestans* transformants expressing reporter genes have been employed in only a few studies. The β-glucuronidase (GUS) gene was used as a reporter or marker gene to investigate sexual oospore formation (Judelson, 1997b), and to monitor disease progression *in planta* (Kamoun et al., 1998). In this thesis (Chapter 4), we describe the use of the β-glucuronidase gene to study temporal and spatial expression patterns of the *in planta*-induced *ipiO* gene. A major disadvantage of GUS is that it is difficult to monitor gene expression in living tissue. Although non-destructive GUS assays have been described and used successfully in plants (Martin et al., 1992), other, more recently developed reporter gene systems exploiting luciferase (LUC) or green-fluorescent protein (GFP) have the advantage that gene expression can be followed *in vivo* during development of a single cell (Millar et al., 1992; Spellig et al., 1996). We showed that *luc* can be expressed as a reporter gene in *P. infestans*, but unfortunately only a small percentage of the transformants showed LUC activity (Chapter 4). Recently, it has been shown that GFP can be expressed and used as a reporter gene in other *Phytophthora* species (Bottin et al., 1999; van West et al., 1999).

Gene Silencing

In numerous plant pathogenic fungi and bacteria, directed mutagenesis is feasible, mainly by gene disruption. In contrast, a directed mutagenesis protocol was not described for oomycetes and this has severely hampered the elucidation of molecular processes underlying pathogenicity of oomycetes. Even though stable integrative DNA transformation of *P. infestans* and some other *Phytophthora* species such as *P. sojae*, *P. parasitica*, and *P. palmivora* has been accomplished (Judelson et al., 1991; 1993; Bottin et al., 1999; van West et al., 1999), direct disruption of a target gene has not been reported. Apparently, the frequency of homologous recombination is very low. Moreover, the diploid nature of oomycetes requires successive rounds of homologous recombination at the target gene to obtain homozygous mutants.

As an alternative to gene disruption, gene silencing can be used to obtain mutants. In several organisms it has been shown that introduction of one or multiple copies of homologous transgenes into the genome often leads to gene silencing of the transgenes and of the homologous host gene copies (Matzke & Matzke, 1995; Baulcombe & English, 1996; Meyer & Saedler, 1996). In plants, animals, and fungi, a reduction in mRNA levels is caused by a variety of epigenetic processes. However, the exact mechanisms underlying gene silencing are often poorly defined. Nevertheless, gene silencing technologies have been instrumental in unravelling gene function in many organisms, particularly in plants, and have been used to generate stable transgenic lines with desirable characteristics. For example, in tomato, gene silencing has been used to analyse the *in vivo* function of several genes involved in fruit ripening and antisense tomato lines with altered ripening properties have been

developed (Bird et al., 1991; Picton et al., 1995). In addition, transgenic plants containing virus-derived sequences have been shown to be resistant to virus infections through silencing of viral genes (Baulcombe, 1996; Prins & Goldbach, 1996).

To investigate the feasibility of gene silencing in *Phytophthora*, we selected the *inf1* gene of *P. infestans* as a target gene (Chapter 5). Efficient *inf1* gene silencing was found after stable integration of *inf1* gene constructs in the *P. infestans* genome.

Scope of This Thesis

Despite extensive studies on *P. infestans* and on various aspects of the interaction with its host plant, relatively little is known about compounds produced by the pathogen during the interaction and factors involved in determining compatibility or incompatibility. In this thesis, the characterisation of four *P. infestans* genes, *ipiO*, *ipiB*, *ric1*, and *inf1*, is described (Chapters 2, 4 & 7). These genes all encode proteins expected to be involved in the plant pathogen interaction.

Three different strategies were used to isolate and clone these genes. The *ipiB* and *ipiO* genes were isolated by differential screening of a genomic library of *P. infestans* with probes derived from mRNA isolated from infected leaves, and mRNA isolated from *in vitro*-cultured *P. infestans* (Pieterse et al., 1993a). Both genes were selected as specific *in planta*-induced genes, with the idea that such genes may play a role in pathogenesis. The characterisation of *ipiB* and *ipiO* is described in Chapter 2. In Chapter 7, a small scale expressed sequence tag (EST) approach was used to isolate genes that might play a role in the interaction. By sequencing a few randomly isolated cDNA clones from an interaction cDNA library we isolated *ric1*, which appeared to code for a stress-induced protein with putative membrane-spanning domains. The fourth gene, *inf1*, was cloned by heterologous hybridisation of a cDNA library with a probe from an elicitin gene of *P. parasitica* (Chapter 4). From previous data, it was hypothesised that the INF1 protein could function as an avirulence factor by limiting the host range of *P. infestans*.

Expression of *ipiO* was studied in detail by making use of the GUS reporter gene (Chapter 3). *IpiO* appears to be highly expressed in the hyphal tips at the edge of the expanding lesion where the pathogen is invading healthy plant cells, suggesting that IPI-O is localised at the interface between the invading hyphae and the plant cells, and thus could play a role in pathogenicity.

Strong evidence for involvement of a particular protein in the interaction can be obtained with stable mutants deficient in the production of such a protein. By homology-dependent gene silencing, expression of the *inf1* gene was abolished (Chapter 5) which allowed the functional analysis of this gene. The INF1-deficient mutants appear to have an extended host range and this suggests a role for INF1 as a host specific

avirulence factor (Chapter 6). Using gene silencing in an efficient manner and as a general method to obtain mutants requires insight into the mechanism of gene silencing in *P. infestans*. Therefore, the INF1-deficient mutants were used to analyse whether *inf1*-silencing is a transcriptional or post-transcriptional event (Chapter 5).

Finally in Chapter 8 the relevance of these studies to unravel key processes during development of late blight is discussed.

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Chapter 2

Structure and Genomic Organisation of the *ipiB* and *ipiO* Gene Clusters of *Phytophthora infestans*

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Summary

Two *in planta*-induced (*ipi*) genes, designated *ipiB* and *ipiO*, of the potato late blight pathogen, *Phytophthora infestans* (Mont.) de Bary, were isolated from a genomic library by a differential hybridisation procedure (Pieterse et al., 1993a). Both genes are expressed at high levels in the early phases of the pathogenic interaction of *P. infestans* with its host plant potato, suggesting that their gene products have a function in the early stages of the infection process. Here, we describe the nucleotide sequence and genomic organisation of *ipiB* and *ipiO*. The *ipiB* gene belongs to a small gene family consisting of at least three genes, designated *ipiB1*, *ipiB2* and *ipiB3*, which are clustered in a head-to-tail arrangement. The three *ipiB* genes are highly homologous throughout the coding regions and 5' and 3' flanking regions. The *P. infestans* genome contains two very similar *ipiO* genes, *ipiO1* and *ipiO2*, which are closely linked and arranged in an inverted orientation. The *ipiB* genes encode three novel, highly similar glycine-rich proteins of 301, 343 and 347 amino acids, respectively. The glycine-rich domains of the IPI-B proteins are predominantly composed of two repeats with the core sequences, A/V-G-A-G-L-Y-G-R and G-A-G-Y/V-G-G. The *ipiO* genes code for two almost identical 152-amino acid proteins which do not have any homology with sequences present in data libraries. IPI-B and IPI-O contain putative signal peptides of 20 and 21 amino acids, respectively, suggesting that they are transported out of the cytoplasm. In the promoter regions of *ipiB* and *ipiO*, a 16-nucleotide sequence motif, matching the core sequence GCTCATTYYNCAWTTT, was found. This sequence motif appears to be present around the transcription start site (*tss*) of seven out of eight oomycete genes for which the *tss* have been determined, suggesting that oomycetes have a sequence preference for transcription initiation.

Introduction

The oomycete *Phytophthora infestans* (Mont.) de Bary is the causal agent of the devastating late blight disease on potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.). Molecular studies on the potato-*P. infestans* interaction have demonstrated that pathogen attack activates genes in the host plant (Fritzemeier et al., 1987; Hahlbrock et al., 1989; Taylor et al., 1990; Choi et al., 1992; Schröder et al., 1992; Martini et al., 1993). Many of these genes encode products which are thought to be involved in the inhibition of pathogen development. Also in the pathogen, interaction with the host plant is accompanied by the activation of certain genes (Pieterse et al., 1991; 1992; 1993a; 1993b). Products of these so-

called *in planta*-induced (*ipi*) genes may be necessary for establishment and maintenance of basic pathogenicity or for the increase of disease severity. Characterisation of *P. infestans* genes of which the expression is specifically induced *in planta* may therefore lead to the identification of so far unknown pathogenicity factors.

Recently, we described the selection of nine *in planta*-induced genes by differential screening of a genomic library of *P. infestans* DNA using first strand cDNA probes synthesised on (i) mRNA isolated from *P. infestans*-infected potato leaves and (ii) mRNA isolated from *P. infestans* grown *in vitro* (Pieterse et al., 1993a). A detailed characterisation of two of these *in planta*-induced genes, *ubi3R* and *calA*, showed that they encode polyubiquitin and calmodulin, respectively (Pieterse et al., 1991; 1993b). Ubiquitin plays a key role in several cellular processes such as selective degradation of intracellular proteins, maintenance of chromatin structure, regulation of gene expression and modification of cell-surface receptors (Monia et al., 1990). Calmodulin is a calcium-binding protein which is known to play an essential role in basic cellular processes such as signal transduction, ion transport and cytoskeleton function (Cheung, 1980). Both *ubi3R* and *calA* are expressed during growth of *P. infestans in vitro* but during pathogenesis on potato, the expression levels are consistently five-fold higher. In contrast to *ubi3R* and *calA*, two other *in planta*-induced genes, *ipiB* and *ipiO*, show a transient expression pattern during pathogenesis with the highest expression level in the early stages of infection (Pieterse et al., 1993a). It appears that both *ipiB* and *ipiO* belong to small, clustered gene families. In this paper we describe the molecular characterisation and genomic organisation of the members of the *ipiB* and *ipiO* gene clusters.

Results and Discussion

Isolation and Genomic Organisation of the *ipiB* and *ipiO* Genes

With the aim to select *P. infestans* genes whose expression is induced or significantly increased during pathogenesis on potato, a genomic library of *P. infestans* DNA was constructed in λEMBL3 and differentially screened as described previously (Pieterse et al., 1993a). The differential screening resulted in the selection of several genomic clones. Two of these differentially hybridising clones (DHCs), DHC-B and DHC-O, contain *in planta*-induced genes which are highly expressed in the early stages of infection (Pieterse et al., 1993a). The *in planta*-induced genes located on DHC-B and DHC-O were designated *ipiB* and *ipiO*, respectively. The approximate location of the coding regions of the *ipiB* and *ipiO* genes on DHC-B and DHC-O was assessed by Southern blot analyses. Blots containing digested DNA of DHC-B and DHC-O were hybridised with a labelled first strand cDNA probe which was synthesised on poly(A)$^+$RNA isolated from

P. infestans-infected potato leaves, two days post-inoculation (interaction cDNA probe). In this way, the DHC-B and DHC-O fragments containing transcribed sequences which correspond to the coding regions of the *ipiB* and *ipiO* genes were identified (indicated with a closed line in Figures 1A & 1C). These fragments were subcloned and a detailed restriction endonuclease profile of the DNA surrounding the *ipiB* and *ipiO* genes was determined (Figures 1B & 1D). In both cases, repetition of specific endonuclease profiles was observed. Cross-hybridisation experiments showed that these repeated areas are highly homologous suggesting the presence of a cluster of similar genes.

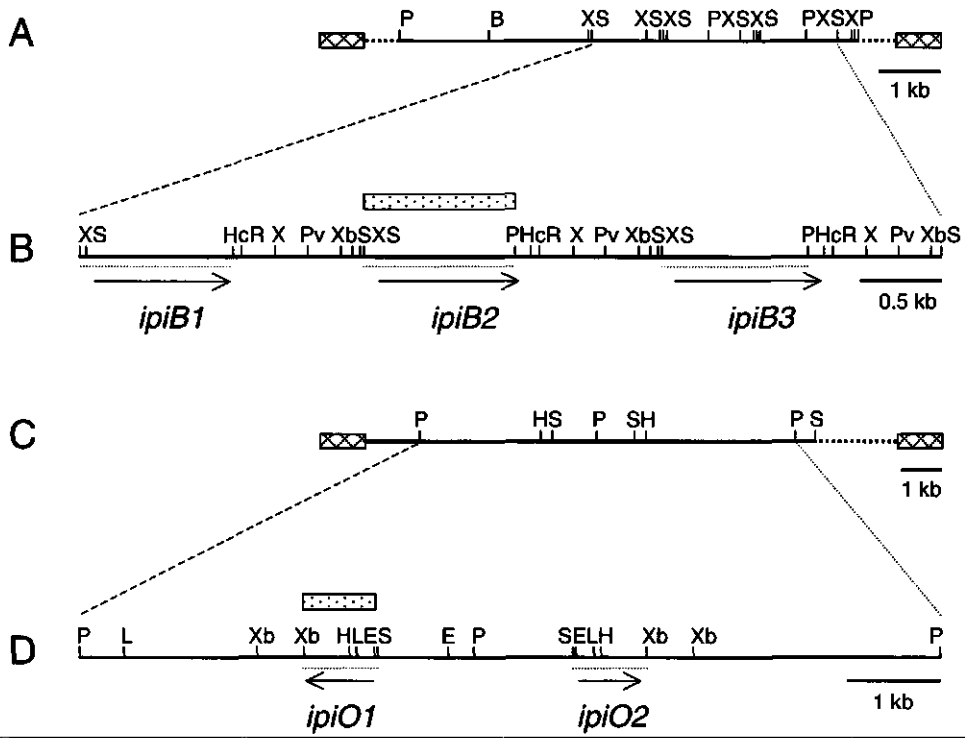

Figure 1. Organisation of the *P. infestans ipiB* and *ipiO* Genes

(A & C). Partial restriction map of λEMBL3 recombinant phages DHC-B (A) and DHC-O (C) which were isolated from a genomic library of *P. infestans* DNA by differential hybridisation as described by Pieterse et al. (1993a). Crosshatched bars represent λEMBL3 arms. Discontinuous lines represent DNA regions of unknown length and restriction endonuclease profile. Solid lines indicate the approximate position of the coding regions of the *ipiB* and *ipiO* genes as identified by Southern blot analysis of DHC-B and DHC-O restriction fragments, using as probe first-strand cDNA synthesised on poly(A)$^+$RNA isolated from *P. infestans*-infected potato leaves, two days post-inoculation (interaction cDNA probe). RNA isolation and cDNA synthesis were performed as described previously (Pieterse et al., 1993a).

(B & D). Restriction map of DNA regions from DHC-B and DHC-O in which *ipiB* and *ipiO* genes are located. Dotted lines show the positions of the coding regions of the *ipiB* (B) and *ipiO* (D) genes, respectively. This was determined by Southern blot analysis of blots containing insert DNA of a number of subclones derived from the shown DNA region, hybridised with labelled interaction cDNA as probe. Arrows indicate positions and directions of the coding sequences of the *ipiB* and *ipiO* genes as

assessed by dideoxy sequencing. Stippled bars represent the DNA fragments which were used as probes for hybridisation of genomic Southern blots and for the isolation of the *ipiO1* cDNA clone from the λZAP cDNA library. Restriction sites indicated are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Hin*cII (Hc), *Sal*I (L), *Pst*I (P), *Pvu*II (Pv), *Eco*RV (R), *Sst*I (S), *Xho*I (X), *Xba*I (Xb).

To determine which restriction fragments contain the coding regions of the *ipiB* and *ipiO* genes, Southern blots of digested DHC-B and DHC-O subclones were hybridised using labelled interaction cDNA as probe. Of the DHC-B subclones, one *Sst*I-*Hin*cII fragment of 0.9 kb and two 0.98 kb *Sst*I-*Pst*I fragments hybridised with the interaction cDNA probe whereas of the DHC-O subclones, two 0.63 kb *Sst*I-*Xba*I fragments hybridised (indicated with dotted lines in Figures 1B & 1D). This indicates that the coding regions of the *ipiB* and *ipiO* genes are constrained within these respective DNA fragments. On northern blots containing RNA isolated from *P. infestans*-infected potato leaves, probes derived from all three DHC-B fragments hybridised to a mRNA transcript of 1200 nucleotides (nt) in length (Pieterse et al., 1993a). Probes derived from the two DHC-O fragments both hybridised to a mRNA transcript of 650 nt in length. Considering the repetitive restriction endonuclease profile, the cross-hybridisation, and the size of the DNA fragments in comparison to the length of the hybridising mRNA transcripts, it can be concluded that DHC-B as well as DHC-O contain gene clusters with three and two highly homologous genes, respectively. The genes located on DHC-B were designated *ipiB1*, *ipiB2* and *ipiB3* (Figure 1B), the ones on DHC-O *ipiO1* and *ipiO2*, respectively (Figure 1D). There is no cross-hybridisation between the *ipiB* and *ipiO* genes.

Southern Blot Analyses of Genomic DNA from *P. infestans*

To determine the copy number of the *ipiB* and *ipiO* genes in the *P. infestans* genome, Southern blot analyses were performed. Blots containing digested genomic *P. infestans* DNA were hybridised with the *ipiB2* containing 0.98 kb *Sst*I-*Pst*I fragment from DHC-B and with the *ipiO1* containing 0.63 kb *Sst*I-*Xba*I fragment from DHC-O, respectively (indicated with dotted bars in Figures 1B & 1D). The *ipiB2* probe hybridised to approximately ten *Pst*I, *Xho*I and *Sst*I fragments (Figure 2). Only the 7.0 and 1.8 kb *Pst*I, the 1.4 and 1.2 kb *Xho*I and the 1.8 and 1.7 kb *Sst*I fragments correspond to restriction fragments present in DHC-B indicating that there are other *ipiB* genes or *ipiB*-like sequences present in the *P. infestans* genome. The *ipiO1* probe hybridised to two *Pst*I fragments of 4.3 kb and 5.0 kb in length and to two *Sst*I fragments of 6.5 kb and 4.5 kb in length (Figure 2). These restriction fragments match with those found in DHC-O and two overlapping λ clones, DHC-O' and DHC-O"(data not shown). It can thus be concluded that the *ipiO* gene cluster present in DHC-O is unique in the *P. infestans* genome. Under the hybridisation conditions used, the *ipiB* nor the *ipiO* probe hybridised to potato DNA (Figure 2) indicating that there are no highly similar sequences present in the potato genome.

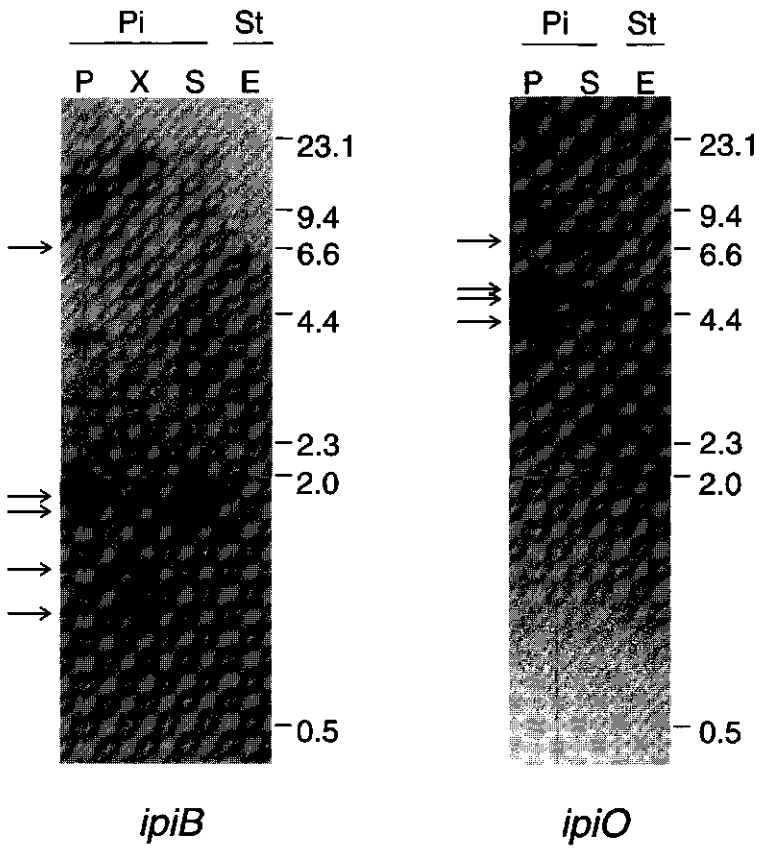

Figure 2. Autoradiographs of Southern blots Containing Genomic DNA of (Pi) *P. infestans* (strain 88069) and (St) Potato (cultivar 'Ajax'), Hybridised with the *ipiB* and *ipiO* Probes
Genomic DNA was isolated as described by Pieterse et al. (1991). Genomic DNA (10 mg) was digested with *Eco*RI (E), *Pst*I (P), *Sst*I (S) or *Xho*I (X). After electrophoresis on a 0.7% agarose gel, Southern blotting was performed on Hybond-N^+ membranes (Amersham) according to the manufacturers instructions. Blots were hybridised with probes derived from the 0.98 kb *Sst*I-*Pst*I fragment of DHC-B and the 0.63 kb *Sst*I-*Xba*I fragment of DHC-O as indicated by the stippled bars in Figures 1B & 1D, respectively. Probes were labelled by random primer labelling (Feinberg & Vogelstein, 1983). Hybridisation was performed overnight in 0.5 M Na_2HPO_4/NaH_2PO_4 (pH 7.2)/7% SDS/1 mM EDTA at 65 °C. Blots were subsequently washed in 0.2x SSC/0.1% SDS at 65 °C and exposed to Kodak X-OMAT S film. Molecular size markers are indicated in kb. Arrows indicate hybridising DNA fragments present in DHC-B and DHC-O, respectively.

Nucleotide Sequence of the *ipiB* Genes

The nucleotide sequence of the *Xho*I-*Sst*I fragment of 5424 bp in length (Figure 1B), comprising the coding regions of *ipiB1*, *ipiB2* and *ipiB3*, was determined by dideoxy sequencing and is shown in Figure 3. Three highly homologous open reading frames (ORFs) of 903, 1029 and 1041 nucleotides were found at positions where the coding regions of the *ipiB* genes were predicted (Figure 1B). The lengths of the ORFs are in agreement with the size of the 1200 nt *ipiB* mRNA when adding 5' and 3' non-

translated regions. The distances between the ORFs of *ipiB1* and *ipiB2*, and between *ipiB2* and *ipiB3* are 820 and 819 nt, respectively. When allowing gaps for optimal alignment, the coding regions of the *ipiB* genes are 96% identical, whereas the 0.82 kb intergenic DNA sequences are for 98% the same. A DNA region highly homologous to the 0.82 kb intergenic DNA sequences is also present immediately downstream of the *ipiB3* coding region, suggesting the presence of a fourth gene succeeding the *ipiB3* gene. However, the DNA regions surrounding the sequenced 5424 bp *Xho*I-*Sst*I fragment do not hybridise to the *ipiB2* probe (data not shown) indicating that there are no additional *ipiB* genes in the direct vicinity of this *ipiB* gene cluster. The 2.2 kb *Bam*HI-*Sst*I fragment preceding the 5424 bp *Xho*I-*Sst*I fragment shows cross-hybridisation with a probe derived from the 0.82 kb intergenic region, suggesting that the 5' regulatory sequences of *ipiB1* are similar to those of *ipiB2* and *ipiB3* (data not shown).

Nucleotide Sequence of the *ipiO* Genes

The DNA sequence of the 3440 bp *Xba*I fragment, comprising the coding regions of *ipiO1* and *ipiO2*, was determined by dideoxy sequencing (Figure 4). In addition, the DNA sequence of a partial *ipiO* cDNA clone was assessed. This *ipiO* cDNA clone was isolated from a λZAP cDNA library representing poly(A)$^+$RNA from *P. infestans*-infected potato leaves, two days post inoculation. The library was screened with the 0.63 kb *Sst*I-*Xba*I fragment from DHC-O (Figure 1D). In Figure 4, lines indicated by (a) show the nucleotide sequence of the *Xba*I-*Pst*I fragment on which *ipiO1* is located. Lines indicated by (b) show the nucleotide sequence of the adjacent *Pst*I-*Xba*I fragment containing *ipiO2*. Two ORFs of 456 nt were found at positions where the coding regions of the *ipiO* genes were predicted (Figure 1D). The 518 nt sequence of the partial *ipiO* cDNA clone is identical to the *ipiO1* sequence from nt +51 relative to the ATG start codon up to 110 nt downstream of the TAG stop codon (Figure 4). The ORF representing *ipiO1* is located at a distance of 2224 bp upstream of the ORF of *ipiO2*. The orientations of the ORFs are inverted (Figure 1D). The nucleotide sequences of *ipiO1* and *ipiO2* show 99% identity from 637 nt upstream of the ATG start codon, throughout the coding sequence, up to at least 152 nt downstream of the TAG stop codon. The restriction endonuclease profile suggests that the similarity extends even further since both genes have a cross-hybridising 0.6 kb *Xba*I fragment downstream of their coding regions (Figure 1D). In the 5' regions, the DNA sequences diverge upstream of positions –637, resulting in a unique 950 nt intergenic DNA region.

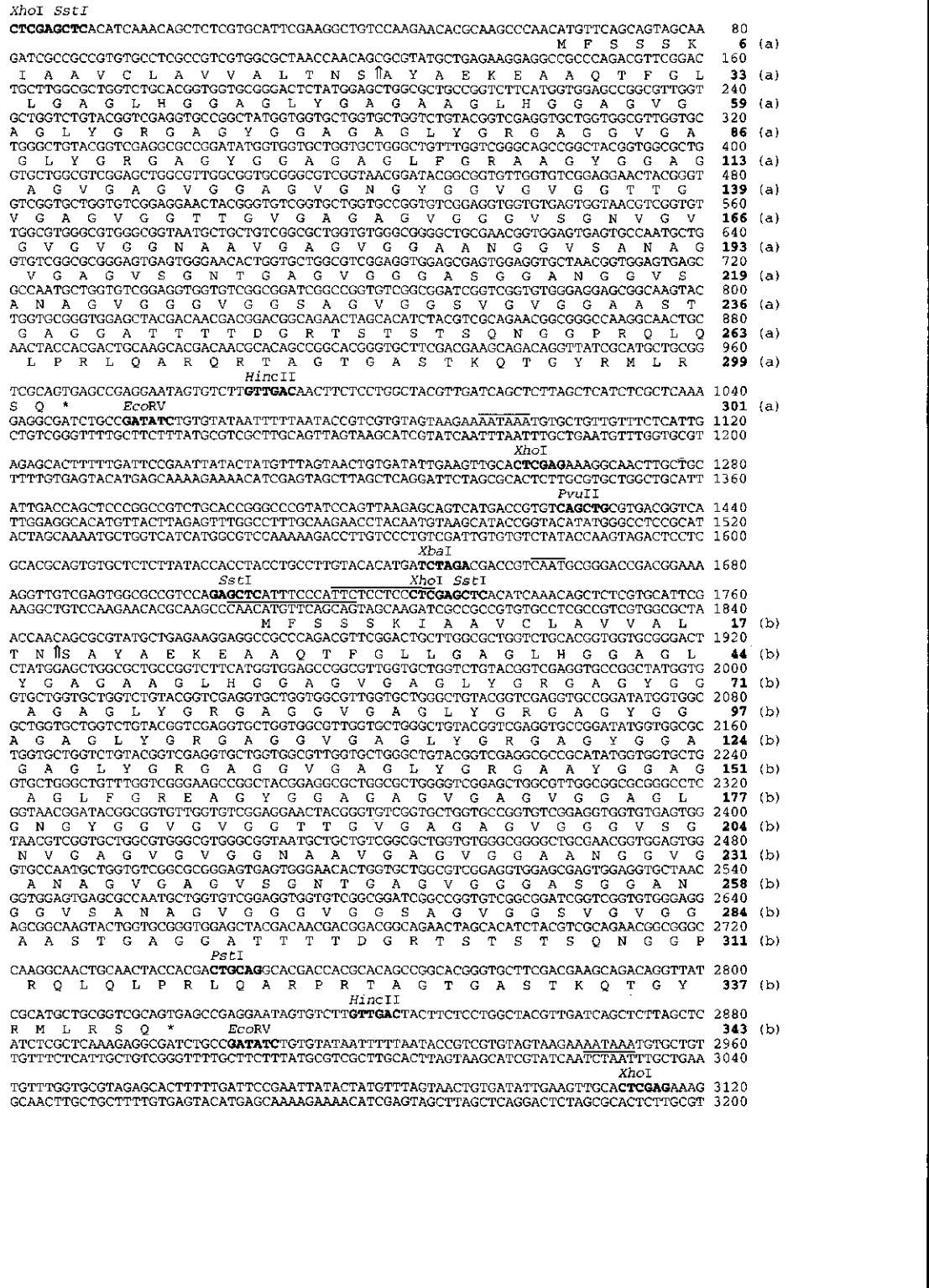
XhoI SstI
CTCGAGCTCACATCAAACAGCTCTCGTGCATTCGAAGGCTGTCCAAGAACACGCAAGCCCAACATGTTCAGCAGTAGCAA 80
M F S S S K 6 (a)
GATCGCCGCCGTGTGCCTCGCCGTCGTGGCGCTAACCAACAGCGCGTATGCTGAGAAGGAGGCCGCCCAGACGTTCGGAC 160
I A A V C L A V V A L T N S ⇑A Y A E K E A A Q T F G L 33 (a)
TGCTTGGCGCTGGTCTGCACGGTGGTGCGGGACTCTATGGAGCTGGCGCTGCCGGTCTTCATGGTGGAGCCGGCGTTGGT 240
L G A G L H G G A G L Y G A G A A G L H G G A G V G 59 (a)
GCTGGTCTGTACGGTCGAGGTGCCGGCTATGGTGGTGCTGGTCTGGTCTGTACGGTCGAGGTGCTGGTGGCGTTGGTGC 320
A G L Y G R G A G Y G G A G A G L Y G R G A G G V G A 86 (a)
TGGGCTGTACGGTCGAGGCGCCGGATATGGTGGTGCTGGTGCTGGGCTGTTTGGTCGGGCAGCCGGCTACGGTGGCGCTG 400
G L Y G R G A G Y G G A G A G L F G R A A G Y G G A G 113 (a)
GTGCTGGCGTCGGAGCTGGCGTTGGCGGTGCGGGCGTCGGTAACGGATACGGCGGTGTTGGTGTCGGAGGAACTACGGGT 480
A G V G A G V G G A G V G N G Y G G V G V G G T T G 139 (a)
GTCGGTGCTGGTGTCGGAGGAACTACGGGTGTCGGTGCTGGTGCCGGTGTCGGAGGTGGTGTGAGTGGTAACGTCGGTGT 560
V G A G V G G T T G V G A G A G V G G G V S G N V G V 166 (a)
TGGCGTGGGCGTGGGCGGTAATGCTGCTGTCGGCGCTGGTGTGGGCGGGGCTGCGAACGGTGGAGTGAGTGCCAATGCTG 640
G V G V G G N A A V G A G V G G A A N G G V S A N A G 193 (a)
GTGTCGGCGCGGGAGTGAGTGGGAACACTGGTGCTGGCGTCGGAGGTGGAGCGAGTGGAGGTGCTAACGGTGGAGTGAGC 720
V G A G V S G N T G A G V G G G A S G G A N G G V S 219 (a)
GCCAATGCTGGTGTCGGAGGTGGTGTCGGCGGATCGGCCGGTGTCGGCGGATCGGTCGGTGTGGGAGGAGCGGCAAGTAC 800
A N A G V G G G V G G S A G V G G S V G V G G A A S T 236 (a)
TGGTGCGGGTGGAGCTACGACAACGACGGACGGCAGAACTAGCACATCTACGTCGCAGAACGGCGGGCCAAGGCAACTGC 880
G A G A T T T T D G R T S T S T S Q N G G P R Q L Q 263 (a)
AACTACCACGACTGCAAGCACGACAACGCACAGCCGGCACGGGTGCTTCGACGAAGCAGACAGGTTATCGCATGCTGCGG 960
L P R L Q A R Q R T A G T G A S T K Q T G Y R M L R 299 (a)
HincII
TCGCAGTGAGCCGAGGAATAGTGTCTTGTTGACAACTTCTCCTGGCTACGTTGATCAGCTCTTAGCTCATCTCGCTCAAA 1040
S Q * EcoRV 301 (a)
GAGGCGATCTGCCGATATCTGTGTATAATTTTTAATACCGTCGTGTAGTAAGAAATAAATGTGCTGTTGTTTCTCATTG 1120
CTGTCGGGTTTTGCTTCTTTATGCGTCGCTTGCAGTTAGTAAGCATCGTATCAATTTAATTTGCTGAATGTTTGGTGCGT 1200
XhoI
AGAGCACTTTTTGATTCCGAATTATACTATGTTTAGTAACTGTGATATTGAAGTTGCACTCGAGAAAGGCAACTTGCTGC 1280
TTTTGTGAGTACATGAGCAAAAGAAAACATCGAGTAGCTTAGCTCAGGATTCTAGCGCACTCTTGCGTGCTGGCTGCATT 1360
PvuII
ATTGACCAGCTCCCGGCCGTCTGCACCGGGCCCGTATCCAGTTAAGAGCAGTCATGACCGTGTCAGCTGCGTGACGGTCA 1440
TTGGAGGCACATGTTACTTAGAGTTTGGCCTTTGCAAGAACCTACAATGTAAGCATACCGGTACATATGGGCCTCCGCAT 1520
ACTAGCAAAATGCTGGTCATCATGGCGTCCAAAAAGACCTTGTCCCTGTCGATTGTGTGTCTATACCAAGTAGACTCCTC 1600
XbaI
GCACGCAGTGTGCTCTCTTATACCACCTACCTGCCTTGTACACATGATCTAGACGACCGTCAATGCGGGACCGACGGAAA 1680
SstI XhoI SstI
AGGTTGTCGAGTGGCGCCGTCCAGAGCTCATTTCCCATTGTCCTCCCTCGAGCTCACATCAAACAGCTCTCGTGCATTCG 1760
AAGGCTGTCCAAGAACACGCAAGCCCAACATGTTCAGCAGTAGCAAGATCGCCGCCGTGTGCCTCGCCGTCGTGGCGCTA 1840
M F S S S K I A A V C L A V V A L 17 (b)
ACCAACAGCGCGTATGCTGAGAAGGAGGCCGCCCAGACGTTCGGACTGCTTGGCGCTGGTCTGCACGGTGGTGCGGGACT 1920
T N ⇑S A Y A E K E A A Q T F G L L G A G L H G G A G L 44 (b)
CTATGGAGCTGGCGCTGCCGGTCTTCATGGTGGAGCCGGCGTTGGTGCTGGTCTGTACGGTCGAGGTGCCGGCTATGGTG 2000
Y G A G A A G L H G G A G V G A G L Y G R G A G Y G G 71 (b)
GTGCTGGTGCTGGTCTGTACGGTCGAGGTGCTGGTGGCGTTGGTGCTGGGCTGTACGGTCGAGGTGCCGGATATGGTGGC 2080
A G A G L Y G R G A G G V G A G L Y G R G A G Y G G 97 (b)
GCTGGTGCTGGTCTGTACGGTCGAGGTGCTGGTGGCGTTGGTGCTGGGCTGTACGGTCGAGGTGCCGGATATGGTGGCGC 2160
A G A G L Y G R G A G G V G A G L Y G R G A G Y G G A 124 (b)
TGGTGCTGGTCTGTACGGTCGAGGTGCTGGTGGCGTTGGTGCTGGGCTGTACGGTCGAGGCGCCGCATATGGTGGTGCTG 2240
G A G L Y G R G A G G V G A G L Y G R G A A Y G G A G 151 (b)
GTGCTGGGCTGTTTGGTCGGGAAGCCGGCTACGGAGGCGCTGGCGCTGGGGTCGGAGCTGGCGTTGGCGGCGCGGGCCTC 2320
A G L F G R E A G Y G G A G A G V G A G V G A G L G 177 (b)
GGTAACGGATACGGCGGTGTTGGTGTCGGAGGAACTACGGGTGTCGGTGCTGGTGCCGGTGTCGGAGGTGGTGTGAGTGG 2400
G N G Y G G V G V G V G G T T G V G A G A G V G G G V S G 204 (b)
TAACGTCGGTGCTGGCGTGGGCGTGGGCGGTAATGCTGCTGTCGGCGCTGGTGTGGGCGGGGCTGCGAACGGTGGAGTGG 2480
N V G A G V G V G G N A A V G A G V G G A A N G G V G 231 (b)
GTGCCAATGCTGGTGTCGGCGCGGGAGTGAGTGGGAACACTGGTGCTGGCGTCGGAGGTGGAGCGAGTGGAGGTGCTAAC 2560
A N A G V G A G V S G N T G A G V G G G A S G G A N 258 (b)
GGTGGAGTGAGCGCCAATGCTGGTGTCGGAGGTGGTGTCGGCGGATCGGCCGGTGTCGGCGGATCGGTCGGTGTGGGAGG 2640
G G V S A N A G V G G G V G G S A G V G G S V G V G G 284 (b)
AGCGGCAAGTACTGGTGCGGGTGGAGCTACGACAACGACGGACGGCAGAACTAGCACATCTACGTCGCAGAACGGCGGGC 2720
A A S T G A G G A T T T T D G R T S T S T S Q N G G P 311 (b)
PstI
CAAGGCAACTGCAACTACCACGACTGCAGGCACGACCACGCACAGCCGGCACGGGTGCTTCGACGAAGCAGACAGGTTAT 2800
R Q L Q L P R L Q A R P R T A G T G A S T K Q T G Y 337 (b)
HincII
CGCATGCTGCGGTCGCAGTGAGCCGAGGAATAGTGTCTTGTTGACTACTTCTCCTGGCTACGTTGATCAGCTCTTAGCTC 2880
R M L R S Q * EcoRV 343 (b)
ATCTCGCTCAAAGAGGCGATCTGCCGATATCTGTGTATAATTTTTAATACCGTCGTGTAGTAAGAAAATAAATGTGCTGT 2960
TGTTTCTCATTGCTGTCGGGTTTTGCTTCTTTATGCGTCGCTTGCACTTAGTAAGCATCGTATCAATCTAATTTGCTGAA 3040
XhoI
TGTTTGGTGCGTAGAGCACTTTTTGATTCCGAATTATACTATGTTTAGTAACTGTGATATTGAAGTTGCACTCGAGAAAG 3120
GCAACTTGCTGCTTTTGTGAGTACATGAGCAAAAGAAAACATCGAGTAGCTTAGCTCAGGACTCTAGCGCACTCTTGCGT 3200

Figure 3. Nucleotide Sequence of the *P. infestans ipiB* Gene Cluster

The nucleotide sequence starts at the most left *Xho*I site shown in Figure 1B and extends up to the most right *Sst*I site depicted in the same Figure. Deduced amino acid sequences of IPI-B1 (a), IPI-B2 (b) and IPI-B3 (c) are indicated below the ORFs of *ipiB1*, *ipiB2*, and *ipiB3*, respectively. Noted in the Figure are: CAAT-motifs (overlined); sequences matching the conserved sequence motif GCTCATTYYNCAWTTT (underlined); C+T-rich regions (overlined); putative signal sequence cleavage sites (⇑); and potential polyadenylation signals AATAAA (overlined). The *ipiB1*, *ipiB2*, and *ipiB3* sequences will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession numbers L24206, L23937 and L23936, respectively. For sequencing, overlapping subclones were made in pTZ19u using standard procedures (Sambrook et al., 1989). Sequencing was performed on double-stranded DNA by the dideoxynucleotide termination method (Sanger et al., 1977) using the Multiwell Microtitre Plate Sequencing System (Amersham) and [α-^{35}S]dATP as a label. Analyses of sequence data and alignment of nucleotide and amino acid sequences as shown in Figures 3, 4, 5, 6 & 7 were performed using the Sequence Analysis Software Package, version 7.1 of the Genetics Computer Group (GCG), Madison, WI, USA (Devereux et al., 1984).

