Differential gene expression in *Phytophthora infestans* during pathogenesis on potato

Differentiële genexpressie in *Phytophthora infestans* tijdens de pathogenese op aardappel

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Proefschrift

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Stellingen

1. De relatief hoge mate van homologie tussen de aminozuurvolgorde van calmoduline van *Phytophthora infestans* en die van hogere eukaryoten, doet vermoeden dat oömyceten geen "echte" schimmels zijn, hetgeen tevens wordt bevestigd door fylogenetische studies.

   Dit proefschrift.
   Wainright *et al.* (1993). *Science* **260**: 340-342.
   Gunderson *et al.* (1987). *Proc. Natl. Acad. Sci. USA* **84**: 5823-5827.

2. De sequentie GCTCATTYYNCA(C/A)TTT speelt waarschijnlijk een rol bij de initiatie van transcriptie van genen van oömyceten.

   Dit proefschrift.

3. Expressie van pathogeniteitsgenen in bladpathogenen kan worden geïnduceerd onder limiterende nutriëntenvoorzieningen tijdens de vroege fasen van het infectieproces.

   Dit proefschrift.

4. De anti-sense RNA techniek biedt goede mogelijkheden voor de manipulatie van genexpressie in *Phytophthora infestans*.

   Dit proefschrift.

5. De tegenstrijdige bevindingen van Bailey *et al.* enerzijds en Judelson *et al.* anderzijds over het al dan niet functioneren van heterologe promoters in diverse *Phytophthora* soorten, doet vermoeden dat er verschillen bestaan in het mechanisme van genactivering binnen het geslacht *Phytophthora*.

   Bailey *et al.* (1991). *Nucleic Acids Research* **19**: 4273-4278.
   Bailey *et al.* (1993). *Current Genetics* **23**: 42-46.
   Judelson *et al.* (1991). *Molecular Plant-Microbe Interactions* **4**: 602-607.
   Judelson *et al.* (1992). *Molecular and General Genetics* **234**: 138-146.

6. De waarneming van Tooley en Carras dat chromosomen van *Phytophthora infestans* niet of nauwelijks migreren tijdens gelelectroforese, bevestigt het vermoeden dat het genoom van *P. infestans* aanzienlijk groter is dan dat van andere oömyceten.

Tooley en Carras (1992). *Experimental Mycology* **16**: 188-196.

7. De ontdekking dat ook in planten RGD-afhankelijke adhesieprocessen optreden, biedt perspectieven voor toekomstig onderzoek aan plant-pathogeen interacties.

Schindler *et al.* (1989). *Journal of Cell Biology* **108**: 1955-1965.
Zhu *et al.* (1993). *The Plant Journal* **3**: 637-646.

8. Calcium helpt vet om zeep.

9. Het zonder toestemming refereren naar andermans "unpublished results" kan er toe leiden dat er onzin wordt gepubliceerd.

Shaw (1991). *Advances in Plant Pathology* **7**: 131-170.

10. Wetenschappers worden graag aangehaald maar hebben niet per definitie een hoge aaibaarheidsfactor.

11. De uitspraak "druk druk druk" welke in toenemende mate wordt gebezigd door mensen met het zogenaamde TATT-syndroom, wekt de indruk dat men de beschikbare tijd niet efficiënt gebruikt.

12. Het bezit van een autoradio-cd-spelertasje werkt statusverhogend.

13. Wie de waarheid vertelt hoeft minder te onthouden.

Stellingen behorend bij het proefschrift:

Differential gene expression in *Phytophthora infestans*
during pathogenesis on potato

Wageningen, 27 oktober 1993   Corné Pieterse

Some cock their glasses up to their eye,
And mushrooms in the cells descry,
But we, my Lords, have looked as well,
And think such notions are a sell;
Decaisne in France, in Germany Kützing,
Have sought the rot all manner of roots in,
And proved that those have looked with a loose eye,
Who said 'twas caused by fungi and fuci.

Sure, never since the days of Plato,
Was there such a row about a rotten potato.

Lyric from "An anthology of the potato" by R. McKay (1961).

Contents

chapter 1

Scope of this thesis

Scope of this thesis

In 1843, 150 years ago, the potato late blight disease, caused by the fungus *Phytophthora infestans* (Mont.) de Bary, showed up in the northeastern part of the USA (Bourke, 1964). Two years later, the late blight epidemic devastated potato crops throughout northwestern Europe. The tremendous losses of the main source of food for millions of people led to a tragic famine in Ireland (Salaman, 1949), which not only caused the death of thousands of people but also resulted in the emigration of 1.5 million people from Ireland to the USA. The disastrous effect of plant diseases on human food supply greatly stimulated research on plant diseases. In 1861, after a long lasting controversy on the cause of the potato late blight epidemic (Bourke, 1991), de Bary proved experimentally that a fungus was responsible for the disease and named it *Phytophthora infestans* in 1876. In the past decades, various aspects of the potato-*P. infestans* interaction have been studied extensively (for overviews see Erwin *et al.*, 1983, Ingram and Williams, 1991, and Lucas *et al.*, 1991). Unfortunately, this has not yet solved the problem of late blight in potato. Despite the efforts of the breeders to develop resistant cultivars, a tight regime of fungicide application is still necessary to control the late blight disease. Merely in The Netherlands, the annual costs of late blight control are approximately 100 million Dutch guilders (Davidse *et al.*, 1989), indicating that the economic impact of the late blight disease worldwide is immense. Besides the fact that fungicide application can lead to the development of fungicide resistant isolates in the field (Davidse *et al.*, 1981), it also creates environmental concerns. In the era of environmental awareness, the need to develop safer, specialized methods to control plant diseases becomes stronger. Therefore, it is essential to have a profound understanding of the resistance mechanisms in potato as well as in the processes involved in pathogenicity of the fungus. Not only the economic importance but also the biological aspects of the disease stimulates the generation of detailed knowledge of the potato-*P. infestans* interaction. Such a specific plant-pathogen interaction provides a model system to study physiological and molecular aspects of plant defence, host-specificity, gene-for-gene interactions and fungal pathogenicity.

In the past decade, studies on the molecular aspects of the potato-*P. infestans* interaction were initiated by several research groups (chapter 2). Especially the molecular processes involved in the defence response of potato have been subject of many studies. In 1988, when the research described in this thesis started, molecular biological research on oomycetous fungi was still in its infancy. Hence, the molecular basis underlying pathogenicity of *P. infestans* was hardly studied. General pathogenicity factors, such as phytotoxins and cell wall degrading enzymes, had been suggested to play a role in pathogenesis but their importance in disease development has never been established convincingly.

In this thesis, a novel and unbiased approach is described to study the molecular processes involved in pathogenicity of plant pathogenic fungi (chapter 3). It is based on the assumption that, by analogy with several other plant-microbe interactions, the expression of genes which are necessary for the establishment and maintenance of a compatible interaction

is specifically induced or significantly increased upon infection. These *in planta* induced genes, which encode putative pathogenicity factors, are selected by differential screening of a genomic library of *P. infestans* DNA. Characterization of the corresponding gene products and their function during pathogenesis may lead to the identification of new pathogenicity factors. The strategy does not prejudge the biochemical nature of possible pathogenicity factors and thus allows the isolation of pathogenicity genes of which the involvement in pathogenesis can not be predicted by other methods. Although the technique of differential screening of DNA libraries is not new, it was never utilized to isolate putative pathogenicity genes from plant pathogenic fungi.

By differential screening of a genomic library of *P. infestans* DNA, nine *in planta* induced genes of *P. infestans* were isolated (chapter 3). Four of these genes have been characterized, e.g. *ubi3R* encoding polyubiquitin (chapter 4), *calA* encoding calmodulin (chapter 5), and *ipiB* and *ipiO* (chapter 6 and 7). The *ipiB* and *ipiO* genes are both members of small gene clusters and code for novel proteins.

To study the function and regulation of these genes, a DNA-mediated transformation system is a prerequisite. In 1991, the first successful transformation of *P. infestans* was described by Judelson *et al.* This transformation procedure was used to analyze the activity of the promoters of the *ubi3R* gene and various other oomycetous genes and to test a strategy for manipulating gene expression in *P. infestans* by antisense inhibition (chapter 8).

The mechanism of transcriptional activation of the characterized *P. infestans* genes and the possible role of their gene products in pathogenesis are discussed in chapter 9.

References

Bourke, P.M.A. (1964). Emergence of potato late blight, 1843-46. *Nature* **203**: 805-808.

Bourke, P.M.A. (1991). Potato late blight in Europe in 1845: the scientific controversy. *In*: Lucas, J.A., Shattock, R.C., Shaw, D.S. and Cooke, L.R. (Eds.), *Phytophthora*, pp. 12-24. Cambridge University Press, Cambridge.