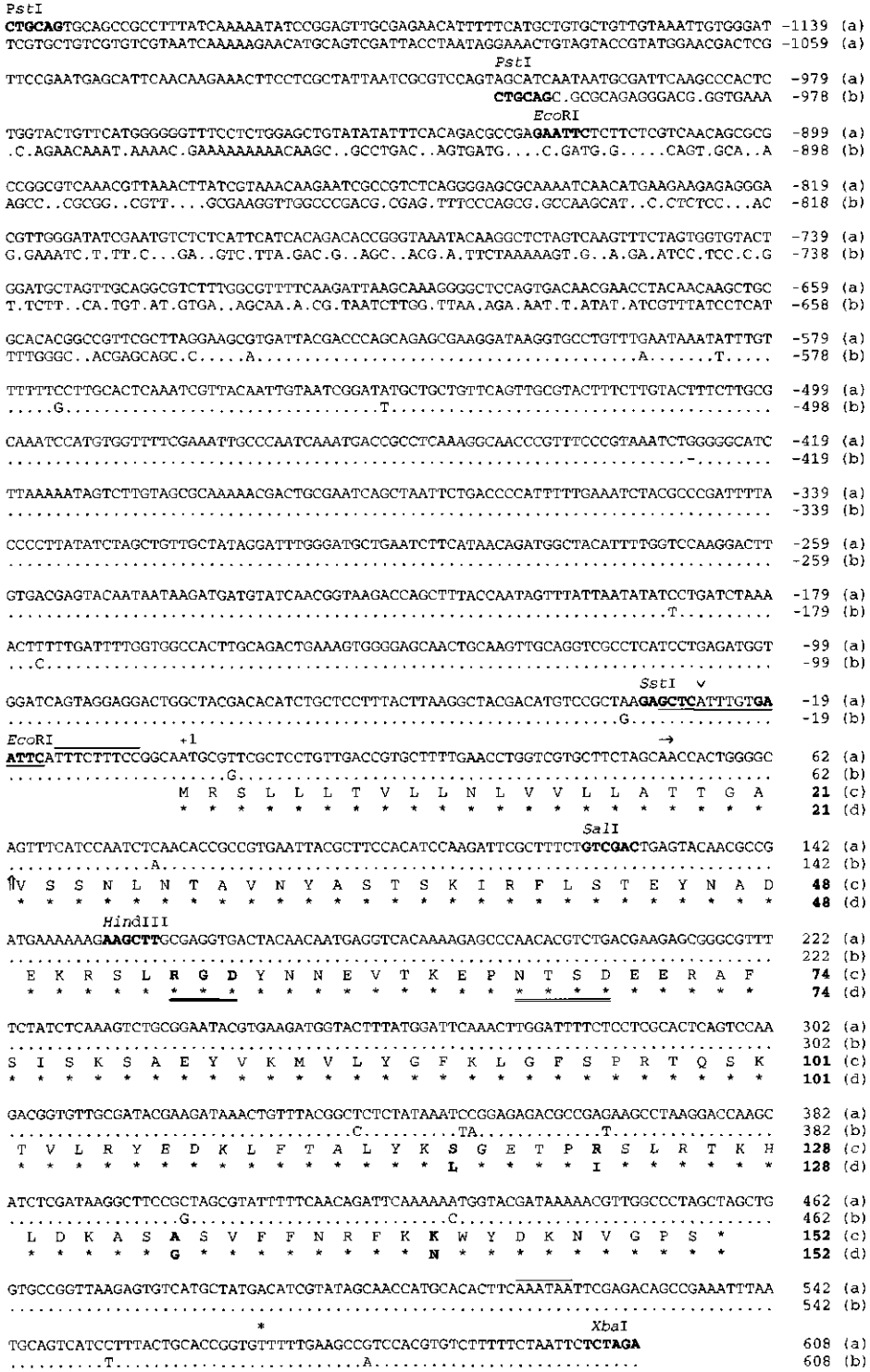
PstI
CTGCAGTGCAGCCGCCTTTATCAAAAATATCCGGAGTTGCGAGAACATTTTTCATGCTGTGCTGTTGTAAATTGTGGGAT -1139 (a)
TCGTGCTGTCGTGTCGTAATCAAAAAGAACATGCAGTCGATTACCTAATAGGAAACTGTAGTACCGTATGGAACGACTCG -1059 (a)
PstI
TTCCGAATGAGCATTCAACAAGAAACTTCCTCGCTATTAATCGCGTCCAGTAGCATCAATAATGCGATTCAAGCCCACTC -979 (a)
CTGCAGC.GCGCAGAGGGACG.GGTGAAA -978 (b)
EcoRI
TGGTACTGTTCATGGGGGGTTTCCTCTGGAGCTGTATATATTTCACAGACGCCGAGAATTCTCTTCTCGTCAACAGCGCG -899 (a)
.C.AGAACAAAT.AAAAC.GAAAAAAAAACAAGC..GCCTGAC..AGTGATG....C.GATG.G.....CAGT.GCA..A -898 (b)
CCGGCGTCAAACGTTAAACTTATCGTAAACAAGAATCGCCGTCTCAGGGGAGCGCAAAATCAACATGAAGAAGAGAGGGA -819 (a)
AGCC..CGCGG..CGTT....GCGAAGGTTGGCCCGACG.CGAG.TTTCCCAGCG.GCCAAGCAT..C.CTCTCC...AC -818 (b)
CGTTGGGATATCGAATGTCTCTCATTCATCACAGACACCGGGTAAATACAAGGCTCTAGTCAAGTTTCTAGTGGTGTACT -739 (a)
G.GAAATC.T.TT.C...GA..GTC.TTA.GAC.G..AGC..ACG.A.TTCTAAAAAGT.G..A.GA.ATCC.TCC.C.G -738 (b)
GGATGCTAGTTGCAGGCGTCTTTGGCGTTTTCAAGATTAAGCAAAGGGGCTCCAGTGACAACGAACCTACAACAAGCTGC -659 (a)
T.TCTT..CA.TGT.AT.GTGA..AGCAA.A.CG.TAATCTTGG.TTAA.AGA.AAT.T.ATAT.ATCGTTTATCCTCAT -658 (b)
GCACACGGCCGTTCGCTTAGGAAGCGTGATTACGACCCAGCAGAGCGAAGGATAAGGTGCCTGTTTGAATAAATATTTGT -579 (a)
TTTGGGC..ACGAGCAGC.C.....A.....A.....T..... -578 (b)
TTTTTCCTTGCACTCAAATCGTTACAATTGTAATCGGATATGCTGCTGTTCAGTTGCGTACTTTCTTGTACTTTCTTGCG -499 (a)
.....G.....T..... -498 (b)
CAAATCCATGTGGTTTTCGAAATTGCCCAATCAAATGACCGCCTCAAAGGCAACCCGTTTCCCGTAAATCTGGGGGCATC -419 (a)
.....-..... -419 (b)
TTAAAAATAGTCTTGTAGCGCAAAAACGACTGCGAATCAGCTAATTCTGACCCCATTTTTGAAATCTACGCCCGATTTTA -339 (a)
..... -339 (b)
CCCCTTATATCTAGCTGTTGCTATAGGATTTGGGATGCTGAATCTTCATAACAGATGGCTACATTTTGGTCCAAGGACTT -259 (a)
..... -259 (b)
GTGACGAGTACAATAATAAGATGATGTATCAACGGTAAGACCAGCTTTACCAATAGTTTATTAATATATCCTGATCTAAA -179 (a)
.....T..... -179 (b)
ACTTTTTGATTTTGGTGGCCACTTGCAGACTGAAAGTGGGGAGCAACTGCAAGTTGCAGGTCGCCTCATCCTGAGATGGT -99 (a)
...C..... -99 (b)
SstI
GGATCAGTAGGAGGACTGGCTACGACACATCTGCTCCTTTACTTAAGGCTACGACATGTCCGCTAAGAGCTCATTTGTGA -19 (a)
.....G..... -19 (b)
EcoRI +1 →
ATTCATTTCTTTCCGGCAATGCGTTCGCTCCTGTTGACCGTGCTTTTGAACCTGGTCGTGCTTCTAGCAACCACTGGGGC 62 (a)
.....G..... 62 (b)
M R S L L L T V L L N L V V L L A T T G A 21 (c)
* 21 (d)
SalI
AGTTTCATCCAATCTCAACACCGCCGTGAATTACGCTTCCACATCCAAGATTCGCTTTCTGTCGACTGAGTACAACGCCG 142 (a)
.....A..... 142 (b)
V S S N L N T A V N Y A S T S K I R F L S T E Y N A D 48 (c)
* 48 (d)
HindIII
ATGAAAAAAGAAGCTTGCGAGGTGACTACAACAATGAGGTCACAAAAGAGCCCAACACGTCTGACGAAGAGCGGGCGTTT 222 (a)
..... 222 (b)
E K R S L R G D Y N N E V T K E P N T S D E E R A F 74 (c)
* 74 (d)
TCTATCTCAAAGTCTGCGGAATACGTGAAGATGGTACTTTATGGATTCAAACTTGGATTTTCTCCTCGCACTCAGTCCAA 302 (a)
..... 302 (b)
S I S K S A E Y V K M V L Y G F K L G F S P R T Q S K 101 (c)
* 101 (d)
GACGGTGTTGCGATACGAAGATAAACTGTTTACGGCTCTCTATAAATCCGGAGAGACGCCGAGAAGCCTAAGGACCAAGC 382 (a)
.....C.....TA.....T..... 382 (b)
T V L R Y E D K L F T A L Y K S G E T P R S L R T K H 128 (c)
* * * * * * * * * * * * * * * * L * * * * I * * * * * * 128 (d)
ATCTCGATAAGGCTTCCGCTAGCGTATTTTTCAACAGATTCAAAAAATGGTACGATAAAAACGTTGGCCCTAGCTAGCTG 462 (a)
.....G.....C..... 462 (b)
L D K A S A S V F F N R F K K W Y D K N V G P S * 152 (c)
* * * * * G * * * * * * * * N * * * * * * * * * * 152 (d)
GTGCCGGTTAAGAGTGTCATGCTATGACATCGTATAGCAACCATGCACACTTCAAATAATTCGAGACAGCCGAAATTTAA 542 (a)
..... 542 (b)
*
XbaI
TGCAGTCATCCTTTACTGCACCGGTGTTTTTGAAGCCGTCCACGTGTCTTTTTCTAATTCTCTAGA 608 (a)
.....T.....A..... 608 (b)

Figure 4. Nucleotide Sequence of the *P. infestans ipiO* Gene Cluster and Deduced Amino Acid Sequences of the IPI-O1 and IPI-O2 proteins
(a) The nucleotide sequence of *ipiO1* starting from the *Pst*I site in the middle of the intergenic region and extending to the *Xba*I site downstream of *ipiO1* (Figure 1D); (b) The nucleotide sequence of *ipiO2* starting from the same *Pst*I site and extending to the *Xba*I site downstream of *ipiO2* (Figure 1D). Nucleotides identical to those in *ipiO1* are indicated by dots. To allow optimal alignment, one dash (-) is introduced at nucleotide position -427 in the *ipiO2* nucleotide sequence. (c) Deduced amino acid sequence of the IPI-O1 protein. (d) Deduced amino acid sequence of the IPI-O2 protein. Amino acid residues identical to those in the IPI-O1 sequence are indicated by asterisks. Noted in the figure are: sequence matching the conserved sequence motif GCTCATTYYNCAWTTT (single underline); *tss* (v); C+T-rich region (overlined); putative polyadenylation signal AAATAA (overlined); 5' end of the partial *ipiO1* cDNA clone (→); start poly(A) tail in partial *ipiO1* cDNA clone (*); putative signal sequence cleavage site (⇑); RGD cell adhesion motif (bold and underlined); putative N-glycosylation site (double underlined). The nucleotide sequences of *ipiO1* and *ipiO2* will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession numbers L23939 and L23938. For sequencing, overlapping subclones and deletion clones were made in pTZ19u using standard procedures (Sambrook et al., 1989). Deletion clones were generated by partial *Sau*3AI digestion of master subclones followed by electrophoresis of digestion products on a 0.7% agarose gel along with linearised master subclone DNA as marker. Singly digested, linearised DNA was isolated from the gel and digested to completion with *Bam*HI. DNA fragments were then circularised by ligation and transferred to *Escherichia coli* cells. The interaction cDNA library from which the partial *ipiO1* cDNA clone was isolated was constructed in λZAP (Stratagene, La Jolla, Ca, USA) according to the manufacturers instructions. As template, poly(A)$^+$RNA isolated from *P. infestans*-infected potato leaf tissue was used. The infected tissue was obtained from a zone of 1 cm in width at the outer edge of lesions surrounding the infection site on leaves of potato cultivar 'Ajax', 3 days after spot inoculation with 10 ml of a suspension of sporangia from *P. infestans* strain 88069 ($5{\cdot}10^5$ sporangia/ml).

Structural Features of the *ipiB* and *ipiO* Genes

The transcription start site (*tss*) of the *ipiO* genes was determined by primer extension. A 5' end labelled oligonucleotide complementary to nucleotides +86 to +102 in *ipiO1* and *ipiO2* was annealed to and extended on poly(A)$^+$RNA isolated from infected potato leaves, two days post-inoculation. A single primer extension product of 128 nt was found indicating that the *tss* corresponds to the A at position -26 relative to the ATG start codon (marked by a v in Figure 4). A direct comparison of the nucleotide sequence surrounding the major *tss* of eight distinct oomycete genes, in which the *tss* have been determined, revealed that these oomycete genes have a sequence preference for transcription initiation. In seven out of eight genes, the major *tss* is located within the sequence motif 5'-GCTCATTYYNCAWTTT (Table 1), which is invariably situated within the first 100 nt upstream of the ATG start codon. The *P. infestans actB* gene encoding actin (Unkles et al., 1991), does contain the conserved motif but the five *tss* of this gene are located 70 to 114 nt upstream of this sequence. The conserved sequence motif is also present in the 5' flanking regions of the *ipiB* genes, 69 to 84 nt upstream of the ATG start codon (Table 1). However, in this study the *tss* of the *ipiB* genes were not determined. The *P. infestans* genome may contain more *ipiB*-like genes in addition to the ones characterised here and so far it is not known whether the *ipiB* genes we have isolated are the ones which are transcribed. The presence of a conserved sequence motif surrounding the major *tss* of the oomycete genes suggests that the motif is important for transcription initiation. Since this motif is not conserved in genes of fungi, plants or animals, the 5'-

GCTCATTYYNCAWTTT sequence motif can be considered to be a consensus sequence for transcription initiation in oomycete genes.

In the majority of genes identified in higher eukaryotes, the consensus 'core promoter' sequences TATAAA and CAAT are found around 30 bp and 70-90 bp upstream of the major transcription initiation site, respectively. Interestingly, the significance of these motifs in transcription initiation in filamentous fungi has never been convincingly established (Gurr et al., 1987; Unkles, 1992). In the 5' flanking regions of *ipiB2* and *ipiB3* there are no typical TATAAA-like motifs. A CAAT motif is present at nucleotides -139 to -136 relative to the ATG start codon (overlined in Figure 3). In the 5' flanking regions of the *ipiO* genes, no TATAAA or CAAT-like motifs are present near the transcription start. The *ipiB* as well as the *ipiO* genes contain a C+T-rich region directly downstream of the transcription initiation consensus sequence (overlined in Figures 3 & 4). C+T-rich regions are commonly found in the vicinity of transcription initiation sites of filamentous fungal genes and are thought to be important for determining the position of transcription initiation (Unkles, 1992). The nucleotide sequence surrounding the translation start codons of the *ipiB* and *ipiO* genes (CCAACATGTT and CGGCAATGCG, respectively) follow the Kozak consensus sequence for translation initiation (Kozak, 1984), the most highly conserved nucleotide at position -3 being a purine.

The 3' terminus of the *ipiO* genes was determined by dideoxy sequencing of a partial *ipiO1* cDNA clone. In the *ipiO1* cDNA sequence, the poly(A) tail starts 110 nt downstream of the TAG stop codon which corresponds to nt +569 of the *ipiO1* genomic sequence (indicated by a * in Figure 4). A putative polyadenylation signal AAATAA (consensus AATAAA) was found 48 nt upstream of the poly(A) tail in the cDNA sequence (overlined in Figure 4). In the genomic sequence of the *ipiB* genes, potential polyadenylation signals (AATAAA) are present 126 nt downstream of the TGA stop codons (overlined in Figure 3).

The ORFs of the three *ipiB* genes as well as the ORFs of the two *ipiO* genes are not interrupted by introns. In contrast to most filamentous fungal genes, 68% of which contains introns (Gurr et al., 1987; Unkles, 1992), the oomycete genes studied to date predominantly lack introns. Among all oomycete genes reported so far (Judelson & Michelmore, 1989; 1990; LéJohn, 1989; Dudler, 1990; Pieterse et al., 1991; 1993b; Unkles et al., 1991; Moon et al., 1992) there is only one gene which contains introns, i.e. the *Phytophthora parasitica trp1* gene encoding indole-3-glycerolphosphate synthase-N-(5'-phosphoribosyl) anthranilate isomerase (Karlovsky & Prell, 1991). The presence of introns in this gene is surprising since all homologous *trp* genes from other eukaryotes tend to lack introns. In addition, the nucleotide sequence of the *P. infestans niaA* gene encoding nitrate reductase (Pieterse et al., 1995) suggests the presence of an intron in this gene as well.

Table 1. Alignment of the DNA Regions Surrounding the Conserved Sequence Motif GCTCATTYYNCAWTTT which Is Present within the First 100 Nucleotides Upstream of the ATG Start Codon of Eight Oomycete Genes [a]

gene	nt sequence surrounding the GCTCATTYYNCAWTTT motif [b]			
P. infestans ipiB[c,d]	-94	GCCGTCCAGA	GCTCATTTCCCATTCT	CCTCCCT -62
			********** *	
P. infestans ipiO1[e]	-40	TCCGCTAAGA	GCTCATTTGTGAATTC	ATTTCTT -8
			******** * ****	
P. infestans ubi3R	-66	CGCCTCCTTT	GCTCATTTTCCATTTT	GAGCGGA -34
			**********	
P. infestans calA	-62	TTTTGGATGG	GATCATTGTTGGATTT	CCCTCGA -30
			* ***** ** ****	
P. infestans actA	-84	TCCCTCTTTG	GCTCATTTCCC/TTTT	CTTCCAG -53
			********** ****	
P. infestans actB[f]	-60	GTGTCAAAGT	TCTCATTCTGCATTTT	GTCTCGA -28
			**********	
P. megasperma actin	-71	GGACCTTGCT	CGTCATTCCGCAATTT	GCTGCCA -39
			**********	
B. lactucae Ham34	-85	CGATCGGAAG	GCTCATTCTCC/TTTT	CACTCTC -54
			********** ****	
B. lactucae hsp70	-78	TCTCAAGTTT	GCTCACTTTGAAATTT	TCCATCT -46
			***** **** *****	

[a] *Phytophthora infestans* genes *ipiB2* and *ipiB3* (Figure 3), *ipiO1* and *ipiO2* (Figure 4), *ubi3R* (Pieterse et al., 1991), *calA* (Pieterse et al., 1993b), *actA* and *actB* (Unkles et al., 1991), the *P. megasperma* actin gene (Dudler, 1990), and the *Bremia lactucae* genes *Ham34* (Judelson & Michelmore, 1990) and *hsp70* (Judelson & Michelmore, 1989).

[b] Numbers refer to the position of the first and last nucleotide of the depicted sequence relative to the ATG start codon of the respective gene. Nucleotides marked with an asterisk match with the consensus sequence GCTCATTYYNCAWTTT. In the *P. infestans actA* sequence and the *B. lactucae Ham34* sequence, a one nt gap, indicated by a slash (/), is introduced in the conserved motif to obtain optimal alignment. Underlined nt show(s) the position of the *tss*.

[c] The nucleotide sequence shown, is present in the promoter region of the *P. infestans ipiB2* and *ipiB3* gene. The nucleotide sequence at positions -62 to -94 relative to the ATG start codon of *ipiB1* was not determined.

[d] The position of the *tss* has not been determined.

[e] The nucleotide sequence at position -8 to -40 relative to the ATG start codon of *ipiO1* and *ipiO2* is nearly identical. Only at position -34, the A in *ipiO1* is a G in *ipiO2*.

[f] Five *tss* have been found in the *P. infestans actB* promoter region which are all located more upstream of the depicted sequence between positions -120 and -164 relative to the ATG start codon (Unkles et al., 1991).

Analysis of the IPI-B Amino Acid Sequences

The IPI-B1 (301 amino acids), IPI-B2 (343 amino acids) and IPI-B3 (347 amino acids) proteins encoded by the ORFs of *ipiB1*, *ipiB2* and *ipiB3*, have a calculated molecular weight of 25.8, 29.6, and 30.2 kDa and a predicted isoelectric point of 11.2, 11.1, and 10.9, respectively. When allowing gaps for optimal alignment the proteins are 96-98% identical (Figure 5). The IPI-B proteins have a high content of glycine (Gly) residues and their Gly-rich domains show up to 47% identity to the Gly-rich domains of several plant Gly-rich proteins. Most plant Gly-rich proteins are characterised by their repetitive primary structure consisting of up to 70% Gly residues which are arranged in short amino acid repeats. Furthermore, they usually have a N-terminal signal sequence for transport out of the cytoplasm (Showalter, 1993). Analysis of the predicted amino acid sequences revealed that the IPI-B proteins share these characteristics with the plant Gly-rich proteins. The IPI-B proteins are composed of four domains. The first 20 amino acids at the N-terminal

end (domain I) comprise a putative signal sequence for secretion which can be recognised by a hydrophobic region in the hydropathy plot shown in Figure 6A. The signal sequence cleavage site, predicted according to Von Heijne (1986), is located between Ser^{20} and Ala^{21}. The hydropathy plot shows that the putative signal sequence is followed by a short hydrophilic region of 10 amino acids (domain II). Domain III, comprising 74-80% of the protein, consists of a large Gly-rich region with up to 47% Gly residues (IPI-B1 43%; IPI-B2 47%; IPI-B3 46%). The primary structure of the Gly-rich domain is highly repetitive with two typical repeats (Figure 7). The first repeat is characterised by the core sequence A/V-G-A-G-L-Y-G-R, the second repeat by G-A-G-Y/V-G-G. The C-termini of the predicted IPI-B proteins are composed of a 46-amino acid hydrophilic region (domain IV).

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MFSSSKIAAVCLAVVALTNSAYAEKEAAQTFGLLGAGLHGGAGLYGAGAAGLHGGAGVGAGLYGRGAGYG   70 IPI-B1
MFSSSKIAAVCLAVVALTNSAYAEKEAAQTFGLLGAGLHGGAGLYGAGAAGLHGGAGVGAGLYGRGAGYG   70 IPI-B2
MFSSSKIAAVCLAVVALTNSAYAEKEAAQTFGLLGAGLHGGAGLYGAGAADLHGGASVGAGLYGRGAGYG   70 IPI-B3

GAGAG.....LYGRGAGGVGAGLYGRGAGYGGAGAGLFGRA.....                          106 IPI-B1
GAGAG.....LYGRGAGGVGAGLYGRGAGYGGAGAGLYGRGA..GGVGAGLYGRGAGYGGAGAG        127 IPI-B2
GAGAGLYAGYGGAGAVLYGRGAGGAGAGLYGRGAGYGGAGAGLYGRGAGYGGAGAGLY...AGYGGAGAG   137 IPI-B3

.............................AGYGGAGAGVGAGVGGAGVGNGYGGVGVGGTTGVGAGVG        145 IPI-B1
LYGRGAGGVGAGLYGRGAAYGGAGAGLFGREAGYGGAGAGVAAGVGGAGLGNGYGGV.....GVG        187 IPI-B2
LYGRGAGGVGAGLYGRGAAYGGAGAGLFGREAGFGGAGAGVAAGVGPAGLGNAYGGV.....GVG        197 IPI-B3

GTTGVGAGAGVGGGVSGNVGVGVGVGGNAAVGAGVGGAANGGVSANAGVGAGVSGNTGAGVGGGASGGAN   215 IPI-B1
GTTGVGAGAGVGGGVSGNVGAGVGVGGNAAVGAGVGGAANGGVGANAGVGAGVSGNTGAGVGGGASGGAN   257 IPI-B2
GTTGVGAGAGVGGGVSGNVGAGVGVGGNAAVGAGVGGAANGGVSANAGVGAGVSGNTGAGVGGGASGGAN   267 IPI-B3

GGVSANAGVGGGVGGSAGVGGSVGVGGAASTGAGGATTTTDGRTSTSTSQNGGPRQLQLPRLQARQRTAG   285 IPI-B1
GGVSANAGVGGGVGGSAGVGGSVGVGGAASTGAGGATTTTDGRTSTSTSQNGGPRQLQLPRLQARPRTAG   327 IPI-B2
GGVSANAGVGGGVGGSA.....GVGGAASTGVGGATKTTDGKTSTSTSQNGGPRQLQLPRLQARQRTAG   331 IPI-B3