Davidse, L.C., Looyen, D., Turkensteen, L.J. and van der Wal, D. (1981). Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in Dutch potato fields. *Netherlands Journal of Plant Pathology* **87**: 65-68.

Davidse, L.C., Henken, J., van Dalen, A., Jespers, A.B.K. and Mantel, B.C. (1989). Nine years of practical experience with phenylamide resistance in *Phytophthora infestans* in the Netherlands. *Netherlands Journal of Plant Pathology* **95**, supplement 1: 197-213.

de Bary, A. (1861). Die gegenwärtig herrschende kartoffelkrankheit, ihre ursache und ihre verhütung. Felix, Leipzig.

de Bary, A. (1876). Researches into the nature of the potato fungus, *Phytophthora infestans*. *Journal of the Royal Agricultural Society of England* **7**: 357-391.

Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.) (1983). *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. American Phytopathological Society, St. Paul, Minnesota.

Ingram, D.S. and Williams, P.H. (Eds.) (1991). Advances in Plant Pathology, volume 7; *Phytophthora infestans*, the Cause of Late Blight of Potato. Academic press, London, San Diego.

Judelson, H.S., Tyler, B.M. and Michelmore, R.W. (1991). Transformation of the oomycete pathogen, *Phytophthora infestans*. *Molecular Plant-Microbe Interactions* **4**: 602-607.

Lucas, J.A., Shattock, R.C., Shaw, D.S. and Cooke, L.R. (Eds.) (1991). *Phytophthora*. Cambridge University Press, Cambridge.

Salaman, R.N. (Ed.) (1949). The History and Social Influence of the Potato. Cambridge University Press, Cambridge.

chapter 2

Molecular aspects of the potato-*Phytophthora infestans* interaction

Introduction

Potato late blight caused by the fungus *Phytophthora infestans* (Mont.) de Bary (Peronosporales, Oomycetes) is one of the most important and devastating diseases of potato (*Solanum tuberosum* L.). The fungus attacks both foliage and tubers and is capable to rapidly develop and spread through host tissue causing a destructive necrosis. *P. infestans* is a hemibiotrophic pathogen with a rather narrow host range, all host plants being *Solanaceae*. From the crop plants, potato is economically the most important host but also tomato (*Lycopersicon esculentum* Mill.) can be infected. The specificity of *P. infestans* for *Solanaceae*, in particular potato, implies that there is a specific recognition mechanism between pathogen and host which consequently involves the exchange of signals between the two organisms. On the one hand, recognition of *P. infestans* by its host leads to the induction of defence responses directed against the pathogen. On the other hand, contact of the pathogen with a potential host activates a number of responses in the pathogen which seem to be involved in pathogenesis.

The extremely destructive nature of the potato late blight disease and its worldwide distribution make the potato-*P. infestans* interaction a favorite topic of research for many plant pathologists. Since the first account of the life history of the potato late blight fungus (Berkeley, 1846) and the description of its life cycle by de Bary (1863), who coined its name *Phytophthora* ("plant destroyer") *infestans* in 1876, various aspects of the potato-*P. infestans* interaction have been studied extensively (Erwin *et al.*, 1983; Ingram and Williams, 1991; Lucas *et al.*, 1991). In recent years, the tools of molecular biology and molecular genetics have been applied to study the potato-*P. infestans* interaction in greater detail. Here we review the current knowledge concerning the molecular and genetic aspects of this interaction.

The disease cycle

The disease cycle starts when a sporangium comes into contact with a potato leaf or tuber (Fig. 1). On moist surfaces, the sporangium germinates directly or develops into a zoosporangium with motile biflagellate zoospores which germinate after encystment. Germinating sporangia and cysts form appressoria, and infection tubes emerging from the appressoria penetrate the host cells (Pristou and Gallegly, 1954). In leaves, penetration occurs preferably in the region of stomata, immediately adjacent to stomatal guard cells (Gees and Hohl, 1988). The infection tubes enter the epidermal cell and form hyphal structures. Subsequently, the hyphae grow into the mesophyll cell layers, both intra- and intercellularly, occasionally forming haustoria (Coffey and Wilson, 1983). When the leaf tissue is fully colonized, sporangiophores emerge from the stomata forming massive amounts of sporangia which can be dispersed by wind or rain and eventually reinfect plants nearby or over long distances. In leaves, the first macroscopic sign of lesion development is the appearance of water-soaked areas. While the fungus grows and sporulates at the advancing
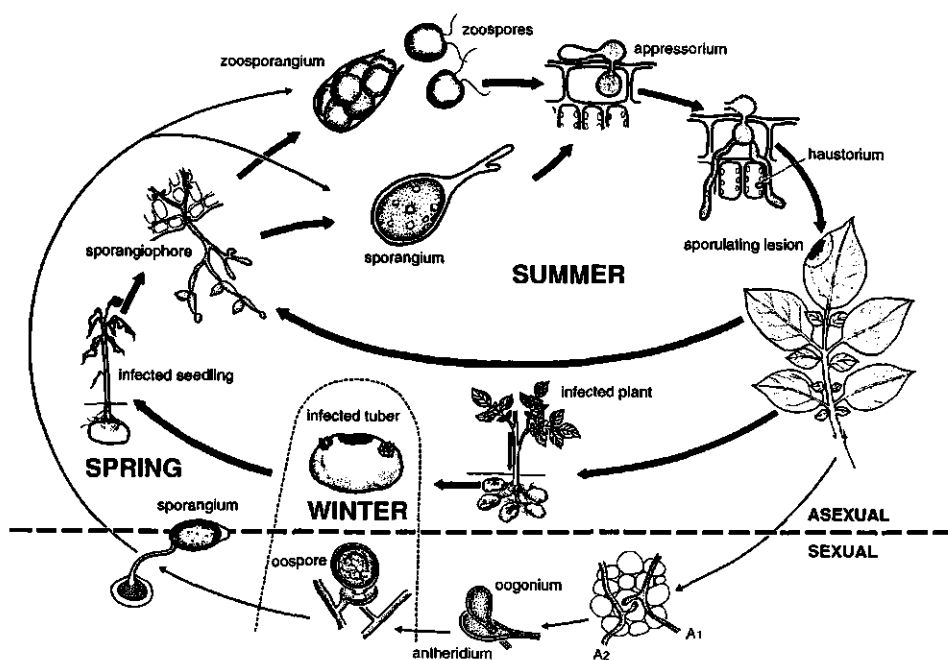
Figure 1. Sexual and vegetative life cycle of the potato late blight fungus *Phytophthora infestans*.

edges of the developing lesion, the centre of the spreading lesion becomes necrotic, eventually leading to complete destruction of foliage and consequently in reduction of tuber yields. Tuber infection itself can also cause severe losses, especially during storage.

Although in nature *P. infestans* reproduces mainly asexually, sexual reproduction has been described as well (Niederhauser, 1956; Smoot *et al.*, 1958). *P. infestans* is a heterothallic organism with two known mating types, A1 and A2, which are both required for sexual reproduction. When thalli of opposite mating types meet, the formation of male and female reproductive organs, antheridia and oogonia, is induced (Fig. 1). The oogonium grows through the antheridium and develops after fertilization into a thick walled sexual spore, the zygotic oospore which is able to survive for long periods in the soil under extreme conditions (Elliot, 1983). Until 1980, central Mexico, the centre of origin of *P. infestans*, was the only site where oospores of the fungus were found in nature. In all other regions where potato late blight occurs only isolates of the A1 mating type were present. In the late seventies, the *P. infestans* population in Europe was displaced by a new population consisting of A1 and A2 mating types (Spielman *et al.*, 1991; Fry *et al.*, 1992; 1993). Recently, A. Drenth in our laboratory demonstrated that also in The Netherlands oospore formation can occur in the field (A. Drenth, unpublished). Presumably sexual reproduction is one of the causes of the drastic increase in genetic variation which has been observed in Dutch

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P. infestans isolates that were collected in the last decade (A. Drenth, unpublished). Sexual reproduction may lead to an acceleration of evolutionary adaptation of the fungal population to resistance introduced in potato cultivars or to fungicides. Therefore, the elucidation of the contribution of oospores to the development of late blight epidemics is of great importance.

The genetic basis of host resistance to *Phytophthora infestans*

Genetically controlled disease resistance to plant pathogens can be classified as either race-specific of race-nonspecific. By classical breeding, both types of resistance to *P. infestans* have been introduced into potato (Wastie, 1991).

Race-specific resistance. In the potato-*P. infestans* pathosystem, race-specific resistance, also called vertical resistance, is based on a gene-for-gene relationship (Black *et al.*, 1953). The gene-for-gene concept was first proposed by Flor (1942; 1956) for the flax-*Melampsora lini* interaction and later formally defined by Person *et al.* (1962). To date, the gene-for-gene recognition system has been demonstrated or suggested for many plant-pathogen interactions (de Wit, 1992; Thompson and Burdon, 1992). In this recognition system, the products of avirulence genes of the pathogen, the so called race-specific elicitors, are thought to interact directly or indirectly with the products of the matching major resistance genes (R-genes) of the plant (de Wit, 1991; 1992). This results in triggering of a hypersensitive response (HR) which is characterized by rapid cell death of primary invaded host cells and a limited number of cells surrounding the infection site (Tomiyama, 1963). In this way, the biotrophic fungus is arrested in growth and remains confined to the focus of infection resulting in an incompatible interaction. Races lacking functional avirulence genes corresponding to R-genes in the host do not trigger a HR and are able to colonize the plant successfully, resulting in a compatible interaction.

Little is known about the genetic and molecular basis of avirulence/virulence in *P. infestans* and race-specific resistance/susceptibility in potato. In most plant pathogens avirulence is dominant over virulence but there are exceptions (Shaw, 1991). Spielman *et al.* (1989; 1990), who analyzed the segregation of avirulence loci in the progeny of genetic crosses, showed that in *P. infestans* avirulence towards the potato resistance genes R3 and the tomato resistance gene Ph1 is dominant. On the other hand they found that avirulence towards R2 and R4 inherits as a recessive treat. Whether dominant inhibitor genes, which mask the expression of avirulence, are involved is not known. Race-specific resistance, governed by the R-genes, is single gene based (Mastenbroek, 1953; Malcolmson and Black, 1966). To date, 11 dominant R-genes, all originating from *Solanum demissum* Lindl., have been introduced into the cultivated potato by breeding (Wastie, 1991). Mapping of some of these R-genes in potato and the corresponding avirulence genes in *P. infestans* on molecular genetic linkage maps is in progress in several laboratories. For *P. infestans* this work is in its infancy (Dyer *et al.*, 1992) but for potato, a detailed genetic linkage map containing more than 300 restriction fragment length polymorphism (RFLP) loci has been constructed

(Gebhardt *et al.*, 1989; 1991). Recently, Gebhardt and coworkers have determined the map position of resistance genes R1 (Leonards-Schippers *et al.*, 1992) and R3 (A. El-Khrbotly, personal communication), which is a first step towards the isolation of R-genes from potato by a map based cloning approach. An alternative way to clone R-genes from potato is by means of transposon tagging, an approach recently initiated by Pereira *et al.* (1992). The isolation and characterization of R-genes from potato and the corresponding avirulence genes from *P. infestans* will lead to a better understanding of the mechanisms involved in race-specific resistance and in the induction of the defence responses.

Race-nonspecific resistance. In contrast to race-specific resistance where a single R-gene is sufficient to protect the plant completely against particular races of *P. infestans*, race-nonspecific resistance, also called horizontal resistance or field resistance, is assumed to be multiple gene based (Umaerus *et al.*, 1983). In race-nonspecific resistant cultivars, the rate of spread of the disease through the crop is reduced due to a combined set of many parameters such as lower infection efficiency, slower tissue colonization and reduced sporulation rate (Umaerus, 1970). The level of field resistance varies among cultivars but is effective against all races of *P. infestans*.

Race-nonspecific resistance is durable and thus commercially more attractive than race-specific resistance. In the latter, new virulent races evolve rapidly, rendering the R-genes ineffective. Presently, breeding efforts aim at introducing durable field resistance into commercial potato cultivars. But since the molecular and genetic basis underlying race-nonspecific resistance is basically unknown, breeders still have to rely on empirical methods. However, this may change in the near future. In attempts to localize quantitative trait loci (QTLs) on the potato RFLP linkage map, Gebhardt and coworkers were able to identify six regions in the potato genome which contribute to quantitatively inherited resistance (C. Gebhardt, personal communication). Molecular markers linked to these QTLs can be very useful in breeding programmes aimed at selection of durable resistance.

The plant response

Cytology. The rate of development of the potato late blight disease is dependent on the aggressiveness of the pathogen and the defence response of the host. Spores of all races germinate with equal efficiency on resistant and susceptible cultivars and also the frequency of penetration is not significantly different (Gees and Hohl, 1988). From this point on, the development of the infection process is divergent. In a susceptible host, *P. infestans* spreads throughout the tissue intercellularly as well as transcellularly while in a resistant host the fungus remains confined to the focus of infection (Hohl and Suter, 1976). In compatible as well as incompatible interactions, the initially invaded host cells react hypersensitively and produce callose-like material within a few hours after inoculation (Cuypers and Hahlbrock, 1988). However, in the incompatible interaction a HR is triggered in epidermal and mesophyll cells adjacent to those invaded by *P. infestans* and they become necrotic within

5 hours after inoculation. In the compatible interaction, the cells do not necrotize within the first 12-24 hours (Cuypers and Hahlbrock, 1988). In general, it is obvious that in comparison to compatible interactions, in incompatible interactions host cells respond much faster to *P. infestans* attack by activating a HR. This leads to rapid browning and subsequent death of host cells in the vicinity of the invading pathogen shortly after penetration. As a result, further development of the biotrophic fungus is arrested. In a susceptible host, the rapid HR reaction does not occur. Apparently the fungus evades recognition by the host (Kombrink *et al.*, 1991).

Physiological and molecular aspects. Upon pathogen attack in both susceptible and resistant plants, a complex defence mechanism is activated which involves transcriptional activation of numerous defence-related genes (Collinge and Slusarenko, 1987; Hahlbrock *et al.*, 1989). This leads to synthesis and accumulation of pathogenesis-related (PR) proteins and secondary metabolites such as phytoalexins and lignin (Hahlbrock, 1987; Kombrink *et al.*, 1991).

Phytoalexins. Phytoalexins are antimicrobial molecules which are produced by the plant upon infection, elicitor treatment or wounding (Bailey and Mansfield, 1982). The phytoalexin concept was proposed by Müller and Börger (1941). Since then, the role of phytoalexins in plant defence has been studied extensively (Bailey and Mansfield, 1982). In potato tubers, phytoalexin production occurs primarily via the terpenoid biosynthesis pathway resulting in sesquiterpenes such as rishitin, phytuberin and lubimin (Kúc, 1982). Upon infection of potato tubers with *P. infestans*, sesquiterpenoid phytoalexins are produced in healthy tuber tissue surrounding the infection site and are then transported into browning cells where they accumulate to high concentrations (Sato *et al.*, 1971). Rohwer *et al.* (1987) studied phytoalexin accumulation in a compatible and an incompatible interaction. In potato tubers, they found a more rapid accumulation of sesquiterpenoid phytoalexins during the first 24-48 hours of infection in the incompatible interaction compared to the compatible one. In infected leaves sesquiterpenoid phytoalexins have not been detected. This suggests that at least in leaves, sesquiterpenoid phytoalexins are not necessary for defence against *P. infestans*. However, this does not rule out the possibility that other, as yet unknown, phytoalexins are produced in potato leaves.

One of the key enzymes in the sesquiterpenoid phytoalexin biosynthesis pathway is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), which catalyses the conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonic acid, the rate-limiting step in terpenoid biosynthesis (Bach, 1987). In potato tubers, HMGR activity increases in response to wounding and fungal infection (Oba *et al.*, 1985). Several research groups have shown that increased activity of HMGR in potato tubers upon pathogen challenge, wounding, or treatment with arachidonic acid, an elicitor of sesquiterpenoid phytoalexin synthesis (Bostock *et al.*, 1981), is due to induced gene expression (Choi *et al.*, 1992; Stermer *et al.*, 1991; Yang *et al.*, 1991). In potato HMGR is encoded by a small gene family of which distinct members are differentially activated in response to *P. infestans* infection and elicitor treatment, but are suppressed upon wounding (Choi *et al.*, 1992). This indicates that the

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signals leading to activation of HMGR genes upon pathogen challenge and wounding are diverged.

Phenylpropanoid pathway. Another major plant response to pathogen attack is the induction of expression of the phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4-CL) genes. PAL and 4-CL are both key enzymes in the phenylpropanoid pathway, the biosynthesis pathway of lignin and other wall-bound phenolics in the plant cell (Hahlbrock and Scheel, 1989). Accumulation of these phenylpropanoid compounds and their deposition in the cell wall is most likely involved in the rapid browning process of invaded host cells, a process which is associated with HR in incompatible interactions (Tomiyama, 1983). Fritzemeier *et al.* (1987) studied PAL and 4-CL gene expression in compatible and incompatible potato-*P. infestans* interactions and found that in incompatible interactions the expression of both genes is induced within several hours post-inoculation whereas in compatible interactions this is slightly delayed. This difference in timing of PAL mRNA accumulation was also demonstrated by Cuypers *et al.* (1988) who used the *in situ* hybridization technique. In an incompatible interaction, a clear halo of accumulating PAL mRNA around the penetration site appeared within 3 hours after inoculation and disappeared within the following three hours. In the compatible interaction, however, the halo of accumulating PAL mRNA did not appear earlier than six hours post-inoculation.