TGASTKQTGYRMLRSQ                                                        301 IPI-B1
TGASTKQTGYRMLRSQ                                                        343 IPI-B2
TGASTKQTGYRMLRSQ                                                        347 IPI-B3
```

Figure 5. Comparison of the Predicted Amino Acid Sequences of IPI-B1, IPI-B2 and IPI-B3
Gaps are introduced in the sequence to obtain optimal alignment. Numbers indicate position of last amino acid in each line.

To our knowledge, this is the first report on genes encoding fungal or oomycete Gly-rich proteins. Many plant genes encoding Gly-rich proteins have been characterised (Condit & Keller, 1990; Showalter, 1993) and most of them have been implicated to be cell wall proteins (Keller et al., 1988; 1989a; 1989b; Condit et al., 1990). Whether the IPI-B proteins of *P. infestans* are structural proteins associated with the cell wall needs to be investigated. Since the *ipiB* mRNA is detectable at high

levels in the early stages of infection, it is tempting to speculate that the IPI-B proteins are involved in the development of infection structures. Although the IPI-B proteins share some characteristics with fungal hydrophobins (Wessels, 1992; Stringer & Timberlake, 1993), e.g. putative cell wall location, hydrophobicity and the presence of a signal peptide, they do not contain the conserved cysteine motif and their lengths are not within the known size range for hydrophobins (96 to 157 amino acids).

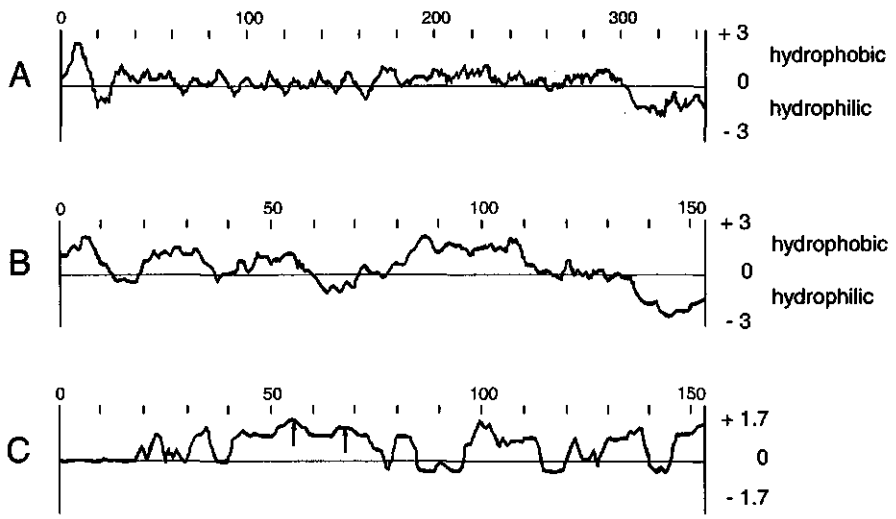

Figure 6. Hydropathy Plot of the Deduced Amino Acid Sequence of IPI-B2 (A) and IPI-O1 (B), and Antigenic Index of the Predicted Amino Acid Sequence of IPI-O1 (C)
Hydrophobicity was determined by the method of Kyte & Doolittle (1982). Along the co-ordinate, amino acid positions in the proteins are indicated. In (A) and (B), regions above the base line are hydrophobic, regions below this line are hydrophilic. The antigenic index was predicted according to the algorithm described by Jameson & Wolf (1988). Positions of the 'RGD' cell attachment sequence and the putative N-glycosylation site are indicated by arrows. The hydropathy plots of IPI-B1, IPI-B3 and IPI-O2, as well as the antigenic index of IPI-O2, are not shown since they are highly similar to those of IPI-B2 and IPI-O1, respectively.

Analysis of IPI-O Amino Acid Sequences

The *ipiO* genes encode a 152-amino acid, 17.2 kDa protein (Figure 4) with a predicted isoelectric point of 10.5. The deduced amino acid sequences of IPI-O1 and IPI-O2 are 97% homologous. They differ in only 4 single amino acid residues at positions 117, 122, 134 and 143. The predicted IPI-O1 and IPI-O2 amino acid sequences show no significant similarity with any sequence present in data libraries. The proteins contain a putative N-terminal signal sequence which suggests that they are secreted (see Figure 6B for hydropathy plot). According to the rules of von Heijne (1986), the signal sequence cleavage site was predicted between Ala^{21} and Val^{22}. A putative cell attachment sequence, recognised by the Arg-Gly-Asp (RGD) tripeptide,

is located at positions 54-56. At positions 66-69, a potential N-glycosylation site (NTSD) is present. From the surface probability plot (Jameson & Wolf, 1988) it appears that the RGD tripeptide as well as the NTSD sequence are located on potentially exposed surface peaks (Figure 6C), indicating that their function as cell attachment sequence or N-glycosylation site, respectively, is not hindered.

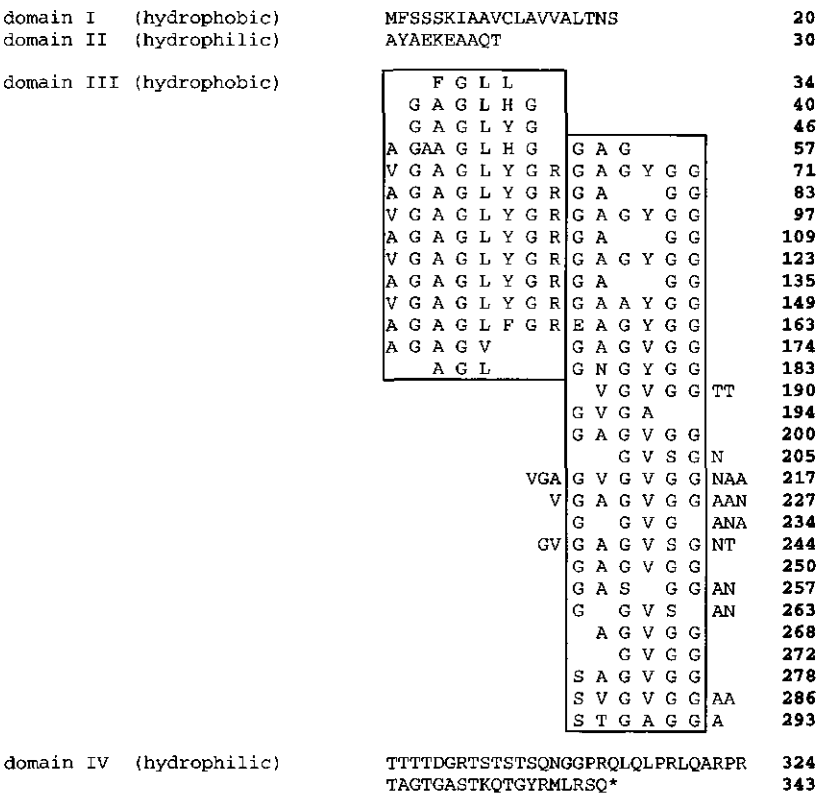

Figure 7. The Four Domains in the IPI-B2 Protein
In domain III, the Gly-rich repeats with the core sequence A/V-G-A-G-L-Y-G-R and G-A-G-Y/V-G-G are boxed. Numbers indicate position of last amino acid in each line.

Conclusions

(1) The phage clones DHC-B and DHC-O, which were isolated from a *P. infestans* genomic library in a screen for *in planta*-induced genes, both contain a small gene cluster. The *ipiB* gene cluster contains three members whereas on other locations in the genome additional *ipiB* or *ipiB*-like genes are present. The *ipiO* gene cluster consists of two members. Within these gene clusters, the members are highly homologous throughout the coding sequences and the regulatory 5' and 3' flanking regions.
(2) In seven out of eight distinct oomycete genes in which the *tss* have been determined, transcription initiation occurs in the conserved sequence motif GCTCATTYYNCAWTTT.
(3) The coding regions of the *ipiB* and the *ipiO* genes are not interrupted by introns, a feature which is observed in most oomycete genes studied so far.
(4) The members of the *ipiB* gene cluster encode three novel, highly homologous Gly-rich proteins. The IPI-B proteins have a putative signal sequence for transport out of the cytoplasm and a highly repetitive Gly-rich domain, both features which are often found in plant cell wall Gly-rich proteins.
(5) The two *ipiO* genes code for two almost identical proteins which have no significant similarity with any sequence in the data libraries. The IPI-O proteins have an N-terminal signal sequence. In addition, they contain a RGD motif which might function as a cell attachment sequence, and a putative N-glycosylation site.

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Chapter 3

The *ipiO* Gene of *Phytophthora infestans* Is Highly Expressed in Invading Hyphae during Infection

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Summary

The expression of the *in planta*-induced gene *ipiO* of the potato late blight pathogen *Phytophthora infestans* was analysed during various developmental stages of its life cycle. *ipiO* mRNA was detected in zoospores, cysts, germinating cysts and in young mycelia, but not in sporangia or in old mycelia grown *in vitro*. *ipiO* is not only expressed in stages prior to infection but also during colonisation of potato and tomato leaves. In disease lesions, *ipiO* mRNA was detected in the water-soaked area and the healthy-looking plant tissue surrounding it. In contrast, *ipiO* mRNA was not found in necrotised tissue and sporulating areas of a lesion. To determine more precisely the location and time of *ipiO* gene expression *in planta*, cytological assays were performed using a *P. infestans* transformant expressing a transcriptional fusion between the *ipiO1* promoter and the β-glucuronidase (GUS) reporter gene. GUS staining was specifically found in the subapical and vacuolated area of tips of invading hyphae. The histochemical GUS assays demonstrate that *ipiO* is expressed during biotrophic stages of the disease cycle.

Introduction

The oomycete, *Phytophthora infestans* (Mont.) de Bary, is the causal agent of late blight. Infection of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.) by a virulent strain of this plant pathogen initially causes rapidly expanding water-soaked lesions followed by necrosis and complete collapse of infected tissue. Our aim is to unravel molecular processes that occur during the disease cycle of *P. infestans* by identifying factors of the pathogen that are required for pathogenicity. Pathogenicity factors are generally regarded as components of the pathogen which are essential for disease development (reviewed by Oliver & Osbourn, 1995). Knowledge about the nature and the role of pathogenicity factors of *P. infestans* might enable the development of new strategies to control potato late blight.

Previously, we demonstrated that during the interaction of *P. infestans* with its host, a number of genes of *P. infestans* are activated (Pieterse et al., 1993). When comparing *in vitro* growth and *in planta* growth, expression of these genes is either increased or specifically induced *in planta*. These so-called *in planta*-induced (*ipi*) genes might encode pathogenicity factors which enable *P. infestans* to establish and maintain a compatible interaction with its host or to increase disease severity. One of these *in planta*-induced genes is *ipiO*. *P. infestans* has two *ipiO* genes, *ipiO1* and *ipiO2*, which are closely linked and located in a reverse orientation on a 2.5 kb genomic fragment. The nucleotide sequences of the promoter and terminator regions

of the two *ipiO* genes are almost identical, indicating that both genes are probably regulated in a similar way. The putative IPI-O protein contains 152 amino acid residues. IPI-O1 and IPI-O2 differ at only four amino acid residues and have no homology with known proteins (Pieterse et al., 1994b). The presence of a putative signal peptide at the N-terminus suggests that the mature IPI-O protein of 131 amino acids resides extracellularly. Expression studies showed that *ipiO* is transiently expressed *in planta*, with the highest mRNA levels in the early stages of infection. *IpiO* mRNA was found *in vitro* during germination of cysts but not in mycelia. Based on these observations, a role for IPI-O during the onset of the interaction was suggested (Pieterse et al., 1994a).

Unravelling the role of *ipiO* and other genes which are specifically expressed during the interaction might give more insight into the molecular processes which take place during the interaction of *P. infestans* and its hosts. Little is known about these events. In contrast, processes which occur at the cytological level and which can be observed microscopically, have been studied in more detail (reviewed by Coffey & Wilson, 1983). Under moist conditions, when sporangia come into contact with a potato leaf or tuber, they germinate directly or develop into zoosporangia which release motile biflagellate zoospores. When the zoospores touch the surface of the leaf, they encyst and germinate. Subsequently, the germ tube of either a sporangium or a cyst forms an appressorium. From the appressorium a penetration peg emerges which pierces the cuticle and penetrates an epidermal cell. Here, the biotrophic phase of the disease cycle starts. An infection vesicle is produced in the epidermal cell and hyphae grow into the mesophyll cell layers both intra- and intercellularly. Occasionally, haustoria are formed. After three to four days, *P. infestans* starts to grow saprophytically in the necrotised centre of the growing lesion. Hyphae emerge through the stomata and sporangiophores are formed which produce numerous new sporangia.

Here we describe the expression of *ipiO* during various developmental stages that can be distinguished cytologically. Expression was analysed by northern blot hybridisations and by histochemical localisation of GUS activity in a *P. infestans* transformant carrying the *ipiO1* promoter fused to the β-glucuronidase reporter gene.

Results

Expression of *ipiO* during Differentiation of *P. infestans in Vitro*

During *in vitro* growth of *P. infestans* the following cell types can be distinguished: coenocytic hyphae, hyphal tips, sporangiophores, sporangia, germinating sporangia, zoospores, cysts and germinating cysts. From most of these cell types relatively pure fractions can be collected for RNA isolation. *P. infestans* tissue consisting of more than 90% of the desired cell type (as determined by microscopy) was obtained from zoospores, cysts, germinating cysts, sporangia and mycelia. Mycelial tissue was

taken from twenty-hour-old germinated sporangia which contain many hyphal tips but no sporangiophores or sporangia (young mycelia), and from four to seven-day-old mycelia (mycelia). The latter consists mainly of coenocytic hyphae with a minor proportion of sporangiophores and sporangia. Total RNA was isolated, transferred to membranes and hybridised with the *ipiO1* probe which detects mRNA transcribed from the *ipiO1* as well as the *ipiO2* gene. A low level of *ipiO* mRNA was detected in zoospores (Figure 1A). An increased level of *ipiO* mRNA was found after encystment of zoospores. This level increased slightly more in germinating cysts. *IpiO* mRNA was not found in sporangia nor in old mycelia, whereas in young mycelia a low level of *ipiO* mRNA was detected. Hybridisation with a probe derived from the constitutively expressed actin gene, *actA* (Unkles et al., 1991), was performed to check the amounts of RNA loaded on the gel.

In order to analyse the expression of *ipiO* during mycelial growth *in vitro* in more detail, a time course experiment was performed. Cysts were incubated for two hours in water. Subsequently, the germinated cysts were either starved by transferring them to water or they were grown in complete medium by transferring them to liquid rye sucrose medium. After transfer, the germinated cysts were allowed to grow for 1, 3 or 5 days in water and 1, 2, 3, 5 or 7 days in medium. In water, little mycelial growth was observed in the first three days. After four days, the mycelia did not grow further, most likely due to the lack of nutrients. Continuous mycelial growth was observed in medium. Total RNA was isolated, transferred to membranes and hybridised with *ipiO* and actin probes. One day after transfer to water or medium, the *ipiO* mRNA level was strongly decreased when compared to the level found in germinating cysts (Figure 1B). Two days after transfer, *ipiO* mRNA was not detectable anymore. Approximately equal amounts of intact actin mRNA were found in all RNA samples. However, slightly less actin mRNA was noted in cysts germinated in water.

To exclude the possibility that the expression pattern of *ipiO* is strain specific, we analysed *ipiO* expression in another *P. infestans* isolate (90128). Also in this strain *ipiO* mRNA was found only in germinating cysts and not in sporangia or mycelia (Figure 1C).

These results show that different cell types contain different levels of *ipiO* mRNA. This suggests that there is differential expression of *ipiO* in the various developmental stages of *P. infestans in vitro* with the highest expression observed in germinating cysts, moderate expression in zoospores and cysts, and low expression in young mycelia. *IpiO* is neither expressed in hyphae of maturing mycelia nor in sporangia.

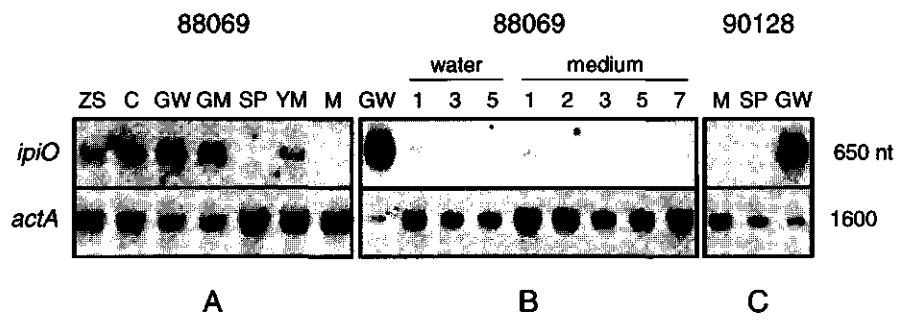

Figure 1. Expression of *ipiO* during Growth and Differentiation of *Phytophthora infestans in Vitro*
(A). Autoradiograph of a northern blot containing total RNA (10 µg) isolated from *P. infestans* isolate 88069 zoospores (ZS), cysts (C), cysts germinating in water for 2.5 hours (GW), cysts germinating in ALBA medium for 2.5 hours (GM), sporangia (SP), young mycelia (YM) and mycelia (M), and hybridised with *ipiO* and *actA* probes.
(B). Autoradiograph of a northern blot containing total RNA (10 µg) isolated from *P. infestans* 88069 germinating cysts in water (GW) and mycelia grown in water for 1, 3 and 5 days; and mycelia grown in rye sucrose medium for 1, 2, 3, 5 and 7 days, and hybridised with *ipiO* and *actA* probes. Mycelia grown in medium or water for 1 day represents the material that is indicated in the text as young mycelia.
(C). Autoradiograph of a northern blot containing total RNA (10 µg) isolated from *P. infestans* 90128 mycelia (M), sporangia (SP) and cysts germinating in water for 2.5 hours (GW), and hybridised with *ipiO* and *actA* probes. Transcript lengths in nucleotides are indicated on the right.

Expression of *ipiO* in *P. infestans* during Infection of Potato Leaves

During the disease cycle of *P. infestans* most growth occurs *in planta*. Also here, several developmental stages can be distinguished. Prior to infection, sporangia land on the surface of a leaf, germinate or produce zoospores which encyst and produce germ tubes. These pre-infection stages up to appressorium formation are comparable to the *in vitro* stages described above. They are followed by appressorium formation and penetration peg formation, which can also be induced *in vitro* on artificial surfaces. However, it appeared very difficult to obtain sufficient material that consisted of more than 50% of appressoria or penetration pegs. Therefore, we decided to look in more detail at the expression of *ipiO* in the next step of the disease cycle, that is in expanding lesions. Previously, we found that *ipiO* is highly expressed *in planta* (Pieterse et al., 1994a) but these studies were limited to time-course experiments without taking into account the differentiation of *P. infestans in planta*. Now, we performed spot-inoculations with only one inoculation spot per leaf. In this way *P. infestans* can develop from one starting point in all directions without being hampered by growing hyphae emerging from neighbouring lesions. This enabled us to characterise more accurately the distinct growth stages of *P. infestans* during the interaction with its host. Sixteen hours after inoculation, small brown spots appeared beneath the inoculation droplet where a lesion started to develop. After two days, a water-soaked area developed at the edge of the growing lesion. Three to six days after inoculation, the centre of the lesion became necrotic. At day six, large areas of the leaf were fully colonised by the pathogen. Sporulating hyphae emerged through the stomata in the necrotic centre and in the area

surrounding it. Four concentric zones could be distinguished in a lesion: (I) the necrotic centre, (II) the sporulating zone, (III) the water-soaked area and (IV) the outer zone which consisted of green, healthy-looking tissue (Figure 2).

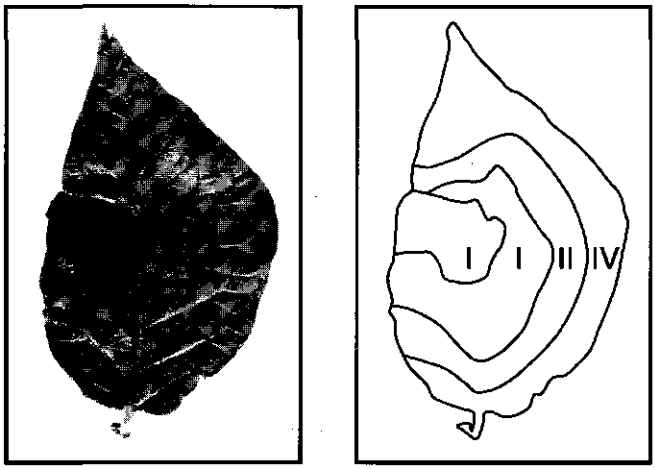

Figure 2. Lesion of a Spot-Inoculated Potato Leaflet (cv. Bintje) Six Days after Inoculation with *P. infestans* Isolate 88069
Four distinct zones are indicated in the schematic drawing on the right, the necrotic zone (I), the sporulating zone (II), the water-soaked zone (III), and the zone surrounding the water-soaked zone which contains predominantly green tissue (IV).

Two parallel experiments were performed to follow the expression of *ipiO* in developing lesions. In the first experiment, leaf discs with a fixed diameter and with the inoculation spot in the centre were harvested from infected leaves. Leaf discs harvested one day after inoculation contained relatively little *P. infestans* biomass. They consisted for the major part of healthy plant tissue, whereas leaf discs harvested at day five or later, contained mainly necrotic plant tissue and a large amount of sporulating hyphae. After five days, the water-soaked area extended beyond the edge of the leaf disc. From the leaf discs, total RNA was isolated and northern blot analyses were performed (Figure 3A). Hybridisation with the *ipiO* probe showed high levels of *ipiO* mRNA on two, three, and four days after inoculation. Five days after inoculation little *ipiO* mRNA was observed and at day six, seven and eight it was not detected anymore.

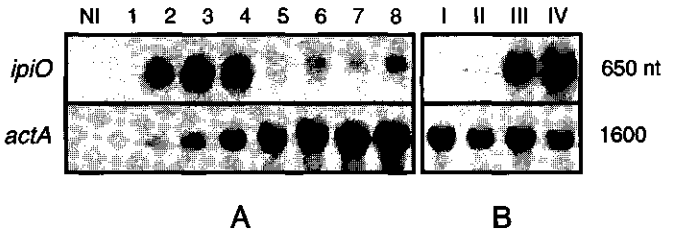

Figure 3. Expression of *ipiO* during Differentiation and Growth of *P. infestans in Planta*
(A). Autoradiograph of a northern blot containing total RNA (15 µg) isolated from potato leaves (cv. Bintje) one to eight days after spot-inoculation with *P. infestans* isolate 88069 (1-8) and from non inoculated potato leaves (NI), and hybridised with *ipiO* and *actA* probes.
(B). Autoradiograph of a northern blot containing total RNA (15 µg) isolated from potato leaves (cv. Bintje) six days after spot-inoculation with *P. infestans* isolate 88069. Total RNA was isolated from the zones indicated in Figure 2 (I-IV) and hybridised with *ipiO* and *actA* probes. Transcript lengths, in nucleotides, are indicated on the right.

In the second experiment, leaves were spot-inoculated and six days later, lesions were cut and divided in the four visually distinguishable zones described above (Figure 2). From the tissue of the four separated zones, total RNA was isolated and northern blot analyses were performed (Figure 3B). *IpiO* mRNA was predominantly found in zones III and IV (Figure 3B), which represent the water-soaked area and the green zone surrounding the water-soaked area. *IpiO* mRNA was not detected in zone I and only a little in zone II. Instead, another transcript (approximately 700 nucleotides), was found in the necrotic centre of the lesion. This transcript was also detected in the time course experiment (Figure 3A). It first appeared at day five and the level continuously increased from day five to day eight. Even though the hybridisation signal is much lower than the one observed with the *ipiO* transcript, higher stringency washing of the blot did not remove the signal. In other experiments the same cross-hybridising transcript was observed. Further characterisation of the cDNA or the gene encoding this transcript is required to determine what the origin is of the transcript. It might be derived from a distantly related member of the *ipiO* gene or there might be an alternative transcription start site in the *ipiO* genes. Hybridisation of the same blots with the *actA* probe showed that the four zones contain equal amounts of actin mRNA. In the time course experiment, the actin mRNA level increased up to five days after inoculation and then remained constant. This reflects the increase of *P. infestans* biomass during infection.

These results show that there is differential expression of *ipiO* during colonisation of plant tissue. *IpiO* is expressed in hyphae growing in the water-soaked area and beyond, but expression is not observed in hyphae growing in the necrotised and sporulating zones of the lesion.

Transformation of *P. infestans* and Selection of *ipiO-gus* Transformants

A transcriptional fusion of the *ipiO* promoter with the coding sequences of the β-glucuronidase gene (*uid*A encoding GUS), pPIN13, was introduced into *P. infestans* strain 88069, by co-transformation with pHAMT34N which contains the geneticin resistance gene (*nptII*) as a selection marker. PCR screening and Southern blot analysis showed that twenty of forty four putative co-transformants contained the *ipiO gus* construct. Two transformants, CX4 and CX7, with low pPIN13 copy number and no GUS activity in *in vitro*-grown mycelia, were selected for further analysis. *P. infestans* transformants that express the *uid*A gene constitutively, were obtained by co-transformation of strain 88069 with pHAMT35G, a construct which contains the constitutive *Ham34* promoter of *Bremia lactucae* (Judelson & Michelmore 1991), and a geneticin resistance plasmid (pTH209). *Ham34-gus* transformant EY6 showed high GUS activity *in vitro* and was still pathogenic in various pathogenicity assays. Hence, EY6 was used as a GUS positive control in the experiments.

CX4 and CX7 were tested for GUS activity in cysts and germinating cysts. Both transformants showed GUS activity in cysts, but significantly higher GUS activity was found in germinating cysts, up to 65-fold in CX7 and 72-fold in CX4 (Table 1). Very high GUS activity was found in cysts and germinated cysts of the constitutive *Ham34-gus* transformant EY6. However, only a 2.6 fold increase of GUS activity was found when cysts and germinated cysts of EY6 were compared. Wild type strain 88069 did not show any GUS activity (data not shown). These results demonstrate that the *ipiO1* promoter of the integrated GUS constructs is significantly more activated during germination of cysts than the *Ham34* promoter.

Table 1. GUS Activity[a] in Protein Extracts of Cysts and Germinated Cysts of *P. infestans* Transformants

P. infestans transformant	transgene	plasmid	cysts[b]	germinated cysts[b]
CX4	*ipiO-gus*	pPIN13	10.4 ± 0.3	752 ± 11
CX7	*ipiO-gus*	pPIN13	2.8 ± 0.5	180 ± 11
EY6	*Ham34-gus*	pHAMT35G	1625 ± 165	4225 ± 184

[a] The amount of MU (μM) produced by β-glucuronidase present in 1 mg of total protein per hour at 37°C.
[b] Values represent the average of four independent experiments (n=4).

In addition we checked GUS activity of CX4 and CX7 *in vitro* on solidified rye sucrose medium during a time course experiment (Figure 4A). Little staining was observed in the hyphae of CX7 (data not shown) and even less staining was noted in the hyphae of CX4. Colonies of the non-transformed strain 88069 did not stain at all. EY6 however, showed very strong staining in all hyphae. The very faint blue staining observed in CX4 suggests that there is a very low level of expression of the *ipiO gus* construct.

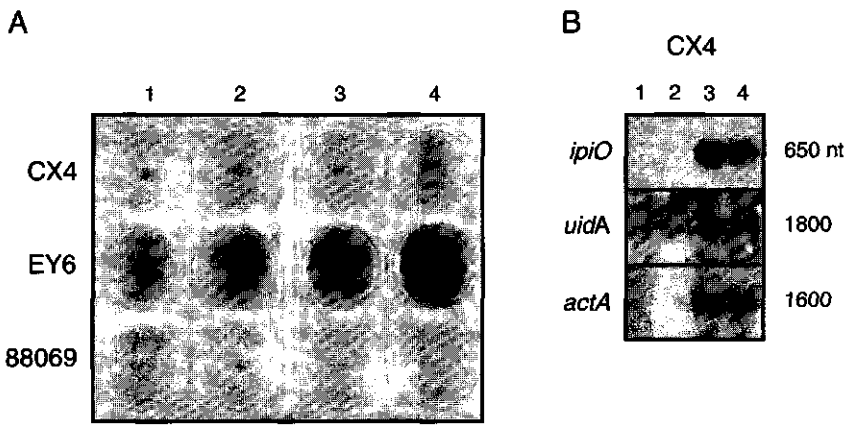

Figure 4. *In Vitro* and *in Planta* Expression Studies of *ipiO*-gus Transformant CX4
(A). Histochemical localisation of GUS activity in *in vitro*-grown mycelia of *P. infestans ipiO-gus* transformant CX4, *Ham34-gus* transformant EY6 and wild type 88069. Mycelia was grown on rye-sucrose medium for 1, 2, 3, and 4 days.
(B). Autoradiograph of a northern blot containing total RNA (15 µg) isolated from potato leaves (cv. Bintje) one to four days after spot-inoculation with *ipiO-gus* transformant CX4 (1-4) and hybridised with *ipiO*, *uidA* and *actA* probes.

In planta expression studies of *ipiO-gus* transformant CX4 were performed during a time course to compare the appearance of *gus* mRNA, *ipiO* mRNA and *actA* mRNA during lesion development (Figure 4B). The hybridisation patterns obtained on northern blots with *uidA* and *ipiO* probes were comparable. Activation of the integrated *uidA* gene coincided with the activation of the *ipiO* gene. Lesions caused by CX4 appeared a little later and were somewhat smaller when compared to lesions caused by the wild type strain (data not shown). Despite the slightly reduced aggressiveness of CX4, we used this *ipiO-gus* transformant to localise expression of *ipiO in planta*. The expression pattern of the *ipiO-gus* construct in cysts, germinating cysts and mycelia is similar to the expression pattern of the endogenous *ipiO* genes and, equally important, the promoter of the *ipiO-gus* construct in CX4 is induced *in planta* and not in mycelia cultured *in vitro*.

Localisation of *ipiO* Expression *in Planta*

Histochemical GUS assays were performed on potato leaves infected with CX4 during a time course of four days. *P. infestans* wild type strain 88069 and EY6 were used as negative and positive controls, respectively. Leaf tissue of spot-inoculated leaves was harvested, stained for GUS activity and analysed microscopically. On day one, germinated cysts and appressoria of CX4 showed GUS activity (Figure 5A). Two days after inoculation, the pathogen had produced some inter- and extracellularly growing hyphae. At that stage, hyphae were only present in the vicinity of the inoculation site. Most of these hyphae stained blue (data not shown). Three

and four days after inoculation, a visible lesion started to develop as fresh plant tissue became colonised by invading hyphae. GUS activity was specifically found in these invading hyphae. A characteristic example is shown in Figure 5C, where blue staining can be seen in a small zone surrounding the growing lesion. At higher magnification, in most cases, GUS activity was absent in the apical region (up to 3 μm) of the hyphal tip. A blue-stained area was noted approximately 3-10 μm from the hyphal tip in the subapical region. GUS activity was found in a zone varying from 0.3 to 4 mm in width which corresponds to the water-soaked area and the green area surrounding the water-soaked area (zone III and IV, Figure 2). When *in vitro*-grown mycelium was microscopically checked for GUS activity, such a zone of blue staining could not be observed (shown macroscopically in Figure 4A). Four days after inoculation, the centre of the lesion did not show any GUS activity (Figure 5E). Here, in the necrotised area, *P. infestans* was growing saprophytically with hyphae growing on the surface of the leaf and sporulating hyphae emerging through stomata. Occasionally, it appeared that physical barriers, like veins, arrested the growth of the expanding hyphae for some time. When the pathogen was able to overgrow these barriers, "escaping" hyphae started to invade non-infected tissue. These hyphae showed GUS activity as well (Figure 5G). Similar patterns of GUS activity were observed in lesions caused by *ipiO-gus* transformant CX7 (data not shown). In contrast, in lesions caused by the *Ham34-gus* transformant, EY6, a completely different staining pattern was found. Here, GUS activity was present throughout the disease cycle. Germinating cysts, appressoria (Figure 5B), invading hyphae (Figure 5D), hyphae on the leaf surface (Figure 5F), sporangiophores, and sporangia stained blue. GUS activity was noted in the centre of the lesion and proceeded to hyphal tips where the blue staining was slightly more intense. GUS activity was also found close to the hyphal tips of EY6 (4-10 μm). In leaves infected by wild type strain 88069 no blue staining was observed (data not shown).

These *in situ* studies clearly demonstrate that during the interaction of *P. infestans* with potato, expression of *ipiO* is limited to a restricted zone in invading hyphae, close to the hyphal tip.

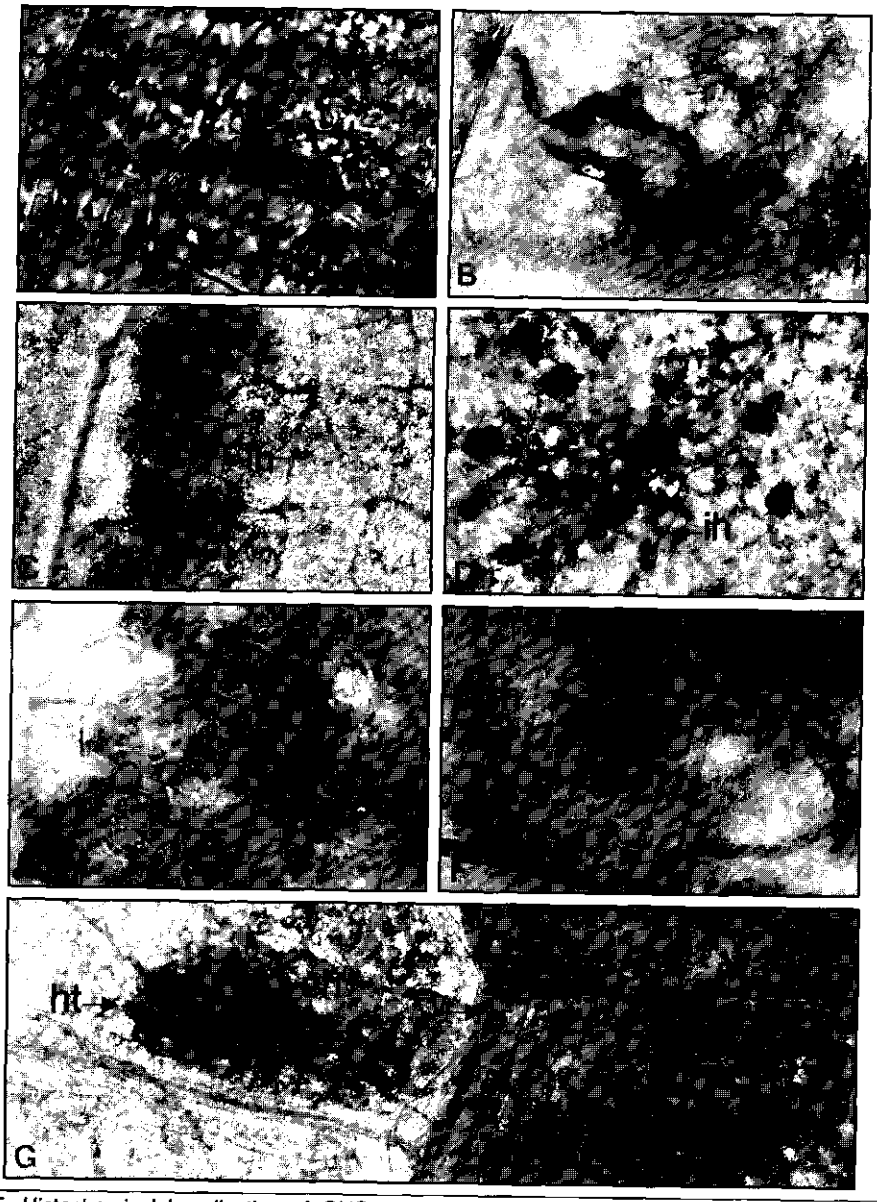

Figure 5. Histochemical Localisation of GUS Activity in Leaves of Potato (cv. Bintje) Infected by *P. infestans ipiO-gus* Transformant CX4 (A, C, E & G) and *Ham34-gus* Transformant EY6 (B, D & F)
(A & B). Germinating cyst (c) with appressorium (a), at the abaxial side of a potato leaf, one day after inoculation (1000x).
(C & D). Invading hyphae (ih) growing in a potato leaf, four days after inoculation (C: 300x, D: 600x).
(E & F). Hyphae (h) emerging through stomata (s) at the abaxial side of a potato leaf near the site of inoculation, four days after inoculation (1200x).
(G). Escaping hyphae (eh) and hyphal tips (ht) after crossing a vein (v) in the potato leaf, four days after inoculation (800x).

Discussion

In this paper we describe the expression pattern of the *ipiO* genes of the plant pathogen *P. infestans* during various developmental stages of its life cycle. We analysed expression during growth of the pathogen *in vitro* and *in planta*. Northern blot analyses and histochemical GUS staining of a *P. infestans* strain carrying the *ipiO1* promoter fused to the β-glucuronidase reporter gene, showed that *ipiO* is expressed in zoospores, cysts and germinating cysts but not in sporangia. It is expressed weakly in young mycelia, but not at all in older mycelia. Apparently *ipiO* expression is restricted to particular stages of the life cycle of *P. infestans*. In expanding lesions, *ipiO* expression is specifically detected in the outer zones, but not in the centre, where the leaf is necrotised and *P. infestans* grows saprophytically. Since expression is specifically found in hyphae invading the still healthy-looking plant tissue at the edge of the lesion, we conclude that *in planta* expression of *ipiO* is restricted to the biotrophic stage of the disease cycle.

Previous analyses of spray-inoculated potato leaves showed a transient expression pattern of *ipiO*, with the highest levels of *ipiO* mRNA being found on day one and two after inoculation. This was followed by a rapid decline in accumulation of *ipiO* mRNA at day three and four (Pieterse et al., 1994a). However, in the experiments described here, leaves were spot-inoculated and *ipiO* mRNA was found up to at least six days after inoculation (Figure 3B). The observed differences in expression pattern are probably due to the method of infection, i.e. spray- or spot-inoculation. By using spray-inoculation, the leaf is fully colonised within three to four days and the *ipiO* mRNA level rapidly decreases as the disease progresses to the later saprophytic stages. During spot-inoculation however, biotrophic growth takes place for a longer period. The pathogen can invade fresh tissue continuously until the leaf is fully colonised and subsequently, *ipiO* mRNA can be detected as long as the lesion expands.

IpiO gene expression was also quantitatively determined, in cysts and germinated cysts by making use of *ipiO-gus* transformants. GUS activity was up to seventy-fold higher in protein extracts from germinating cysts than in extracts from cysts (Table 1). This demonstrates a rapid induction of expression of *ipiO* during germination of cysts. In fact, in all *in vitro* growth stages which have been analysed, *ipiO* gene expression was the highest in germinating cysts. Previously, we reported a similar high expression level in *in vitro*-grown mycelia which had been transferred from rich medium to water (Pieterse et al., 1994a). At that time, we concluded that nutrient starvation is inductive for *ipiO* gene expression and we hypothesised that germinating cysts on the leaf surface encounter the same conditions. Here, we found that transfer of germinating cysts to medium or water resulted in rapid decrease of *ipiO* expression. Apparently, starvation conditions encountered by the germinating cysts upon transfer

to water are not inductive. Hence, we conclude that starvation per se does not result in induction or maintenance of expression.

The *in planta* studies in which *ipiO* expression was localised by making use of *ipiO-gus* transformants, showed that GUS activity was specifically localised in the subapical zone of the hyphal tip. Ultrastructural studies of hyphal tip growth in several oomycetes like *Pythium ultimum, Saprolegnia ferax* and *Phytophthora parasitica*, revealed that in young hyphae three distinct regions can be detected: an apical zone (3-5 µm), a subapical zone (40-100 µm) and a zone of vacuolation. The subapical zone contains dense accumulation of protoplasmic components such as nuclei, mitochondria, ribosomes, endoplasmic reticulum and Golgi bodies, which are not present in the apical zone (Hemmes & Hohl 1969; Grove et al., 1970; Heath & Kaminskyj 1989). During hyphal tip growth, hyphae extend by incorporation of vesicles which contain wall material, in the plasma-membrane at the tip (Heath & Kaminskyj, 1989). The packaging of proteins in vesicles by the endoplasmic reticulum and Golgi bodies requires the presence of a signal peptide in the pre-protein. The *ipiO-gus* construct that was used to transform *P. infestans*, does not contain a signal peptide. Hence, GUS activity is not observed in the apical zone. *In vitro* translation assays of *ipiO* mRNA in the presence of microsomal membranes resulted in cleavage of the signal peptide of IPI-O, which indicates that the signal sequence is functional (unpublished results). Thus, in contrast to GUS, IPI-O could be transported to the apical region and secreted through the hyphal tip of invading hyphae. This would suggest that IPI-O protein is localised at the interface between the invading hyphae and plant cells, and could play a role as a pathogenicity factor.

In this paper we have shown for the first time that promoter activity of an endogenous gene can be studied in *P. infestans* using β-glucuronidase as a reporter gene. With the GUS reporter system, we were able to show the temporal and spatial expression pattern of *ipiO* during interaction of *P. infestans* with its host. However, it would be more convenient and more informative to have a non-destructive reporter gene system which allows monitoring promoter activity *in vivo* without the need to grind or fix tissue. Although non-destructive GUS assays have been described and used successfully in plants (Martin et al., 1992), other, more recently developed systems exploiting luciferase and green fluorescent protein (GFP), have many advantages above GUS (Millar et al., 1992; Spellig et al., 1996). We tested whether the luciferase gene could be used as a reporter gene in *P. infestans* and found that *P. infestans* can synthesise active luciferase. However, only a small percentage of the transformants showed luciferase activity. In most cases the activity was very low despite the fact that expression was regulated by a highly constitutive promoter (unpublished results). To use luciferase as an efficient reporter system in oomycetes, the system has to be optimised.

The *ipiO* gene was isolated by differential screening which was devised to select for *in planta*-induced genes of *P. infestans*. A number of *in planta*-induced genes from several fungal plant pathogens show expression patterns which resemble the expression pattern of *ipiO*. The pathogenicity gene *MPG1*, a hydrophobin encoding gene from the rice blast fungus *Magnaporthe grisea*, is also highly expressed prior to infection (Talbot et al., 1993). However, in contrast to *ipiO* expression, *MPG1* expression is down-regulated after penetration. During lesion development and especially during conidia formation, *MPG1* expression increases again. Disruption of the *MPG1* gene showed a lack of appressorium formation and a reduced ability to disperse conidia from aerial hyphae (Talbot et al., 1996). A pathogenicity gene of the fruit rot- and anthracnose- causing plant pathogen *Colletotrichum gloeosporioides*, *cap20*, is specifically expressed during appressorium formation but not during germination of conidia (Hwang et al., 1995). Moreover, in infected tomato fruits *cap20* expression is detected specifically at the infection front but not in fruit layers which have already been colonised by the fungus. This resembles the *in planta* expression pattern of *ipiO*, which also shows localised expression in invading hyphae colonising fresh tissue, and no expression in hyphae present in the necrotic centre. Disruption of the *cap20* gene resulted in decreased virulence on tomato fruits. The *in planta*-induced genes *ecp*1 and *ecp2*, which have been isolated from the tomato leaf mould pathogen *Cladosporium fulvum*, are active throughout all stages of interaction with the host. Increased expression is found in the vicinity of vascular tissue (Wubben et al., 1994). In initial studies of gene-disruption mutants of *ecp2* no clear change in pathogenicity phenotype was found (Marmeisse et al., 1994). However, recently it was shown that the *ecp*1 and *ecp2* mutants have a reduced fitness on tomato plants (Laugé et al., 1997).

Like *ipiO*, the genes described above have been selected as *in planta*-induced genes and it is interesting to note that disruption of these particular genes has an effect on the virulence characteristics of the pathogens. Whether also *ipiO* has a function in virulence is still unknown. However, the specific expression pattern of *ipiO* *in planta* as well as *in vitro*, strongly suggests a requirement for IPI-O in the pre-infection stage and in the invasion of host tissue. The predicted amino acid sequence does not reveal a specific function of IPI-O (Pieterse et al., 1994b). The possibly secreted protein contains an RGD tripeptide, a motif which is known to be involved in cell attachment properties of other proteins (D'Souza et al., 1991; Hynes, 1992). The putative N-glycosylation site and the serine- and threonine-rich nature suggest that IPI-O is glycosylated. To determine whether *ipiO* is essential for pathogenicity, it would be useful to test transformants in which the gene is inactivated. However, a gene disruption technique for *P. infestans* has not been developed yet. Complicating factors are the diploid nature of *P. infestans*, and the fact that homologous recombination has not been reported up till now.

Experimental Procedures

Phytophthora infestans Strains, Culture Conditions and Developmental Stages

P. infestans strains 88069 (A1 mating type, race 1.3.4.7.) and 90128 (A2 mating type, race 1.3.4.6.7.8.10.11.) from the Netherlands were used throughout this study. All transgenic isolates have an 88069 background. Isolates were routinely grown on rye agar medium supplemented with 2% (w/v) sucrose (Caten & Jinks, 1968) in the dark at 18 °C. Mycelia for isolation of DNA and RNA was obtained by growing *P. infestans* in ALBA medium (Bruck et al., 1980) or rye medium amended with 2% w/v sucrose for four to seven days. To obtain sporangia, sporulating mycelia growing on rye sucrose medium was flooded with water (10 ml per Petri dish) and gently rubbed with a sterile glass rod. The suspension of sporangia was then transferred to tubes and sporangia were collected by centrifugation (1200 rpm). To obtain zoospores, flooded mycelia was incubated at 4 °C for one to two hours. The water containing mainly zoospores was filtered through a 10 µm mesh to remove sporangia and mycelial pieces. Zoospores were collected by very gentle centrifugation (400 rpm) to prevent encystment at this stage. Cysts were obtained by continuously shaking a zoospore suspension for two minutes followed by centrifugation. Cysts were allowed to germinate by incubation in water or ALBA medium at 18 °C for 2-4 hours. To obtain young mycelia, sporangia were allowed to germinate in ALBA medium at 18 °C for 20 hours.

Plant Assays

Detached potato leaves of cv. Bintje were placed with the abaxial side up in Petri dishes containing 1.5% (w/v) water agar and inoculated with *P. infestans* zoospores. Droplets (10 µl) containing approximately 1000 zoospores were spotted on the abaxial side of a leaflet. The Petri dishes with the inoculated leaves were subsequently placed under 100% relative humidity in the dark at 18 °C. After 16 hours, the Petri dishes were transferred to a growth chamber (15-18 °C) with cool fluorescent light for 16 hours and darkness for eight hours per day. Circular leaf discs, with a diameter of 15 mm and with the inoculation spot in the centre, were cut from the inoculated leaves every day up to eight days after inoculation. In addition, lesions developed six days after spot-inoculation were cut and divided in four visual distinguishable zones as depicted in Figure 2.

Southern and Northern Blot Analysis

Genomic DNA of *P. infestans* was isolated from mycelia as described by Pieterse et al., (1991). Total RNA from *P. infestans* and from infected and non-infected potato leaves was isolated using the guanidine hydrochloride extraction method (Logemann et al., 1987). For northern blot analyses, 10-15 µg of total RNA was denatured at 50 °C in 1 M glyoxal, 54% (v/v) DMSO and 10 mM sodium phosphate buffer (pH 7.0), separated on a 1.4% (w/v) agarose gel in 10 mM sodium phosphate buffer (pH 7.0), and transferred to Hybond N$^+$ membranes (Amersham International place., Little Chalfont, Buckinghamshire, UK) by capillary transfer in 25 mM sodium phosphate buffer (pH 7.0) (Ausubel et al., 1987; Sambrook et al., 1989). Hybridisations with radiolabelled probes were performed at 65 °C in 0.5 M sodium phosphate buffer, 7% SDS and 1 mM EDTA (pH 7.2). The filters were washed at 65 °C in 0.5x SSC (75 mM NaCl and 7.5 mM sodium citrate) and 0.5% SDS and exposed to Kodak X-OMAT, AR films (Eastman Kodak Co., Rochester, NY, USA).

DNA Probes

As DNA templates for probe synthesis the following fragments were used: the 589 bp *Eco*RI-*Xba*I fragment containing the coding region of *ipiO1* (Pieterse et al., 1994b), the 796 bp *Hin*dIII fragment from pSTA31 containing the coding region of the *P. infestans* actin gene, *actA* (Unkles et al., 1991), the 1870 bp *Nco*I-*Sma*I fragment of pHAMT35G (Judelson et al., 1992) containing the coding region of the *uidA* gene. All DNA templates were gel purified with the Qiaex II Agarose Gel Extraction kit (Qiagen GmbH., Hilden, Germany). The probes were radiolabelled with [α-^{32}P]dATP by using the Random Primers DNA Labelling System (Gibco BRL, Gaithesburg, MD, USA). To remove non-incorporated nucleotides, the Qiaquick Nucleotide Removal Kit (Qiagen GmbH., Hilden, Germany) was used.

Plasmids for Transformation of *P. infestans*

Plasmid pPIN13, which contains the coding region of the β-glucuronidase gene (*uidA*) of *E. coli* fused to the *ipiO* promoter, was constructed as follows. A *Hin*dIII-*Eco*RI fragment from pRAJ275 (kindly provided by R.A. Jefferson) was inserted into pBluescriptSK⁻ resulting in pGUS-1. The *uidA* gene was excised from pGUS-1 with *Hin*dIII and *Sma*I and this fragment was used to replace the *Ham34* promoter of *Bremia lactucae* and the *hpt*II coding region in pHAMT34H (Judelson et al., 1991) resulting in pPIN12. A 1219 bp long fragment of the *ipiO1* promoter was generated by a PCR using oligonucleotides PIET2 (GCGGATCCATGGCCGGAAAGAAATGAAT), and M13 Forward Sequencing Primer (-40) 17 MER (New England Biolabs, Beverly, MA, USA) on pSO2.6, a plasmid containing a *Pst*I-*Hin*dIII fragment with the promoter and part of the coding region of *ipiO1* (Pieterse et al., 1994b) inserted in pTZ19u. The PCR fragment was inserted in *Pst*I and *Bam*HI digested pTZ19u (pPIN10). Subsequently, the *ipiO1* promoter was excised from pPIN10 as a *Hin*dIII-*Nco*I fragment and inserted in pPIN12 resulting in pPIN13. pTH209 and pHAMT34N (Judelson et al., 1991) consist of the *hsp70* and the *Ham34* promoter of *Bremia lactucae*, respectively, fused to the coding sequences of the neomycin phosphotransferase (*nptII*) gene and the *Ham34* terminator. The promoters of *hsp70* and *Ham34* are constitutively active promoters in *P. infestans*. pHAMT35G (Judelson & Michelmore 1991), consists of the *Ham34* promoter fused to the *uidA* gene and the *Ham34* terminator. *E. coli* strain DH5α was used for all cloning experiments and was routinely grown at 37 °C in Luria-Bertania (LB) media (Sambrook et al., 1989). Plasmid DNA was isolated and purified with Qiagen/Filter Plasmid Midi Kit (Qiagen GmbH., Hilden, Germany). For transformation non-linearised plasmids were used.

Transformation of *P. infestans*

Stable transformation was conducted according to the protocol of Judelson (1993) with some modifications. Two weeks after mycelial transfer, sporangia of strain 88069 were isolated from Petri dishes containing rye sucrose medium by flooding the plates with sterile water and gently rubbing the mycelia with a rod of glass. The sporangial suspension was diluted with water to 2.10^5 sporangia per ml and mixed with two times concentrated ALBA medium. After growth for 30-48 hours at 18 °C, young mycelia were harvested by filtration. The mycelia retained on a nylon filter (50 µm mesh) were washed in KC osmoticum (0.64 M KCl and 0.2 M $CaCl_2$) for 5 minutes and gently centrifuged (1200 rpm) for 2 minutes. The pelleted mycelia were resuspended in KC containing 2.5 mg/ml Novozym 234 (Novo Laboratories, Wilton, CT, USA) and 2.5 mg/ml cellulase (Onozuka, Kinki Yakult Mfg. Co., Ltd., Nishinomiya, Japan) (5-10 ml per 1 ml of pelleted mycelia) and incubated at room temperature for 15-20 minutes to generate protoplasts. To remove debris, the protoplast suspension was filtered though a nylon filter (50 µm mesh). After centrifugation (2 min., 1200 rpm) the soft pellet was washed in KC (30 sec.), centrifuged (2 min., 1200 rpm), washed in KC-MT (0.32 M KCl, 0.1 M $CaCl_2$, 0.5 M mannitol and 5 mM Tris/HCl pH 7.5) (30 sec.), centrifuged (2 min., 1200 rpm) washed in MT (1 M mannitol and 10 mM Tris/HCl pH 7.5) (30 sec.) and centrifuged (2 min., 1200 rpm). All steps were performed at room temperature. Finally the protoplast suspension was diluted to 5.10^6-1.10^7 protoplasts per ml MT and 1 ml of the protoplast suspension was gently mixed with a pre-incubated Lipofectin-DNA mixture. The Lipofectin-DNA mixture was prepared by mixing 50 µl water containing 30 µg of the non-selectable plasmids, pHAMT35G or pPIN13 and 15 µg of the selectable plasmids pTH209 or pHAMT34N 60 µl of Lipofectin reagent (Gibco-BRL, Gaithesburg, MD, USA) in polystyrene tubes and incubating it for 15 minutes at room temperature. The protoplasts with the Lipofectin-DNA mixture were kept at room temperature for 5 minutes after which 1 ml of 50% PEG 3350 (Sigma Chemical Co. St. Louis, MO. USA) in 20 mM $CaCl_2$ and 10 mM Tris/HCl, pH 7.5 was added very slowly. Five minutes later, the suspension was gently mixed and 10 ml of clarified liquid rye sucrose medium containing 1 M mannitol was added slowly. Ten minutes later, this mixture was poured in Petri dishes containing 15 ml liquid clarified rye sucrose medium with the antibiotics, ampicillin, vancomycin (Sigma Chemical Co. St. Louis, MO, USA), and pimaricin (Duchefa Biochemie BV., Haarlem, The Netherlands) at final concentrations of 50, 50 and 25 µg/ml, respectively. After incubation for 20-24 hours at 18 °C, the mixture, now containing regenerated protoplasts (~15% of the input), was gently centrifuged and the pellet was resuspended in a total volume of 1-2 ml clarified rye sucrose medium containing 1 M mannitol. The suspension was very gently spread on rye sucrose agar containing 5 µg/ml geneticin (Gibco BRL, Gaithesburg, MD, USA). Colonies appeared within 6-12 days and were propagated on rye sucrose medium containing 10 µg/ml geneticin. Overall, a transformation efficiency of two transformants per µg selection plasmid DNA was achieved.

Selection of *ipiO-gus* and *Ham34-gus* Transformants

Integration of the *ipiO-gus* construct in the *P. infestans* genome was examined by PCR and Southern blot analysis. Twenty of the forty four putative co-transformants (49%) contained the *ipiO-gus* construct. Subsequently, *ipiO-gus* transformants were tested for GUS activity *in vitro* by transferring them to rye sucrose medium containing 25 µg/ml X-gluc. Seven of these stained blue, indicating aberrant expression of *gus*. These were not used for further experiments. From the remaining *ipiO-gus* transformants, only two transformants with a low pPIN13 copy number, CX4 and CX7, were used in further experiments. Twenty eight putative *Ham34-gus* transformants were tested for β-glucuronidase activity by transferring them to rye sucrose medium containing 25 µg/ml X-gluc. Fifteen transformants stained blue after fourteen days of growth. Transformant EY6 showed high GUS activity and was used as a positive GUS control.

GUS-assays

Sectors containing late blight lesions were cut from infected Bintje leaves during a time course and were vacuum infiltrated for 10 minutes with a β-glucuronidase (GUS) staining solution (0.5 mg/ml 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide (X-gluc, (Biosynth AG, Staad, Switzerland) in 100 mM sodium phosphate (pH 7.0), 1% Triton X-100, 1% DMSO and 10 mM EDTA). Infiltrated leaf material was kept in the staining solution for 48 hours at 37 °C. Chlorophyll was removed by washing the tissue four times with 70% (v/v) ethanol. Subsequently, leaf material was washed twice with 50% (v/v) glycerol to remove the ethanol and embedded in 50% glycerol for long term storage. Microscopic analysis was performed with a Zeiss Axioscope microscope and photographs were taken using a Zeiss MC-100 camera unit. *In vitro* GUS activity of *ipiO-gus* transformant CX4 and CX7, *Ham34-gus* transformant EY6 was checked on solidified rye sucrose medium by transferring agar plugs containing mycelia to fresh medium in six-well microtiter plates and incubated for 1, 2, 3 or 4 days. Five ml of GUS staining buffer was layered over each colony and after vacuum infiltration the microtiter plates were incubated at 37 °C overnight. Quantitative measurements of β-glucuronidase activity in protein extracts of cysts and germinated cysts were performed according to Jefferson (1987), by using a fluorogenic assay with 4-methyl-umbelliferyl-β-D-glucuronide (MUG) (Biosynth AG, Staad, Switzerland) as a substrate. Values obtained were normalised to µM MU produced by β-glucuronidase per µg of total protein per hour at 37 °C.

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Chapter 4

A Gene Encoding a Protein Elicitor of *Phytophthora infestans* Is Down-Regulated during Infection of Potato

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Summary

Most species of the genus *Phytophthora* produce 10 kDa extracellular protein elicitors, collectively termed elicitins. Elicitins induce a hypersensitive response in a restricted number of plants, particularly in the genus *Nicotiana* within the Solanaceae family. A cDNA encoding INF1, the major secreted elicitin of *Phytophthora infestans*, a pathogen of solanaceous plants, was isolated and characterised. The expression of the corresponding *inf1* gene during the disease cycle of *P. infestans* was analysed. *Inf1* was shown to be expressed in mycelium grown in various culture media, whereas it was not expressed in sporangia, zoospores, cysts, and germinating cysts. *In planta*, during infection of potato, particularly during the biotrophic stage, expression of *inf1* was down-regulated compared to *in vitro*. The highest levels of expression of *inf1* were observed in *in vitro*-grown mycelium and in late stages of infection when profuse sporulation and leaf necrosis occur. The potential role of INF1 as an elicitor in interactions between *P. infestans* and *Solanum* species was investigated. Nineteen lines, representing nine solanaceous species with various levels of resistance to *P. infestans*, were tested for response to an *Escherichia coli* expressed INF1. Within the genus *Solanum*, resistance to *P. infestans* did not appear to be mediated by a defence response elicited by INF1. However, INF1 recognition could be a component of non-host resistance of tobacco to *P. infestans*.

Introduction

Interactions between plants and pathogens can be either compatible, leading to successful infection by the pathogen (plant is susceptible), or incompatible, leading often to localised cell death through a hypersensitive response (HR) of the plant (plant is resistant). Both types of interactions are believed to involve an exchange of molecular signals between the plant and the pathogen, and the nature of such signals would lead to either disease or resistance (Lamb et al., 1989; Dixon & Lamb, 1990; Ebel & Scheel, 1992; de Wit, 1992; Staskawicz et al., 1995). A simple and well characterised illustration of this model lies in incompatible interactions mediated by specific elicitor molecules. In such interactions, elicitors produced directly or indirectly by avirulence genes of the pathogen induce a defence response only in plants that contain the corresponding *R*-gene. These *R*-genes are thought to encode receptors that bind to the elicitors and activate an array of plant genes leading to a HR and inhibition of pathogen growth.

In this paper, we describe studies on the role of an elicitor protein in the *Phytophthora infestans*-Solanaceae pathosystem. *P. infestans*, an oomycete pathogen, causes late blight, economically the most important disease of potato world-wide. The oomycetes have traditionally been included in the Kingdom Fungi. However, based on biochemical characteristics, oomycetes are now classified along with golden-brown algae (chrysophytes) in the Kingdom Protoctista (Protista) (Corliss, 1984; Dick, 1990; Margulis, 1996). Among the notable oomycete pathogens, members of the genus *Phytophthora* (order Peronosporales) cause destructive diseases on thousands of plant species (Erwin et al., 1983).

The molecular basis of host-specificity of *Phytophthora* is not known (Judelson, 1996). However, in recent years, a family of extracellular elicitor proteins, termed elicitins, has been identified and evidence has accumulated for a role of these molecules in delimiting the host-range of *Phytophthora* species (Yu, 1995). Elicitins are highly conserved 10 kDa proteins that are secreted by all tested *Phytophthora* and *Pythium* species (Pernollet et al., 1993; Kamoun et al., 1994; Huet et al., 1995). These proteins induce defence responses including a HR on a restricted number of plant species, specifically in the Solanaceae and Cruciferae families (Ricci et al., 1989; Kamoun et al., 1993b). The basic hypothesis behind studies on elicitins is that responsive plants display higher levels of resistance to elicitin-producing isolates of *Phytophthora* than to elicitin-deficient ones. The role of elicitins as avirulence factors that trigger plant defence responses leading to resistance has been examined in detail in the *Phytophthora parasitica-Nicotiana* pathosystem. In *P. parasitica*, the absence of elicitin production correlates with high virulence on tobacco, a plant species that strongly responds to elicitins. In contrast, isolates that produce elicitins either cause a mild brown rot disease or are completely avirulent on tobacco (Bonnet et al., 1994; Kamoun et al., 1994). The response of tobacco to elicitins also leads to acquired resistance against otherwise virulent *P. parasitica* isolates (Ricci et al., 1989; Kamoun et al., 1993b; Keller et al., 1996), and in a sexual progeny of *P. parasitica*, elicitin production segregates with low virulence on tobacco (Kamoun et al., 1994), suggesting that elicitins indeed can function as avirulence factors. However, despite the availability of cloned elicitin genes (Kamoun et al., 1993a; Panabieres et al., 1995), direct demonstration of the role of elicitins through transformation of elicitin deficient strains has not been achieved due to difficulties in the genetic manipulation of *P. parasitica*.

The late blight pathosystem is an attractive model system to investigate the role of elicitins in *Phytophthora*-plant interactions. *P. infestans* produces elicitins (Huet et al., 1994; Kamoun et al., 1994) and displays a complex range of interactions with solanaceous plants. A great deal of information on the life cycle, the infection process, and the cytology of both compatible and incompatible interactions is available (Coffey & Wilson, 1983; Gees & Hohl, 1988; Coffey & Gees, 1991; Freytag et al., 1994), and methods for measurements of gene expression *in planta* have

been developed (Pieterse et al., 1991; 1994a). Moreover, molecular manipulation as well as stable DNA transformation is well established for *P. infestans* (Judelson et al., 1991, Judelson, 1996). For these reasons and because DNA transformation is a prerequisite for unequivocal demonstration of the role of elicitins in *Phytophthora*-plant interactions, we decided to exploit *P. infestans* for functional analyses of elicitins.

We present here the cloning and characterisation of cDNAs from *P. infestans* encoding the elicitin INF1. The expression of the corresponding elicitin gene (*inf1*) in various developmental stages of *P. infestans* and during interaction of *P. infestans* with potato was investigated. Finally, *inf1* was expressed in *Escherichia coli* and the elicitor activity of the recombinant protein was tested in several solanaceous plants which differ in levels of resistance to *P. infestans*.

Results

Cloning and Molecular Analysis of cDNAs Encoding *P. infestans* Elicitin

A cDNA library constructed in the phagemid vector, λZAP, from RNA isolated from leaves of potato cultivar Ajax three days after inoculation with *P. infestans* 88069 (Pieterse et al., 1994b) was hybridised with a 0.3 kb fragment internal to the open reading frame (ORF) of the *parA1* gene of *P. parasitica* (Kamoun et al., 1993a). A total of nineteen positive clones was isolated, and plasmids were rescued by *in vivo* excision. Based on restriction enzyme mapping and Southern blot hybridisation, two classes of clones were identified. One class consisted of six clones of which the inserts hybridised weakly to the *parA1* probe. DNA sequencing revealed that these clones contain cDNA sequences of two novel elicitin-like genes that will be described elsewhere. The remaining thirteen clones hybridised strongly to the *parA1* probe and appeared to contain sequences highly homologous to the *parA1* gene. These cDNA clones were further characterised. DNA sequence analysis revealed no sequence polymorphism between the overlapping regions of the thirteen cDNA clones indicating that they all encode the same protein. A 354 bp ORF was found (Figure 1), which encodes a protein composed of a 20 amino acid signal peptide, followed by a 98 amino acid sequence that is identical to the amino acid sequence of a *P. infestans* elicitin published by Huet et al. (1994). We have designated the *P. infestans* elicitin INF1 and consequently the cDNA was named *inf1* cDNA.

In all thirteen *inf1* cDNA clones, a 164 bp 3' untranslated region and a stretch of poly(A) followed the ORF. This suggests that transcriptional termination occurs at one site which is probably governed by the putative polyadenylation signal AATAAA (underlined in Figure 1). The longest cDNA clone obtained, pFB7, possesses a 36 bp untranslated 5' region, which is 20 bp shorter than the 5' untranslated region of

parA1 (Kamoun et al., 1993a). The size of the cDNA insert in pFB7 (554 bp) corresponds to the estimated size of the *inf1* mRNA (550 nt) as described below.

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                                                    TGTTATCCCCACCCACTCCTCCTCACTCCGTCCACG  -1

ATGAACTTTCGTGCTCTGTTCGCTGCCACCGTTGCTGCCCTCGTCGGCTCCACTTCCGCCACCACGTGCACCACCTCGCA   80
M  N  F  R  A  L  F  A  A  T  V  A  A  L  V  G  S  T  S  A  T  T  C  T  T  S  Q   20

GCAGACCGTAGCGTACGTGGCGCTCGTAAGCATCCTCTCGGACACHTCGTTTAATCAGTGCTCGACGGACTCCGGCTACT 160
 Q  T  V  A  Y  V  A  L  V  S  I  L  S  D  T  S  F  N  Q  C  S  T  D  S  G  Y  S  47
                                                    SalI
CGATGCTGACGGCCACCTCGCTGCCCACGACGGAGCAGTACAAGCTCATGTGCGCGTCGACGGCGTGCAAGACGATGATC 240
  M  L  T  A  T  S  L  P  T  T  E  Q  Y  K  L  M  C  A  S  T  A  C  K  T  M  I   73

AACAAGATCCTGTCGCTCAACGCTCCCGACTGCGAGCTGACGGTGCCAACTAGTGGCCTGGTACTCAACGTGTACTCGTA 320
N  K  I  V  S  L  N  A  P  D  C  E  L  T  V  P  T  S  G  L  V  L  N  V  Y  S  Y  100

CGCGAACGGGTTCTCGTCTACGTGTGCGTCGCTATGAGTGGACTTGACTCAGCGCGCTCAGCCGCTGAGTCAGCGTCCAT 400
 A  N  G  F  S  S  T  C  A  S  L  *                                              118

TGGCCGTATCATCTTGTCTTTCATCGACGATTAGGCTTTGTTCTCCGCGTCTGTTGCTATTACTGTAATAGTGTAGCAGC 480

TGCGGTATAAAGCTATTTGAATAAAAATTCTTGTATGCCAT                                        521
```