In potato, lignin and other soluble and wall-bound phenolics are the defence related endproducts of the phenylpropanoid pathway (Hahlbrock and Scheel, 1989). Ampomah and Friend (1988) found a correlation between PAL activity, deposition of lignin or lignin-like material and susceptibility to *P. infestans* infection in potato tuber discs. In tuber discs of cultivars with a high degree of field resistance more lignin and lignin-like material was deposited than in tuber discs of susceptible cultivars. Treatment with an inhibitor of PAL decreased the deposition of lignin and lignin-like material and the tuber discs became more susceptible to fungal infection. Thus the endproducts of the phenylpropanoid pathway may play a role in plant defence but their significance in the ultimate resistance reaction remains to be demonstrated.

Pathogenesis-related proteins. PR proteins are a characteristic group of plant proteins which accumulate after infection, elicitor treatment and sometimes during senescence. Most PR proteins share some characteristic properties, such as low molecular weight (M, ranging from 10,000-40,000), acid solubility, resistance to proteinases and predominant accumulation in the apoplastic space of the leaf (van Loon, 1985).

Kombrink *et al.* (1988) identified a number of PR proteins which accumulate in the intercellular space of potato leaves following inoculation with *P. infestans*. Eight of these proteins have been purified. Six appeared to have chitinase activity and two 1,3-ß-glucanase activity. Chitinases and 1,3-ß-glucanases have been suggested to be involved in the defence response of plants to pathogens since they are potentially able to degrade fungal cell walls (Boller *et al.*, 1983; Mauch *et al.*, 1988). Schröder *et al.* (1992) identified the temporal and spatial patterns of 1,3-ß-glucanase and chitinase induction in compatible and incompatible interactions by immunochemical and *in situ* hybridization methods. They showed that,

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compared to PAL and 4-CL, protein and mRNA accumulation of 1,3-ß-glucanase and chitinase is relatively slow, long lasting and systemically activated. In addition, no significant differences could be observed between compatible and incompatible interactions indicating that it is unlikely that the two hydrolases play a decisive role in race-specific resistance of potato.

In addition to genes encoding hydrolases, other pathogenesis-related genes have been isolated and characterized. Taylor *et al.* (1990) isolated a cDNA clone of a potato gene, designated *prp-1*, which shows induced expression very early after infection by *P. infestans* and which has a striking homology with a gene encoding a heat shock protein in soy-bean. The expression pattern of *prp-1*, which belongs to a small gene family, is similar to that of the PAL and 4-CL genes in both compatible and incompatible interactions. However, in contrast to other defence related genes, *prp-1* mRNA accumulates selectively upon fungal infection and not in response to abiotic environmental stimuli such as wounding (Martini *et al.*, 1993). This suggests that a specific signal, originating from the fungus and/or infected host cells causes this specific response (Martini *et al.*, 1993). One of the *prp-1* loci was mapped on the RFLP map of potato (Gebhardt *et al.*, 1991). Interestingly, one of the QTLs associated with horizontal resistance against *P. infestans* maps in the same region (C. Gebhardt, personal communication) suggesting that the *prp-1* gene product may be involved in this type of resistance. Matton and Brisson (1989) selected two other cDNA clones of potato genes encoding PR proteins. The expression of these genes reaches a maximum within 24 hours after elicitor treatment. The two proteins encoded by these genes differ by only six amino acids and show a high degree of similarity with PR protein sequences from pea and parsley.

The induced expression of genes encoding PR proteins and the subsequent accumulation of these proteins upon infection suggests that they are part of a plant defence mechanism. Some PR proteins, e.g. 1,3-ß-glucanases and chitinases are potentially able to degrade cell walls of fungi. A major component of the *P. infestans* cell wall is 1,3-ß-glucan but chitin is lacking. Therefore only 1,3-ß-glucanases are potentially capable of hydrolysing the cell wall of *P. infestans*. However, there is no direct evidence that these proteins, as well as the other identified PR proteins, are involved in resistance against *P. infestans*. The only protein that has been described to have inhibitory activity towards *P. infestans* is the thaumatin-like protein osmotin, which was isolated from tobacco and tomato (Woloshuk *et al.*, 1991). Purified osmotin causes lysis of sporangia and inhibition of hyphal growth *in vitro*, which indicates that osmotin might play a role in the plant defence mechanism. However, it still remains to be established whether or not this protein has the same effect *in vivo*.

In summary. In the incompatible interaction the plant reacts within a few hours with a very rapid and highly localized HR in the immediate vicinity of the invading fungus. To date, the molecular events causing HR are unknown. The HR is accompanied or immediately followed by a rapid, transient and local activation of various genes, e.g. PAL, 4-CL and *prp-1*, around the penetration site. Subsequently, there is another, relatively slow, long-lasting and systemic

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response, e.g. the activation of genes encoding several PR proteins, and the accumulation of phytoalexins (in tubers only). The rapid response may be aimed at an efficient localization and growth inhibition of the invading fungus, whereas the slower, long-lasting and systemically activated responses may facilitate protection against subsequent infections. In the compatible interaction similar genes are activated but there are differences in timing and site of expression. Whether these differences are decisive for the ultimate outcome of the resistance reaction is unknown. Also the relative contribution of the various synthesized compounds to the resistance reaction remains to be established. It is likely that a combination of compounds, produced at the right time and the right place, ultimately determines compatibility or incompatibility. However, it can not be ruled out that other, so far unknown, defence mechanisms are involved.

Pathogenicity of *Phytophthora infestans*

P. infestans belongs to the ecologically obligate biotrophs which means that the fungus normally does not exist in an independent mycelial form in the field (Lewis, 1973). Independent of the host, encysted zoospores can germinate in water and even form appressoria when an appropriate artificial surface is provided (E. Schmelzer, personal communication). In initial stages of the interaction, the fungus uses its food reserves. Once these reserves are exhausted, the host provides all the nutrients essential for growth and development of the fungus. To obtain these nutrients, *P. infestans* has to colonize the host tissue. Hence, the pathogen must possess the machinery required for growth and development in the host environment. For several fungal and bacterial plant pathogens it has been shown that they produce certain compounds which damage host cells and facilitate pathogen entry, in this way providing an optimal environment for growth and development (Keen and Yoshikawa, 1983). For example, cutinase produced by several plant pathogenic fungi, has been implicated in the enzymatic penetration of the host cuticle (Kolattukudy, 1985). In the obligate parasitic rust fungus *Uromyces viciae-fabae*, cutinase has been shown to be involved in the attachment of uredospores to the host cuticle (Deising *et al.*, 1992), an essential prepenetration process that determines the success of infection of several fungi (Nicholson and Epstein, 1991). In contrast, other investigations question the importance of cutinase in fungal penetration. Disruption of cutinase genes in *Nectria haematococca* (anamorph: *Fusarium solani* f.sp. *pisi*) (Stahl and Schäfer, 1992) and in the rice blast fungus *Magnaporthe grisea* (Sweigard *et al.*, 1992) did not reduce pathogenicity or virulence in comparison to wild type strains. To our knowledge, the involvement of cutinase in the potato-*P. infestans* interaction has never been studied. Moreover, for *P. infestans* it is unlikely that cutinase is required for penetration. *P. infestans* forms an appressorium and is able to penetrate host cells directly. Enzymatic breakdown of the cuticle and epidermal cell walls is probably not required.

In several phytopathogenic fungi and bacteria, pathogenesis is associated with the production of extracellular hydrolytic enzymes (Wood, 1967). Fungi typically have a

resorptive mode of nutrition uptake (Hohl, 1991). Therefore, hydrolytic extracellular enzymes are important prerequisites for growth on complex, high molecular weight substrates. The biotrophic nature of *P. infestans* suggests that the cell wall degrading enzyme system of the fungus is under tight control. Cell walls may be degraded to some extent to facilitate the fungal hyphae to grow through the tissue, but the host cells should not be destroyed. During growth of *P. infestans in vitro*, several hydrolytic enzymes are excreted in the culture medium (Moreau and Seibles, 1985). Several extracellular enzymes have been isolated and characterized. Among those are endocellulases, 1,3-ß-glucanases, ß-glucosidases, pectin-esterases, polygalacturonases and galactanases (Bodenmann *et al.*, 1985; Cole, 1970; Förster, 1988; Jarvis *et al.*, 1981), but none of these show massive cell wall degrading activity. In tubers, *P. infestans* produces a dry rot (Cole, 1970), which implies that cell walls do not contribute substantially to the nutrition of the pathogen (Hohl, 1991). This suggests that also extracellular hydrolytic enzymes do not play a decisive role in pathogenesis of *P. infestans*.

Phytotoxins are a diverse group of low molecular weight secondary metabolites which have been shown to be pathogenicity factors of several plant pathogens (Scheffer, 1976). Phytotoxic metabolites have been isolated from culture fluids of *P. infestans* (Seidel, 1961) but their importance in disease development still remains to be demonstrated. In view of the biotrophic nature of *P. infestans*, it is unlikely that phytotoxins play a decisive role in pathogenesis.

It is clear that the importance of the few potential pathogenicity factors studied so far in the potato-*P. infestans* interaction, has not been established convincingly. Besides, little is known about the molecular basis underlying pathogenicity of *P. infestans*. The specificity of *P. infestans* for solanaceous hosts cannot simply be explained by the action of general pathogenicity factors described above. This specificity implies a mechanism for recognition which involves the exchange of signals between host and pathogen. Successful colonization most likely requires the expression of particular pathogenicity genes, i.e. genes of the pathogen which are essential for the establishment and maintenance of a compatible interaction. It is feasible that, like in other symbiotic or pathogenic plant-microbe interactions, signal molecules originating from the host, or environmental stimuli present in the host, induce the expression of genes in the micro-organism which are necessary for the establishment of a successful interaction (Innes *et al.*, 1985; Peters and Verma, 1990; Stachel *et al.*, 1986; Willis *et al.*, 1991; Woloshuk and Kolattukudy, 1986). Hence, one approach to gain more insight in the molecular processes involved in pathogenesis, is based on the characterization of *P. infestans* genes which show induced expression during a compatible interaction with the host plant. Once these genes are isolated, the gene products can be identified and their role in pathogenicity can be studied. In our laboratory we isolated nine distinct *in planta* induced genes of *P. infestans* by differential screening of a genomic library (Pieterse *et al.*, 1993b; chapter 3). One of these *in planta* induced genes, designated *ubi3R*, appeared to encode polyubiquitin (Pieterse *et al.*, 1991; chapter 4). Another *in planta* induced gene, named *calA*, encodes calmodulin (Pieterse *et al.*, 1993d; chapter 5) whereas two other

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genes characterized so far, *ipiB* and *ipiO*, both belong to small, clustered gene families encoding glycine-rich proteins and as yet unknown proteins, respectively (Pieterse *et al.*, 1993a; 1993c; chapter 6; chapter 7). In the following chapters of this thesis the possible role of these *in planta* induced genes in pathogenicity will be discussed.

Molecular genetics of *Phytophthora infestans*

Until five years ago, molecular genetic approaches were not applied extensively on *P. infestans* nor any other plant pathogenic oomycete. The first report on the molecular cloning of an oomycete gene did not appear until 1989 (Judelson and Michelmore, 1989). The first successful transformation of a member of the oomycetes, the water mould *Achlya ambisexualis* J.R. Raper, was already reported in 1988 by Manavathu *et al.* However, detailed analyses of the transformants showed that the *APHII* gene, which confers resistance to the neomycin analogue G-418, was not stably integrated in the genome but was maintained on episomal DNA. Moreover, the SV40 promoter used to activate the *APHII* gene did not function correctly since transcription of the *APHII* gene appeared to be under the control of regulatory elements other than the SV40 promoter. Initial attempts to transform *P. infestans* were reported by Kinghorn *et al.* (1991), who used various heterologous transformation vectors of which several had been proven to be successful in a diverse array of filamentous fungi including plant pathogenic fungi (Punt *et al.*, 1987; Garber *et al.*, 1989). Despite extensive attempts in which both CaCl$_2$/PEG treatment and electroporation was used to mediate DNA uptake into the protoplasts, stable transformants could not be achieved. This led to the assumption that transformation of *P. infestans* requires transformation vectors in which expression is regulated by oomycete regulatory sequences. Judelson and Michelmore (1991) assayed transient expression of the GUS reporter gene (Jefferson *et al.*, 1987) in *P. infestans* using promotor and transcription termination sequences from the *hsp70* gene (Judelson and Michelmore, 1989) and the *ham34* gene (Judelson and Michelmore, 1990) of the oomycet *Bremia lactucae* Regel, the causal agent of lettuce downy mildew. This approach appraised both function of gene construction and methods of delivering DNA into the protoplasts. By using optimized transformation conditions deduced from the transient expression assays and vectors containing chimaeric genes consisting of transcriptional regulatory sequences from the *ham34* or *hsp70* genes and the coding region of the bacterial HPG or NPTII gene conferring resistance to hygromycin B or G-418 respectively, Judelson *et al.* (1991) were the first to obtain stable transformants of *P. infestans*. By using the same procedure and the same transformation vectors (kindly provided by H. Judelson) but a different recipient strain, we also obtained stable transformants of *P. infestans* (C.M.J. Pieterse, unpublished). Integrative DNA transformation has now been reported for several other *Phytophthora* species, e.g. *P. parasitica*, *P. capsici*, *P. citricola*, *P. cinnamomi*, *P. citrophthora* and *P. megasperma* f.sp. *glycinea* (Bailey *et al.*, 1991, 1993; Judelson *et al.*, 1993). Although transformation efficiencies are still very low, this achievement will surely stimulate efforts to isolate and characterize pathogenicity and

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avirulence genes of these pathogens.

Lately, several *P. infestans* genes were cloned. Kinghorn and coworkers isolated the *P. infestans* actin genes *actA* and *actB* (Unkles *et al.*, 1991) and the *gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase (Moon *et al.*, 1992). In this thesis, the isolation and detailed characterization of the *P. infestans* genes *ubi3R*, *calA*, *ipiB*, and *ipiO* is described (Pieterse *et al.*, 1991; Pieterse *et al.*, 1993a; Pieterse *et al.*, 1993b; Pieterse *et al.*, 1993c; Pieterse *et al.*, 1993d; chapter 3-7). In addition, we isolated the *P. infestans niaA* gene encoding nitrate reductase, the *P. infestans* EF-1α gene encoding translation elongation factor 1α, and a *P. infestans* rDNA operon (C.M.J. Pieterse, unpublished). Table 1 summarizes the *P. infestans* genes which have been characterized so far. Basically all these genes lack introns. The same holds for other oomycetous genes, i.e. the *B. lactucae* heat-shock gene *hsp70* (Judelson and Michelmore, 1989), the highly expressed *B. lactucae ham34* gene (Judelson and Michelmore, 1990) and the calmodulin gene of the water mould *Achlya klebsiana* (LéJohn, 1989). There are only two oomycetous genes known which may have introns, i.e. the *P. infestans niaA* gene (C.M.J. Pieterse, unpublished) and the *trp1* gene of *P. parasitica* encoding an enzyme of the tryptophan biosynthetic pathway (Karlovsky and Prell, 1991). However, conclusive evidence for this still has to be provided. Other characteristics of the structure of fungal genes in general and of oomycetous genes in particular will be discussed in chapters 4, 5 and 6.

Table 1. List of *Phytophthora infestans* genes currently characterized.

gene	gene product	reference
actA	actin (ACTA)	Unkles *et al.*, 1991
actB	actin (ACTB)	Unkles *et al.*, 1991
gpdA	glyceraldehyde-3-phosphate dehydrogenase	Moon *et al.*, 1992
ubi3R	polyubiquitin	Pieterse *et al.*, 1991; chapter 4
calA	calmodulin	Pieterse *et al.*, 1993d; chapter 5
ipiO1	IPIO1	Pieterse *et al.*, 1993c; chapter 6
ipiO2	IPIO2	Pieterse *et al.*, 1993c; chapter 6
ipiB1	glycine-rich protein (IPIB1)	Pieterse *et al.*, 1993c; chapter 6
ipiB2	glycine-rich protein (IPIB2)	Pieterse *et al.*, 1993c; chapter 6
ipiB3	glycine-rich protein (IPIB3)	Pieterse *et al.*, 1993c; chapter 6
niaA	nitrate reductase	C.M.J. Pieterse, unpublished
EF-1α	translation elongation factor 1α	C.M.J. Pieterse, unpublished
rDNA	rDNA operon encoding 5.8S, 18S and 27S rRNA	C.M.J. Pieterse, unpublished

Concluding remarks

Although there have been extensive studies on various aspects of the interaction between *P. infestans* and potato there are still considerable gaps in our knowledge. For example the processes involved in molecular signalling which lead to compatibility and incompatibility are poorly understood. Potato resistance genes and the corresponding avirulence genes of *P. infestans*, which are certainly involved in this signalling, have to be isolated and characterized in order to get a better understanding of the gene-for-gene relationship. In the host plant potato, all the defence responses which have been studied at the molecular level can more or less be mimicked with race-nonspecific elicitors and apparently there is no strict association with R-gene mediated resistance. The significance of these responses in plant defence is not yet very well understood and further investigations are needed.