Figure 1. Nucleotide Sequence of the *inf1* cDNA Clone
Nucleotide sequence of pFB7 from *Phytophthora infestans* isolate 88069 (GenBank accession number U50844), and the deduced amino acid sequence of the pre-INF1 protein. The mature secreted INF1 elicitin is shown in bold. The putative polyadenylation sequence AATAAA is underlined.

Occurrence of *inf1* Sequences in the *P. infestans* Genome

In order to determine the number of copies of the cloned *inf1* sequences that occur in the *P. infestans* genome, Southern blot hybridisations were performed. *Bam*HI and *Sal*I digested DNA of *P. infestans* isolate 88069 was hybridised with the entire insert of *inf1* cDNA. One *Bam*HI fragment of approximately 2.3 kb and three *Sal*I fragments of 7.0, 3.6, and 2.5 kb were detected (Figure 2). Since there is one *Sal*I site and no *Bam*HI site in the cloned *inf1* cDNA (Figure 1), this result indicate that at least two copies of the *inf1* gene occur in the *P. infestans* genome. These two copies could correspond to two *inf1* alleles polymorphic for a flanking *Sal*I site, or could correspond to conserved repeated sequences of *inf1*. Hybridisation of the same blot with a probe containing the 3' noncoding region of the *inf1* cDNA (see Experimental Procedures) yielded a slightly different result. Although, the same 2.3 kb *Bam*HI fragment and the 7.0 kb *Sal*I fragment were detected, the 3.6 and 2.5 kb *Sal*I fragments were not detected (data not shown), suggesting that the 3.6 and 2.5 kb *Sal*I fragments contain the 5' half of the *inf1* gene. Southern blot analyses with two other *P. infestans* isolates, IPO-r0 and 90128, revealed the same hybridisation pattern as *P. infestans* 88069 with both the entire cDNA probe (Figure 2) and the 3' end probe (data not shown) even though these isolates are polymorphic at other loci (data not shown). This suggest that *inf1* sequences are conserved between the three tested *P. infestans* isolates. No cross-hybridisation with other elicitin-like genes was observed under the hybridisation conditions used in these experiments.

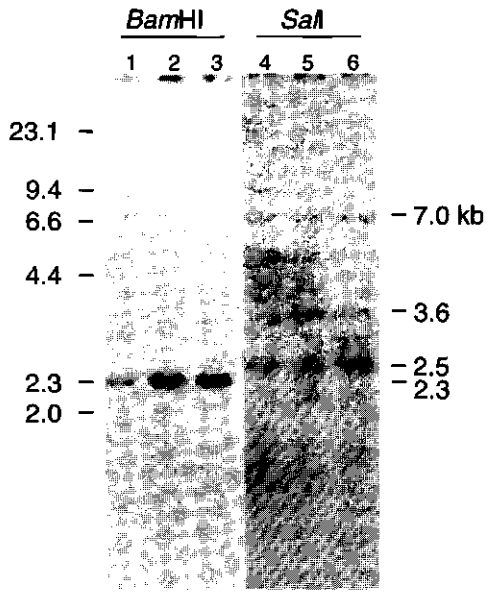

Figure 2. Occurrence of *inf1* Sequences in *Phytophthora infestans*
Total DNA (20 µg) of *P. infestans* isolates 88069 (lanes 1 and 4), IPO-r0 (lanes 2 and 5), and 90128 (lanes 3 and 6) was digested with *Bam*HI (lanes 1-3) or *Sal*I (lanes 4-6) and hybridised with a probe containing the *inf1* cDNA. The numbers on the left indicate the sizes in kb of the molecular size marker fragments. On the right, the approximate sizes in kb of the three hybridising *Sal*I fragments are indicated.

Expression of *inf1* during Various Developmental Stages of *P. infestans*

In order to determine the patterns of expression of the *inf1* gene, northern blot analyses were performed, and accumulation of *inf1* mRNA in various developmental stages of *P. infestans* 88069 was determined. No *inf1* mRNA was detected in RNA extracts of zoospores, cysts, germinating cysts (2.5 hours in either water or Lima bean medium), and sporangia. However, *inf1* mRNA was detected in extracts of mycelium obtained 20 hours after germination of sporangia in Lima bean medium (Figure 3). Additionally, *inf1* mRNA was consistently detected at high levels in extracts of mycelium cultured in rye sucrose or other standard media (data not shown, see also Figure 4) suggesting that the *inf1* gene is exclusively expressed in mycelium. Control hybridisations with a probe of the constitutively expressed *actA* gene (Unkles et al., 1991) showed that all lanes contained similar amounts of total RNA (Figure 3).

Expression of *inf1 in Planta*

To analyse the expression of *inf1* during the interaction of *P. infestans* with its host plant potato, total RNA was isolated from leaves of potato cv. Bintje 1, 2, 3, 4, 5, 6, 7, and 8 days after inoculation with *P. infestans* 88069 and from *P. infestans* mycelium grown in rye sucrose medium. A northern blot containing these samples was hybridised with probes of the *P. infestans inf1* and *actA* genes (Figure 4). Because

the total RNA extracted from infected leaves consists of a mixture of *P. infestans* and plant RNA, the signals obtained on northern blots with probes of differentially expressed genes should be normalised to actual *Phytophthora* RNA levels as determined by the signals obtained with a probe of a constitutively expressed gene.

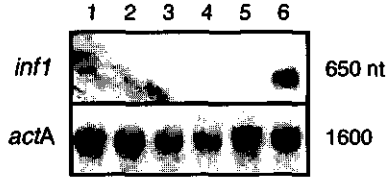

Figure 3. Expression of *inf1* in Various Developmental Stages of *Phytophthora infestans*
Total RNA from *P. infestans* 88069 zoospores (1), cysts (2), cysts germinating for 2.5 hours in water (3), cysts germinating for 2.5 hours in Lima bean medium (4), sporangia (5), and mycelium obtained from sporangia germinating in Lima bean medium for 20 hours (6) was sequentially hybridised with probes from the *inf1* and *actA* genes. The approximate sizes of the *inf1* and *actA* transcripts are shown on the right.

Consistent with increases in *P. infestans* biomass during the infection, the mRNA of the constitutively expressed *actA* gene was first detected at day 2 and its level increased in the following days until reaching a maximal level at days 6, 7, and 8. In contrast, *inf1* mRNA was first detected at day 3 after inoculation and reached the highest level at days 5 and 6, when extensive sporulation of *P. infestans* and leaf necrosis occurred. Subsequently, at days 7 and 8, when little additional sporulation occurred, the level of *inf1* mRNA decreased. In contrast to the *actA* mRNA, the levels of the *inf1* mRNA observed *in planta* were always lower than the levels observed *in vitro*, particularly during the early biotrophic stages of infection (days 2 to 4) and the late post-sporulation stages (days 7 and 8). These results suggest that expression of *inf1* is down-regulated during infection of potato. Similar results were obtained when the northern blot was hybridised with the 3' end *inf1* probe instead of the full cDNA probe.

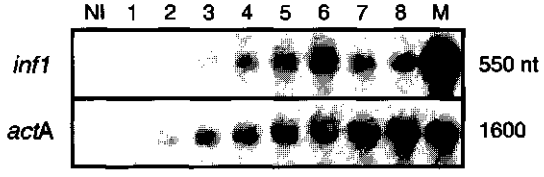

Figure 4. Time Course of Expression of *inf1* and *actA* of *Phytophthora infestans* During Infection of Potato cv. Bintje
Total RNA isolated from infected leaves of potato, 1, 2, 3, 4, 5, 6, 7, or 8 days after inoculation, from non-infected leaves (P), and from *P. infestans* mycelium grown in rye-sucrose medium (M) was sequentially hybridised with probes from the *inf1* and *actA* genes. The approximate sizes of the transcripts are shown on the right.

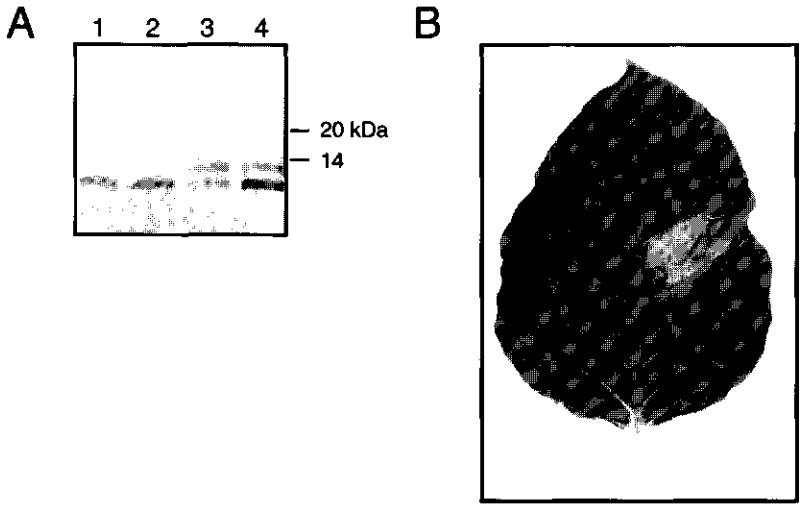

Figure 5. Bacterial Expression of the Cloned *inf1* cDNA
(A) Western blot immunodetection of recombinant proteins in SDS-PAGE of culture supernatants (lanes 1 and 2) and soluble cellular fractions (lanes 3 and 4) of *Escherichia coli* strain pFB52 (FLAG-INF1*S3*, lanes 1 and 3) and strain pFB53 (FLAG-INF1, lanes 2 and 4). FLAG M2 monoclonal antibody was used for detection. Molecular weight standards are shown on the left.
(B) Leaf infiltration of tobacco cv. Xanthi with 100 nM solutions of purified FLAG-INF1*S3* (left side of the leaf) and FLAG-INF1 (right). The photograph was taken 5 days after infiltration but the response was already visible 24 hours after infiltration.

Bacterial Expression of *inf1* cDNA

In order to determine whether the protein encoded by the *inf1* cDNA is biologically active as an elicitor molecule and to determine the host specificity of INF1, the *inf1* cDNA was subcloned into pFLAG-ATS, a vector which allows isopropyl-β-D-thiogalactopyranoside (IPTG) induced expression of the inserted gene in *E. coli*. The product is secreted by *E. coli* as a fusion protein containing a signal peptide derived from the *ompA* gene followed by the epitope tag, FLAG, at the amino-terminus. Two plasmids were constructed in which the coding sequence of the processed form of the INF1 protein is inserted downstream of the epitope tag. pFB53 contains a wild type *inf1* sequence (FLAG-INF1), whereas pFB52 contains a mutated *inf1* sequence, in which the cysteine codon at position 23 (or position 3 of the processed protein) is mutated into a serine (FLAG-INF1*S3*). The mutant is expected to serve as a negative control, since it should lack one of the three disulphide bridges and is predicted to have reduced elicitor activity (Ricci et al., 1989). Western blots containing soluble cellular extracts and culture supernatants of IPTG-induced *E. coli* strains bearing pFB52 or pFB53 were incubated with FLAG monoclonal antibodies (Figure 5A). Two bands of approximately 12 and 14 kDa reacted with the FLAG antibody in the cellular fraction, whereas only the lower band was present in the supernatant fractions suggesting that secretion of the fusion proteins is accompanied by the proper removal of the OMPA signal peptide.

Table 1. Specificity of Hypersensitive Response Induced by INF1 Elicitin in the Solanaceae

Species (cv. or accession no.)	Resistance level[a]	Response to[b] INF1	INF1 *S3*
Nicotiana tabacum			
Xanthi	fully resistant	+	-
Solanum berthaulti			
BGRC 10.063-9	fully resistant	-	-
BGRC 10.063-11	partial resistant	-	-
S. circaeifolium ssp. *circaeifolium*			
BGRC 27.057	partial resistant	-	-
S. microdontum			
BGRC 24.981-167	partial resistant	-	-
BGRC 24.981-178	partial resistant	-	-
S. microdontum ssp. *giganthophyllum*			
BGRC 18.570-265	susceptible	-	-
S. nigrum			
SN18	fully resistant	-	-
S. sucrense			
BGRC 27.370-23	partial resistant	-	-
BGRC 27.370-71	partial resistant	-	-
S. tuberosum			
Bintje	susceptible	-	-
Desiree	susceptible	-	-
Ehud	susceptible, *R*-1	-	-
Estima	susceptible, *R*-10	-	-
Premiere	susceptible, *R*-10	-	-
Surprise	partial resistant	-	-
Robijn	partial resistant	-	-
S. vernei			
BGRC 24.733-530	partial resistant	-	-
Lycopersicon esculentum			
Moneymaker	susceptible	-	-

[a] The resistance data were obtained from Colon & Budding, 1988; Colon et al., 1992; 1995; Colon, 1994.
[b] 100 nM of purified fusion proteins FLAG-INF1 and FLAG-INF1 *S3* were infiltrated in attached leaves of the listed plants: + indicates a confluent hypersensitive response, and – indicates no visible response.

Following affinity chromatography purification of the fusion proteins from *E. coli* supernatant fractions, a single band corresponding to either FLAG-INF1 or FLAG-INF1*S3* was detected on silver stained SDS-PAGE gels or on western blot incubated with FLAG antibodies (data not shown). Infiltration of tobacco (cv. Xanthi) leaves with 100 nM of purified FLAG-INF1 consistently induced a hypersensitive response, whereas infiltration with FLAG-INF1*S3* did not induce any visible response (Figure 5B). Infiltration with the various buffers and culture supernatant from *E. coli* bearing the cloning vector induced no visible response. These results demonstrate that the cloned *inf1* cDNA encodes an active elicitor protein.

Response of *Solanum* Plants to INF1 and Relation to Late Blight Resistance

In order to determine whether INF1 elicitin plays a role as an elicitor in the interaction between solanaceous plants and *P. infestans*, the response to INF1 of nineteen lines representing nine solanaceous species was examined (Table 1). Besides tobacco,

all tested plants failed to respond to infiltration with 100 nM solutions of purified FLAG-INF1 and none of the tested plants responded to FLAG-INF1*S3*. Previously, the response of these lines to INF1-producing strains of *P. infestans* was determined in both field and growth chamber experiments (Colon and Budding, 1988; Colon et al., 1992; 1995; Colon, 1994) and ranged from full resistance to susceptible (Table 1). These results confirm the narrow specificity of elicitins (Kamoun et al., 1993b), and indicate that resistance to *P. infestans* within the genus *Solanum* is not determined by the recognition and response to INF1 elicitin.

Discussion

Molecular Structure of the Elicitor cDNA *inf1* of *P. infestans*

In this paper, we report the molecular cloning of a *P. infestans* cDNA, *inf1*, encoding a host-specific elicitor protein of the elicitin family. The DNA sequence of the *inf1* cDNA revealed a 354 bp ORF encoding a pre-INF1 protein of 118 amino acids. Processing of the 20 amino acid N-terminal signal peptide results in the mature 98 amino acid INF1 protein (Figure 1). The deduced amino acid sequence of INF1 is identical to the sequence of an acidic elicitin protein purified from another isolate of *P. infestans* (Huet et al., 1994). Sequence comparisons indicate a high degree of homology between the coding sequence of *inf1* of *P. infestans* and those of other characterised elicitin genes, *parA1* of *P. parasitica* (Kamoun et al., 1993a), and cryptogein A1-B14, cryptogein B-X24, *hae*-B20 and *hae*-B26 of *P. cryptogea* (Panabieres et al., 1995). *Inf1* is more similar to the genes encoding acidic elicitins, *parA1* (92% identity at the DNA level over the coding region) and cryptogein A1-B24 (85%), than to the basic elicitin gene cryptogein B-X24 (81%).

Using Southern blot analyses, at least two genomic copies of the *inf1* gene could be detected in three different *P. infestans* isolates. These two copies are both contained in a conserved 2.3 kb *Bam*HI fragment and could correspond to two alleles of *inf1*. Even though additional elicitin-like sequences were isolated from *P. infestans* (unpublished data), no cross-hybridisation was noted between these genes and the *inf1* probes under the Southern and northern blot hybridisation conditions used in this study. This indicates that the *inf1* transcript detected in the northern blot analyses must be derived from one or both genomic copies of the *inf1* gene.

The *Inf1* Gene is Down-Regulated During Infection of Potato

The pattern of expression of the *inf1* gene was followed throughout the disease cycle and in various stages of the life cycle of *P. infestans*. During the early stages of infection, expression of *inf1* does not occur prior to the penetration of plant cells. This contrasts to the expression pattern of the *in planta*-induced genes *ipiB* and *ipiO* of *P. infestans* (Pieterse et al., 1994a), which are already expressed at high levels in

germinating cysts. Expression of *inf1* occurs later during infection although at a reduced level. The highest levels of *inf1* expression occur at later stages concurrent with the onset of extended leaf necrosis, saprophytic growth and profuse sporulation. The expression of *inf1* is then repressed in sporangia and zoospores until a new infection cycle is initiated. The *inf1* gene reaches maximal levels of expression in sporulating mycelium whether growing *in vitro* or *in planta.* Comparison of the expression patterns of *inf1* and *actA* indicate that *inf1* is down-regulated during infection of potato.

Based on their abundant secretion and primary amino acid structure, elicitins have been proposed to function as structural proteins in *Phytophthora* (Tempelton et al., 1994; Yu, 1995). The high expression of *inf1* during the onset of the saprophytic stage of infection and in mycelium growing *in vitro* supports this hypothesis. Both stages are characterised by abundant sporulation. Increased synthesis of structural proteins might be required in sporulating mycelium, particularly in sporangia, in order to sustain the additional amounts required for spore formation. Additional experiments, including cellular localisation of elicitins in *Phytophthora* should help unravel the biological function of these proteins.

The products of elicitor or avirulence genes trigger defence responses in plants that are ultimately deleterious to the pathogen. Therefore these genes are under selective pressure to mutate to inactive forms leading the pathogen into an evolutionary race with the plant (Staskawicz et al., 1995). The down-regulation of the expression of the *inf1* gene during infection of potato, noted in this study, could therefore be an adaptation of *P. infestans* to evade plant defence responses. However, since no known host plant of *P. infestans* has been shown to respond to elicitins (Table 1), the expression pattern of *inf1* might simply reflect the potential role of elicitins as structural proteins in *Phytophthora*.

Host Specificity of Elicitins

Purified elicitin proteins from several *Phytophthora* species were shown to induce defence responses in two plant families. In the Solanaceae, response to elicitins appears to be restricted to the genus *Nicotiana*, whereas in the Cruciferae, cultivar-specific response was noted for radish, turnip and rape (Bonnet et al., 1996; Kamoun et al., 1993b). The results obtained in this study with INF1 confirm the narrow specificity of elicitins. In the Solanaceae, no INF1-responsive plant was identified outside the genus *Nicotiana* (Table 1).

It should be noted that conflicting results have been published on host specificity of elicitins (discussed by Yu, 1995). Pernollet and co-workers (1993) described elicitins as toxic molecules capable of inducing necrosis on all tested plants. In their studies, solanaceous plants including tomato, pepper and potato responded similarly to tobacco after elicitin treatment (Huet et al., 1994; Pernollet et al., 1993). These results are particularly intriguing considering that systemic necrosis should then be

observed during compatible interactions between *Phytophthora* and plants. This is generally not the case, for example in pepper plants infected by *Phytophthora capsici*, elicitins were detected systemically but no corresponding necrosis was observed (Devergne et al., 1994). In studies by various laboratories, specificity in the response to elicitins has been reported not only for whole plants, but also for cell cultures (Blein et al., 1991; Yu 1995). Additionally, specificity was observed after treatment with elicitins purified by various biochemical methods from either *Phytophthora* or *E. coli*, and applied to plants by either leaf infiltration or a number of other uptake methods (Bonnet et al., 1996; Kamoun et al., 1993b) (this study). The conclusion that elicitins are non-specific toxins involved in pathogenesis of *Phytophthora* and *Pythium* species (Huet et al., 1994; Pernollet et al., 1993) is therefore inconsistent with reports from several laboratories, and will not be further considered in this paper.

Role of INF1 Elicitin in the Interaction Between *P. infestans* and Solanaceous Plants

Similar to other elicitors, elicitins induce a number of plant responses that can be attributed to defence mechanisms indicating that these proteins function as classical elicitor molecules that trigger defence responses in a restricted range of plant genotypes (Milat et al., 1991a; 1991b; Kamoun et al., 1993; Keller et al., 1994). In this paper, we show that the INF1 elicitin protein of *P. infestans* does not induce a HR on potato and tomato, two major host plants. Expression of the *inf1* gene during infection of potato occurs, albeit at a down-regulated level. Therefore, even though INF1 is produced during infection, it is not detected by compatible host plants and does not appear to play a role in restricting the disease process. Several *Solanum* species, with either partial or full resistance to *P. infestans*, also failed to respond to INF1, indicating that the observed resistance involves other molecular mechanisms than elicitin recognition. On the other hand, resistance of tobacco to *P. infestans* could involve to some degree the response to elicitins. Future experiments using *P. infestans* transformants altered in INF1 production should help determine whether elicitin recognition is a significant component of the resistance of tobacco to *P. infestans*. Additionally, biotechnological manipulation of potato to acquire recognition and response to elicitin molecules is predicted to yield plants with enhanced resistance to *P. infestans*.

Experimental Procedures

Phytophthora Strains and Culture Conditions

P. infestans 88069 (A1 mating type, race 1.3.4.7), a tomato isolate from Bennekom, NL was used throughout this study. *P. infestans* 90128 (A2 mating type, race 1.3.4.7.8.9.10.11, potato isolate, NL) and IPO-r0 (A1 mating type, race 0, unknown origin) were also used in Southern blot analyses. *P. infestans* strains were routinely grown on rye agar medium supplemented with 2% sucrose (Caten & Jinks, 1968) or modified Plich medium (Kamoun et al., 1993b). To isolate sporangia, sporulating

mycelium in rye sucrose medium was flooded with water (10 ml per Petri dish) and gently rubbed with a sterile glass rod. Sporangia were then pipetted with the water. Zoospores were obtained after incubation of similarly flooded plates at 4 °C for two hours. After pipetting the solution, the zoospores were separated from the remaining sporangia by filtration through a 10 µm nylon mesh. Encystment was induced by continuous shaking for 2 min. Germinating sporangia and cysts were obtained by incubation for various times in water or media at 18 °C. All three *P. infestans* isolates used in this study produce INF1 abundantly when grown in liquid Plich medium.

Bacterial Strains and Plasmids

E. coli XL1-Blue and DH5α were used in most experiments and were routinely grown at 37 °C in Luria-Bertani (LB) media (Sambrook et al., 1989). Phage manipulations were conducted according to the protocols provided by Stratagene (La Jolla, CA). Helper phage VCS-M13 was used in *in vivo* excision experiments (Stratagene, La Jolla, CA).

Plasmids pFB52 and pFB53 were constructed by cloning polymerase chain reaction (PCR) amplified DNA fragments corresponding to the *inf1* ORF into the *Hind*III site of pFLAG-ATS (IBI-Eastman Kodak, New Haven, CT). The oligonucleotides used in the PCR are SK-F (5'-GCGAAGCTTACCACGTGCACCACCTCG-3') and SK-R1 (5'-GCGAAGCTTATAGCGACGCACACGTAGA-3') for the fragment cloned in pFB53, and SK-F1S (5'-GCGAAGCTTACCACG**A**GCACCACCTCG-3') and SK-R1 for the fragment cloned in pFB52. The introduced *Hind*III restriction sites are underlined and the T to A mutation in SK-F1S is shown in bold. Partial nucleotide sequencing of the cloned fragments (including the mutated site) in pFB52 and pFB53 fully matched the predicted sequence. The N-terminal sequence of the processed recombinant FLAG-INF1 protein of pFB53 is "DYKDDDDKDKVKI ***TTCTTSQQTV***...". The FLAG antibody binding site is underlined, and the first ten amino acids of mature INF1 are shown in bold with the mutated cysteine of pFB52 shown in bold italics.

DNA Manipulations

DNA manipulations were conducted essentially as described elsewhere (Ausubel et al., 1987; Sambrook et al., 1989). Total DNA of *P. infestans* was isolated from mycelium grown in liquid culture as previously described (Pieterse et al., 1991). Alkaline DNA transfer to Hybond N^+ (Amersham, Arlington Heights, IL) and Southern hybridisations were performed at 65 °C as described elsewhere (Ausubel et al., 1987; Sambrook et al., 1989). Filters were washed at 65 °C in 0.5x SSC (75 mM NaCl and 7.5 mM sodium citrate). *Dideoxy chain-termination sequencing was carried out using an AmpliCycle* sequencing kit (Perkin Elmer, Foster City, CA). Sequence analysis was made using software DNA Strider 1.0 (C. Marck, Institut de Recherche Fondamentale, France). Database homology searches were conducted using the BLAST software package (Altschul et al., 1990) as available through the Internet.

Screening of the cDNA Library

The screened cDNA library was constructed in the phagemid vector, λZAP (Stratagene, La Jolla, CA), from leaves of potato cultivar Ajax three days after inoculation with sporangia of *P. infestans* (Pieterse et al., 1994b). A total of 30,000 plaques was screened with the 0.3 kb insert fragment of a subclone of pELC100 containing nucleotides 61 to 358 of the *parA1* gene of *P. parasitica* (Kamoun et al., 1993a). Positive plaques were purified, subjected to *in vivo* excision of pBluescript SK^- (Stratagene, La Jolla, CA), and characterised by restriction enzyme digestion, Southern blot hybridisation, and DNA sequencing.

RNA Manipulations

Total RNA from *P. infestans* and from infected potato leaves was isolated using the guanidine *hydrochloride extraction method* (Logemann et al., 1987). For northern blot analyses, 10-15 µg of total RNA was denatured at 50 °C in 1 M glyoxal, DMSO, and 10 mM sodium phosphate, electrophoresed, and transferred to Hybond N^+ membranes (Amersham, Arlington Heights, IL) (Ausubel et al., 1987; Sambrook et al., 1989). Hybridizations were conducted at 65 °C in 0.5 M sodium phosphate buffer, 7% SDS and 1 mM EDTA. Filters were washed at 65 °C in 0.5x SSC (75 mM NaCl and 7.5 mM sodium citrate).

Southern and Northern Blot Hybridisation Probes

Gel purified DNA fragments containing the full *inf1* cDNA insert from pFB7, or the *actA* gene from pSTA31 (Unkles et al., 1991) were used as probes and radiolabelled with α-^{32}P-dATP using a random primer labelling kit (Gibco-BRL, Bethesda, MD). In order to obtain a probe specific to the *inf1*

sequence, single stranded, radiolabelled probe complementary to the 3' end untranslated region of the *inf1* mRNA was generated by extending a single primer, INF2-F1, from the gel purified *inf1* insert from pFB7. The primer INF1-F1 (5'-CTATGAGTGGACTTGACTC-3') binds to the region surrounding the TGA stop codon (underlined) of the *inf1* open reading frame. The probe was generated by incubating in a final volume of 20 µl, 0.2 µg of primer INF2-F1, 10 ng of *inf1* cDNA template, 0.0166 mM each of dCTP, dGTP, and dTTP, 50 µCi of α-^{32}P-dATP, 1x Taq PCR buffer, and 3 units of Taq polymerase (as supplied by Perkin Elmer) in a Perkin Elmer 9600 thermocycler for 2 min at 95 °C followed by 25 cycles of 30 sec at 95 °C, 30 sec at 55 °C, and 30 sec at 72 °C.

Expression of *inf1* in *E. coli*

Expression of *inf1* in pFLAG-ATS and immuno-affinity purification of FLAG fusion proteins were conducted following the protocols provided by the manufacturer (IBI-Eastman Kodak, New Haven, CT). Overnight cultures of *E. coli* DH5α containing either pFB52 or pFB53 were diluted (1:100) in LB medium containing ampicillin (50 µg/ml) and incubated at 37 °C. When the OD_{600} of the cultures reached 0.6, IPTG was added to a final concentration of 0.4 mM. The cultures were further incubated for 3-4 hours before processing. Immuno-affinity purification was performed using a FLAG M2 antibody affinity gel. Elution of fusion proteins from the affinity column was obtained after treatment with 0.1 M glycine (pH 3.0). Proteins were then diluted in water and protein concentration determined by a Bradford assay (Bradford, 1976).

SDS-PAGE and Western Blot Analyses

Proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Sambrook et al., 1989; Schagger & von Jagow, 1987). Following electrophoresis, gels were silver stained following the method of Merril et al. (1981) or the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Corporation, Bedford, MA) using a Mini Trans-Blot apparatus (BioRad Laboratories, Richmond, CA). Detection of antigen-antibody complexes was carried out with a western blot alkaline phosphatase kit (BioRad Laboratories, Richmond, CA).

Plant Assays

Inoculation of potato with *P. infestans* in time course experiments was conducted by placing 10 µl water droplets containing approximately 1000 zoospores on the underside of detached potato leaves. The leaves were placed in petri dishes containing water agar (15 g/litre) to maintain high humidity. Late blight symptoms were scored daily, and leaf disks of similar sizes were dissected around the inoculated area and used for RNA extractions. Induction of hypersensitivity was determined by infiltration of elicitin solutions into attached leaves as described previously (Kamoun et al., 1993b). Virulence of *P. infestans* on the examined collection of solanaceous plants was determined in field tests, in growth chamber assays, and in detached leaf assays (Colon & Budding, 1988; Colon et al., 1992; 1995; Colon, 1994) (data not shown). Plants were routinely grown in greenhouses or in regulated growth chambers (16 hour photoperiod, 18 °C). The infiltrations described in Table 1 were conducted on plants cultivated in the growth chamber.

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Chapter 5

Internuclear Gene Silencing in *Phytophthora infestans*

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Summary

Transformation of the diploid, oomycete plant pathogen *Phytophthora infestans* with antisense, sense and promoter-less constructs of the coding sequence of the elicitin gene *inf1* resulted in transcriptional silencing of both the transgenes and the endogenous gene. Since heterokaryons obtained by somatic fusion of an *inf1*-silenced transgenic strain and a wild type strain displayed stable gene silencing, *inf1* silencing is dominant and acts in *trans*. *Inf1* remained silenced in non-transgenic homokaryotic progeny from the silenced heterokaryons, thereby demonstrating that the presence of transgenes is not essential for maintaining the silenced status of the endogenous *inf1* gene. These findings support a model reminiscent of paramutation and involving a *trans*-acting factor that is capable of transferring a silencing signal between nuclei.

Introduction

Introduction of transgenes into eukaryotic genomes often leads to silencing of expression of both the transgenes and the homologous host genes (Matzke & Matzke, 1995; Meyer & Saedler, 1996; Baulcombe & English, 1996, Pal-Bhadra et al., 1997). As a result, accumulation of specific mRNAs is affected. However, the molecular mechanisms that trigger this so-called homology-dependent gene silencing remain largely unknown.

Gene silencing can be regulated at the transcriptional or post-transcriptional level. Transcriptional silencing in plants and fungi is often found to be correlated with cytosine methylation of promoter sequences and/or coding sequences (Meyer & Saedler, 1996; Park et al., 1996; Schuurs et al., 1997; Selker, 1997). Also condensation of chromatin may play a role (Ye & Signer, 1996; van Blokland et al., 1997). In the case of co-suppression, where homology-dependent gene silencing is regulated at the post-transcriptional level, a high turnover of RNAs is thought to be responsible (Dehio & Schell, 1994; van Blokland et al., 1994; Dougherty & Parks, 1995; English et al., 1996; Metzlaff et al., 1997; Vaucheret et al., 1997). If the transgene is a viral gene, silencing can result in resistance to virus infection. It was proposed that synthesis of aberrant RNA molecules provokes specific degradation of all homologous RNAs in the cytoplasm. The aberrant RNAs may also function as templates for the synthesis of copy-RNA (cRNA) molecules produced by endogenous RNA-dependent RNA polymerases (Sijen et al., 1996; Metzlaff et al., 1997). It has been postulated that these cRNAs bind to the mRNAs and that the so formed double stranded RNAs (dsRNAs) will subsequently be degraded by double strand-specific RNases (Lindbo et al., 1993; Dougherty & Parks, 1995). Support for a model in which

dsRNA plays an essential role was recently found in *Caenorhabditis elegans*, where efficient silencing of a target gene was accomplished following injection of cells with dsRNA (Fire et al., 1998). This dsRNA interference appears to cross cellular boundaries and requires only a few molecules of dsRNA per affected cell.

Quelling, a transgene-induced gene silencing phenomenon found in the ascomycete fungus *Neurospora crassa*, resembles post-transcriptional gene silencing in plants (Cogoni & Macino, 1997b). Interestingly, quelling is shown to be a dominant trait in heterokaryotic strains containing a mixture of transgenic and non-transgenic nuclei (Cogoni et al., 1996). Production of aberrant RNA molecules caused by transcription of the transgene is thought to trigger degradation of homologous mRNAs derived from the transformed nucleus and the wild type nucleus (Cogoni & Macino, 1997a).

The non cell-autonomous gene silencing recently found in *C. elegans* (Fire et al., 1998) has previously been reported in plants (reviewed by Jorgensen et al., 1998). Palauqui et al. (1997) found silencing in scions of grafted plants consisting of a silenced stock and an initially non-silenced scion containing the transgene. Such systemic spread of co-suppression, or systemic acquired silencing, was also found by Voinnet & Baulcombe (1997) in transgenic plants producing green-fluorescent protein (GFP). After local infection by *Agrobacterium tumefaciens* carrying the GFP-reporter gene, the whole plant became GFP-silenced. These findings illustrate that a gene specific diffusible signal is capable of transmitting silencing.