The molecular basis of pathogenicity of the pathogen *P. infestans* has hardly been studied. By characterizing the proteins encoded by *P. infestans* genes which are specifically expressed during pathogenesis we might be able to identify factors which are required for pathogenesis. Now that a DNA mediated transformation system for *P. infestans* has been developed, the involvement of the *in planta* induced genes in pathogenesis can be studied in more detail. Hopefully this will lead to our ultimate goal, the elucidation of the molecular basis underlying pathogenicity of *P. infestans*.

References

Ampomah, Y.A. and Friend, J. (1988). Insoluble phenolic compounds and resistance of potato tuber disc to *Phytophthora* and *Phoma*. *Phytochemistry* 27: 2533-2541.

Bach, T.J. (1987). Synthesis and metabolism of mevalonic acid in plants. *Plant Physiology and Biochemistry* 25: 163-178.

Bailey, A.M., Mena, G.L. and Herrera-Estrella, L. (1991). Genetic transformation of the plant pathogens *Phytophthora capsici* and *Phytophthora parasitica*. *Nucleic Acids Research* 19: 4273-4278.

Bailey, A.M., Mena, G.L. and Herrera-Estrella, L. (1993). Transformation of four pathogenic *Phytophthora spp* by microprojectile bombardment on intact mycelia. *Current Genetics* 23: 42-46.

Bailey, J.A. and Mansfield, J.W. (Eds.) (1982). Phytoalexins. Blackie, Glasgow, London.

Berkeley, M.J. (1846). Observations, botanical and physiological, on the potato murrain. *Journal of the Horticultural Society of London* 1: 9-34.

Black, W., Mastenbroek, C., Mills, W.R. and Peterson, L.C. (1953). A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives. *Euphytica* 2: 173-179.

Bodenmann, J., Heiniger, U. and Hohl, H.R. (1985). Extracellular enzymes of *Phytophthora infestans*: endo-cellulase, ß-glucosidases, and 1,3-ß-glucanases. *Canadian Journal of Microbiology* 31: 75-82.

Boller, T., Gehri, A., Mauch, F. and Vögeli, U. (1983). Chitinases in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta* 157: 22-31.

Bostock, R.M., Kúc, J.A. and Laine, R.A. (1981). Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiterpenes in the potato. *Science* 212: 67-69.

Choi, D., Ward, B.L. and Bostock, R.M. (1992). Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. *The Plant Cell* 4: 1333-1344.

Coffey, M.D. and Wilson, U.E. (1983). Histology and cytology of infection and disease caused by *Phytophthora*. *In*: Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.), *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology, pp. 289-301. American Phytopathological Society, St. Paul, Minnesota.

Cole, A.L.J. (1970). Pectic enzyme activity from *Phytophthora infestans*. *Phytochemistry* 9: 337-340.

Collinge, D.B. and Slusarenko, A.J. (1987). Plant gene expression in response to pathogens. *Plant Molecular Biology* 9: 389-410.

Cuypers, B. and Hahlbrock, K. (1988). Immunohistochemical studies of compatible and incompatible interactions of potato leaves with *Phytophthora infestans* and of the nonhost response to *Phytophthora megasperma*. *Canadian Journal of Botany* 66: 700-705.

Cuypers, B., Schmelzer, E. and Hahlbrock, K. (1988). *In situ* localization of rapidly accumulated phenylalanine ammonia-lyase mRNA around penetration sites of *Phytophthora infestans* in potato leaves. *Molecular Plant-Microbe Interactions* 1: 157-160.

de Bary, A. (1863). Recherches sur le développment de quelques champignons parasites. *Annals des Sciences Naturelles* 20: 5-148.

de Bary, A. (1876). Researches into the nature of the potato fungus, *Phytophthora infestans*. *Journal of the Royal Agricultural Society of England* 7: 357-391.

Deising, H., Nicholson, R.P., Haug, M., Howard, R.J. and Mendgen, K. (1992). Adhesion pad formation and the involvement of cutinase and esterases in the attachment of uredospores to the cuticle. *The Plant Cell* 4: 1101-1111.

de Wit, P.J.G.M. (1991). Functional models to explain gene-for-gene relationships in plant pathogen interactions. *In*: Boller, T. and Meins, F. (Eds.), Genes Involved in Plant Defense. Plant Gene Research, volume 8, pp. 25-47. Springer Verlag, New york.

de Wit, P.J.G.M. (1992). Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. *Annual Review of Phytopathology* 30: 391-418.

Dyer, A.T., Tooley, P.W., Govers, F., Carras, M., Galindo, J.J. and Fry, W.E. (1992). A preliminary genetic linkage map of *Phytophthora infestans*. *Phytopathology* 82: 1140.

Elliot, C.G. (1983). Physiology of sexual reproduction in *Phytophthora*. *In*: Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.), *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology, pp. 71-80. American Phytopathological Society, St. Paul, Minnesota.

Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.) (1983). *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. American Phytopathological Society, St. Paul, Minnesota.

Flor, H.H. (1942). Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology* 32: 653-669.

Flor, H.H. (1956). The complementary genetic systems in flax and flax rust. *Advanced Genetics* 8: 29-54.

Förster, H. (1988). Pectinesterases from *Phytophthora infestans*. *Methods in Enzymology* 16: 355-361.

Fritzemeier, K.-H., Cretin, C., Kombrink, E., Rohwer, F., Taylor, J., Scheel, D. and Hahlbrock, K. (1987). Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. *Plant Physiology* 85: 34-41.

Fry, W.E., Goodwin, S.B., Matuszak, J.M., Spielman, L.J., Milgroom, M.G. and Drenth, A. (1992). Population genetics and intercontinental migrations of *Phytophthora infestans*. *Annual Review of Phytopathology* 30: 107-129.

Fry, W.E., Goodwin, S.B. Dyer, A.T., Matuszak, J.M., Drenth, A., Tooley, P.W., Sujkowski, L.S., Koh, Y.J., Cohen, B.A., Spielman, L.J., Deahl, K.L., Inglis, D.A. and Sandlan, K.P. (1993). Historical and recent migrations of *Phytophthora infestans*: chronology, pathways and implications. *Plant Disease* 77: 653-661.

Garber, R.C., Turgeon, B.G. and Yoder, O.C. (1989). Transformation of plant pathogenic fungi. *Plant Molecular Biology Manual* A10: 1-13.

Gebhardt, C., Ritter, E., Debener, T., Schachtschabel, U., Walkemeier, B., Uhrig, H. and Salamini, F. (1989). RFLP analysis and linkage mapping in *Solanum tuberosum*. *Theoretical and Applied Genetics* 78: 65-75.

Gebhardt, C., Ritter, E., Barone, A., Debener, T., Walkemeier, B., Schachtschabel, U., Kaufmann, H., Thompson, R.D., Bonierbale, M.W., Ganal, M.W., Tanksley, S.D. and Salamini, F. (1991). RFLP maps of potato and their alignment with the homeologous tomato genome. *Theoretical and Applied Genetics* 83: 49-57.

Gees, R. and Hohl, H.R. (1988). Cytological comparison of specific (R3) and general resistance to late blight in potato leaf tissue. *Phytopathology* 78: 350-357.

Hahlbrock, K. and Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 347-369.

Hahlbrock, K., Cretin, C., Cuypers, B., Fritzemeier, K.-H., Hauffe, K.-D., Jahnen, W., Kombrink, E., Rohwer, F., Scheel, D., Schmelzer, E., Schröder, M. and Taylor, J. (1987). Tissue specificity and

dynamics of disease resistance responses in plants. *In*: von Wettstein, D. and Chua, N.H. (Eds.), Plant Molecular Biology. NATO ASI Series, volume A140, pp. 399-406. Plenum Press, New york.

Hahlbrock, K., Arabatzis, N., Becker-André, M., Joos, H.-J., Kombrink, E., Schröder, M., Strittmatter, G. and Taylor, J. (1989). Local and systemic gene activation in fungus-infected potato leaves. *In*: Lugtenberg, B.J.J. (Ed.), Signal Molecules in Plants and Plant-Microbe Interactions. NATO ASI Series, volume H36, pp. 241-249. Springer Verlag, Berlin, Heidelberg.

Hohl, H.R. (1991). Nutrition. *In*: Ingram, D.S. and Williams, P.H. (Eds.), *Phytophthora infestans*, the Cause of Late Blight of Potato. Advances in Plant Pathology, volume 7, pp. 53-83. Academic press, London, San Diego.

Hohl, H.R. and Suter, E. (1976). Host-parasite interfaces in a resistant and a susceptible cultivar of *Solanum tuberosum* inoculated with *Phytophthora infestans*: leaf tissue. *Canadian Journal of Botany* 54: 1956-1970.

Ingram, D.S. and Williams, P.H. (Eds.) (1991). Advances in Plant Pathology, volume 7; *Phytophthora infestans*, the Cause of Late Blight of Potato. Academic press, London, San Diego.

Innes, R.W., Kuempel, P.L., Plazinski, J., Cante-Cremers, H., Rolfe, B.G. and Djordjevic, M.A. (1985). Plant factors induce expression of nodulation and host-range genes in *Rhizobium trifolii*. *Molecular and General Genetics* 201: 426-432.

Jarvis, M.C., Threlfall, D.R. and Friend, J. (1981). Potato cell wall polysaccharides: degradation with enzymes from *Phytophthora infestans*. *Journal of Experimental Botany* 32: 1309-1319.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987). GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* 6: 3901-3907.

Judelson, H.S. and Michelmore, R.W. (1989). Structure and expression of a gene encoding heat-shock protein *Hsp70* from the oomycete fungus *Bremia lactucae*. *Gene* 79: 207-217.

Judelson, H.S. and Michelmore, R.W. (1990). Highly abundant and stage-specific mRNAs in the obligate pathogen *Bremia lactucae*. *Molecular Plant-Microbe Interactions* 3: 225-232.

Judelson, H.S. and Michelmore, R.W. (1991). Transient expression of genes in the oomycete *Phytophthora infestans* using *Bremia lactucae* regulatory sequences. *Current Genetics* 19: 453-459.

Judelson, H.S., Tyler, B.M. and Michelmore, R.W. (1991). Transformation of the oomycete pathogen, *Phytophthora infestans*. *Molecular Plant-Microbe Interactions* 4: 602-607.

Judelson, H.S., Coffey, M.D., Arredondo, F.R. and Tyler, B.M. (1993). Transformation of the soybean pathogen *Phytophthora megasperma* f.sp. *glycinea* occurs by DNA integration into single or multiple chromosomes. *Current Genetics* 23: 211-218.

Karlovsky, P. and Prell, H.H. (1991). The *TRP1* gene of *Phytophthora parasitica* encoding indole-3-glycerolphosphate synthase-N-(5'-phosphoribosyl)anthranilate isomerase: structure and evolutionary distance from homologous fungal genes. *Gene* 109: 161-165.

Keen, N.T. and Yoshikawa, M. (1983). Physiology of disease and the nature of resistance to *Phytophthora*. *In*: Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.), *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology, pp. 279-287. American Phytopathological Society, St. Paul, Minnesota.

Kinghorn, J.R., Moon, R.P., Unkles, S.E. and Duncan, J.M. (1991). Gene structure and expression in *Phytophthora infestans* and the development of gene-mediated transformation. *In*: Lucas, J.A., Shattock, R.C., Shaw, D.S. and Cooke, L.R. (Eds.), *Phytophthora*, pp. 295-311. Cambridge University Press, Cambridge.

Kolattukudy, P.E. (1985). Enzymatic penetration of the plant cuticle by fungal pathogens. *Annual Review of Phytopathology* 23: 223-250.

Kombrink, E., Schröder, M. and Hahlbrock, K. (1988). Several "pathogenesis-related" proteins in potato are 1,3-ß-glucanases and chitinases. *Proceedings of the National Academy of Sciences USA* 85: 782-786.

Kombrink, E., Hahlbrock, K., Hinze, K. and Schröder, M. (1991). Molecular responses of potato to infection by *Phytophthora infestans*. *In*: Smith, C.J. (Ed.), Biochemistry and Molecular Biology of Plant-Pathogen Interactions, pp. 237-254. Clarendon Press, Oxford.

Kúc, J. (1982). Phytoalexins from the Solanaceae. *In*: Bailey, J.A. and Mansfield, J.W. (Eds.), Phytoalexins, pp. 81-105. Blackie, Glasgow, London.

LéJohn, H.B. (1989). Structure and expression of fungal calmodulin gene. *Journal of Biological Chemistry* 264: 19366-19372.

Leonards-Schippers, C., Gieffers, W., Salamini, F. and Gebhardt, C. (1992). The R1 gene conferring race-specific resistance to *Phytophthora infestans* in potato is located on potato chromosome V. *Molecular and General Genetics* 233: 278-283.

Lewis, D.H. (1973). Concepts in fungal nutrition and the origin of biotrophy. *Biological Reviews* 48: 261-278.

Lucas, J.A., Shattock, R.C., Shaw, D.S. and Cooke, L.R. (Eds.) (1991). *Phytophthora.* Cambridge University Press, Cambridge.

Malcolmson, J.F. and Black, W. (1966). New R-genes in *Solanum demissum* Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary. *Euphytica* **15**: 199-203.

Manavathu, E.K., Suryanarayana, K., Hasnain, S.E. and Leung, W. (1988). DNA-mediated transformation in the aquatic filamentous fungus *Achlya ambisexualis. Journal of General Microbiology* **134**: 2019-2028.

Martini, N., Egen, M., Rüntz, I. and Strittmatter, G. (1993). Promoter sequences of a potato pathogenesis-related gene mediate transcriptional activity selectively upon fungal infection. *Molecular and General Genetics* **236**: 179-186.

Mastenbroek, C. (1953). Experiments on the inheritance of blight immunity in potatoes derived from *Solanum demissum* Lindl. *Euphytica* **2**: 197-206.

Matton, D.P. and Brisson, N. (1989). Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Molecular Plant-Microbe Interactions* **2**: 325-331.

Mauch, F., Mauch-Mani, B. and Boller, T. (1988). Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. *Plant Physiology* **88**: 936-942.

Moon, R.P., Unkles, S.E., Duncan, J.M., Hawkins, A.R. and Kinghorn, J.R. (1992). Sequence of the *Phytophthora infestans* glyceraldehyde-3-phosphate dehydrogenase-encoding gene (*gpdA*). *Plant Molecular Biology* **18**: 1209-1211.

Moreau, R.A. and Seibles, T.S. (1985). Production of extracellular enzymes by germinating cysts of *Phytophthora infestans. Canadian Journal of Botany* **63**: 1811-1816.

Müller, K.O. and Börger, H. (1941). Experimentelle untersuchungen über die *Phytophthora*-resistenz der kartoffel. *Arbeiten aus der Biologischen Reichsanstalt für Land- und Forstwirtschaft. Berlin-Dahlem* **23**: 189-231.

Nicholson, R.L. and Epstein, L. (1991). Adhesion of fungi to the plant surface: Prerequisite for pathogenesis. *In:* Cole, G.T. and Hoch, H.C. (Eds.), The Fungal Spore and Disease Initiation in Plants and Animals, pp. 3-23. Plenum Press, New York.

Niederhauser, J.S. (1956). The blight, the blighter, and the blightest. *Transaction of the New York Academy of Sciences* **19**: 55-63.

Oba, K., Kondo, K., Doke, N. and Uritani, I. (1985). Induction of 3-hydroxy-methylglutaryl CoA reductase in potato tuber after slicing, fungal infection or chemical treatment, and some properties of the enzyme. *Plant Cell Physiology* **26**: 873-880.

Pereira, A., Jacobs, J.M.E., te Lintel-Hekkert, W., Rutgers, E., Jacobsen, E. and Stiekema, W.J. (1992). Towards the isolation of resistance genes by transposon targeting in potato. *Netherlands Journal of Plant Pathology* **98**, supplement 2: 215-221.

Person, C., Samborski, D.J. and Rohringer, R. (1962). The gene-for-gene concept. *Nature* **194**: 561-562.

Peters, N.K. and Verma, D.P.S. (1990). Phenolic compounds as regulators of gene expression in plant-microbe interactions. *Molecular Plant-Microbe Interactions* **3**: 4-8.

Pieterse, C.M.J., Risseeuw, E.P. and Davidse, L.C. (1991). An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Molecular Biology* **17**: 799-811.

Pieterse, C.M.J., Derksen, A.-M.C.E., Folders, J. and Govers, F. (1993a). Expression of the putative pathogenicity genes *ipiB* and *ipiO* of *Phytophthora infestans in planta* and *in vitro.* Submitted.

Pieterse, C.M.J., Riach, M.B.R., Bleker, T., van den Berg-Velthuis, G.C.M. and Govers, F. (1993b). Isolation of putative pathogenicity genes of the potato late blight fungus *Phytophthora infestans* by differential hybridization of a genomic library. *Physiological and Molecular Plant Pathology,* in press.

Pieterse, C.M.J., van West, P., Verbakel, H.M., Brassé, P.W.H.M., van den Berg-Velthuis, G.C.M. and Govers, F. (1993c). Structure and genomic organization of the *ipiB* and *ipiO* gene clusters of *Phytophthora infestans. Gene,* in press.

Pieterse, C.M.J., Verbakel, H.M., Hoek Spaans, J., Davidse, L.C. and Govers, F. (1993d). Increased expression of the calmodulin gene of the late blight fungus *Phytophthora infestans* during pathogenesis on potato. *Molecular Plant-Microbe Interactions* **6**: 164-172.