In this study we describe a novel transcriptional gene silencing phenomenon in the diploid oomycete *Phytophthora infestans*, an important plant pathogen that is also known as 'the Irish potato famine fungus'. Despite the fact that *P. infestans* shows filamentous growth it is considered to be a protoctist eukaryotic organism and, as such, it is more related to golden-brown algae than to higher fungi. The mycelium of oomycetes is coenocytic and hence mycelial cells may contain multiple nuclei that can differ genetically resulting in heterokaryotic strains. In the present study we take full advantage of the unique features of *P. infestans* to address the following questions: (i) is gene silencing dominant in multinucleated cells, (ii) can the silenced state be transmitted from nucleus to nucleus, and (iii) can stable gene silencing of an endogene occur in a non-transformed nucleus?

In order to address these questions, the *inf1* gene of *P. infestans* was selected as a target gene. This gene encodes the secreted protein INF1, a member of the elicitin family (Kamoun et al., 1997a, 1997b). Elicitins induce defence responses in plants and recently we demonstrated that INF1 restricts the host-range of *Phytophthora infestans* (Kamoun et al., 1998). Since *inf1* is a single locus gene that is highly expressed during vegetative growth *in vitro* and of which the gene product INF1 is easily detectable, the *inf1* gene appeared ideal for unravelling the mechanism of gene silencing. The novel gene silencing phenomenon described here involves internuclear transfer of signals from transgenic silenced nuclei to wild type nuclei

leading to stable gene silencing in the wild type nuclei. Once gene silencing is induced in wild type nuclei it is maintained in progeny, even in the absence of nuclei carrying transgenes.

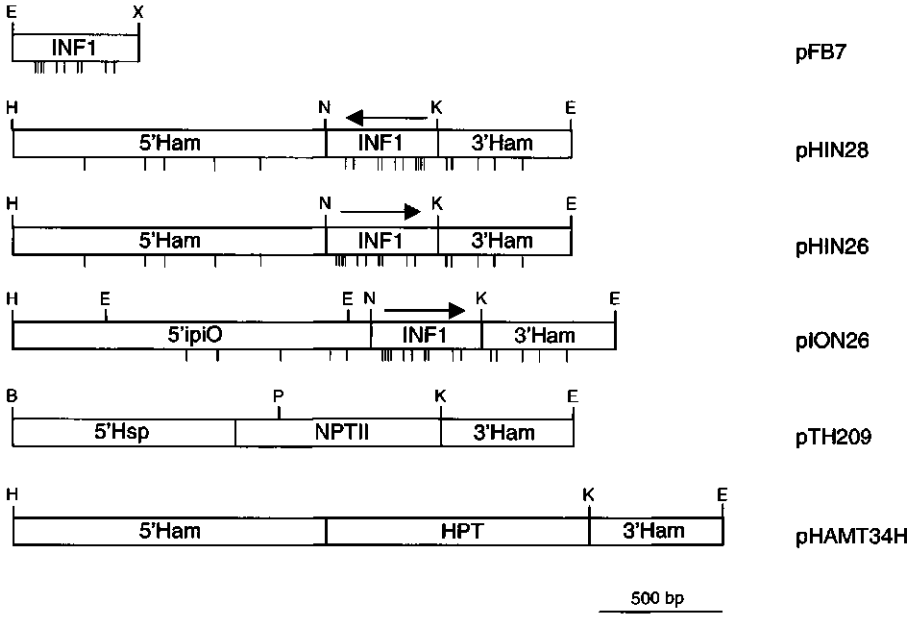

Figure 1. Transformation Constructs
pFB7 is an *inf1* cDNA clone. pHIN26 and pHIN28 contain the *inf1* coding sequence (INF1), either in sense orientation (→) or antisense orientation (←), fused to the promoter (5' Ham) and terminator (3' Ham) of the *Ham34* gene of *Bremia lactucae*. pION26 contains a transcriptional fusion of the coding sequence of *inf1* in sense orientation and the *in planta*-induced *ipiO1* promoter (5' ipiO) of *P. infestans*. pTH209 and pHAM34H contain coding sequences of the selectable antibiotic resistance genes neomycine phosphotransferase (NPTII) and hygromycine B (hyg.B) respectively, flanked by oomycete promoter and terminator sequences. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; P, *Pst*I; X, *Xho*I. Restriction sites checked for cytosine and adenosine methylation are indicated in the constructs containing *inf1* (I).

Results

INF1 Is Absent in Culture Filtrates of Antisense and Sense Transformants

To investigate whether we could engineer *P. infestans* mutants deficient in the production of the secreted protein INF1, strain 88069 was transformed with the geneticin resistance construct pTH209 and co-transformed with constructs containing *inf1* in either sense (pION26 or pHIN26) or antisense (pHIN28) orientation (Figure 1). It has been described that integration of multiple copies at the same locus enhances

the probability of silencing (Meyer, 1996). Therefore, we aimed at increasing the chance of tandem integrations of the transgenes by linearising the plasmid DNA with *Eco*RI (pHIN26 and pHIN28) or *Hin*dIII (pION26). Culture filtrates from 56 co-transformants were screened for the absence of INF1. Six out of thirty antisense transformants (PY23, PY31, PY37, PY47, PY53 and PY57) and three out of twenty-six sense transformants (OY1, OY8 and QY1) failed to produce INF1 or produced significantly reduced amounts of INF1 (Figure 2). Twenty control transformants containing only the geneticin resistance construct (Y10 and Y15 are shown in Figure 2), and 47 co-transformants (of which 10 are shown in Figure 2) produced INF1 in similar amounts as the wild type recipient strain 88069. Obviously introduction of *inf1* sense and antisense constructs can lead to deficiency in INF1 production in 11 to 20 % of the transformants.

Furthermore, we checked whether integration of a promoter-less construct also leads to silencing of *inf1*. Transformants were generated with one or more integrations of plasmid pFB7 containing a full length *inf1* cDNA sequence. Sixteen co-transformants were obtained and culture filtrates were screened for absence of INF1. Four transformants (SY2, SY6, SY21 and SY27) failed to produce INF1 or produced significantly lower amounts of INF1 (data not shown) demonstrating that promoter sequences in the *inf1* transgene construct are not required to induce gene silencing in *P. infestans*.

inf1 mRNA Is Not Detected in INF1-Deficient Transformants

To determine whether absence of secreted INF1 protein correlates with absence of *inf1* mRNA in the mycelium, total RNA was isolated from *in vitro*-grown strains and northern blot analyses were performed. High levels of *inf1* mRNA were detected in the recipient strain 88069, in the INF1-producing co-transformants, and in the control transformants (Y10 and Y15). In contrast, reduced levels of *inf1* mRNA were detected in the INF1-non-producing antisense, sense (Figure 2) and promoter-less transformants (data not shown). Indeed, endogenous *inf1* mRNA was only observed in a few transformants upon long exposure of the autoradiographs (data not shown). Hybridisation of the same blots with a single stranded antisense *inf1* probe, resulted in the same hybridisation pattern, whereas with a single stranded sense *inf1* probe, no antisense transcripts were detected in any of the tested co-transformants (data not shown). Hybridisation with a probe of the constitutively expressed actin gene resulted in signals with similar intensity in all lanes, indicating that each lane contained equal amounts of total RNA. We conclude that absence of the INF1 protein is caused by a deficiency in *inf1* mRNA. Apparently, introduction of *inf1* gene constructs has caused silencing of both the endogenous *inf1* gene and the *inf1* transgenes.

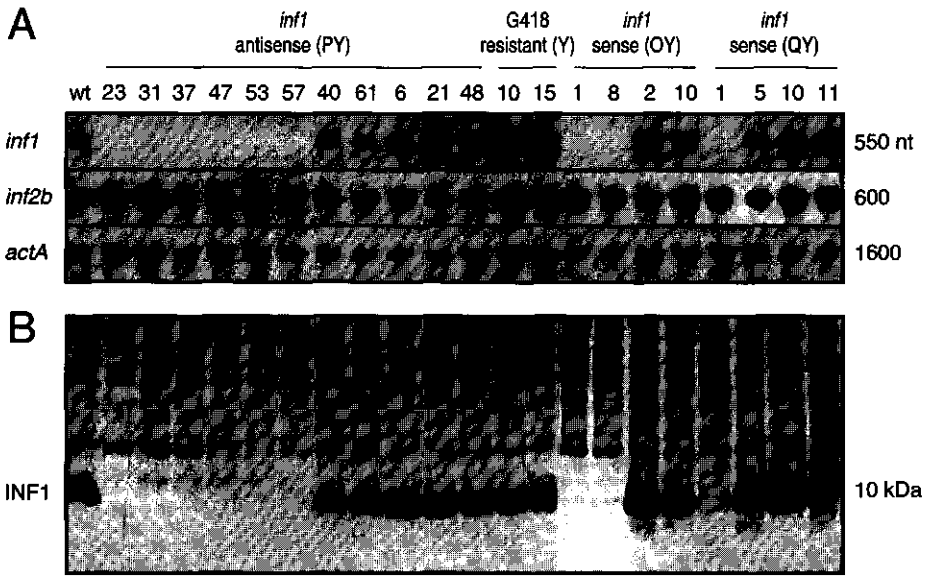

Figure 2. *P. infestans* Transformants Deficient in *inf1* mRNA and INF1 Protein Production
Analysis of *inf1* mRNA production (A) and INF1 protein production (B) in the wild type recipient strain (wt), in transgenic antisense (PY) and sense (OY and QY) transformants, and in G418 resistant non-co-transformed (Y) strains.
(A) Northern blots containing in each lane 15 µg total RNA isolated from mycelium grown *in vitro* for 10 days, were hybridised with probes derived from *inf1*, *inf2b* and the actin gene *actA*. Transcript lengths in nucleotides (nt) are indicated on the right.
(B) Proteins present in culture medium of 7-day-old cultures were separated by Tricine-SDS-PAGE and visualised by silver staining. The position of the 10 kDa INF1 protein is indicated (INF1).

To investigate whether the gene silencing observed in the *inf1* silenced transformants is gene sequence specific, expression of the related *inf2b* gene was analysed by hybridising the northern blot with an *inf2b* probe (Figure 2). The *inf2b* gene belongs to a separate class within the elicitin gene family, but shares 60% DNA homology to *inf1* over the homologous region of the open reading frames (Kamoun et al., 1997a). Equal levels of *inf2b* mRNA were detected in all tested transformants as well as in the wild type strain, suggesting that expression of the *inf2b* gene was not affected by the presence of the transgenic *inf1* gene sequences. Hence, we conclude that *inf1* gene silencing in *P. infestans* is gene sequence specific.

Silencing Is Not Due to Gene Disruption

To determine whether deficiency in *inf1* mRNA is associated with disruption or displacement of the endogenous *inf1* gene, genomic DNAs of the transformants were analysed on Southern blots. Hybridisation of *Bam*HI digested DNA with an *inf1* probe resulted in a 2.3 kb hybridising fragment in all tested transformants and in the recipient strain (Figure 3A). This fragment corresponds to the endogenous single locus *inf1* gene,

thereby demonstrating that in all transformants the endogenous *inf1* gene remained intact. Multiple hybridising fragments, representing integrations of the transgene constructs, were only observed in the co-transformants. Apparently, the observed *inf1* silencing is not based on inactivation due to disruption.

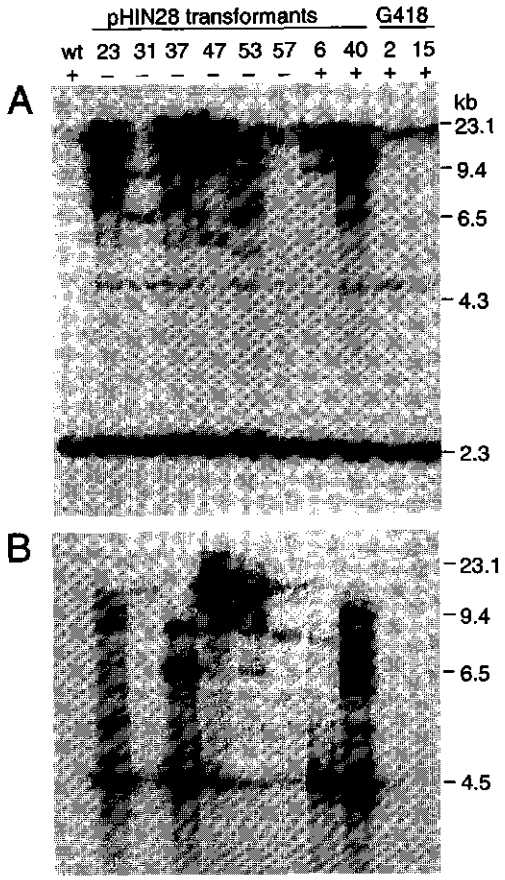

Figure 3. The Endogenous *inf1* Gene Is Not Disrupted in *inf1*-Silenced Transformants
Southern blot analysis of genomic DNA isolated from INF1 producing (+) and INF1-deficient strains (-). *Bam*HI digested (Figure 3A) and *Eco*RI digested (Figure 3B) DNA from the wild type recipient strain (wt), antisense transformants and G418 resistant non-co-transformed strains, was separated by electrophoresis and blotted onto a membrane. The blot in A was hybridised with a probe of the *inf1* gene. The 2.3 kb hybridising band represents the endogenous *inf1* single locus gene. The blot in B was hybridised with a probe from the *Ham34* promoter. The 4.5 kb hybridising band represents tandem integrations of the pHIN28 construct.

Southern blots containing genomic DNA digested with enzymes that recognise single restriction sites in the transgenic plasmid DNA, were hybridised with probes specific for the promoters of the transgene constructs (*Ham34* for pHIN26 and pHIN28, and *ipiO* for pION26). This resulted in a strong hybridising band of the size of the linearised

plasmids in almost all transformants (Figure 3B) indicating that tandem integration of the constructs had occurred. However, the strong hybridising band was not observed in the *inf1*-silenced transformants PY31, PY57 (Figure 3B) and OY8 (data not shown) suggesting that these strains contain only one or more single integrations of the construct. Therefore, *inf1* silencing is independent of the number of transgene integrations at a single site.

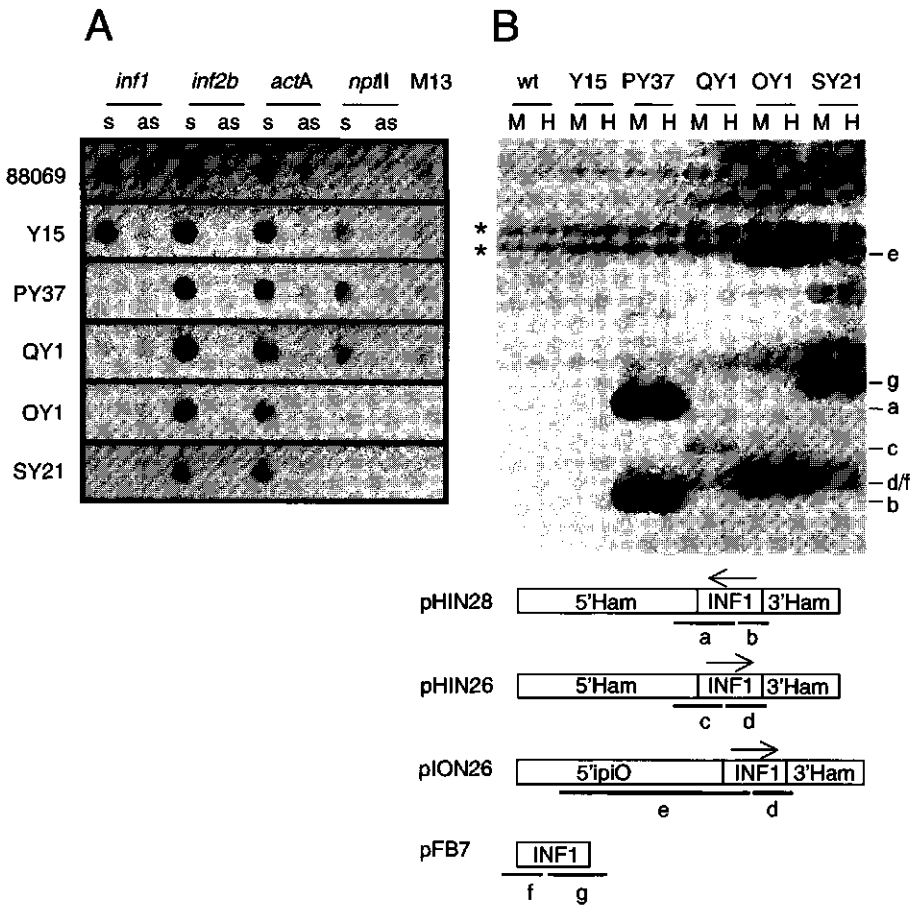

Figure 4. *Inf1*-Silencing Is Regulated at the Transcriptional Level and Not Due to Hypermethylation
(A) Transcriptional activity of *inf1* analysed by nuclear run-on assays.
Autoradiographs of dot-blot filters, containing per dot 1 µg of gene-specific single-stranded DNAs hybridised to ^{32}P-labeled nascent RNA synthesised *in vitro* in nuclei isolated from 5-day-old mycelia of wild type strain 88069, non-co-transformant Y15, antisense transformant PY37, sense transformants QY1 and OY1, and promoter-less transformant SY21. The gene specific single stranded DNAs synthesised from M13-based recombinant phages hybridise to sense (s) or antisense (as) RNAs derived from *inf1*, *inf2b*, *actA*, and *nptII*. The filters contain M13 DNA as a control.
(B) Analysis of cytosine methylation at the *inf1* locus.
Southern blot analysis of genomic DNA isolated from INF1-producing and INF1-deficient strains. DNA

from the wild type recipient strain 88069 (wt), a non-co-transformed strain (Y15), an antisense transformant (PY37), two sense transformants (QY1 and OY1), and a promoter-less transformant (SY21), was digested with the isoschizomeric restriction enzymes *Msp*I (M) and *Hpa*II (H), separated by electrophoresis, blotted and hybridised with a *inf1* probe. *Hpa*II does not digest when a internal C residue in the recognition site CCGG is methylated. Endogenous *inf1* DNA fragments are indicated with an asterisk (*). The strong hybridising bands a-g correspond to DNA fragments indicated by a-g in the transformation constructs shown at the bottom.

Silencing Is Not Based on High Turnover of *inf1* mRNA

Absence of *inf1* mRNA in the silenced transformants, may result from either reduced transcription of *inf1* or from reduced mRNA stability. To distinguish between these two possibilities, nuclear run-on assays were performed. Nuclei of mycelia of wild type strain 88069, control transformant Y15, antisense transformant PY37, sense transformants QY1 and OY1, and a promoter-less transformant SY21, were isolated. The transcripts present in the nuclei were extended and ^{32}P-UTP-labeled. Filters, containing single-stranded DNA fragments of *inf1*, *inf2b*, the actin gene *actA*, and the neomycin phosphotransferase gene *nptII* in antisense and sense orientations, were hybridised with the nuclear transcripts. As shown in Figure 4A, the control transformant Y15 produced similar steady state levels of nuclear *inf1* RNA as wild type strain 88069, whereas the silenced strains contained no or very low levels of nuclear *inf1* RNA. Quantification of the hybridisation signals showed that the silenced transformant PY37 does not contain sense *inf1* mRNA whereas QY1 contains very few *inf1* primary transcripts, only up to 5% compared to the levels in 88069 and Y15. QY1 also showed small amounts of INF1 protein on silver-stained gels (Figure 2). *Inf1* antisense RNA could not be detected in any of the silenced transformants. *Inf2b* and actin mRNA levels were similar in all strains tested and antisense RNAs were absent. These results demonstrate that *inf1* silencing in *P. infestans* is not based on a high turn-over of *inf1* mRNA. Instead, inhibition of transcription is more likely.

Silencing Is Not Correlated with Hypermethylation of DNA

To investigate a possible role of DNA methylation in transcriptional silencing of *inf1*, genomic DNAs isolated from the wild type strain, a control transformant (Y15) and four silenced transformants (QY1, OY1, PY37 and SY21), were digested with restriction enzymes suitable to detect cytosine or adenosine methylation and analysed by Southern blot hybridisation with *inf1* probes. The following isoschizomeric restriction enzyme pairs were used: *Hpa*II / *Msp*I (recognise CCGG) *Sau*3A / *Mbo*I (recognise GATC) and *Dpn*I / *Mbo*I (recognise GATC). *Hpa*II and *Sau*3A are sensitive to cytosine methylation and *Mbo*I is sensitive to adenosine methylation. Also restriction enzymes *Alu*I (recognises AGCT), *Hha*I (recognises GCGC), both cytosine methylation sensitive, and adenosine methylation sensitive *Rsa*I (recognises GTAC), were used. Methylation sites that were tested in *inf1* and in the transformation constructs are shown in Figure 1. By comparing the hybridisation patterns of the *inf1*-silenced strains with the wild type and Y15 strains, no shifts in hybridising bands representing the endogenous *inf1* gene were noted, suggesting

absence of cytosine and adenosine hyper-methylation at the sites tested (Figure 4B and data not shown). Similarly, the sizes of the hybridising bands representing the *inf1* transgenes did not change, indicating that also at the transgenes methylation is absent. From these data we conclude that hyper-methylation of the endogenous *inf1* gene sequence is not involved in the silencing mechanism.

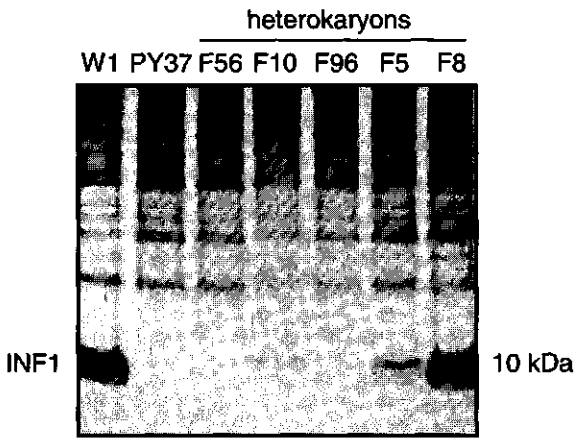

Figure 5. INF1 Deficiency in Heterokaryons
Analysis for the presence or absence of INF1 protein in culture filtrate of heterokaryons (F3, F5, F8, F10, F56, and F96) obtained by fusion of a hygromycin B resistant INF1-producing strain (W1) and a G418 resistant *inf1*-silenced transformant (PY37). The heterokaryons are G418 and hyg.B resistant. Proteins present in culture medium of 7-day-old cultures were separated by Tricine-SDS-PAGE and visualised by silver staining. The position of the 10 kDa INF1 protein is indicated (INF1).

Gene Silencing in Heterokaryons of *P. infestans*

To determine whether gene silencing is dominant in heterokaryotic strains, forced heterokaryons of *inf1*-silenced transformants and a non-silenced wild type strain were generated by protoplast fusion. Protoplasts of geneticin (G418) resistant, *inf1*-silenced transformants QY1, PY23 or PY37 were fused with protoplasts of W1, a hygromycin B (hyg.B) resistant transformant. W1 is a derivative of the wild type strain 88069 obtained after transformation with pHAMT34H, a vector containing the hygromycin phosphotransferase gene (*hpt*) (Judelson et al., 1991). W1 produces normal levels of INF1 protein (Figure 5). In total, 100 heterokaryotic somatic fusion products of PY37 and W1 were selected and analysed for INF1 production. Seven heterokaryotic isolates failed to produce INF1 whereas thirty-four showed reduced levels of INF1 protein, when compared to the levels produced by the wild type strain or W1. F56 and F96 are examples of heterokaryons that fail to produce INF1 (Figure 5). Silencing in the heterokaryons remained stable during vegetative growth on medium containing both G418 and hyg.B for at least nine months (data not shown). Similar results were obtained upon fusion of PY23 with W1, and QY1 with W1 (data not shown). In all cases the

heterokaryons represented the whole range of INF1 phenotypes: some produced no INF1 at all, whereas others produced reduced or similar amounts as the wild type. These results demonstrate that *inf1* gene silencing in heterokaryotic strains can be dominant and act *in trans*.

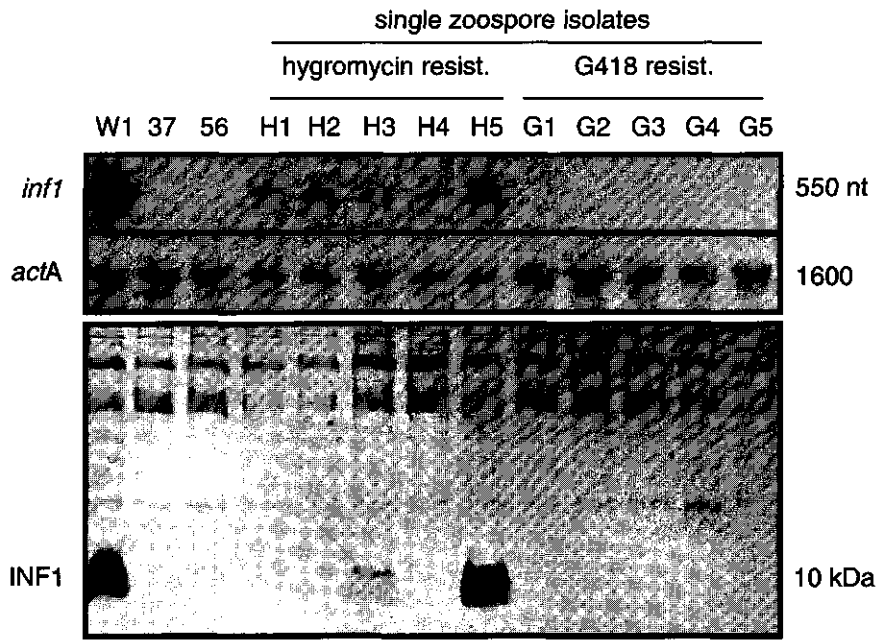

Figure 6. *Inf1* Silencing in Homokaryotic Progeny from a Silenced Heterokaryon
Analysis of *inf1* mRNA production (upper panel) and INF1 protein production (lower panel) in homokaryotic single zoospore isolates (H1-H5, G1-G5) derived from the INF1-deficient heterokaryon F56. F56 was obtained by fusion of a hyg.B resistant INF1-producing strain (W1) and a G418 resistant *inf1*-silenced transformant (PY37). F56 is G418 and hygromycin B resistant whereas G1-G5 are G418 resistant, hyg.B sensitive and H1-H5 are G418 sensitive, hyg.B resistant.
(Upper panel) Northern blots containing in each lane 15 µg total RNA isolated from mycelium grown *in vitro* for 10 days, were hybridised with probes derived from *inf1* and *actA*. Transcript lengths in nucleotides (nt) are indicated on the right.
(Lower panel) Proteins present in culture medium of 7-day-old cultures were separated by Tricine-SDS-PAGE and visualised by silver staining. The position of the 10 kDa INF1 protein is indicated (INF1).

Internuclear Transfer of the Silenced State

To investigate whether the silenced state can be transmitted from nucleus to nucleus, single unicellular and uninucleate zoospore cultures were obtained from the silenced heterokaryons. Zoospores from heterokaryons F56 and F96 (fully silenced), from heterokaryon F10 (partly silenced), and from the parental strains PY37 and W1 were plated onto medium without antibiotics and on medium containing either G418, either hyg.B, or both G418 and hyg.B. No colonies resistant to both antibiotics were

recovered, suggesting that karyogamy had not occurred in these heterokaryons. In all cases, equal numbers of colonies grew on plates containing hyg.B and on plates containing G418. In general, the total number of colonies growing on these plates, matched the total number of colonies growing on plates without antibiotics, indicating a nuclear distribution of 1:1 of the two nuclear types in the heterokaryotic mycelia.

Subsequently, the homokaryotic strains derived from the heterokaryons F56, F96, and F10 were screened for INF1 protein production and *inf1* gene expression. All *inf1* transgenic (G418-resistant) homokaryotic strains derived from the silenced heterokaryotic strains F56, F96, and F10, remained fully silenced (homokaryons G1-5 derived from F56 are shown in Figure 6). In the G418-resistant single zoospore isolates G1-5, the *inf1* mRNA levels were less than 0.1% of the wild type level, which is comparable to the reduction found in the heterokaryotic strain F56 and the parental strain PY37. Surprisingly, silencing was also observed in all homokaryotic isolates resistant to hyg.B (homokaryons H1-5 derived from F56 are shown in Figure 6). In these single zoospore isolates *inf1* mRNA levels were reduced varying from 3% (H1) to 18% (H5) of the wild type level and little or no INF1 protein was found.

In order to confirm that karyogamy did not occur in the heterokaryons, *Bam*HI digested genomic DNA of the parental strains W1 and PY37, heterokaryotic strain F56 and its derived homokaryotic single zoospore isolates (H1-5 and G1-5) was hybridised to a probe of the *hpt* gene (Figure 7B). One hybridising band was observed in the hyg.B resistant homokaryotic single zoospore strains and in F56 and W1, but not in the silenced parental strain PY37 or the G418-resistant homokaryotic single zoospore isolates (G1-5). Hybridisation with a probe derived from the *nptII* gene revealed hybridising bands in G1-5, F56 and PY37, but not in the parental strain W1 or the single zoospore isolates H1-5 (Figure 7C). The endogenous *inf1* gene visualised by hybridisation with the *inf1* probe was detected in all strains, whereas transgenic *inf1* sequences could only be found in the G418-resistant strains (Figure 7A). Southern blot analysis of the single zoospore homokaryotic strains derived from F10 and F96 gave similar results (data not shown). Apparently, karyogamy did not occur in the heterokaryotic strains tested.

To investigate whether mutations in the DNA sequence of the endogenous *inf1* gene are responsible for loss of INF1 production in the silenced strains, the non-transgenic *inf1*-silenced single zoospore isolates resistant to hyg.B (H1-5), appeared to be ideal. In those strains the endogenous *inf1* gene can be amplified by PCR from genomic DNA without interference of transgenic *inf1* sequences. Sequence analysis of PCR fragments corresponding to endogenous *inf1* derived from four silenced homokaryotic strains (H1, 3, 4 and 5) did not reveal any consistent base pair mutation (data not shown).

These results demonstrate that *inf1* in wild type nuclei remains silenced in the absence of transgenic *inf1* sequences, indicating that internuclear transfer of the silenced state must have occurred.

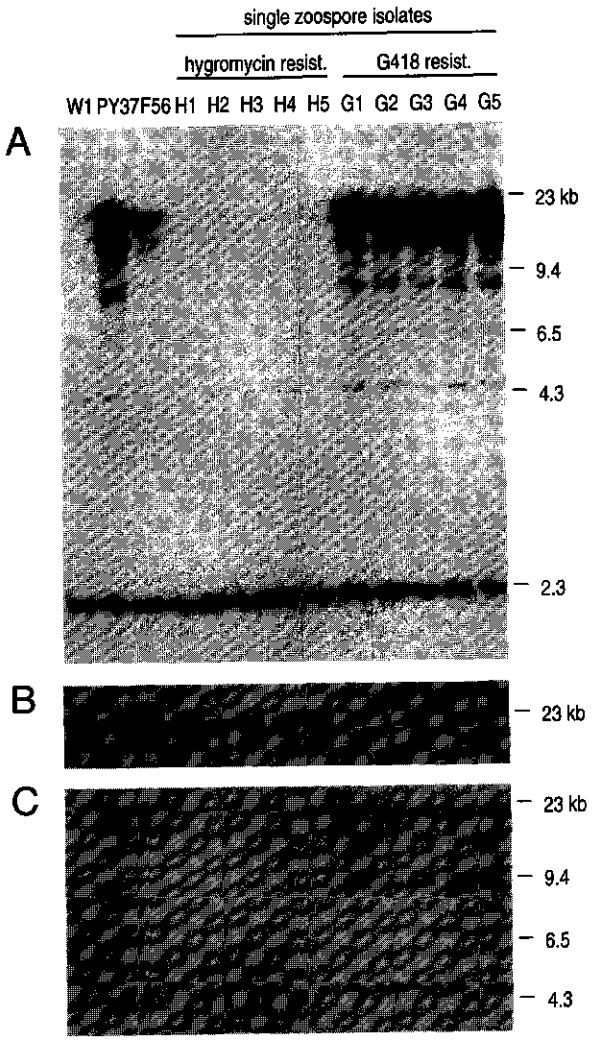

Figure 7. *Inf1* Silencing in the Absence of *inf1* Transgenes
Southern blot analysis of genomic DNA isolated from homokaryotic hyg.B resistant (H1-5), and G418 resistant (G1-5) progeny from the INF1-deficient heterokaryon F56. F56 was obtained by somatic fusion of INF1-producing strain W1 and *inf1*-silenced transformant PY37. *Bam*HI digested DNA was separated by electrophoresis, blotted onto a membrane, and hybridised with a ^{32}P-labeled probe of (A) *inf1*, (B) the hyg.B resistance gene *hpt*, and (C) the G418 resistance gene *nptII*.
(A) The 2.3 kb hybridising fragment, present in all lanes, represents the endogenous *inf1* single locus gene. Other hybridising fragments, visible in lanes containing genomic DNA of PY37, F56, and G1-5, represent *inf1* transgenes.
(B) The 23 kb hybridising fragment contains the transgenic *hpt* gene(s) present in the hyg.B resistant strains W1 and F56, and the homokaryons H1-H5.
(C) Hybridising fragments contain transgenic *nptII* sequences present in the G418 resistant strains PY37 and F56, and the homokaryons G1-G5.

Discussion

Here we describe a novel gene silencing phenomenon in the oomycete *Phytophthora infestans*. Stable silencing of an endogenous target gene was achieved following transformation with antisense, sense and promoter-less gene constructs. Efficient silencing was also manifested in heterokaryotic mycelia obtained by protoplast fusion of a transgenic silenced strain and a non-silenced strain, suggesting the involvement of a *trans*-acting silencing signal. Furthermore, we discovered that the presence of nuclear transgenic sequences is not essential to retain silencing of an endogenous gene: homokaryotic strains, obtained from uninuclear spores from silenced heterokaryotic strains, maintained the silenced phenotype even in the absence of transgenes.

Inf1 Silencing Is Gene Sequence Specific

The introduction of various DNA constructs of the *inf1* target gene into the *P. infestans* genome resulted in a complete suppression of INF1 protein production in up to 20% of the co-transformants and this suppression is the result of absence of *inf1* mRNA. Similar to what has been found in other systems (Matzke & Matzke, 1995), this gene silencing in *P. infestans* seems to be gene sequence specific. In the silenced transformants only accumulation of *inf1* mRNA is affected, but not the mRNA levels of a related *inf2b* elicitin gene.

Since only a portion of the co-transformants (up to 20%) shows silencing, introduction of *inf1* transgenes in itself does not seem to be the trigger for gene silencing nor is tandem integration of transgenes. While in most silenced transformants the transgene was integrated in tandem repeats with high copy numbers, we obtained several silenced transformants with single integrations. In all silenced transformants the endogenous *inf1* gene is still intact and sequence analyses of the endogenous *inf1* coding sequence in silenced homokaryotic strains revealed no specific (point) mutations, demonstrating that the observed gene silencing is not the result of mutations or gene disruptions.

Transcriptional *Inf1* Silencing Is Not Due to *de Novo* methylation

Nuclear run-on assays demonstrated that *inf1* silencing occurs at the transcriptional level. A feature, often found to be associated with transcriptional silencing in plants as well as in filamentous fungi, is cytosine methylation of repeated sequences (Selker, 1990; Rossignol & Faugeron, 1994; Meyer, 1996; Schuurs et al., 1997). This DNA methylation can either be the cause or the consequence of gene inactivation. Here we show that the endogenous and transgenic *inf1* sequences are not hyper-methylated and conclude that methylation can not be responsible for the *inf1* silencing.

Internuclear Gene Silencing

A major advantage of using a fungus or a fungus-like organism for dissecting gene silencing mechanisms is that individual nuclei can easily be separated and multiplied by regenerating mycelium from single vegetative spores. Subsequently, fusion of protoplasts derived from different homokaryotic strains will result in stable heterokaryotic strains carrying nuclei with different characteristics. Here we report efficient gene silencing in heterokaryons of *P. infestans* containing *inf1* transgenic and non-transgenic nuclei, thereby demonstrating that silencing is dominant and acts *in trans* to silence the target gene in both transformed and untransformed nuclei. Moreover, *inf1* silencing is stably maintained in homokaryotic strains obtained following nuclear separation of the silenced heterokaryons, even in the absence of *inf1* transgenes. Since karyogamy could not be demonstrated it is unlikely that the silenced state of the *inf1* gene is transmitted from one nucleus to the other by specific DNA-DNA interactions. Also transitory interactions between DNA of *inf1* transgenic and non-transgenic nuclei during simultaneous mitotic divisions seem unlikely, since the nuclear envelope and nuclear matrix remain fully intact throughout mitosis in most fungal and oomycete species (Heath, 1980). We propose a novel silencing phenomenon in which a diffusible silencing factor is involved in inducing stable gene silencing, and we call this phenomenon internuclear gene silencing.

Even though a silencing phenomenon seemingly similar to what we observed in heterokaryons of *P. infestans* was found in the ascomycete fungus *Neurospora crassa*, the mechanisms are not the same. While analysing the mechanism of quelling in *N. crassa,* Cogoni et al. (1996) found that fusion of a wild type orange coloured strain and an *al-1* silenced strain with the 'albino' phenotype, resulted in 'albino' heterokaryons. However, after nuclear separation of the quelled heterokaryons, the recovered homokaryotic strains containing only wild type nuclei were orange again and *al-1* was not silenced anymore. This demonstrated that, in contrast to *P. infestans,* the silenced state in *N. crassa* is not heritably transmitted from nucleus to nucleus. The presence of transgenes in quelled strains of *N. crassa* seems to be a prerequisite for maintenance of the silenced state. Furthermore, quelling is regulated at the post-transcriptional level and, therefore, quelling in *N. crassa* and internuclear gene silencing in *P. infestans* must be based on different mechanisms.

Internuclear silencing in *P. infestans* is also clearly different from yet another silencing phenomenon observed in ascomycetes and that is MIP (Methylation Induced Premeiotically), a process extensively studied in *Ascobolus immersus* (Colot et al., 1996). MIP involves transfer of DNA methylation between homologous alleles, is most likely based on DNA-DNA interaction and takes place during a particular stage in the sexual cycle when the haploid nuclei are in a common cytoplasm. In contrast to MIP, internuclear gene silencing involves neither methylation nor DNA-DNA contact and is an event occurring in diploid nuclei during asexual stages of the life cycle.

Is Internuclear Gene Silencing in *P. infestans* Related to Paramutation?

Interestingly, the internuclear silencing observed in *P. infestans* is a *trans*-inactivation phenomenon that shares similarities with paramutation. Paramutation is an epigenetic phenomenon involving either allelic interactions or interactions between homologous unlinked loci, e.g. endogenes and transgenes, and resulting in persistent changes in expression even after the interacting alleles or genes segregate in the progeny (Meyer et al., 1993; Hollick et al., 1997). Alleles that are sensitive to paramutation are termed 'paramutable' and alleles that incite paramutation are termed 'paramutagenic'. Examples of paramutation are so far limited to plant genes, but there are indications that paramutation occurs in a wide variety of biological systems (Hollick et al., 1997).

We speculate that in the *inf1*-silenced *P. infestans* heterokaryons, the duplicated *inf1* gene sequences in the transgenic nucleus, represent the paramutagenic loci (or silencer loci), and that the endogenous *inf1* gene loci in the transgenic nuclei and in the wild type nuclei represent the paramutable loci. These endogenous *inf1* gene loci have become *trans*-inactivated and converted into paramutant, *inf1*-silenced loci. Following separation of the transgenic and wild type nuclei, the paramutant *inf1* loci in the homokaryotic single zoospore isolates containing a wild type nucleus retain the reduced level of expression. Among the heterokaryons and homokaryons we found variation in INF1 protein production ranging from 82 to 97% reduction. Similarly, variation in expression of paramutant loci has been found in plants (Meyer & Saedler, 1996).

The most extensively studied examples of paramutation concern plant genes that determine flower or seed colour, two phenotypes that, when mutated, are easily recognised in sexual progeny of the primary mutants (reviewed by Hollick et al., 1997). In general, paramutation is defined as an epigenetic change that is meiotically inheritable. Nevertheless, the paramutation itself takes place in somatic cells and besides being meiotically stable, the paramutant loci are also stable during mitosis. Silencing of *inf1* in *P. infestans* is incited in somatic cells by introduction of homologous transgenes. The presumed *inf1* paramutation in non-transgenic nuclei also occurs in somatic cells. As demonstrated, the paramutant *inf1* loci are stable during mitosis and the silenced phenotype is maintained after the interacting genes are inherited separately in asexual progeny. Whether the epigenetic change at the *inf1* locus is inherited in sexual progeny and is meiotically stable remains to be determined.

The molecular mechanisms underlying paramutation are not clear. In fact, every example of paramutation is different and each example might reflect a distinct mechanism (Hollick et al., 1997). For instance some cases seem to be associated with DNA methylation while others are not. One intriguing aspect of the mechanism is the basis of the allelic interaction. Our findings imply that the *inf1*-silenced state is transmitted *in trans* without direct DNA-DNA contact between the paramutagenic *inf1*

locus and the paramutable loci. In the heterokaryons karyogamy could not be demonstrated and in coenocytic mycelium where the nuclear envelope remains intact during mitosis (Heath, 1980), pairing of chromosomes present in different nuclei seems very unlikely. Also in maize, where paramutation occurs at several loci, there is no evidence for chromosomal pairing in somatic cells (Heslop-Harrison & Bennett, 1990). It has been postulated that protein factors produced by transposable elements mediate *trans*-interactions that cause heritable changes in gene activity in genes located in the vicinity of transposable elements (Martienssen, 1996; Matzke et al., 1996; Hollick et al., 1997). In such a model physical contact between paramutagenic and paramutable loci is not required. Instead, a *trans*-acting factor mediates silencing from one locus to the other. The heritable change that influences neighbouring gene activity could well be a conformational change in chromatin structure.

We conclude that the strongly reduced transcription in the homokaryotic non-transgenic *P. infestans* strains, has to be the consequence of paramutation causing an inheritable change in *inf1* expression possibly brought about by changes in chromatin structure.

What is the *trans*-acting Silencing Factor?

If indeed changes in chromatin structure bring about the heritable change in *inf1* expression, the proposed *trans*-acting silencing factor that moves from nucleus to nucleus might be a protein. In *Drosophila* and yeast it has been shown that changes in chromatin structure and histone modifications are responsible for the silenced state of several genes and these processes involve many different proteins that constitute chromatin silencing factors (reviewed by Pirrotta, 1997; 1998; Sherman & Pillus, 1997). Examples are the Polycomb group (PcG) proteins that interact with several genes in *Drosophila* as well as in vertebrates (Pirrotta, 1998), and the Sir1p-4p proteins from yeast that establish and maintain silencing at the silent mating type loci and at the telomeres. Sir2p is known to affect levels of histone deacetylation while Sir3p and Sir4p are limiting structural components important for remodelling and establishing silenced chromatin (Sherman & Pillus, 1997).

However, to account for the sequence specificity of the internuclear gene silencing, the *trans*-acting silencing factor must be able to recognise the paramutable *inf1* alleles. It is feasible that one of the components of the *trans*-acting silencing factor is *inf1* RNA by which the target gene is recognised. Indeed, interactions between RNA and genomic DNA, combined with propagation of changes along the chromatin, have been suggested to play a role in transcriptional gene silencing, not only in plants (Wassenegger et al., 1994; Matzke & Matzke, 1995) and fungi (Schuurs et al., 1997) but also in mammalian X-chromosome inactivation (Latham, 1996) and in gene inactivation in *C. elegans* (Fire et al., 1998).

Based on our results and in conjunction with the gene silencing concepts described above, we speculate that the proposed diffusible *trans*-acting silencing

factor in *P. infestans* is either a protein, an aberrant RNA molecule or a complex consisting of RNA and protein. At first, RNA-mediated interference at the level of chromatin structure or transcription seems unlikely, since in the run-on assays no (aberrant) *inf1* transcripts have been detected. However, the proposed silencing factor does not need to be produced in large amounts. Even very low quantities could be effective, since the molecule would act at the DNA level (Fire et al., 1998). Moreover, if the silencing factor is a small RNA molecule it can be easily transported from nucleus to nucleus to facilitate changes in DNA structure of the target gene in non-transgenic nuclei in heterokaryotic strains. However, as long as the *trans*-acting silencing factor is not characterised, its exact nature remains a matter of speculation.

Experimental Procedures

Phytophthora Strains and Culture Conditions

P. infestans strain 88069 was used in all transformation experiments. Cultures were routinely grown in the dark at 18 °C on rye agar medium supplemented with 2% (w/v) sucrose (RS-medium) as described before (van West et al., 1998). Mycelium for isolation of DNA and RNA was obtained by growing cultures of *P. infestans* in liquid RS-medium or modified Plich medium (containing per litre 0.5 g KH_2PO_4, 0.25 g $MgSO_4$, 1.0 g asparagine, 1.0 mg thiamine, 0.5 g yeast extract, 10 mg β-sitosterol and 25 g glucose).

Plasmid Constructions and Transformation of *P. infestans*

Plasmid pFB7, containing a 557 bp *inf1* cDNA, was used as a promoter-less transformation construct (Kamoun et al., 1997b). Plasmids pION26 and pHIN26, which contain the coding region of *inf1* fused to the *ipiO1* promoter (Pieterse et al., 1994) or the *Ham34* promoter (Judelson et al., 1991), respectively, were constructed as follows. A 354 bp fragment of the complete *inf1* coding sequence was generated by polymerase chain reaction (PCR) using oligonucleotides PIET25 (5'-CCGATATCCATGGACTTTCGTGCT-CTGTTCGC-3'), and PIET26 (5'-GGCCCCGGGTACCTCATAGCGACGCACACGT-AG-3') and plasmid pFB7. Following digestion, the amplified fragment was inserted in *Nco*I and *Kpn*I digested pPIN13 (van West et al., 1998) or pHAMT35G (Judelson et al., 1991), which resulted in pION26 and pHIN26, respectively. Plasmid pHIN28, was obtained as described by Kamoun et al. (1998). Stable *P. infestans* transformation was conducted according to van West et al. (1998) using linearised plasmid DNA.

M13 recombinant phages used for generation of single stranded DNA probes for the detection of sense and antisense *inf1*, *inf2b*, *actA*, and *nptII* RNA were constructed using standard techniques. Basically, full-length coding sequences of *inf1*, *inf2b*, *actA*, and *nptII* were amplified using complementary PCR primers extended with appropriate restriction sites. The amplified fragments were inserted in pGEM-T (Promega) and recloned in M13mp18 RF and M13mp19 RF (Gibco BRL).

Protoplast Fusion

Protoplasts from two parental transformants either resistant to hygromycin B (hyg.B) or resistant to geneticin (G418) were obtained and regenerated as described by van West et al. (1998). The parental protoplast suspensions were diluted to an equal concentration (10^7-10^8 protoplasts per ml). To 1 ml of the protoplast mix, 1 ml of 50% PEG 3350 (Sigma) in 20 mM $CaCl_2$ and 10 mM Tris/HCl, pH 7.5 was added very slowly. Selection of heterokaryons was performed on RS-medium containing 10 µg/ml G418 and 50 µg/ml hyg.B. Heterokaryons able to grow under these conditions, were further subcultured on RS-medium containing 5 µg/ml G418 and 25 µg/ml hyg.B. Uninuclear zoospores were collected as described by van West et al. (1998). To obtain homokaryotic isolates 100 µl of a diluted zoospore suspension ($1x10^2$ per ml) was gently spread on RS-medium containing 5 µg/ml G418 or 25 µg hyg.B. Colonies appeared within 4-10 days and were propagated on RS-medium containing the appropriate antibiotic.

Southern and Northern Blot Analysis

Genomic DNA of *P. infestans* was isolated from mycelium as described by Raeder & Broda (1985) with minor modifications. Genomic DNA for methylation studies was digested with a 5-10 times excess of restriction enzyme. Total RNA from *P. infestans* was isolated, blotted and hybridised as previously described (van West et al., 1998).

DNA templates for probe synthesis were a 354 bp *NcoI-KpnI* fragment and a 920 bp *HindIII-NcoI* fragment of pHIN26 containing the *inf1* coding sequence and the *Ham34* promoter sequence respectively, a 796 bp *HindIII* fragment from pSTA31 containing the *actA* coding sequence, and a 343 bp PCR fragment containing the *inf2b* coding sequence. Probes were radiolabelled with [α-^{32}P]dATP by using a Random Primers DNA Labelling System (Gibco BRL).

Protein Analysis

Protein concentrations in culture filtrates of *P. infestans* isolates were determined by the Bradford method with bovine serum albumin as a standard (Bradford, 1976). Equal amounts of protein were electrophoresed on Tricine sodium dodecyl sulphate-polyacrylamide gels and the separated proteins were silver-stained as described before (Kamoun et al., 1998).

Nuclei Isolation and Nuclear Run-ons

Suspensions of 2.10^5 sporangia per ml were mixed with two times concentrated ALBA medium (van West et al. 1998). After growth for 5 days, mycelial pellets were harvested by filtration, and frozen in liquid nitrogen. From approximately 3 g of frozen mycelia sufficient nuclei can be obtained to perform a nuclear run-on assay. The isolation of nuclei and the run-on assays were conducted as described by van Blokland et al. (1994). Aliquots of labelled nuclear RNA were taken for scintillation counting to determine the incorporation of labelled nucleotides, which varied from $1x10^6$ to $2.2x10^6$ c.p.m. per batch of nuclei.

One µg of the single stranded DNAs prepared from the M13 recombinant phages (diluted in 200 µl of 10 x SSC) were applied to Hybond N^+ membranes (Amersham) using a dot blot apparatus (Biorad). The membranes were hybridised with the radioactive labelled nuclear run-on RNA ($1x10^6$ c.p.m.) as described by van Blokland et al. (1994).

Transcription levels of the endogenous *inf1* gene were quantified by determining the amount of radioactive labelled RNA hybridising to the membranes with a Fujix Bio-imaging analyser (BAS 2000). To normalise for incorporation differences between separate run-ons we determined the ratio of the signals of the *inf1* sense probe to that of the *actA* sense probe and used that as internal control. The percentage of *inf1* transcripts in the silenced transformants was determined by dividing the *inf1* to *actA* signal ratios of the *inf1*-silenced transformants by the average *inf1* to *actA* signal ratio of 88069 and Y15, multiplying this by 100% and subtracting M13 background hybridisation (5%).

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Chapter 6

Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is Mediated by the Recognition of the Elicitor Protein INF1

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Summary

***Phytophthora infestans*, the agent of potato and tomato late blight disease, produces a 10 kDa extracellular protein, INF1 elicitin. INF1 induces a hypersensitive response in a restricted number of plants, particularly those of the genus *Nicotiana*. In virulence assays with different *P. infestans* isolates, five *Nicotiana* species displayed resistant responses. In all of the interactions, after inoculation with *P. infestans* zoospores, penetration of an epidermal cell was observed, followed by localised necrosis typical of a hypersensitive response. To determine whether INF1 functions as an avirulence factor in these interactions, we adopted a gene silencing strategy to inhibit INF1 production. Several transformants deficient in *inf1* mRNA and INF1 protein were obtained. These strains remained pathogenic on host plants. However, in contrast to the wild type and control transformant strains, INF1-deficient strains induced disease lesions when inoculated on *Nicotiana benthamiana*. These results demonstrate that the elicitin INF1 functions as an avirulence factor in the interaction between *N. benthamiana* and *P. infestans*.**

Introduction

Microbial plant pathogens often exhibit high degrees of specialisation and can only infect a limited number of plant species (Agrios, 1988). Pathogen specialisation results when a complex set of preformed and induced mechanisms is put into motion to defend a plant against invading pathogens. In some interactions, preformed physical barriers and antimicrobial compounds in the plant help to ward off pathogens (Osbourn, 1996a; 1996b). In other interactions, perception by the plant of signal molecules, namely elicitors, produced by the avirulent pathogen leads to the induction of effective defence responses, including a programmed cell death response termed hypersensitive response (HR) (Lamb et al., 1989; Dixon & Harrison, 1990; Ebel & Scheel, 1992; Baker et al., 1997; Morel & Dangl, 1997). This model has been genetically defined by Flor's gene-for-gene hypothesis (Flor, 1956; 1971). According to this hypothesis, a resistance reaction is determined by the simultaneous expression of a pathogen avirulence (*Avr*) gene with the corresponding plant resistance (*R*) gene (Staskawicz et al., 1995). In recent years, the gene-for-gene hypothesis has received tremendous experimental support through the identification and functional characterisation of both *Avr*- and *R*-genes. A number of *Avr*-genes from fungi, bacteria, and viruses were shown to encode specific elicitor proteins. This was demonstrated directly by infiltration of AVR proteins into plant leaves or indirectly by expression of *Avr*-genes in plant cells containing the corresponding *R*-gene (Culver & Dawson, 1991; de Wit, 1995; Alfano & Collmer, 1996; Knogge, 1996;

Bonas & van den Ackerveken, 1997; van den Ackerveken & Bonas, 1997). Elicitor treatment or *Avr*-gene expression triggers the HR and related defence responses in plants that mimic the response induced by avirulent pathogens (Hahlbrock et al., 1995; Hammond-Kosack & Jones, 1996). *R*-genes, on the other hand, are thought to encode specific receptors that interact directly or indirectly with elicitors, thereby initiating signal transduction pathways that lead to the HR and expression of disease resistance response (Staskawicz et al., 1995; Bent, 1996; Hammond-Kosack & Jones, 1996; 1997; Baker et al., 1997). One remarkable feature is the occurrence of similar structural domains in the products of *R*-genes, suggesting conserved mechanisms of pathogen recognition and signalling of defence responses in the plant kingdom (Dangl, 1995; Staskawicz et al., 1995; Bent, 1996; Hammond-Kosack & Jones, 1997). It has now become apparent that mechanisms of pathogen-induced cellular defences of plants share some analogies with the immune response of vertebrates and insects (Baker et al., 1997).

Most examples of pathogen-triggered resistance responses in plants have been examined at a subspecific or varietal level. However, it has been suggested that mechanisms of gene-for-gene recognition may also determine resistance at higher taxonomic levels, namely, species, genus, or family (Newton & Crute, 1989; Keen, 1990; Heath, 1991; Crute & Pink, 1996). Several bacterial and fungal pathogens contain avirulence genes or produce elicitors that condition avirulence toward a resistant species (Keen, 1990; Dangl et al., 1992; Kamoun et al., 1993,1997b; Kang et al., 1995; Sweigard et al., 1995; Leach & White, 1996). Similarly, functional conservation of *R*-genes in unrelated species has been noted, and it also contributes to a restriction of host-range (Whalen et al., 1991; Dangl et al., 1992; Innes et al., 1993; Bent, 1996). These findings suggest that the traditional separation between "host" and "non-host" resistance in plant-pathogen interactions may not reflect fundamentally different mechanisms of action. A complex overlay of gene-for-gene recognitions may therefore mediate interactions between pathogens and their non-host plants. Durable and stable resistance responses may have evolved in non-host plants through the accumulation of an arsenal of *R*-genes governing the recognition of multiple and/or essential avirulence molecules in the pathogen (Heath, 1991; Crute & Pink, 1996).

Phytophthora infestans, a hemibiotrophic oomycete plant pathogen, causes late blight, an economically devastating disease of potato and tomato (Anonymous, 1996; Fry & Goodwin, 1997a, 1997b). The life cycle and infection process of *P. infestans* are well known (Pristou & Gallegly, 1954; Hohl & Suter, 1976; Coffey & Wilson, 1983; Judelson, 1997). 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 neighbouring cells through the intercellular space. Later,

infected tissue necrotises, 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* has been noted in all examined interactions, including those with plant species unrelated to the solanaceous hosts (Gross et al., 1993; Schmelzer et al., 1995; Naton et al., 1996; V.G.A.A. Vleeshouwers, F. Govers & L. Colon, unpublished data). Fully resistant plants, such as some of the potato lines bearing *R*-genes or the non-hosts *Solanum nigrum* and parsley, display a typical localised HR at all infection sites (Gees & Hohl, 1988; Colon et al., 1992; Gross et al., 1993; Freytag et al., 1994; Schmelzer et al., 1995; Naton et al., 1996), suggesting that the classical model of pathogen elicitor recognition by a plant receptor and the subsequent activation of signal transduction pathways leading to a HR could mediate these interactions.

P. infestans is generally considered a specialised pathogen. Only sporadic reports of natural infection of plants outside of the genera *Solanum* and *Lycopersicon* have been provided (Erwin & Ribeiro, 1996). The molecular basis of host-specificity of *P. infestans* is poorly understood (Judelson, 1996, 1997). To date, no late blight resistance gene of *Solanum* spp. or race-specific avirulence gene of *P. infestans* has been isolated. However, in recent years, a family of extracellular protein elicitors, termed elicitins, has been identified in *P. infestans* and other *Phytophthora* species. Evidence that these molecules play a role in delimiting the host-range of *Phytophthora* is accumulating (Yu, 1995; Grant et al., 1996). Elicitins are highly conserved 10 kDa proteins that are secreted by all tested *Phytophthora* and *Pythium* species (Kamoun et al., 1993; Pernollet et al., 1993; Huet et al., 1995). Elicitins induce defence responses, including HR, on a restricted number of plants, specifically *Nicotiana* species within the Solanaceae family (Kamoun et al., 1993; Bonnet et al., 1996). In *Phytophthora parasitica*, the absence of elicitin production correlates with virulence on tobacco, a plant species that exhibits a strong response to elicitins (Ricci et al., 1989; Kamoun et al., 1993). Moreover, in a sexual progeny of *P. parasitica*, elicitin production segregates with low virulence (Kamoun et al., 1994), suggesting that elicitins function as avirulence factors in *P. parasitica*-tobacco interactions (Yu, 1995). Similarly, elicitin recognition has been proposed to be a component of non-host resistance of *Nicotiana* species to *P. infestans* and other elicitin-producing *Phytophthora* species (Yu, 1995; Kamoun et al., 1997b). This recognition 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). However, no direct assessment of the role of elicitins as avirulence factors through genetic manipulation of elicitin production has been reported to date.

Molecular manipulations and stable DNA transformation of *P. infestans* are well established techniques (Judelson & Michelmore, 1991; Judelson, 1996; 1997; van West et al., 1998). Because DNA transformation is a prerequisite for an unequivocal demonstration of the role of elicitins in *Phytophthora*-plant interactions, we decided to

exploit *P. infestans* for functional analysis of elicitins. In this study we examine in detail the response of five *Nicotiana* species to *P. infestans* and use transgenic *P. infestans* strains deficient in the production of INF1, the major elicitin of *P. infestans*, to determine whether INF1 elicitin acts as an avirulence factor that induces resistance in *Nicotiana* species to *P. infestans.*

Results

Nicotiana Species are Resistant to Wild Type *P. infestans* Isolates

P. infestans is typically considered a host-specific pathogen with a host-range limited to a few solanaceous hosts (Erwin & Ribeiro, 1996). To determine whether *Nicotiana* species are resistant to *P. infestans*, we examined the interaction between four isolates of *P. infestans* and five species of *Nicotiana* by using a well defined virulence bioassay (see Experimental Procedures). Zoospores from *P. infestans* strains 88069, 90128, and ME93-2A isolated from epidemics occurring in the Netherlands and United States, and strain MEX580 isolated from Mexico (described in Table 1), were inoculated on leaves from potato, tomato and seven tobacco cultivars representing the species *N. alata, N. benthamiana, N. clevelandii, N. rustica* and *N. tabacum.* Inoculations of the host plants potato and tomato with all four isolates of *P. infestans* consistently yielded expanding disease lesions accompanied by sporulation. In contrast, resistance responses were observed after inoculations of tobacco plants. Such resistance responses consisted of either localised necrotic spots typical of a HR or no visible macroscopic response, as described in Table 2. These results indicate that the *Nicotiana* species used in this study are highly resistant to several *P. infestans* isolates of diverse origin.

Table 1. *P. infestans* Strains Used in This Study

Strain	Description	Origin
88069	Wild type. Isolated in 1988 from tomato in the Netherlands. A1 mating type. INF1$^+$.	This laboratory[a]
90128	Wild type. Isolated in 1990 from potato in the Netherlands. A2 mating type. INF1$^+$.	This laboratory[a]
ME93-2A	Wild type. Isolated in 1993 from potato in the USA. A2 mating type. INF1$^+$. US-8 genotype.	W.E. Fry[b]
MEX580	Wild type. Isolated in the 1980s from potato in Mexico. A1 mating type. INF1$^+$.	W.E. Fry[b]
Transformants		
Y15	88069 transformed with G418 resistance plasmid pTH209. INF1$^+$.	This study
PY23	88069 cotransformed with pTH209 and *inf1* antisense construct pHIN28. INF1$^-$.	This study
PY37	88069 cotransformed with pTH209 and *inf1* antisense construct pHIN28. INF1$^-$.	This study

[a] *Phytophthora* culture collection of the Laboratory of Phytopathology, Wageningen Agricultural University, The Netherlands.
[b] Cornell University, Ithaca, NY, USA.

A HR Occurs in *Nicotiana* Species Inoculated with *P. infestans*

To determine the cytological basis of the resistance of *Nicotiana* species to *P. infestans*, we examined several representative interactions microscopically by using lactophenol-trypan blue-stained discs of inoculated leaves. As previously observed in other resistance interactions between plants and *P. infestans* (Gross et al., 1993; Schmelzer et al., 1995; Naton et al., 1996; V.G.A.A. Vleeshouwers, F. Govers & L. Colon, unpublished data), penetration of an epidermal cell by a germinating cyst of *P. infestans* was noted in all combinations that were examined. This was followed by a necrotic HR response that varied between different *Nicotiana* species in severity and number of affected cells. The resistance responses of *N. tabacum* and *N. benthamiana* illustrate two typically different responses of *Nicotiana* species to *P. infestans*. Some examples from these interactions are shown in Figure 1. In tobacco, which showed no visible macroscopic response after inoculation with *P. infestans* (Table 2), cellular responses generally were limited to the penetrated epidermal cell and zero to three adjacent epidermal or mesophyll cells (Figure 1A). Granular cytoplasm, condensed nuclei, thickened cell walls, and increased trypan blue-staining were observed in the responding cells, suggesting a typical HR response. Secondary infection hyphae were not visible, suggesting that the pathogen is restricted to the penetrated cell. In *N. benthamiana*, which showed macroscopic necrosis after inoculation with *P. infestans* (Table 2), cellular responses were more extensive than on *N. tabacum*. At 46 hrs after inoculation (Figures 1B & C), cells displaying a HR were visible at some but not all infection spots. Secondary hyphae were formed, and intercellular growth was noted. In most cases, secondary hyphae with protruding haustoria were found between a group of one to ten spongy parenchyma cells displaying increased trypan blue-staining (Figure 1B, data not shown). At 70 hrs after inoculation, the invading hyphae did not appear to have spread much further. They were surrounded by clusters of heavily stained mesophyll cells (Figure 1C). Apparently, the hyphae were restricted to these HR clusters and did not spread further. The observed HR clusters corresponded to the necrotic spots observed macroscopically (Table 2).

Table 2. Response of Different Solanaceous Plants to Four Isolates of *P. infestans*

Species/Cultivar	Response to *P. infestans* [a]	Interaction
N. alata cv Lime Green	No macroscopic response	Resistant
N. benthamiana	Necrotic spots	Resistant
N. clevelandii	No macroscopic response	Resistant
N. rustica var WAU	Necrotic spots	Resistant
N. rustica var Americana	Necrotic spots	Resistant
N. tabacum cv Xanthi	No macroscopic response	Resistant
N. tabacum cv White Burley	No macroscopic response	Resistant
S. tuberosum cv Bintje	Extending lesions with sporulation	Susceptible
L. esculentum cv Moneymaker	Extending lesions with sporulation	Susceptible

[a] *P. infestans* isolates 88069, 90128, ME93-2A and MEX580 were used on all genotypes and gave similar results.

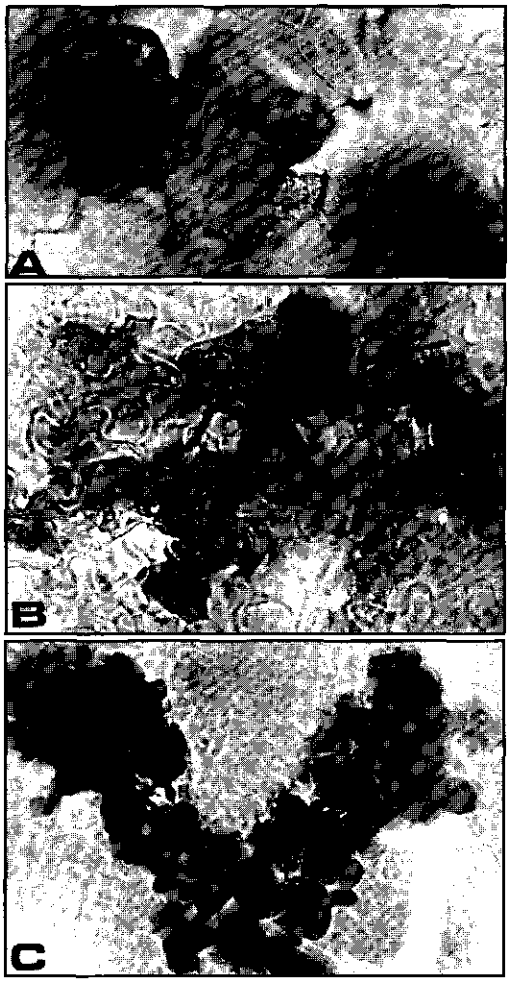

Figure 1. HR of *N. tabacum* and *N. benthamiana* Inoculated with Wild Type Isolates of *P. infestans*
(A). Trypan blue-stained leaf disc of tobacco 70 hours after inoculation with *P. infestans* wild type isolate 88069 showing response of one epidermal cell.
(B & C). Trypan blue-stained *N. benthamiana* leaf discs 46 hours (B) or 70 hours (C) after inoculation with *P. infestans* wild type isolate 88069. Extensive blue-staining in (C) is due to irreversible membrane damage. G, granular cytoplasm; H, hyphae; N, condensed nucleus; P, penetration site; T, increased trypan blue-staining; W, thickened cell wall.

Production of INF1 Elicitin by *P. infestans* and Response of *Nicotiana* species to INF1

To determine whether the resistance and the HR observed in tobacco after inoculation with *P. infestans* could involve the recognition of the protein elicitor INF1, we examined isolates of *P. infestans* for production of INF1. Culture filtrates from the four isolates of *P. infestans* used in virulence assays along with culture filtrates from

63 other isolates from recent epidemics in Europe and North America contained a 10 kDa band that co-migrated with authentic elicitins in SDS-PAGE analyses (Table 1, data not shown). To determine whether our *Nicotiana* species would respond to INF1, we infiltrated a 100 nM solution of the *Escherichia coli* produced FLAG-INF1 protein (Kamoun et al., 1997b) into leaves of the *Nicotiana* species listed in Table 2. Two days later, the leaves were inspected for signs of a HR. All infiltrated plants responded to INF1 with a typical necrotic HR (data not shown) suggesting that the resistance observed in these plants to *P. infestans* could involve the recognition of INF1. In contrast, and as previously shown (Kamoun et al., 1997b), potato and tomato did not respond to infiltrations of INF1 protein.

P. infestans Transformants Silenced in the *Inf1* Gene

A simple consequence of the elicitor-receptor model is that pathogen strains deficient in the production of a specific elicitor are predicted to be more virulent than elicitor-producing strains. To engineer *P. infestans* strains deficient in the production of INF1, we co-transformed strain 88069 with pHIN28, a construct containing *inf1* in an antisense orientation, and the geneticin resistance plasmid pTH209. All putative co-transformants were screened by polymerase chain reaction for presence of the transgenes. Culture filtrates from thirty co-transformants and twenty-six control transformants, containing only the pTH209 plasmid, were screened for the absence of INF1 by using silver-stained polyacrylamide gels. Six of the thirty antisense co-transformants failed to produce INF1, whereas none of twenty-six control transformants was affected in INF1 production and produced INF1 in similar amounts, as did the wild type recipient strain 88069.

To determine whether the absence of the INF1 protein in culture filtrates correlates with absence of *inf1* mRNA in the mycelium, we isolated total RNA from cultures grown *in vitro* and performed RNA blot analyses as shown in Figure 2A. High levels of *inf1* mRNA were detected in the recipient strain 88069 and in a control transformant Y15. In contrast, no *inf1* mRNA was detected in two independent antisense transformants, PY37 and PY23, that do not produce INF1 (Figure 2B). Hybridisation with a probe of the constitutively expressed actin gene resulted in similar signals in all lanes, indicating that equal amounts of RNA were loaded. These results suggest that introduction of an antisense *inf1* construct in *P. infestans* caused silencing of the *inf1* gene.

Genomic DNA of the recipient strain 88069, the control transformant Y15, and the antisense transformants PY37 and PY23 was isolated and analysed using DNA gel blot hybridisations assays (data not shown). Hybridisations with a probe of the *inf1* gene showed that the endogenous *inf1* gene remained intact, suggesting that the silencing of the *inf1* gene obtained in PY37 and PY23 is not due to gene disruption or displacement.

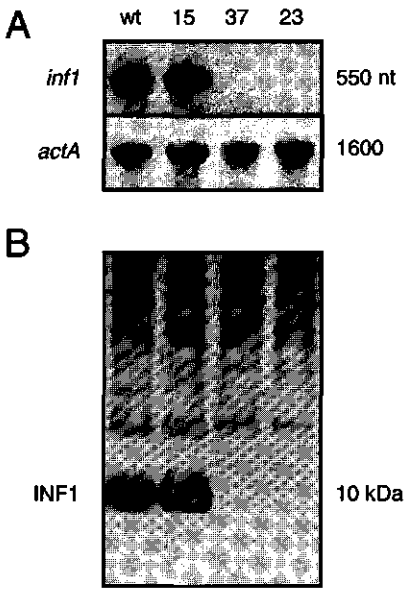

Figure 2. *P. infestans* Transformants Deficient in INF1 Production
(A). Analysis of *inf1* mRNA production in the *P. infestans* wild type recipient strain 88069 (wt), control G418 non-co-transformed Y15 strain (15), and two independent silenced antisense transformants PY37 (37) and PY23 (23). Each lane of the cell contains 15 µg of total RNA isolated from mycelium grown *in vitro* for ten days. The blot was sequentially hybridised with a probe from the *inf1* gene (*inf1*), and a probe from the actin gene (*actA*). Approximate transcript lengths are indicated at the right in nucleotides (nt).
(B). Analysis of INF1 protein production in the same set of strains as given in (A). Protein samples were obtained from filtrated culture medium of seven-day-old cultures, separated by Tricine-SDS-PAGE, and visualised by silver-staining. The position of the 10 kDa INF1 protein is indicated (INF1).

Silencing of *inf1* Is Mitotically Stable under Various Conditions

To determine whether the INF1 non-producing phenotype of the antisense transformants PY37 and PY23 is mitotically stable and allows functional analyses, we cultured the transformants in different media and subjected them to various treatments. Silenced transformants were vegetatively cultured *in vitro* by transferring them monthly to fresh medium over a period of eight months. Regularly, agar plugs containing sporulating mycelia were transferred to liquid media and the culture filtrates were checked for INF1 production. Neither PY37 nor PY23 ever reverted to the wild type state under these or other *in vitro* conditions. Furthermore, we investigated whether the silenced state of PY23 is maintained during growth in the plant. Potato tuber slices (1.0 cm thick) were inoculated on one side; after a week, when mycelia had grown through the tuber slice, young sporulating mycelium were transferred to fresh tuber slices. This procedure was repeated three times, after which mycelium was re-isolated, transferred to liquid medium, and checked for INF1

production. No effect on silencing of *inf1* in PY23 was observed after this treatment (data not shown). These results demonstrate that silencing of *inf1* remains stable through vegetative growth over time *in vitro* and *in planta*. Therefore, the INF1-deficient strains PY23 and PY37 are suitable for functional assays.

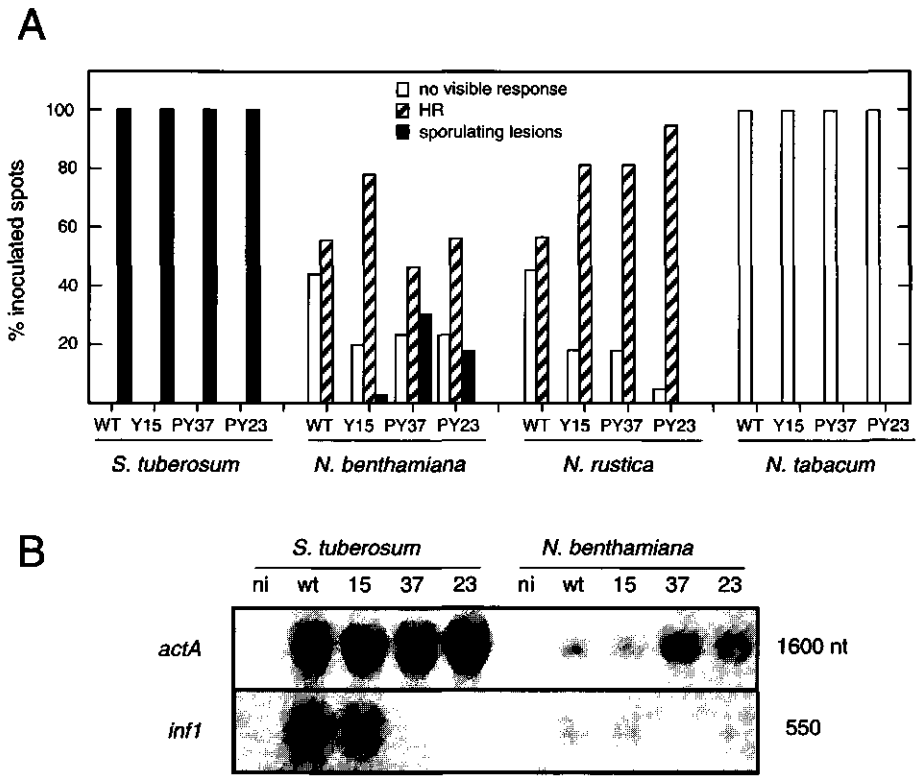

Figure 3. Virulence of *P. infestans* Wild Type and INF1 Deficient Strains on Potato (*S. tuberosum*) and *Nicotiana* species

(A). A histogram showing the percentage of resistance (no visible response or HR) and susceptible (sporulating lesion) responses observed after inoculation of potato (*S. tuberosum*), *N. benthamiana*, *N. rustica* (var. Americana) and *N. tabacum* with the wild type recipient strain 88069 (WT), a control transformant Y15, and two INF1 deficient strains PY23 and PY37. The number of inoculation spots examined per strain was 11 to 23 for potato, 38 to 65 for *N. benthamiana*, 9 to 17 for *N. rustica*, and 16 for *N. tabacum*. Note that *N. alata* and *N. clevelandii* gave results similar to those for *N. tabacum* (no visible response with all strains of *P. infestans*; data not shown).

(B). Analysis of actin and *inf1* mRNA production in infected plant tissue. Each lane of the RNA gel blot contains 15 µg of total RNA isolated from infected potato or *N. benthamiana* leaves 6 days after inoculation with the wild type strain 88069 (wt), the control transformant Y15 (15), and two INF1-deficient strains PY23 (23) and PY37 (37). The blot was sequentially hybridised with probes from the actin gene (*actA*) and the *inf1* gene (*inf1*). The intensity of the *P. infestans* actin signals correlates with the extent of *P. infestans* colonisation in the infected tissue. RNA extractions were conducted from pools of 10 leaf discs showing representative responses as determined in Figure 4A. Approximate transcript lengths are indicated at the right (nt). ni, not inoculated control.

INF1 Deficient Strains Remain Virulent on Potato But Produce Disease Lesions on *N. benthamiana*

To determine whether deficiency in INF1 production alters virulence of *P. infestans* on host and non-host plants, we inoculated the potato and the seven *Nicotiana* lines previously examined (Table 2) with zoospore solutions from the INF1-producing strains, 88069 and Y15, and *inf1* mutants, PY23 and PY37. As illustrated in Figure 3A, inoculated leaves were first examined for macroscopic symptoms of resistance (no response or HR) and susceptibility (disease lesions). Disease lesion formation followed by extensive production of sporangia (sporulation) was observed at all inoculation sites and with all *P. infestans* strains on potato. Resistance responses were observed with 88069, Y15, PY23 and PY37 in *N. alata*, *N. clevelandii*, *N. rustica* and *N. tabacum*, indicating that INF1 is not a major determinant of these resistant responses. In contrast, 20 to 30% of inoculations of *N. benthamiana* with INF1-deficient strains PY23 and PY37 consistently resulted in disease lesion formation accompanied by sporulation; however, inoculations with 88069 always led to resistance reactions, and inoculations with Y15 led to disease lesions in less than 3% of the inoculations.

To assess *P. infestans* biomass in infected leaves, we isolated total RNA from leaves 6 days after inoculation, blotted it, and sequentially hybridised the blot with probes from the actin (*actA*) and *inf1* genes. As shown in Figure 3B, high levels of actin RNA were detected in total RNA isolated from potato leaves infected by all four *P. infestans* strains, whereas *inf1* mRNA was only detected in the INF1-producing strains 88069 and Y15. This suggests that all strains can extensively colonise infected potato leaves independently of their ability to produce *inf1* mRNA. In addition, this result also confirms that the *inf1* gene remains silenced in PY23 and PY37 during growth *in planta*. In total RNA samples isolated from *N. benthamiana* leaves inoculated with PY23 and PY37, significant levels of actin mRNA were detected, whereas *inf1* mRNA was not detected. In contrast, trace amounts of actin and *inf1* mRNA were detected in leaves inoculated with 88069 or Y15. These results confirm the macroscopic observations and indicate that the INF1-deficient strains PY23 and PY37 reach higher levels of colonisation and biomass in leaves of *N. benthamiana* than do INF1-producing strains.

Infection of *N. benthamiana* by INF1 Deficient Strains

To explore in detail the infection of *N. benthamiana* by INF1-deficient strains, we carefully examined inoculated leaves at both the macroscopic and microscopic level, as illustrated in Figure 4.

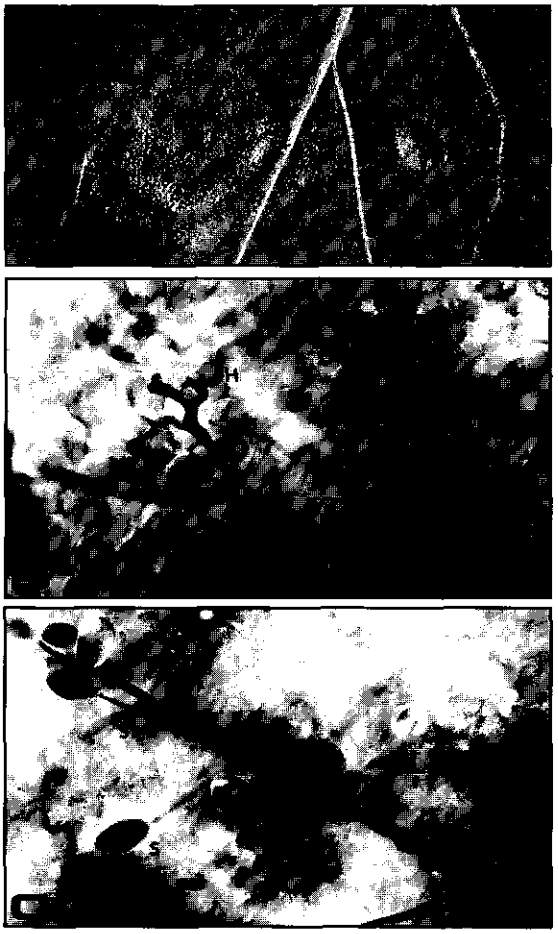

Figure 4. *N. benthamiana* Leaves Infected with an *inf1*-Silenced Strain
(A). A *N. benthamiana* leaf 4 days after inoculation with the *P. infestans* recipient wild type strain 88069 (right) and the INF1-deficient strain PY23 (left). Arrows indicate inoculation sites. Note the expanding lesion area caused by PY23 surrounding the inoculation spot with sporulation visible as a grayish white zone and the local necrosis (HR) caused by 88069. The dried inoculation droplet is visible as a mirroring area around the inoculation spot of 88069.
(B & C). Trypan blue-stained *N. benthamiana* leaf discs 70 hrs after inoculation with *P. infestans inf1* mutant PY37 showing biotrophic growth and sporulation. Compare with Figure 1C. H, hyphae; S, sporangia; SP, sporangiophores.

In numerous side-by-side inoculations of *N. benthamiana* leaves with INF1-producing and INF1-non-producing *P. infestans* strains, a dramatic difference in response was observed. In contrast to wild type strains (Table 2 & Figure 3A), up to 30% of the sites inoculated with *P. infestans* INF1-deficient strains went through a full disease cycle. The first symptoms of colonisation appeared within 2 or 3 days after inoculation, with a rapidly expanding water-soaked zone forming around the

inoculation spot. As early as three days after inoculation, a greyish white sporulation zone became visible on the surface of the infected leaf, in contrast to localised necrosis (HR) that was observed after inoculation with INF1-producing strains (Figure 4A). In contrast to infection of potato plants obtained under the same conditions, little browning or necrosis accompanied such sporulation. Disease lesions expanded on *N. benthamiana* at a rate similar to potato and ultimately covered the entire leave. In microscopic examinations of trypan blue-stained sections of *N. benthamiana* leaves infected by *P. infestans* mutant PY37 (Figures 4B & 4C), extensive biotrophic colonisation of the mesophyll by intercellularly growing hyphae with haustoria was observed 70 hrs after inoculation. In contrast to infections by the wild type strains (Figure 1C), no response of the mesophyll cells surrounding these invading hyphae was observed (Figure 4B). On the surface of the infected leaf, sporangiophores emerging through the stomata and numerous sporangia were readily observed (Figure 4C).

Discussion

Ever since the potato late blight epidemics of the mid-nineteenth century, members of the genus *Phytophthora* have emerged as major pathogens of numerous crops (Erwin & Ribeiro, 1996). Despite the importance of *Phytophthora* species as devastating plant pathogens, little is known about the molecular mechanisms that determine the outcome of interactions between *Phytophthora* and plants (Judelson, 1996; 1997). Elicitins, a family of host-specific elicitor proteins of *Phytophthora*, induce the HR on particular plant species, most notably tobacco and other *Nicotiana* species in the Solanaceae family. Using a single-step transformation procedure with an antisense construct of the *inf1* elicitin gene, we engineered stable *P. infestans* strains deficient in the production of the INF1 elicitin. Based on the observed increased virulence of these INF1-deficient strains on the plant species *N. benthamiana*, we conclude that the recognition of INF1 is a major determinant of the resistance response of *N. benthamiana* to *P. infestans*. These results directly demonstrate the role of a particular molecule in host-specificity of *Phytophthora* and suggest that elicitins are avirulence factors that condition resistance at the species level.

In this study, all of the interactions that we examined between *P. infestans* isolates and *Nicotiana* species resulted in resistance. On the five tested *Nicotiana* species, *P. infestans* zoospores encysted, germinated, and formed appressoria and penetration pegs. Resistance reactions occurred after penetration of an epidermal cell and were always associated with a HR of epidermal and mesophyll cells. This suggests that the interaction of *Nicotiana* species with *P. infestans* follows the classical model of pathogen elicitor recognition by a plant receptor and the

subsequent activation of signal transduction pathways leading to a HR. A candidate elicitor is the INF1 elicitin protein (Kamoun et al., 1997a) which induces a HR on all of the examined *Nicotiana* species but not on the host plants potato and tomato, and which supposedly interacts specifically with a high-affinity binding site in the tobacco plasma membrane, as was shown for the *P. cryptogea* elicitin, cryptogein (Wendehenne et al., 1995).

Even though the HR was always associated with the resistance response of *Nicotiana* to *P. infestans*, the timing, severity, and extent of the HR varied considerably, depending on the examined genotype. Similarly, wild type *P. infestans* reached different levels of colonisation on different *Nicotiana* plants. In tobacco, *P. infestans* was blocked early in the infection after penetration of the epidermal cell, and secondary intercellular hyphae were never observed. In contrast, in *N. benthamiana*, secondary hyphae with haustoria were formed and some level of mesophyll colonisation occurred. Plant response reached a climax three days after inoculation, and clusters of HR cells engulfing the invading hyphae were formed, correlating with the cessation of further *P. infestans* ingress. These observations suggest that several layers of resistance to *P. infestans* occur with various degrees of effectiveness in different *Nicotiana* species. Similar variation in the phenotype of plant resistance to pathogens was observed in interactions between *Arabidopsis thaliana* and the biotrophic oomycetes *Peronospora parasitica* and *Albugo candida* (Holub et al., 1994; Reignault et al., 1996). In these interactions, several classes of resistance reactions were defined on the basis of the strength and appearance of necrotic tissue. As observed in *Nicotiana-P. infestans* interactions, various levels of pathogen ingress correlated with the different necrotic responses (Reignault et al., 1996).

In contrast to the wild type and other *P. infestans* INF1-producing strains, the engineered INF1-deficient strains produced disease lesions with profuse sporulation on *N. benthamiana*. Furthermore, both RNA gel blot hybridisations with infected *N. benthamiana* tissue using a constitutive gene as a probe, and cytological examinations of infected *N. benthamiana* tissue indicated that INF1-deficient strains achieve significant levels of biomass and colonisation in *N. benthamiana*. In contrast, these mutants remained unable to infect other *Nicotiana* species, such as tobacco. This disparity appears to reflect the differences observed by cytological examination of the resistance responses in the *Nicotiana* species. In *N. benthamiana*, wild type INF1-producing *P. infestans* strains can penetrate the leaf as far as the mesophyll, whereas INF1-deficient strains can grow further and fully colonise leaf tissue. In tobacco, the first layer of response to infection by both INF1-producing and INF1-non-producing strains occurs immediately after penetration and can effectively stop further ingress by *P. infestans*. This indicates that resistance to *P. infestans* in *N. benthamiana* is mainly triggered by INF1, whereas the early resistance reaction observed in tobacco is not. Possibly, before recognition of INF1, tobacco responds to

additional host-specific elicitors that are not detected by *N. benthamiana*. Putative candidates are the products of the *inf2A* and *inf2B* genes, both members of the *P. infestans* elicitin gene family (Kamoun et al., 1997a). These two genes are expressed in the plant during infection of potato and *N. benthamiana*, and their products induce a HR on tobacco (S. Kamoun, P. van West & F. Govers, unpublished data). In addition, a 30 kDa glycoprotein identified in several *Phytophthora* species is known to induce defence responses in tobacco (Baillieul et al., 1996). Whether these elicitors induce different responses on tobacco and *N. benthamiana* remains to be tested.

The suggestion that INF1 is not involved in the early resistance response of tobacco does not exclude the possibility that INF1 is effective as an avirulence factor on this plant in a later stage of the disease cycle. This hypothesis is supported by the increase in expression of the *inf1* gene during the latest stages of infection of potato leaves by *P. infestans* (Kamoun et al., 1997b), and is in line with the observation that in *N. benthamiana*, INF1-producing strains are blocked at an advanced stage of colonisation. Future experiments, such as constructing strains of *P. infestans* with multiple mutations, will help to test this hypothesis.

In relation to the observed difference between *N. benthamiana* and the other *Nicotiana* species, *N. benthamiana* is known to anomalously allow infection by numerous plant viruses and plant virus mutants including some with restricted host range (van Dijk et al., 1987; Dawson & Hilf, 1992). Therefore, *N. benthamiana* may have a particular deficiency in its defence response, making it generally more susceptible to plant pathogens, including INF1-producing and INF1-non-producing *P. infestans*, than are other *Nicotiana* species.

Interactions between *P. infestans* and plants are notable for their quantitative nature and for the ambiguous response of the plant to infection by the pathogen. For example, susceptible and partially resistant potato plants display a mosaic pattern of responses to infecting spores: some sites are readily infected, whereas others respond by a typical localised HR, which effectively stops the pathogen at that particular site (Gees & Hohl 1988; Freytag et al., 1994). Similar quantitative aspects were observed in this study in the interaction between *N. benthamiana* and *P. infestans* strains. INF1-deficient strains were able to produce disease lesions on *N. benthamiana* in only 20 to 30% of the inoculated sites. This is reminiscent of the infection efficiencies obtained following inoculation of partially resistant *Solanum* lines by *P. infestans* (Colon et al., 1995) and suggests that *N. benthamiana* may retain a low level of resistance against INF1-deficient *P. infestans* strains. Macroscopically, visible HR varied also quantitatively, because we did not observe lesions at all inoculation sites (Figure 3A). No visible response was observed in 20 to 40% of the spots inoculated with both INF1-producing and INF1-non-producing *P. infestans* strains. Based on the cytological examinations of multiple infection sites (data not shown), we think that the absence of symptoms generally corresponds to aborted

infections, which reflect the observed infection efficiency of *P. infestans* on *N. benthamiana.*

Conflicting results have appeared regarding the host-specificity of elicitins (discussed by Yu, 1995; Kamoun et al., 1997b). It has been suggested by others that elicitins may be non-specific toxins that induce necrosis on all plant species including hosts (Pernollet et al., 1993; Huet et al., 1994). In this study, we show unambiguously that INF1 deficient strains remain capable of infecting potato and tomato. No significant difference in disease severity or symptomology was noted between INF1-producing and INF1-non-producing isogenic strains. This indicates that the *P. infestans* elicitin INF1 is not required for pathogenicity on potato and tomato. It can then be ruled out that INF1 functions as a non-specific toxin essential for virulence.

Based on traditional definitions (Heath, 1991), the *Nicotiana* species examined in this study can be considered as non-hosts of *P. infestans*. Contrary to the assumption that non-host resistance has multiple components and is genetically complex, our results show that resistance of *N. benthamiana* to *P. infestans* involves one major component, the recognition of the elicitor protein INF1. *R*-genes are generally bred from resistant wild species into a cultivated species through conventional methods. Cultivars containing such *R*-genes can then discriminate between genotypes of the pathogen (races). However, there is some evidence, such as the functional conservation of *R*-genes between unrelated species (Whalen et al., 1991; Dangl et al., 1992; Innes et al., 1993; Bent, 1996), that higher taxa specificity may also be a reflection of gene-for-gene interactions. Experimental and particularly genetic characterisation of such non-host interactions is hampered by the absence of variation in plant resistance and in pathogen virulence. In addition, resistance identified in plants that are sexually incompatible with a given susceptible crop plant may not be dissected into discrete components, and *R*-genes from such plants cannot be transferred into isogenic background for further study. Here, we demonstrate that pathogen protein elicitors that induce a HR on non-host plants can function as avirulence factors. Therefore, species-specific elicitors can be used as a tool to identify novel sources of resistance in germplasm unrelated to the host plant, to evaluate resistance levels, and to isolate *R*-genes.

The postulate that elicitins are avirulence factors that restrict the host-range of *Phytophthora* isolates points to a number of biotechnological applications. The ubiquitous occurrence of conserved structural features noted in *R*-genes of diverse origin (Dangl, 1995; Staskawicz et al., 1995; Bent, 1996) suggests that a classic *Nicotiana R*-gene could be involved in the recognition and response to INF1 and other elicitins. Further genetic and biochemical research should help isolate *Nicotiana R*-genes involved in the INF1 response. The results we present in this study further suggest that manipulation of potato and tomato to recognise and response to elicitin molecules is predicted to yield plants with enhanced resistance to *P. infestans.*

Experimental Procedures

Phytophthora Strains and Culture Conditions

The various *P. infestans* isolates used in this study are listed and described in Table 1. Strains were routinely cultured in the dark at 18 °C on rye agar medium supplemented with 2% sucrose (Caten & Jinks, 1968). For INF1 elicitin production, culture filtrates were harvested after growth for 3-4 weeks at 18 °C in still cultures in the synthetic medium described by Kamoun et al. (1994). To isolate zoospores for plant inoculations, sporulating mycelia in rye sucrose medium were flooded with water (10 ml per Petri dish) and incubated at 4 °C for two hrs. The zoospore solution was then gently poured out of the Petri dish and placed on ice until inoculation.

Plasmid Construction and Transformation of *P. infestans*

Plasmid pHIN28, which contains the *inf1* coding sequence in antisense orientation fused to the *Ham34* promoter of *Bremia lactucae* (Judelson & Michelmore, 1991), was constructed by generating a 390 bp polymerase chain reaction fragment of the *inf1* coding sequence with primers PIET28 (5'-TATCGGTACCCACTCCTCCTCACTC-3') AND PIET29 (5'-CGGCCCATGGACGCTGACTC-3'). The amplified fragment was digested with *Kpn*I and *Nco*I and inserted into *Nco*I- and *Kpn*I-digested pHAMT35G (Judelson & Michelmore, 1991). Stable co-transformation was conducted according to van West et al. (1998) by using *Eco*RI linearised pHIN28 and the selection plasmid pTH209 (geneticin (G418) resistance) (Judelson et al., 1991). Polymerase chain reaction amplification of DNA from individual transformants by using the M13/pUC reverse primer (Gibco-BRL) in combination with PIET28 was conducted to discriminate between co-transformants (amplification of a fragment of the expected size) and pTH209 transformants (no amplification product).

DNA Manipulations

Routine DNA manipulations were conducted essentially as described elsewhere (Ausubel et al., 1987; Sambrook et al., 1989). Total DNA of *P. infestans* was isolated from mycelium grown in liquid culture as previously described (Pieterse et al., 1991). Alkaline DNA transfer to Hybond N^+ membranes (Amersham, Arlington Heights, IL) and DNA gel blot hybridisations were performed at 65 °C as described elsewhere (Ausubel et al., 1987; Sambrook et al., 1989). Filters were washed at 65 °C in 0.5x SSC (75 mM NaCl and 7.5 mM sodium citrate) +0.5% SDS.

RNA Manipulations

Total RNA from *P. infestans* and infected plant tissue was isolated using the guanidine-hydrochloride extraction method (Logemann et al., 1987). For RNA gel blot analyses, 10-15 µg of total RNA was denatured at 50 °C in 1 M glyoxal, 50% DMSO, and 10 mM sodium phosphate, electrophoresed, and transferred to Hybond N^+ membranes (Amersham, Arlington Heights, IL) (Ausubel et al., 1987; Sambrook et al., 1989). Hybridizations were conducted at 65 °C in 0.5 M sodium phosphate buffer, 7% SDS and 1 mM EDTA. Filters were washed at 65 °C in 0.5x SSC + 0.5% SDS.

DNA and RNA Blot Hybridisation Probes

Gel-purified DNA fragments containing the full length *inf1* cDNA insert from pFB7 (Kamoun et al., 1997b), the *actA* gene from pSTA31 (Unkles et al., 1991), and the *Ham34* promoter (Judelson & Michelmore, 1991) were used as probes and radiolabelled with α ^{32}P-dATP using a random primer labelling kit (Gibco-BRL, Bethesda, MD).

SDS-PAGE

Culture filtrates were subjected to Tricine-SDS-PAGE as described elsewhere (Schagger & von Jagow, 1987; Sambrook et al., 1989). After electrophoresis, gels were silver-stained following the method of Merril et al. (1981).

Plant Assays

The plant species and cultivars used in this study are listed in Table 2. Plants were grown in growth chambers or a greenhouse for 4 to 8 weeks, depending on the species. Infection assays with *P. infestans* were conducted as described by Turkensteen (1973). Resistance levels of solanaceous plants observed using this assay were shown to correlate with the resistance levels obtained with attached leaves in the field or in the greenhouse (V.G.A.A. Vleeshouwers, F. Govers, & L. Colon, manuscript submitted). In general, the third to sixth leafs from the top, were detached from several plants, and the petioles were fitted in water-saturated florist foam (trademark 'Oasis', V. L. Smithers

A/S, Denmark). The leaves and the foam were then placed in plastic trays lined with wet filter paper and a plastic mesh to prevent direct contact between the leaves and the wet paper. 10 µl droplets containing ca. 500 zoospores were then applied to the underside of the leaves in the middle of a leaf panel. A total of 2 to 10 droplets were placed on each leaf, depending on its size. The trays were tightly fitted with a transparent plastic cover and placed under fluorescent light in a regulated growth chamber (15 °C, 16 hrs photoperiod). Inoculation spots were examined for disease symptoms and necrosis daily for 7 days.

Microscopic Observations and Trypan Blue-Staining

Leaf discs containing the inoculum were excised at various times after inoculation and examined by microscopy for plant response and growth of *P. infestans*. Lactophenol-trypan blue-staining and de-staining with chloral hydrate were performed as described earlier (Wilson & Coffey, 1980; Colon et al., 1992).

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Chapter 7

Ric1, a *Phytophthora infestans* Gene with Homology to Stress-Induced Genes

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Summary

From a set of *P. infestans* cDNA clones that were randomly selected from a potato-*Phytophthora infestans* interaction cDNA library, a relatively high proportion (five out of 22) appeared to be derived from the same gene. The gene was designated *ric1*. *P. infestans* contains two copies of *ric1*. They share 98% homology on nucleotide sequence level and 100% on amino acid level. The nucleotide sequence predicts an open reading frame of 171 bp encoding a 57 amino acid hydrophobic peptide with two potential membrane-spanning domains. The predicted peptide shows high homology to a peptide encoded by plant genes whose expression is specifically induced during stress conditions. Southern blot analysis of genomic DNA of several *Phytophthora* species indicated that most species contain *ric1* homologues. During the life cycle of *P. infestans*, *ric1* was expressed in all developmental stages but the level of expression varied. Sporangia and germinating cysts appeared to contain only very little *ric1* mRNA whereas in mycelium and during *in planta* growth higher levels were detected. Subjecting mycelium to osmotic stress or high pH resulted in increased *ric1* expression.

Introduction

Phytophthora infestans (Mont.) de Bary is the causal agent of potato late blight, one of the most devastating diseases of potato (*Solanum tuberosum* L.). This oomycete plant pathogen is able to infect both foliage and tubers and can spread rapidly through host tissue causing destructive necrosis. Little is known about the molecular processes taking place in the *P. infestans*-potato interaction. Identification of genes expressed during the interaction may help in unravelling cellular signalling processes essential for establishing and maintaining a pathogenic interaction. Previously, we identified several so-called *in planta*-induced (*ipi*) genes selected by differential screening of a genomic DNA library of *P. infestans.* A major advantage of this screening was that it yielded exclusively *in planta*-induced genes of the pathogen and no host genes. A disadvantage was that the selected genomic clones contained large proportions of non-coding and repetitive DNA and might contain multiple genes on a single vector insert (Pieterse et al., 1993a). In order to identify additional *P. infestans* genes that are expressed during the interaction with the host we now screened a so-called interaction cDNA library. The basis for construction of this cDNA library was poly(A^+) RNA isolated from *P. infestans*-infected potato leaves, 3 days post-inoculation. We selected candidate genes by screening the library with random cDNA probes representing on the one hand RNA isolated from non-infected potato leaves and on the other hand RNA isolated from *P. infestans* mycelium. The cDNA clones

hybridising to the *P. infestans* probe were considered for further analysis whereas those hybridising to the potato probe were excluded. Due to the origin of the *P. infestans* probe used for the screening, the cDNA clones represent *in planta*-expressed, but not necessarily *in planta*-induced genes. Even though, the selection was performed in an unbiased manner, five of twenty-two randomly isolated cDNA clones (*ric)* appeared to be derived from the same gene. Here we describe characterisation of this gene which we designated *ric1*. The deduced amino acid sequence shows high homology with a peptide encoded by two stress-induced genes from plants. To investigate whether *ric1* is also a stress-induced gene, *P. infestans* was exposed to stress conditions and *ric1* expression was analysed and compared to *ric1* expression during various developmental stages, *in vitro* and *in planta.* A possible role of the putative RIC1 protein is discussed.

Results

Characterisation of *ric1* cDNA Clones

From the set of twenty-two randomly selected *P. infestans* cDNA clones, five (i.e. pPi101, pPi103, pPi108, pPi109 and pPi121) were strongly related as they all cross-hybridised to each other (data not shown). The nucleotide sequence of three of these was determined. Sequence analysis showed that pPi109 contains a continuous open reading frame of 171 bp (Figure 1). The coding sequence starts with an ATG-start codon 23 bp downstream of the 5' end and encodes a putative protein of 57 amino acids. pPi121 is a partial cDNA clone with a 160 bp of open reading frame and lacking the N-terminal part of the putative protein (Figure 1). pPi101 is even smaller. At the 5' end pPi101 is 45 bp shorter than pPi121 but the overlapping sequences of pPi101 and pPi121 are exactly identical. The nucleotide sequence of the coding region in pPi109 differs only at one position when compared with the nucleotide sequence of pPi101 and pPi121, whereas 14 mismatches are found in the 3' non-coding regions of the cDNA clones. We can not rule out at this stage whether these mismatches are in fact due to sequence mistakes. The gene from which the randomly isolated cDNA clones were derived was designated *ric1*.

Occurrence of *ric1* in the *P. infestans* Genome and in Other *Phytophthora* Species

The observation that DNA sequences of pPi109 and pP1121 are not 100% but 98% homologous suggests the existence of at least two *ric1* copies in the genome of *P. infestans.* Genomic Southern blot analysis (Figure 2A) revealed the presence of two genomic *Hin*cII fragments (1.0 kb and 2.3 kb) hybridising to the *ric1* probe, two *Bam*HI fragments (8.5 kb and 11 kb), and two *Pst*I fragments (0.7 kb and 0.75 kb). In the *Eco*RI digest and the *Hin*dIII digest only one hybridising fragment was found. (10

and 4.0 kb, respectively). Since there is a *Hin*cII restriction site in the *ric1* cDNA clones, two hybridising fragments can be expected even when *ric1* is a single copy gene. However, since there are no *Bam*HI and *Pst*I restriction sites present in pPi109, the two hybridising *Bam*HI and *Pst*I fragments support the suggestion that *P. infestans* has two copies of the *ric1* gene. These two copies either are contained within a 4.0 kb *Hin*dIII fragment and a 10 kb *Eco*R1 fragment, or correspond to two alleles of the *ric1* gene. The *ric1* probe does not hybridise to *Eco*RI digested genomic DNA isolated from potato (cv Ajax) (Figure 2A), thus confirming that the cDNA clones selected from the interaction cDNA library are derived from *P. infestans* and not from potato.

```
      HincII
109   GGCACGAGGCAAACTAGTTAACGATGCCGATTACCTGCGGAGATATCCCTCGTCTGATCT   60
121                                                                  22
 aa                              M  P  I  T  C  G  D  I  P  R  L  I  C   13

109   GCTCAGTCATCATCCCGCCCGTCGGCGTGTTCTTCCAGGTTGGATGCACAAAGGACCTCG  120
121   ------------------------------------------------------------   82
 aa     S  V  I  I  P  P  V  G  V  F  F  Q  V  G  C  T  K  D  L  A   33

109   CGATCAACTGCCTGCTTACGGTTCTCGGCTACATCCCCGGAGTCATTCACGCTGTATACA  180
121   ----------------C-------------------------------------------  142
 aa     I  N  C  L  L  T  V  L  G  Y  I  P  G  V  I  H  A  V  Y  I   53

109   TTTTGATCAAGGAATAGAGCGGCGCCACATCGAAGTAAAAGATGTGGCGCTGTGTTCTCT  240
121   ------------------------------------------------------------  202
 aa     L  I  K  E  *                                                57

109   ATGAGTCTGGGGCAAGTGGCGAAACCTCTTCGGTCGGTGCTAAAATATCGAGATACGACA  300
121   ----------------------------------------G-------------------  262

109   TCGTCGGCGTGGATGGCAGATAGATTCATCGTGGAACAGCTCGCGTTTTAAGTCTCTTGT  360
121   ---------------------------------------------C----------C--  322

109   ACAATGGCGTGTGGTGATTCTCAACGGCTTCTTTCACCGTTTTGTTGCAACGGCTGGGGC  420
121   ------------------------------------------------------------  382

109   ACTTTTTGCACATTGAAGAGGAGTGAAATGCTCTCGTACAATGGAAATTGTGCTTCAGCT  480
121   --------------------T---------------------------------------  442

109   GTGGTGGCTGATCGTGGAGTCGCAAATGCAGGTCGCACACGTACATTGAAGAGGAGGGCT  540
121   -------------------------G-----C-------------------------T-  502

109   AGACAACGGTGACACGGTGTTTCATTTTGTTATTTGAAGAGGTCTGACAGTTGCAGTGGG  600
121   G-----------------------------------------------------------  562

109   CCAAAGTTAATATAAAGATCGATATCGAAAAAAAAAAAAACAAAAAAAAAAAAAAAAAAA  659
121   --T-----------G------------TT------------------------------  621
```

Figure 1. Nucleotide sequence of the *ric1* cDNA clones pPi109 (109) and pPi121 (121) from *P. infestans* isolate 88069 (GenBank accession No. AJ133023), and the deduced amino acid sequence of RIC1 (aa). The *Hin*cII restriction site is indicated. Numbers on the right indicate either nucleotide length or amino acid length (bold).

To determine whether other *Phytophthora* species have *ric1* homologues we analysed genomic DNA of various *Phytophthora* species by Southern blot hybridisation. Representatives of most taxonomic groups within the genus *Phytophthora* were included in the analysis. As shown in Figure 2B, most

Phytophthora species contain one or more genomic fragments that hybridise strongly to *ric1*. The only exception is *P. porri* where no hybridising fragment is detected, even though this lane contains sufficient and adequately digested *P. porri* DNA (data not shown). These results demonstrate that most *Phytophthora* species contain a *ric1* homologue. Whether or not these homologues are actively transcribed genes remains to be determined.

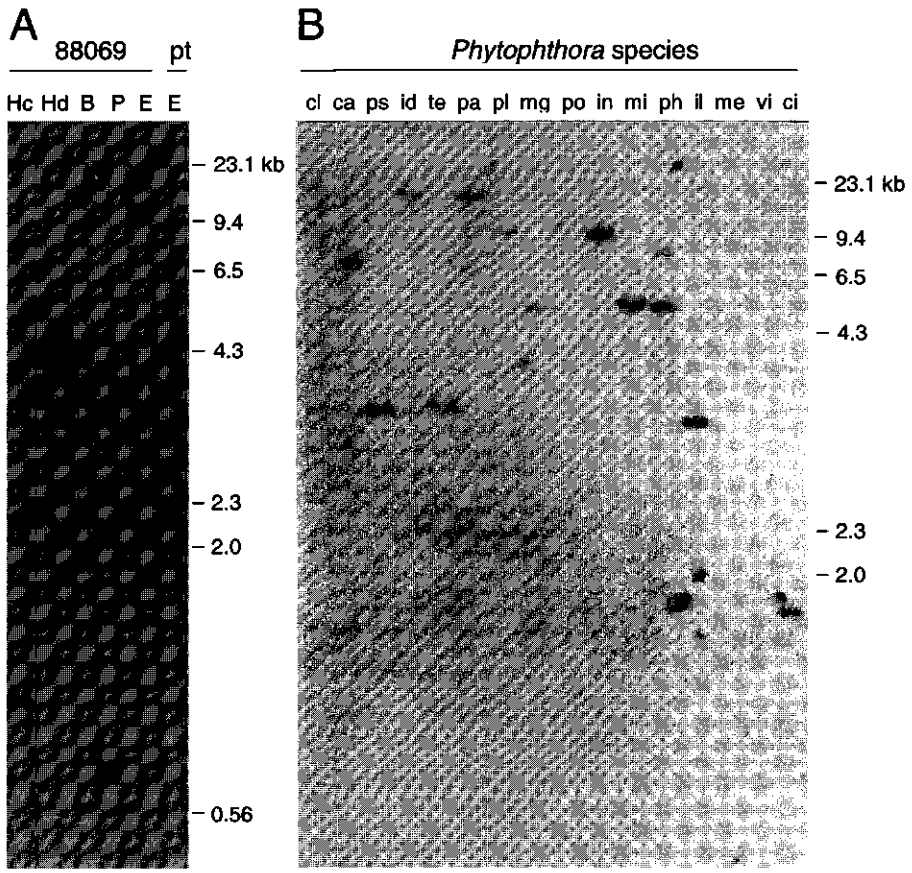

Figure 2A. Occurrence of *ric1* sequences in the *Phytophthora infestans* genome. The Southern blot contains genomic potato DNA of cv Ajax (pt) (15 µg) digested with *Eco*RI (E) and genomic *P. infestans* DNA of strain 88069 (15 µg/lane) digested with *Hin*cII (Hc), *Hin*dIII (Hd), *Bam*HI (B), *Pst*I (P), *Eco*RI (E) that was hybridised with the *ric1* cDNA probe. Molecular-size markers are indicated in kb.
(2B). Occurrence of *ric1* homologues in the genus *Phytophthora*. Southern blot analysis of *Eco*RI digested genomic DNA isolated from *P. clandestina* (cl), *P. cactorum* (ca), *P. pseudotsugae* (ps), *P. idaei* (id), *P. tentaculata* (te), *P. parasitica* (pa), *P. palmivora* (pl), *P. megakarya* (mg), *P. porri* (po), *P. infestans* (in), *P. mirabilis* (mi), *P. phaseoli* (ph), *P. ilicis* (il), *P. megasperma* f. sp. *glycinea* (me), *P. vignae* (vi), and *P. cinnamomi* (ci). The DNA was hybridised with the *ric1* probe. Molecular-size markers are indicated in kilobases (kb).

The RIC1 Peptide

The protein encoded by *ric1* consists of only 57 amino acids. The calculated molecular mass of this peptide is 6137 Da and the pI is 6.51. Hydropathy analysis (Kyte & Doolittle, 1982) suggests that RIC1 is highly hydrophobic and lacks a signal sequence (Figure 3). It contains two potential membrane spanning domains at amino acid positions 11-27 and 33-51, and four cysteine residues, which could be involved in the formation of disulphide bridges.

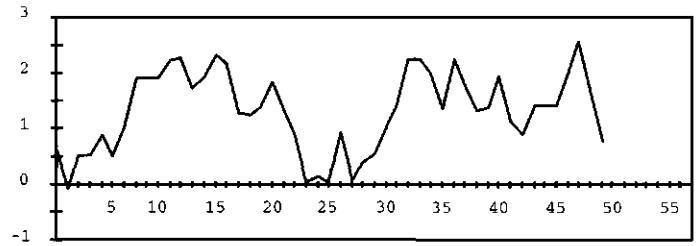

Figure 3. Hydropathy analysis (Kyte & Doolittle, 1982) of the RIC1 peptide sequence

Comparison of the RIC1 amino acid sequence to known sequences available in databases revealed significant similarity with stress-induced proteins from plants (Figure 4). The two identical putative peptides encoded by a cold stress-induced gene from *Hordeum vulgare*, *blt101* (Goddard et al 1993), and a salt stress-induced gene from *Lophopyrum elongatum*, *ESI3* (Gulick et al 1994), share 22 out of the 54 amino acids with RIC1, and an additional 13 amino acid residues are similar. Also two peptides encoded by cold-induced genes from *Arabidopsis*, RC12A and RC12B (Capel et al 1997), share high similarity (22 out of 54). However, the highest homology was found with an Expressed Sequence Tag from the nematode *Caenorhabditis elegans.* Thirty-one amino acids out of 57 are identical and fourteen are similar. Also yeast and *Escherichia coli* appear to have genes encoding proteins homologous to RIC1. Interestingly, these homologous proteins all have similar sizes ranging from 51 to 57 amino acids and the highest homology is found in the putative membrane spanning domains.

High identity was only found in the putative membrane spanning domains of another class of proteins. However, these putative proteins are much larger. The amino acid sequences range from 79 residues in case of a protein from a cyanobacterium *Synechocystis* sp. (Genbank accession No. D64005) to 133 residues of a yeast homologue (Genbank accession No. P14359) (data not shown).

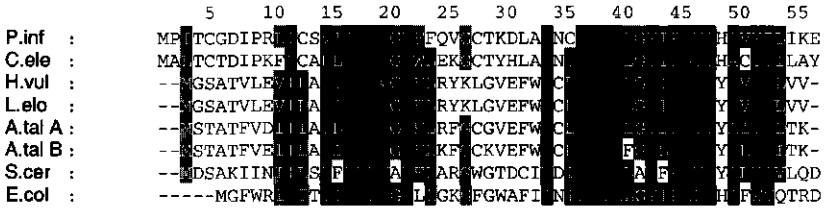

Figure 4. Alignment of the *P. infestans* RIC1 peptide sequence (P.inf) with its homologues CEESP35F from *Caenorhabditis elegans* (C.ele) (GenBank accession No. U39649), *blt101* from *Hordeum vulgare* (H.vul) (GenBank accession No. Z25537), *ESI3* from *Lophopyrum elongatum* (L.elo) (Genbank accession No. U00966), RC12A and RC12B from *Arabidopsis thaliana* (A.tal A and A.tal B) (GenBank accession numbers AAD17302 and AAD17303), a putative protein from *E. coli* (E.col) (GenBank accession No. AE000351) and Sc YDR276c from yeast (S.cer) (GenBank accession No. U51030). Black boxes represent similar or identical amino acids in all peptide sequences. Grey boxes represent identical or similar amino acids in 80% of the peptide sequences.

Expression of *ric1*

Detailed expression studies were performed to determine in which stages of the life cycle of *P. infestans* the *ric1* gene is active. Northern blots containing total mRNA isolated from *in vitro*-grown material such as zoospores, cysts, germinated cysts, sporangia, young mycelia, and old mycelia and from *P. infestans* spot-inoculated potato leaves were prepared as described previously (van West et al., 1998) (Figure 5A). Hybridisation of the RNA with the *ric1* probe showed that *ric1* is expressed in all stages. However, in sporangia and germinating cysts little *ric1* mRNA was detected when compared to the other stages. The highest level was observed in *in vitro*-grown old mycelium. Hybridisation with a probe derived from the constitutively expressed actin gene *actA* (Unkles et al., 1991) was performed to check whether equal amounts of RNA were loaded on the gel.

Expression analysis of *ric1* during *in planta* growth of *P. infestans* on detached potato leaves showed that *ric1* mRNA is first detectable three days after inoculation (Figure 5A). When comparing this to the steady increase in the relative amount of actin mRNA in the total RNA samples in time, a continuous increase in the relative amount of *ric1* mRNA is observed. The relative amount of actin mRNA is considered as a measure for the *P. infestans* biomass in the infected leaf. Since *ric1* mRNA follows the pattern of actin mRNA, we conclude that *ric1* gene expression is not specifically up- or down-regulated during *in planta* growth.

To determine whether *ric1* gene expression is specifically induced during stress conditions, as has been described for its plant homologues *blt101* and *ESI3* (Goddard et al., 1993; Gulick et al., 1994), we subjected *P. infestans* cultures to various treatments that might be experienced as stress conditions. The treatments were applied for two hours and subsequently, total RNA was isolated, and the presence of *ric1* mRNA analysed by northern blot hybridisations (Figure 5B). Physical force like washing of a mycelial mat and transfer to a new Petri dish containing fresh medium did not result in an increase of *ric1* mRNA levels. Moreover, also transfer to water (starvation), or to medium containing heavy metal ions (Pb^{2+}) did not seem to

effect the *ric1* gene expression (data not shown), nor did cold treatment (4 °C) or heat shock (37 °C) (data not shown). Approximately a two fold increase in *ric1* mRNA levels (based on densitometric scanning of the signals) was noted in mycelia transferred to basic medium (pH 9), whereas hardly any increase could be observed after transfer to acidic medium (pH 4.5). An increase in *ric1* mRNA levels was also noted in mycelia exposed to high salt concentrations. In medium with a sodium-chloride concentration of 400 mM, a 60 % increase was observed within only two hours. These results demonstrate that *ric1* gene expression is up-regulated when *P. infestans* mycelium encounters stress conditions induced by high pH or salt.

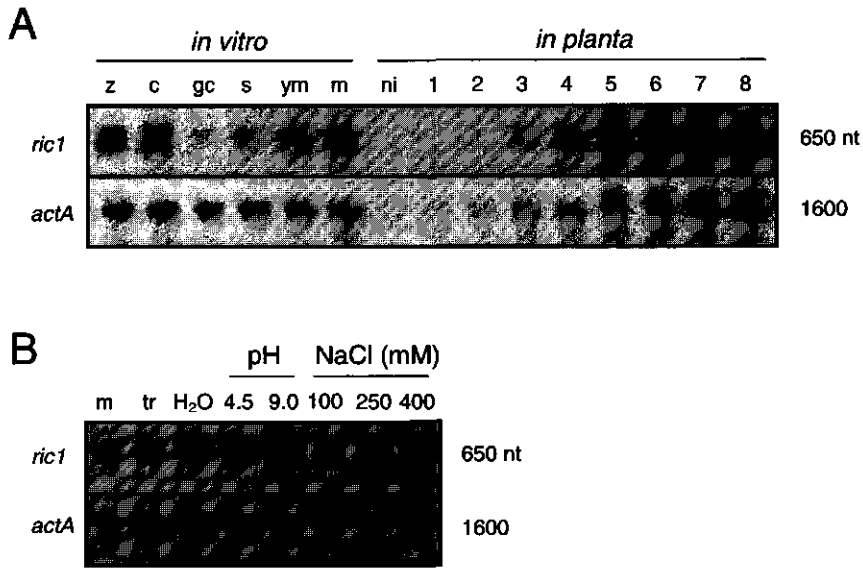

Figure 5A. Expression of *ric1* During Growth and Differentiation of *P. infestans in Vitro* and *in Planta*
Autoradiograph of a northern blot containing total RNA (10 µg) isolated from *P. infestans* 88069 zoospores (z), cysts (c), cysts germinated in water for 2.5 hours (gc), sporangia (s), young mycelium (ym), mycelium (m), from potato leaves (cv Bintje) one to eight days after spot inoculation with *P. infestans* 88069 (1-8) and from non-inoculated potato leaves (ni), and hybridised with *ric1* and *actA* probes. Transcript lengths in nucleotides (nt) are indicated on the right.
(5B). Expression of *ric1* in *in vitro*-grown mycelium of *P. infestans* during various treatments.
Autoradiograph of a northern blot containing total RNA (10 µg) isolated from mycelium that was subjected to various stress treatments and hybridised with *ric1* and *actA* probes. The various treatments were: transfer of medium (tr), starvation in water (H_2O); acidic conditions, pH 4.5 (4.5); and basic conditions, pH 9 (9.0); salt 100 mM NaCl (100), 250 mM NaCl (250) and 400 mM NaCl (400); non-treated mycelium (m). Transcript lengths in nucleotides (nt) are indicated on the right.

Discussion

In this study we describe the isolation and characterisation of the *Phytophthora infestans ric1* gene. Based on the presented cDNA sequences and the detailed genomic Southern blot studies, we assume that *P. infestans* has two copies of *ric1*, which are both expressed. The 171 bp open reading frame encodes a small protein of 57 amino acids with a predicted molecular mass of 6137 Da. The predicted RIC1 protein is a hydrophobic peptide with two putative membrane-spanning domains. This suggests that RIC1 is a membrane component.

Previously we reported the cloning of so-called *in planta*-induced (*ipi*) genes, using a differential screening procedure of a genomic library of *P. infestans* (Pieterse et al., 1993a). Two of these *ipi*-genes, *ubi3R* and *calA*, encode polyubiquitin and calmodulin, respectively (Pieterse et al., 1991; 1993b). *Ubi3R* and *calA* are expressed during *in vitro* growth of mycelia but expression increases significantly during the interaction with host plants when *P. infestans* grows *in planta*. One other *ipi* gene, *ipiO*, is not expressed *in vitro* in mycelium but is highly expressed *in planta* during the (pre-)infection stages and in the tip of invading hyphae during colonisation of the leaf (Pieterse et al., 1994a; van West et al., 1998). The selection procedure that was followed for the isolation of the *ipi*-genes was specifically designed to identify *P. infestans* genes of which the expression is considerably induced during growth on the host. Here, however, we employed an unbiased random selection procedure of expressed genes. Since the cDNA library was derived from infected potato leaves, the genes represent *in planta*-expressed genes. Twenty-two cDNA clones were analysed of which five seemed to originate from the same gene, *ric1*. Expression studies showed that *in vitro, ric1* is predominantly expressed in mycelial stages of the life cycle of *P. infestans*. Low expression was observed in sporangia and germinating cysts. *Ric1* expression increased in young mycelium and reached its highest level in *in vitro*-grown old mycelium. *In planta* expression studies demonstrated that *ric1* gene expression is not specifically increased during growth of *P. infestans* in potato leaves. Hence *ric1* is not an *in planta*-induced gene.

Based on cross-hybridisation studies we conclude that most *Phytophthora* species contain *ric1* homologues. However, *ric1* is not only conserved within the genus *Phytophthora*. Also plants, *C. elegans, S. cerevisiae*, and some prokaryotes contain highly similar homologues. Based on the amino acid alignments, we conclude that RIC1 is closely related to a putative peptide present in plants. Four genes from three different plants species, *blt101*, *ESI3*, RC12A and RC12B encode similar peptides and expression of these genes is induced upon stress (Goddard et al., 1993; Gulick et al., 1994; Capel et al., 1997). Also expression of *ric1* is induced upon stress. The basal level of *ric1* expression in mycelial growth stages throughout the life cycle of *P. infestans* increases considerably when the mycelium is exposed to high salt or high pH. Whether *P. infestans* encounters these conditions during growth *in planta* is

unknown. However, it is likely that pH changes and osmotic changes occur during colonisation of the leaf due to defence responses or leakage of degrading leaf cells in the *inter* and *extra* cellular spaces. We hypothesise that RIC1 is a structural protein that is necessary to maintain membrane integrity, especially during unfavourable conditions. Now that targeted gene silencing in *P. infestans* is feasible (van West et al., 1999), future studies with *ric1* silenced transformants may elucidate the function of the putative RIC1 peptide.

Experimental Procedures

Phytophthora Strains and Culture Conditions

A Dutch *P. infestans* strain 88069 was used throughout this study. This isolate was routinely grown in the dark on rye agar medium supplemented with 2% (w/v) sucrose (RS-medium) (Caten & Jinks, 1968) at 18 °C. The following *Phytophthora* species and strains were analysed for the presence of *ric1* homologous sequences in their genome: *P. clandestina* (UQ 3085), *P. cactorum* (0436), *P. pseudotsugae* (CBS 444.84, PD 95/9141), *P. idaei* (PD 94/959), *P. tentaculata* (CBS 412.96), *P. parasitica* (USA 1751), *P. palmivora* (PD 93/56), *P. megakarya* (UQ 2822), *P. porri* (HH), *P. infestans* (88069), *P. mirabilis* (CBS 150.88), *P. phaseoli* (CBS 556.88), *P. ilicis* (PD 91/595), *P. megasperma* f. sp. *glycinea* (UQ 60), *P. vignae* (20853), and *P. cinnamomi* (2). Mycelium for isolation of DNA and RNA was obtained by growing the *Phytophthora* strains in liquid RS medium for five to ten days. Plant assays to obtain infected potato leaves were performed as described by van West et al. (1998).

Selection of *P. infestans* cDNA Clones

Construction of the λZAP cDNA library made from poly(A^+) RNA extracted from *P. infestans*-infected potato leaves (cv Ajax), three days after spray inoculation, has been described by Pieterse et al. (1994b). Recombinant bacteriophages were plated and incubated according to the Stratagene λZAP manual. Of the resulting plaques two replicas were made on Hybond N^+ membranes and bacteriophage DNA was released according to the manufacturers instructions (Amersham International plc., Little Chalfont, Buckinghamshire, UK). [α-^{32}P]dATP labelled probes used for hybridisation were synthesised on poly(A^+) RNA isolated from *in vitro*-grown mycelia of *P. infestans* and from poly(A^+) RNA isolated from non-infected potato leaves using the Random Primed DNA Labelling System (Gibco BRL, Gaithesburg, MD, USA). The hybridisation patterns were compared. Twenty-two random phages that hybridised to the *P. infestans* probe and not to the potato probe, were propagated and cDNA clones were obtained by *in vivo* excision according to the Stratagene λZAP manual.

RNA and DNA Manipulations

RNA isolation and northern blot analyses were performed as described by van West et al. (1998). Genomic DNA of *Phytophthora* strains and potato (cv Ajax) was isolated as described by Raeder & Broda (1985). Southern blot hybridisations were performed as described by van West et al. (1998). Heterologous hybridisations were performed at 58 °C and the blots were washed to 2X SSC and 0.5% SDS. Dideoxy chain-termination sequencing was carried out using an AmpliCycle sequencing kit (Perkin Elmer, Foster City, CA). Sequence analysis was performed using software DNA Strider 1.0 (C. Marck Institute de Recherche Fondamentale, France). Database homology searches were conducted using BLAST software package as available through the Internet. The amino acid sequence was characterised using the Baylor College Medicine Houston TX internet software package.

DNA Probes

DNA templates for probe synthesis were the 671 bp *Eco*RI-*Xho*I insert of the *ric1* cDNA from pPi109, and a 796 bp *Hin*dIII fragment from pSTA31 containing the coding region of the *P. infestans* actin gene, *actA* (Unkles et al., 1991). All DNA templates were gel purified with the Qiaex II Agarose Gel Extraction kit (Qiagen GmbH., Hilden, Germany). The probes were radiolabelled with [α-^{32}P]dATP by

using the Random Primed DNA Labelling System (Gibco BRL). To remove non-incorporated nucleotides, the Qiaquick Nucleotide Removal Kit (Qiagen GmbH.) was used.

Stress Conditions

An agar plug with sporulating mycelium was transferred to a Petri dish containing liquid RS-medium. After five days of growth at 18 °C, the mycelium was subjected to various treatments for exactly two hours. After the treatment, the mycelium was collected for extraction of total RNA. The various treatments were: (I) mycelium washed in water and subsequently starved in water; (II) mycelium washed with and incubated in RS medium of pH 4.5 or pH 9.0 (the pH of RS-medium is approximately 6.75); (III) mycelium washed with and incubated in RS-medium containing 100 mM, 250 mM or 400 mM NaCl; (IV) mycelium washed with RS-medium and incubated at 4 °C or 37 °C; (V) mycelium washed with and incubated in RS-medium containing 60 ppm $Pb_3(C_6H_5O_7)_2$; and as controls, (VI) untreated mycelium, and (VII) mycelium washed with, and incubated in RS-medium. All washing steps were performed three times.

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Chapter 8

General Discussion

Phytophthora is an important genus of plant pathogens within the class of the oomycetes. Many oomycetes, such as the potato late blight pathogen *Phytophthora infestans* and the forest dieback pathogen *Phytophthora cinnamomi*, are responsible for destructive crop epidemics and destabilising terrestrial ecosystems, respectively (Erwin & Ribeiro, 1996a). The impact of *Phytophthora* on food and fibre production is of increasing concern. Economic damage to crops caused by *Phytophthora* species, already in the United States alone, amounts billions of dollars annually, and world wide the economic losses are manifold (Erwin & Ribeiro, 1996a). Global losses in potato production caused by *Phytophthora infestans* and expenses to control the disease are estimated to be $3 billion annually (Duncan, 1999).

Unfortunately, at present, there are only a few measures available to control *P. infestans*. These include host resistance breeding, chemical control, and prevention by cultural and biological means. However, in many instances, these control measures are no longer effective. For example, resistance in potato to *P. infestans* was thought to be accomplished by introducing single-dominant resistance genes from wild potato species into commercial cultivars by extensive breeding programs. Initially, it was thought that monogenic, race-specific resistance or vertical resistance would successfully control late blight. In practice, however, single gene resistance has been rapidly overcome by the development of new races of *P. infestans* (Erwin & Ribeiro, 1996b). An example of a chemical control measure that is no longer effective is metalaxyl, a phenylamide formerly used to control various downy mildews, *Phytophthora* and *Pythium* species. Many *P. infestans* isolates have become resistant to this chemical (Davidse et al., 1983; 1991). Biological and cultural control measures, such as antagonistic fungi, sanitation, and crop rotation can sometimes be effective in preventing spread of diseases caused by *Phytophthora*. However, in the case of late blight, crop rotation has become less effective after appearance of a new population of *P. infestans* consisting of both A1 and A2 mating types. Presently, sexual reproduction can occur in the field, resulting in the formation of oospores, which are able to remain viable in the soil for several years and serve as a serious inoculum source (Drenth et al., 1995; Zwankhuizen et al., 1998a; 1998b).

From all the control measures against *P. infestans*, host resistance in combination with chemical control is at present regarded as the most successful strategy (Erwin & Ribeiro, 1996b). Nowadays, the emphasis in potato breeding is mainly on the development of non-race specific or horizontal resistance. This type of resistance, which is assumed to be based on multiple resistance genes, seems to be more stable than vertical resistance. However, it does not prevent infection. It is supposed to be effective against all pathogenic races by slowing down the rate of infection and disease development. Hence, chemical control is still needed.

In a world in which people have become increasingly aware of their responsibilities for the environment, it is apparent that chemical crop protection is not the best option for the future. What alternatives are there? In order to answer this question, we need

to explore the *P. infestans*-plant interaction in detail. Basic biological knowledge of *P. infestans* is essential in order to come up with new control strategies. Despite extensive studies on various aspects of the interaction, there are many gaps in our understanding of the molecular events that take place during the interaction. For example, the molecular basis of pathogenicity has hardly been studied. Processes involved in molecular signalling leading to compatibility or incompatibility are unknown, and the molecular bases for non-host resistance have yet to be identified.

A main reason for the lack of basic knowledge of *P. infestans* and other oomycetes is that the development of efficient genetic and molecular biological tools has been slow. Only in 1991, a $CaCl_2$- and polyethylene-glycol-based transformation protocol was developed (Judelson et al., 1991). At that time, transformation and even directed mutagenesis was already demonstrated in some plant pathogenic ascomycetes and basidiomycetes (Kronstad et al., 1989; Stahl & Schafer, 1992). Furthermore, the fact that *P. infestans* is not only taxonomically but also genetically and biologically distinct from ascomycetes and basidiomycetes, implies that studies in true fungi will not necessarily advance the understanding of *P. infestans* (Judelson, 1997b). For example, traditional mutagenesis approaches are not feasible in *P. infestans*, and therefore, alternative strategies need to be investigated to accomplish mutagenesis.

Besides the optimisation of molecular techniques to study *P. infestans*, we also need to identify genes that are important during several stages of pathogenesis. In this thesis we present the characterisation of *in planta*-induced genes which were isolated using a differential hybridisation procedure (Chapter 2). However, other strategies to clone important genes of *P. infestans* are being employed as well. For example, positional cloning is currently being used to identify avirulence genes (van der Lee et al., 1997; 1999), and mating type genes in *P. infestans* (Fabritius & Judelson, 1997). Another method to isolate genes of *P. infestans* is to sequence cDNA clones randomly. These partial sequences are often referred to as expressed sequence tags (EST's). Recently, one thousand cDNAs from a mycelial cell stage of *P. infestans* were sequenced in an EST sequencing project (Kamoun et al., 1999a). It will be extremely informative to get EST's of other developmental stages as well. Furthermore, proteomics which is based on the identification of protein spots on 2D gels, is employed to identify stage specific proteins in *P. infestans* (P. van West & N.A.R. Gow, unpublished data).

The research described in this thesis, involves successful attempts to develop and optimise tools to investigate *P. infestans* at the molecular level. It encompasses the characterisation of four *P. infestans* genes, the optimisation of the β-glucuronidase reporter gene system for analysing gene expression in *P. infestans*, and the development of targeted mutagenesis that allows the functional analysis of genes in *P. infestans*. The mutagenesis approach was based on homology-dependent gene silencing and it resulted in the discovery of an exciting new phenomenon. Below, our

major findings and their implications for future research on *P. infestans* and other oomycetes are discussed.

The *ipiB* Gene Cluster

IpiB was isolated by differential screening of a genomic library of *P. infestans* with probes derived from mRNA isolated from infected leaves, and mRNA isolated from *in vitro*-cultured *P. infestans*. The *ipiB* locus appeared to contain three genes that were designated *ipiB1*, *ipiB2* and *ipiB3* (Chapter 2). They encode three similar glycine-rich proteins. The IPI-B proteins contain a putative signal peptide, suggesting that they are extracellularly located or targeted to a specific organelle. Based on peptide sequence analyses using the PSORT program of ExPaSy (www version 6.4), and assuming that *P. infestans* is closely related to plants, it is expected that the IPI-B proteins are targeted to the vacuole. However, when *P. infestans* is considered to be more closely related to animals, the extracellular matrix seems to be the most probable target site. N-glycosylation sites were not detected in the IPI-B proteins, but nine conceivable O-glycosylation sites were found in the C-terminal region, based on analyses using the NetOGlyc 2.0 software program of ExPaSy (unpublished observations).

Pieterse et al. (1994) demonstrated that the *ipiB* genes show a transient expression pattern, with highest mRNA levels present in germinated cysts, appressoria, and the early stages of infection. The latter was demonstrated by northern analysis of spray inoculated leaves. However, during *in planta* growth of *P. infestans* in spot-inoculated leaves (performed according to the method described in Chapter 3), *ipiB* expression was not detected (P. van West & F. Govers, unpublished data). Furthermore, detailed expression studies of stages prior to germinated cysts showed very high mRNA levels in zoospores and cysts (P. van West & F. Govers, unpublished data). *IpiB* expression was not observed in sporangia or mycelia.

From our studies we speculate that the IPI-B proteins are structural proteins associated with the cell wall, and are possibly involved in the development of early infection structures. Future studies in which the *ipiB* genes are silenced may provide more information concerning the role of the IPI-B proteins in, for example, appressoria formation. *P. infestans* transformants expressing a transcriptional fusion between the *ipiB* promoter and a reporter gene or constructs that encode tagged IPI-B proteins may also give more information on the putative IPI-B function and its location.

The *In Planta*-Induced Gene *ipiO*

A second gene that was obtained by differential screening is *ipiO*. In chapter 2, we described the characterisation of two closely linked copies of the *ipiO* genes, *ipiO1* and *ipiO2*, respectively. Recently, we cloned a third *ipiO* gene, *ipiO3*, from

P. infestans (P. van West & F. Govers, unpublished data). Whether *ipiO3* originates from a different allele or is a third independent member of the *ipiO* gene family needs to be investigated. The *ipiO* genes code for three almost identical proteins which do not have any significant homology with sequences present in data libraries. The three IPI-O proteins contain a putative signal peptide and most likely the IPI-O proteins are targeted to the extracellular matrix. The IPI-O proteins contain one N-glycosylation site and one putative O-glycosylation site. Whether IPI-O is indeed glycosylated is unknown. Furthermore, a putative cell attachment sequence consisting of the three amino acids arginine, glycine and aspartic acid (RGD) was identified. Additional studies to determine whether this RGD sequence indeed functions as a cell attachment sequence for the IPI-O protein are required. However, for these studies, an antiserum that allows detection of IPI-O by immunodetection is required.

Detailed expression studies revealed differential expression of *ipiO* in various developmental stages of the life cycle of *P. infestans* (Chapter 3). *IpiO* mRNA was detected only in zoospores, cysts, and germinating cysts. In disease lesions, *ipiO* mRNA was detected in the water-soaked area and the healthy-looking plant tissue surrounding it. Cytological assays on leaves colonised by *P. infestans* transformants expressing a transcriptional fusion between the *ipiO1* promoter and the β-glucuronidase (GUS) reporter gene allowed a more precise analysis of the spatial and temporal expression of *ipiO in planta*. GUS staining was specifically found in the subapical and vacuolated areas in tips of invading hyphae. From this, we conclude that *ipiO* is expressed during biotrophic stages of the infection process. Since the presence of a signal sequence suggests that the IPI-O protein is secreted, we hypothesise that IPI-O is localised at the interface between the invading hyphae and the plant cells, and thus possibly playing a role in pathogenicity.

We also investigated whether *ipiO* gene sequences are present in other *Phytophthora* species (P. van West & F. Govers, unpublished data). A wide variety of species was analysed by Southern blot hybridisations. Cross-hybridisation with *ipiO* was detected in several additional *Phytophthora* species that cause foliar blights such as: *P. mirabilis, P. ilicis,* and *P. phaseolus*. However, among ten *Phytophthora* species that cause mainly root or stem rot, only *P. cactorum* (Erwin & Ribeiro, 1996c), showed a clear hybridising band. These findings suggest that the IPI-O protein may have a particular function in leaf pathogens.

To determine unambiguously whether IPI-O acts as a pathogenicity factor in host-*P. infestans* interactions, we engineered *P. infestans* strains with the aim to obtain mutants deficient in IPI-O (P. van West & F. Govers, unpublished data). A sense and antisense mediated gene silencing strategy was employed, resulting in several transformants in which the *ipiO* mRNA levels in germinating cysts were reduced up to 95%. Pathogenicity tests showed that some transformants with reduced *ipiO* mRNA levels were less pathogenic on detached potato leaf assays. The transformants caused less disease lesions or the lesions expanded much slower. However, also a

few antisense transformants with normal *ipiO* mRNA levels in germinated cysts showed a similar reduced pathogenicity phenotype. Therefore, it was not possible to conclude that the reduced pathogenicity phenotype was indeed due to a lack of IPI-O. Maybe the transformation itself might have caused an overall loss of fitness. In order to establish whether or not IPI-O acts as a pathogenicity determinant, the level of IPI-O protein in the various transformants has to be determined and the correlation with the reduced pathogenicity phenotype has to be analysed.

Ric1, a Stress-Induced Gene

As an alternative approach to isolate genes that might be involved in the interaction we performed a small scale expressed sequence tag (EST) project (Chapter 7). Several *P. infestans* cDNA clones were randomly selected from a potato-*P. infestans* interaction cDNA library. Five of these clones appeared to be derived from the same gene, *ric1*. There are two copies of the *ric1* gene in *P. infestans* and these encode two identical putative proteins of 57 amino acids. RIC1 is a highly hydrophobic protein that does not have a signal peptide but has two potential membrane spanning domains. The predicted peptide shows high homology to a peptide encoded by plant genes whose expression is specifically induced during stress conditions (Goddard et al., 1993; Gulick et al., 1994; Capel et al., 1997). The *ric1* gene is expressed throughout the life cycle of *P. infestans*, but only small amounts of *ric1* mRNA were found in sporangia, zoospores and cysts. Expression of *ric1* increased considerably during stress conditions such as osmotic stress and high pH. Whether *P. infestans* encounters these conditions during growth *in planta*, is unknown. However, it is likely that pH changes and osmotic changes occur during colonisation of the leaf due to defence responses or leakage of solutes from leaf cell cytoplasm in the inter- and extracellular spaces. We hypothesise that RIC1 is a structural protein that is necessary to maintain membrane integrity, especially during unfavourable conditions.

Inf1, a Species Specific Avirulence Gene

The *inf1* elicitin gene from *P. infestans* was cloned by heterologous hybridisation with a probe derived from the elicitin gene *parA1* from *P. parasitica*. *ParA1* was isolated via a reverse genetics approach (Kamoun et al., 1993a; Chapter 4). There are at least two copies of the *inf1* gene in the *P. infestans* genome. *Inf1* encodes a protein of 118 amino acids including a signal sequence of 20 amino acids. The signal protein targets the INF1 protein to the extracellular matrix. Detailed expression studies demonstrated that the *inf1* gene is down-regulated during the early stages of infection.

It has been shown that elicitins can induce a hypersensitive response (HR) in a restricted number of plants, particularly in the genus *Nicotiana* within the family of Solanaceae (Ricci et al., 1989; Kamoun et al., 1993b; Chapter 4). Therefore, it has been speculated that elicitins may function as plant species-specific avirulence

factors (Yu, 1995). To determine whether INF1 functions as an avirulence factor in interactions between *P. infestans* and *Nicotiana*, we engineered *P. infestans* strains deficient in INF1 production (Chapter 5 & 6). A homology-dependent gene silencing strategy was employed to inhibit INF1 production. In virulence assays, INF1-deficient strains remained pathogenic on the host plants potato and tomato. However, in contrast to the wild type and control transformant strains, INF1-deficient strains induced disease lesions and extensive sporulation when inoculated on *Nicotiana benthamiana*. These results demonstrate that recognition of INF1 elicitin leads to active resistance in *N. benthamiana* to *P. infestans* and that INF1 functions as an avirulence factor in this interaction. In other words, resistance in the non-host plant *N. benthamiana* against *P. infestans* is primarily based on the recognition of a single protein, INF1.

So far, only three species-specific avirulence genes have been cloned and characterised, which include the *inf1* gene from *P. infestans*, and two genes from the rice blast fungus *Magnaporthe grisea*, *PWL1* and *PWL2*, respectively (Kang et al., 1995; Sweigard et al., 1995). These *PWL* genes (pathogenicity towards weeping lovegrass) prevent *M. grisea* from causing disease on weeping lovegrass. The *PWL2* gene was isolated by positional cloning (Sweigard et al., 1995), whereas the *PWL1* gene was cloned by homology to *PWL2* (Kang et al., 1995). Both genes belong to a small gene family encoding hydrophilic proteins with a putative signal peptide, suggesting that the proteins are targeted to the extracellular matrix.

Also the *inf1* gene from *P. infestans* belongs to a gene family. Currently, eight elicitin genes have been identified in *P. infestans*. Two homologous elicitin genes, *inf2a* and *inf2b*, were isolated by heterologous hybridisation of a cDNA library from *P. infestans* (Kamoun et al., 1997). Four additional elicitin genes (*inf4-inf7*) were identified in an EST project described by Kamoun et al. (1999a). *Inf3* was obtained by PCR with degenerated primers (S. Kamoun & F. Govers unpublished data). Whether these new elicitins also induce a HR in some plant species, and play a role as avirulence determinants in *P. infestans*-plant interactions needs further investigation.

The biological function of elicitins is unknown (Kamoun et al. 1999b). However, recently it has been demonstrated that purified elicitins can bind a number of sterols, such as dehydroergosterol, suggesting that they could function as sterol carrier proteins during pathogenesis (Mikes et al., 1997; 1998; Boissy et al., 1999).

A future challenge is to identify the *N. benthamiana* resistance gene involved in this non-host interaction. Genetically engineered potato plants harbouring this resistance gene may result in durable resistance (Kamoun et al., 1999b). However, care must be taken, since such resistance would be based only on a single gene. Therefore, additional *R*-genes, such as partial resistance genes and non-host resistance genes need to be identified as well.

Tools to Study Genes and Gene Function in *P. infestans*

Without the molecular tool of homology-based gene silencing in *P. infestans*, we would not have been able to demonstrate conclusively that INF1 acts as an avirulence factor in the *N. benthamiana-P. infestans* interaction. This technique could only be developed after DNA transformation of *P. infestans* was established (Judelson et al., 1991). Now stable transformation procedures have been developed for several other oomycete species including *Phytophthora sojae* (Judelson et al., 1993a), *Saprolegnia monoïca* (Mort-Bontemps et al., 1997), *Phytophthora palmivora* (van West et al., 1999), and *Phytophthora parasitica* (Bottin et al., 1999). An additional transformation-based technology, that assists in characterising oomycete genes or in inheritance studies, involves introduction of reporter genes encoding β-glucuronidase, β-galactosidase, luciferase and green-fluorescent protein (Judelson, 1993; Judelson et al., 1997a; Chapter 3; Bottin et al., 1999; van West et al., 1999).

In many micro-organisms gene disruption is used to study gene function. However, gene disruption in *P. infestans* is hampered due to the diploid nature of oomycetes. Therefore, we decided to investigate gene silencing as a strategy to suppress expression of the endogenous *inf1* gene in *P. infestans* (Chapter 5). We transformed *P. infestans* with antisense and sense constructs of the *inf1* gene. *Inf1* mRNA or INF1 protein could not be detected in up to 20% of the transformants. Suppression was found in both antisense transformants (antisense-mediated gene silencing) and sense transformants (sense-mediated gene silencing or co-suppression). The silenced state of the *inf1* gene was shown to be mitotically stable under various conditions *in vitro* and *in planta* (Chapter 6). This indicates that gene silencing will enable us to analyse functions of *Phytophthora* genes which are thought to play an important role in the interaction with its hosts. Only a single transformation step was required to establish complete silencing of the *inf1* gene, whereas gene disruption in a diploid organism mostly requires multiple transformation steps, or screening for double mutants.

We anticipate that gene silencing will have broad applications in *Phytophthora* and oomycete research. It provides an excellent basis for future genetic studies by greatly facilitating functional analyses. We expect that antisense- and sense-mediated gene silencing will become essential tools to investigate putative open-reading frames obtained from oomycete genomic-based sequencing programs.

Internuclear Gene Silencing

The development of the gene silencing strategy led to the finding of a new silencing phenomenon, which we named internuclear gene silencing (Chapter 5). This new gene silencing process was found to be regulated at the transcriptional level without hyper-methylation of the *inf1* DNA sequence. We demonstrated that in heterokaryotic cells, *inf1*-silenced nuclei were able to induce *inf1* gene silencing in previously non-silenced nuclei. Therefore, we assume that a diffusible silencing factor is involved in

inducing stable transcriptional gene silencing and we hypothesise that this diffusible *trans*-acting silencing factor is either a protein, an aberrant RNA molecule or a complex consisting of RNA and protein.

For several gene silencing phenomena it has been suggested that they operate through an RNA intermediate (reviewed by Wassenegger & Pélissier, 1998). For example, Wassenegger et al. (1994) demonstrated that *de novo* methylation of DNA sequences can be directed by RNA, and Mette et al. (1999) demonstrated that the production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters *in trans*. Aberrant RNA has also been implicated in post-transcriptional gene silencing phenomena (reviewed by Wassenegger & Pélissier, 1998).

Our findings indicate that the internuclear gene silencing is a *trans*-inactivation phenomenon that shares similarities with paramutation. Paramutation is an epigenetic phenomenon involving either allelic interactions or interactions between homologous unlinked loci, e.g. endogenes and transgenes, and resulting in persistent changes in expression even after the interacting alleles or genes have segregated in the progeny (Meyer et al., 1993; Hollick et al., 1997). Paramutation and transcriptional gene silencing in plants are in general associated with methylation of DNA sequences. Also condensation of chromatin has been demonstrated to play a role in transcriptionally regulated silencing processes (Ye & Signer, 1996; van Blokland et al., 1997). We were not able to detect methylation of the *inf1* gene, and therefore, we speculate that maintenance of the transcriptionally silenced state is associated with chromatin condensation or heterochromatin formation of the *inf1* gene locus. Future experiments are needed to confirm or reject this possibility.

Previously, Judelson et al. (1993b) demonstrated that a β-glucuronidase (GUS) transgene in *P. infestans* could become inactivated after re-transformation with antisense GUS constructs. Effective inhibition of GUS activity was accomplished in transformants which had high levels of antisense mRNA. In our studies, we were not able to detect antisense *inf1* RNA in the *inf1*-silenced strains. This suggests that the mechanism that functions in the *inf1*-silenced transformants is different from the mechanism that causes inhibition of GUS activity in the antisense GUS transformants.

Another feature called spontaneous inactivation of transgenes in *P. infestans*, described by Judelson & Whittaker (1995), could well be a process similar to internuclear gene silencing. They found that during prolonged asexual propagation, transgenes became inactive in 1-3% of the examined transformants during one month of sub-culturing. In these strains, the transgenes were not deleted, methylated, or mutated. Spontaneous phenotypic variation in *P. infestans* has been described by many authors, but the processes underlying this variation are not known yet (Caten & Jinks, 1968; Denward, 1970; Le Grand-Pernot, 1986; 1988). Another possible example of spontaneous inactivation of an endogenous gene may

have been discovered by Kamoun et al. (1998), who found *P. infestans* strains in nature producing very low levels of INF1. It will be interesting to determine whether similar phenomena affecting expression of endogenous genes occur under natural conditions and generate phenotypic variation in *P. infestans*.

Future Perspectives

More than 150 years after the first late blight epidemic, we just begin to discover some of the mysterious processes occurring during infection and colonisation of potato by the notorious late blight pathogen *P. infestans*. Progress has been very slow indeed, but with the help of the molecular tools described in this thesis, we made a start to rapidly expand our basic biological knowledge on *P. infestans* in the next decade. We hope and anticipate, that future employment of these techniques will improve our understanding of the molecular and cellular processes involved in pathogenesis induced by *P. infestans*.

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Summary

The oomycete plant pathogen *Phytophthora infestans* is the causal agent of potato late blight. *P. infestans* is undoubtedly the best known and most studied *Phytophthora* species today. This is mainly because it is such a devastating pathogen that can cause complete destruction of a potato field in only a few days. In this thesis, we describe the characterisation of four *P. infestans* genes with presumed functions in pathogenicity and virulence, and the development of tools to study expression and function of two of these genes in *P. infestans*. The four genes are the *in planta*-induced genes *ipiB* and *ipiO*, the elicitin gene *inf1*, and the stress-induced gene *ric1*. We used the β-glucuronidase reporter gene for expression analysis of the *ipiO* gene, and homology-dependent gene silencing for functional analysis of the *inf1* gene. The latter resulted in the discovery of a new phenomenon, which we named internuclear gene silencing.

In Chapter 2, we describe the characterisation of the *in planta*-induced genes *ipiB* and *ipiO*. *IpiB* constitutes a gene family with at least three members, *ipiB1*, *ipiB2* and *ipiB3*, which are clustered in a head-to-tail arrangement. The *ipiB* genes are highly homologous throughout their promoter, coding and terminator sequences and encode three similar glycine-rich proteins of 301, 343 and 347 amino acids, respectively. The glycine-rich domains of the IPI-B proteins are predominantly composed of two repeats with the core sequences, A/V-G-A-G-L-Y-G-R and G-A-G-Y/V-G-G. The IPI-B proteins contain a putative signal peptide of 20 amino-acids, suggesting that the proteins are targeted to a specific organelle or to the outside of the cell. We speculate that the IPI-B proteins are structural proteins associated with the cell wall that are possibly involved in the development of infection structures. Also *ipiO* is a small gene family. Two members, *ipiO1* and *ipiO2* are closely linked and arranged in an inverted orientation. The *ipiO* genes encode almost identical 152 amino acid-proteins which do not have any significant homology with sequences present in data libraries. The IPI-O proteins contain a putative signal peptide which may target them to the extracellular matrix. A putative cell attachment sequence (RGD), functional in mammalian systems, was identified.

Expression of the *ipiO* gene was analysed during several developmental stages of the life cycle of *P. infestans* (Chapter 3). *IpiO* mRNA was detected in zoospores, cysts, and germinating cysts, but not in sporangia or in mycelia grown *in vitro*. *IpiO* is also expressed during colonisation of potato leaves. In disease lesions, *ipiO* mRNA was detected in the water-soaked area and the surrounding healthy-looking plant tissue. *IpiO* mRNA could not be detected in necrotised tissue and sporulating areas of a lesion. Cytological assays were performed to determine more precisely the location and time of *ipiO* gene expression *in planta*. *P. infestans* transformants expressing a transcriptional fusion between the *ipiO1* promoter and the β-glucuronidase (GUS) reporter gene showed that GUS staining was specifically found in the subapical and vacuolated area of tips of invading hyphae. Therefore, we concluded that *ipiO* is

expressed in hyphae during biotrophic stages of the infection process. We speculate that IPI-O has its function in the hyphal tips at the edge of the expanding lesion where the pathogen is invading healthy leaf cells. The IPI-O protein may well be localised at the interface between the invading hyphae and the plant cells, and could play a role in pathogenicity and/or virulence.

To isolate more genes that might be involved in the interaction between *P. infestans* and potato, we used a small scale expressed sequence tag (EST) approach (Chapter 7). Twenty-two *P. infestans* cDNA clones were randomly selected from a potato-*P. infestans* interaction cDNA library. Five of these clones appeared to be derived from the same gene, *ric1*. Two copies of the *ric1* gene were identified and both genes share 98% homology at the nucleotide sequence level and 100% at the amino acid level. The open reading frame predicts a small protein sequence of 57 amino acids. The highly hydrophobic protein has two potential membrane spanning domains. The deduced amino acid sequence shows high homology with three putative plant proteins encoded by genes of which the expression is specifically induced during stress conditions. Expression of *ric1* increased considerably during osmotic stress and at high pH. We hypothesise that RIC1 is a structural protein that is necessary to maintain membrane integrity, especially during unfavourable conditions.

When culturing various *Phytophthora* species in liquid medium, all examined species secrete highly abundant 10 kDa proteins which have been shown to elicit a hypersensitive response when injected into tobacco leaves. It was hypothesised previously that these 10 kDa proteins, collectively called elicitins, function as plant species specific avirulence factors. In order to test this hypothesis, we set out to clone the elicitin gene *inf1*, of *P. infestans* (Chapter 4) and subsequently generated mutants that no longer produced the INF1 protein (Chapter 5 & 6). An *inf1* cDNA was isolated by heterologous hybridisation of a potato-*P. infestans* interaction cDNA library using *parA1*, the gene encoding the major secreted elicitin of *P. parasitica*, as a probe. *Inf1* encodes a 118 amino acid protein including a 20 amino acid signal peptide. Detailed expression studies show that *inf1* is expressed in mycelium grown in various culture media, whereas expression was not detected in sporangia, zoospores, cysts and germinating cysts. The highest levels of expression of *inf1* are observed in *in vitro*-grown mycelium and *in planta* during the late stages of infection when profuse sporulation and leaf necrosis occur. Expression is, however, down-regulated during early biotrophic stages of the interaction.

To obtain *P. infestans* strains deficient in INF1 production, we explored a homology-dependent gene silencing strategy. Integrative transformation with antisense, sense and promoter-less constructs in *P. infestans* was performed to generate mutants. *Inf1* mRNA and INF1 protein could not be detected in up to 20%

of the transformants. The silenced state of the *inf1* gene was shown to be mitotically stable under various conditions *in vitro* and *in planta*. In pathogenicity assays, the INF1-deficient strains remained pathogenic on the host plants potato and tomato. However, in contrast to the wild type and control transformant strains, INF1-deficient strains induced also disease lesions and extensive sporulation when inoculated on *Nicotiana benthamiana*. These results demonstrate that recognition of INF1 elicitin leads to active resistance in *N. benthamiana* to *P. infestans* and that INF1 functions as a plant species-specific avirulence factor.

In Chapter 5, we describe experiments to identify the mechanism of silencing of the *inf1* gene in *P. infestans*. Nuclear run-on assays showed that *inf1* gene silencing is regulated at the transcriptional level. Interestingly, DNA methylation, a feature often associated with transcriptionally regulated gene silencing could not be detected in the *inf1* gene sequences. Heterokaryons obtained by somatic fusion of an *inf1*-silenced transgenic strain and a wild type strain displayed also stable gene silencing, demonstrating that *inf1* silencing is dominant and acts *in trans*. The *inf1* gene remained silenced in non-transgenic homokaryotic single zoospore isolates released from the silenced heterokaryons. Apparently, the presence of transgenes is not essential for maintaining the silenced status of the endogenous *inf1* gene. Karyogamy was not demonstrated and hence, it is unlikely that the silenced state of the *inf1* gene is transmitted from nucleus to nucleus by specific DNA-DNA interactions. Consequently, we propose a novel silencing phenomenon, called internuclear gene silencing, in which a diffusible silencing factor is involved in inducing stable gene silencing. We speculate that the proposed diffusible *trans*-acting silencing factor is either a protein, an aberrant RNA molecule or a complex consisting of RNA and protein. We envisage that such a molecule is transported from nucleus to nucleus where it may facilitate an inheritable change in *inf1*-expression possibly initiated by changes in chromatin structure of the target gene or regions surrounding the target gene. Internuclear gene silencing is clearly a *trans*-inactivation phenomenon that is reminiscent of paramutation.

In Chapter 8, we discuss how the results presented in this thesis will contribute to a better understanding of the biology and pathogenicity of *P. infestans*. On the long run, increased knowledge of this notorious pathogen will help in developing alternative methods to control potato late blight.

Samenvatting

Phytophthora infestans is de veroorzaker van de aardappelziekte. Jaarlijks zorgt deze ziekte wereldwijd voor een opbrengstderving van meer dan 4 miljard gulden. *P. infestans* lijkt morfologisch op een schimmel en de aardappelziekte wordt dan ook vaak aangeduid als een schimmelziekte. Het geslacht *Phytophthora* behoort echter tot de klasse van de oomyceten en in de huidige taxonomische indeling worden oomyceten ingedeeld in het rijk Protista en niet meer in het rijk der Fungi zoals voorheen. Dit betekent dat oomyceten nauwer verwant zijn aan algen dan aan schimmels.

Sinds de introductie van de aardappelziekte in Europa in het midden van de vorige eeuw is *P. infestans* onderwerp geweest van vele studies. Toch zijn er nog veel vragen onbeantwoord gebleven met betrekking tot de processen en de interacties die plaats vinden op het moleculaire niveau tussen de waardplant en *P. infestans*. Wanneer we meer weten over deze moleculaire processen kunnen we mogelijk, op basis van deze kennis, nieuwe bestrijdingsmethodieken en strategieën ontwikkelen.

Een manier om het moleculaire kat en muis spel tussen *P. infestans* en zijn waardplanten te onderzoeken, is het identificeren en karakteriseren van genen die betrokken zijn bij het infectieproces. Een gen is een DNA fragment dat erfelijke informatie bevat en de basis vormt voor de aanmaak van eiwitten. Eiwitten reguleren bepaalde processen in of buiten de cel en kunnen betrokken zijn bij de bouw, de structuur en het functioneren van een cel. Ongetwijfeld zullen er eiwitten en dus ook genen in *P. infestans* zijn, die een rol spelen in het ziekteproces en die dus bijdragen aan het tot stand komen van een succesvolle (compatibele) interactie met de plant. Daar staat tegenover dat een plant eiwitten zal hebben, die een succesvolle infectie kunnen verhinderen zodat de plant resistent is tegen *P. infestans* (incompatibele interactie).

We hebben verschillende methodes gebruikt om genen van *P. infestans* te isoleren die een rol kunnen spelen tijdens de interactie. Een van de strategieën was gebaseerd op de veronderstelling dat sommige genen van *P. infestans* alleen tijdens de interactie met de plant actief zijn. Mogelijk maken zulke genen eiwitten die essentieel zijn voor succesvolle infectie en kolonisatie van de waardplant. Zulke eiwitten worden ook wel pathogeniteits- of virulentie-factoren genoemd. In Hoofdstuk 2 beschrijven we de karakterisering van twee genen, *ipiB* en *ipiO*, waarvan de activiteit verhoogd is tijdens de interactie met de waardplant. We noemen deze genen daarom *in planta*-geïnduceerde (*ipi*) genen.

Het *ipiB* gen maakt het IPI-B eiwit dat relatief rijk is aan glycine. Dit soort eiwitten worden ook in de celwanden van plantencellen aangetroffen. Mede daarom denken we dat het IPI-B eiwit een soortgelijke functie heeft en dus waarschijnlijk uitgescheiden wordt door *P. infestans*, waar het zijn functie buiten de cel of in de celwand uitoefent.

Het *ipiO* gen is een *in planta*-geïnduceerd gen dat de basis vormt voor de aanmaak van een eiwit met een nog onbekende functie (Hoofdstuk 2 & 3). Genen die op *ipiO* lijken zijn nog niet in een ander organisme gevonden. Het *ipiO* gen is bijzonder actief in de top van koloniserende hyfen, ook wel schimmeldraden genoemd (Hoofdstuk 3). Dit hebben we kunnen aantonen met behulp van een marker gen, het β-glucuronidase gen, dat onder controle stond van de promoter van het *ipiO* gen. β-glucuronidase is een eiwit dat een blauwkleuring veroorzaakt op het moment dat de promoter van het *ipiO* gen actief is en het *ipiO* gen wordt afgelezen. We vermoeden dat *ipiO* een rol speelt in de ruimte tussen de plantencel en de hyfen van *P. infestans*.

Naast de gerichte speurtocht naar genen die specifiek actief zijn tijdens de interactie met de plant, hebben we ook een strategie gebruikt waarbij we een willekeurige set genen analyseren die actief is tijdens de interactie (Hoofdstuk 7). We wisten op voorhand echter niet of deze genen alleen tijdens de interactie actief zijn. Deze strategie heeft geresulteerd in de isolatie van *ric1*, een gen dat continu actief is met de aanmaak van een heel klein eiwitje. Echter tijdens stress condities, zoals hoge zuurgraad of hoge zoutconcentraties, neemt de activiteit van het gen aanzienlijk toe. Daarom noemen we het eiwit dat door het *ric1* gen gemaakt wordt een stress-geïnduceerd eiwit. Het eiwit bevat twee trans-membraan domeinen, hetgeen suggereert dat het waarschijnlijk een membraaneiwit is.

In Hoofdstuk 4 beschrijven we de isolatie van een gen van *P. infestans*, *inf1*, dat zorgt voor de aanmaak van het eiwit INF1. INF1 noemen we een elicitor omdat sommige planten, die INF1 kunnen herkennen, reageren met een overgevoeligheidsreactie. Een overgevoeligheidsreactie is als het ware een resistentiereactie van de plant die verdere groei van de binnendringende ziekteverwekker blokkeert. Infiltratie van het INF1 elicitor eiwit in bladeren van verschillende soorten tabak brengt een dergelijke overgevoeligheidsreactie teweeg. Omdat tabak normaliter geen waardplant is voor *P. infestans*, is het mogelijk dat resistentie in tabak veroorzaakt wordt door herkenning van INF1. In dat geval fungeert INF1 als een avirulentiefactor die herkend wordt door een plant die het bijbehorende resistentiegen heeft. Echter wanneer de plant dit resistentiegen niet heeft, of wanneer het plantenpathogeen de avirulentiefactor niet maakt, leidt de interactie tot een compatibele interactie: de plant wordt geïnfecteerd. Indien het elicitoreiwit herkend wordt door een bepaalde plantensoort, bijvoorbeeld tabak, maar niet door een andere plantensoort, bijvoorbeeld aardappel, spreken we van een soortspecifieke avirulentiefactor.

Om te onderzoeken of het INF1 eiwit van *P. infestans* door tabak wordt herkend als een soortspecifieke avirulentiefactor en waardoor tabak mogelijk resistent is tegen *P. infestans*, hebben we het *inf1* gen in *P. infestans* zodanig gemuteerd dat het INF1 eiwit niet meer gemaakt wordt (Hoofdstuk 5 & 6). Het bleek dat mutanten van

P. infestans die het INF1 eiwit niet meer maken in staat zijn om een bepaalde tabaksoort, *Nicotiana benthamiana*, volledig te infecteren, terwijl een *P. infestans* die normaal INF1 produceert dit niet kan. Dus het *inf1* gen maakt een soortspecifieke avirulentiefactor die *N. benthamiana* resistent maakt tegen *P. infestans* middels herkenning van deze factor.

Het maken van mutanten is van belang om de functie van een bepaald gen en dus van het bijbehorende eiwit te kunnen bestuderen. Bij de meeste schimmels en bacteriën is het gemakkelijk om mutanten te verkrijgen, maar bij *P. infestans* is dit moeilijk. Een belangrijke reden hiervoor is dat *P. infestans*, net als planten en dieren, van ieder gen meestal meerdere kopieën bezit. Je moet dus vaak meerdere genen tegelijk muteren om een effect te krijgen. Om dit probleem te omzeilen, hebben we een methode gebruikt waarbij we tegelijkertijd de activiteit van meerdere genen stil kunnen leggen (Hoofdstuk 5). Dit proces wordt gen-'silencing' genoemd en waarschijnlijk is dit in *P. infestans* gebaseerd op veranderingen in de DNA structuur van een gen.

Tijdens het karakteriseren van de mutanten waarin *inf1* genen werden uitgeschakeld, ontdekten we een fenomeen dat nog niet eerder is beschreven. Het is mogelijk dat *P. infestans* soms meerdere kernen per cel heeft. De kern van een cel bevat het DNA van de genen. Wanneer we kunstmatig één kern van een *inf1* mutant en één 'normale' of niet-gemuteerde kern bij elkaar brengen in één cel gebeurde er iets opmerkelijks. Het bleek dat de gemuteerde kern in staat is om de normale kern ook te muteren. Dit proces hebben we internucleaire gen-silencing genoemd. Hoe dit proces werkt in *P. infestans* is nog onbekend.

In Hoofdstuk 8 bediscussiëren we hoe het onderzoek dat is beschreven in dit proefschrift kan bijdragen aan een beter begrip van het ziekteproces dat door *P. infestans* veroorzaakt wordt. Op de lange termijn kunnen onze bevindingen alsmede nieuwe kennis verkregen over de aardappelziekteverwekker, mogelijk leiden tot de ontwikkeling van nieuwe bestrijdingsmethoden.

Nawoord

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Curriculum Vitae

Pieter van West werd geboren op 27 januari 1969 in de Bilt. In 1988 slaagde hij voor het eindexamen VWO aan de Christelijke Scholengemeenschap 'de Heertganck' te Heerde en in dat zelfde jaar begon hij met de studie plantenziektenkunde aan de Landbouwuniversiteit te Wageningen. De ingenieurstudie, die *cum laude* werd afgerond in november 1993, omvatte afstudeervakken bij de vakgroepen Plantencytologie en –morfologie en Fytopathologie. Een stageperiode werd doorgebracht op het Center for Engineering Plant Resistance Against Pathogens (CEPRAP), Davis, California, USA. Van december 1993 tot januari 1998 werkte hij bij de vakgroep Fytopathologie van de Landbouwuniversiteit Wageningen en de onderzoeksschool Experimentele Plantenwetenschappen aan het onderzoek dat in dit proefschrift beschreven is. Sinds 1 januari 1998 is hij als post-doc werkzaam bij het Department of Molecular and Cell Biology te Aberdeen, Schotland, Verenigd Koninkrijk.

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