Pristou, R. and Gallegly, M.E. (1954). Leaf penetration by *Phytophthora infestans. Phytopathology* **44**: 81-86.

Punt, P.J., Oliver, R.P., Dingemans, M.A., Pouwels, P.H. and van den Hondel, C.A.M.J.J. (1987). Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli. Gene* **56**: 117-124.

Rohwer, F., Fritzemeier, K.-H., Scheel, D. and Hahlbrock, K. (1987). Biochemical reactions of different tissues of potato (*Solanum tuberosum*) to zoöspores or elicitors from *Phytophthora infestans. Planta* **170**: 556-561.

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Sato, N., Kitazawa, K. and Tomiyama, K. (1971). The role of rishitin in localizing the invading hyphae of *Phytophthora infestans* in infection sites at the cut surfaces of potato tubers. *Physiological Plant Pathology* 1: 289-295.

Scheffer, R.P. (1976). Host-specific toxins in relation to pathogenesis and disease resistance. *In*: Heitefuss, R. and Williams, P.H. (Eds.), Physiological Plant Pathology. Encyclopedia of Plant Physiology, volume 4, pp. 247-269. Springer-Verlag, Berlin.

Schröder, M., Hahlbrock, K. and Kombrink, E. (1992). Temporal and spatial patterns of 1,3-ß-glucanase and chitinase induction in potato leaves infected by *Phytophthora infestans*. *The Plant Journal* 2: 161-172.

Seidel, H. (1961). Untersuchungen über den Nährstoffbedarf und die toxinbildung des pilzes *Phytophthora infestans* (Mont.) de Bary in vollsynthetischen nährlösungen. *Phytopathologische Zeitschrift* 41: 1-26.

Shaw, D.S. (1991). Genetics. *In*: Ingram, D.S. and Williams, P.H. (Eds.), *Phytophthora infestans*, the Cause of Late Blight of Potato. Advances in Plant Pathology, volume 7, pp. 131-170. Academic Press, London, San Diego.

Smoot, J.J., Gough, F.J., Lamey, H.A., Eichenmuller, J.J. and Gallegly, M.E. (1958). Production and germination of oospores of *Phytophthora infestans*. *Phytopathology* 48: 165-171.

Spielman, L.J., McMaster, B.J. and Fry, W.E. (1989). Dominance and recessiveness at loci for virulence against potato and tomato in *Phytophthora infestans*. *Theoretical and Applied Genetics* 77: 832-838.

Spielman, L.J., Sweigard, J.A., Shattock, R.C. and Fry, W.E. (1990). The genetics of *Phytophthora infestans*: Segregation of allozyme markers in F2 and backcross progeny and the inheritance of virulence against potato resistance genes R2 and R4 in F1 progeny. *Experimental Mycology* 14: 57-69.

Spielman, L.J., Drenth, A., Davidse, L.C., Sujkowski, L.S., Gu, W., Tooley, P.W. and Fry, W.E. (1991). A second world-wide migration and population displacement of *Phytophthora infestans*? *Plant Pathology* 40: 422-430.

Stachel, S.E., Nester, E.W. and Zambryski, P.C. (1986). A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proceedings of the National Academy of Sciences USA* 83: 379-383.

Stahl, D.J. and Schäfer, W. (1992). Cutinase is not required for fungal pathogenicity on pea. *The Plant Cell* 4: 621-629.

Stermer, B.A., Edward, L.A., Edington, B.V. and Dixon, R.A. (1991). Analysis of elicitor-inducible transcripts encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase in potato. *Physiological and Molecular Plant Pathology* 39: 135-145.

Sweigard, J.A., Chumley, F.G. and Valent, B. (1992). Disruption of a *Magnaporthe grisea* cutinase gene. *Molecular and General Genetics* 232: 183-190.

Taylor, J.L., Fritzemeier, K.-H., Häuser, I., Kombrink, E., Rohwer, F., Schröder, M., Strittmatter, G. and Hahlbrock, K. (1990). Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. *Molecular Plant-Microbe Interactions* 3: 72-77.

Thompson, J.N. and Burdon, J.J. (1992). Gene-for-gene coevolution between plants and parasites. *Nature* 360: 121-125.

Tomiyama, K. (1963). Physiology and biochemistry of disease resistance of plants. *Annual Review of Phytopathology* 1: 295-324.

Tomiyama, K. (1983). Research on the hypersensitive response. *Annual Review of Phytopathology* 21: 1-12.

Umaerus, V. (1970). Studies on field resistance to *Phytophthora infestans*. 5. Mechanisms of resistance and application to potato breeding. *Zeitschrift für Pflanzenzüchtung* 63: 1-23.

Umaerus, V., Umaerus M., Erjefält, L. and Nilsson, B.A. (1983). Control of *Phytophthora* by host resistance: Problems and progress. *In*: Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.), *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology, pp. 315-326. American Phytopathological Society, St. Paul, Minnesota.

Unkles, S.E., Moon, R.P., Hawkins, A.R., Duncan, J.M. and Kinghorn, J.R. (1991). Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* 100: 105-112.

van Loon, L.C. (1985). Pathogenesis-related proteins. *Plant Molecular Biology* 4: 111-116.

Wastie, R.L. (1991). Breeding for resistance. *In*: Ingram, D.S and Williams, P.H. (Eds.), *Phytophthora infestans*, the Cause of Late Blight of Potato. Advances in Plant Pathology, volume 7, pp. 193-224. Academic Press, London, San Diego.

Willis, D.K., Rich, J.J. and Hrabak, E.M. (1991). *hrp* genes of phytopathogenic bacteria. *Molecular Plant-Microbe Interactions* 4: 132-138.

Woloshuk, C.P. and Kolattukudy, P.E. (1986). Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of *Fusarium solani* f.sp. *pisi*. *Proceedings of the National Academy of Sciences USA* 83: 1704-1708.

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Woloshuk, C.P., Meulenhoff, E.J.S., Sela-Buurlage, M., van den Elzen, P.J.M. and Cornelissen, B.J.C. (1991). Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. *The Plant Cell* 3: 619-628.

Wood, R.K.S. (Ed.) (1967). Physiological Plant Pathology. Willmer Brothers, Ltd. Birkenhead, England.

Yang, Z., Park, H., Lacy, G.H. and Cramer, C.L. (1991). Differential activation of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes by wounding and pathogen challenge. *The Plant Cell* 3: 397-405.

chapter 3

Isolation of putative pathogenicity genes of the potato late blight fungus *Phytophthora infestans* by differential hybridization of a genomic library

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Abstract. Plant pathogens produce pathogenicity factors which enable them to parasitize and colonize their host. A strategy for identifying pathogenicity factors involves the isolation and characterization of genes encoding these factors. Potential candidates of pathogenicity genes are genes whose expression is induced during pathogenesis. In order to isolate such genes of the late blight fungus *Phytophthora infestans*, a genomic library of *P. infestans* DNA was differentially hybridized using labeled first strand cDNA probes synthesized on (i) mRNA isolated from *P. infestans* infected potato leaves and on (ii) mRNA isolated from the fungus grown on a basic medium in culture. In total, nine distinct *in planta* induced genes were selected. Expression studies revealed that the mRNA levels of seven of these genes, *ipiA, ipiC, ipiD, ipiJ1, ipiJ2, ipiN* and *ipiQ*, increased 5-10 fold during colonization. The two other genes, *ipiB* and *ipiO*, showed a transient expression pattern with the highest mRNA levels in the early stages of infection.

Introduction

The fungus *Phytophthora infestans* (Mont.) de Bary (Oomycetes) is the causal agent of late blight of potato (*Solanum tuberosum* L.). It is a hemibiotrophic pathogen which infects leaves and tubers of susceptible cultivars, causing rapidly spreading lesions which then become necrotic resulting eventually in complete destruction of infected tissue. Although not confined to potato, the host range of *P. infestans* is rather narrow, all hosts belonging to the Solanaceae with potato and tomato (*Lycopersicon esculentum* Mill.) being the most important economically.

Pathogenesis results from a series of complex interactions between host and pathogen. The few potential pathogenicity factors which have been studied in the potato-*P. infestans* interaction are enzymes that degrade cell walls or other structures, and are thus believed to facilitate pathogen entry and growth through the tissue (Bodenmann *et al.*, 1985; Jarvis *et al.*, 1981). However, it is still not known to what extent these factors contribute to disease development. Moreover, the specificity of *P. infestans* for solanaceous hosts is unlikely to be explained by the action of general cell wall degrading enzymes. The specialization implies much more sophisticated mechanisms for recognition involving the exchange of signals between host and pathogen. In both potato and tomato, pathogen attack has been shown to be accompanied by the induction of the expression of a particular set of genes whose products are thought to be involved in defence (Fritzemeier *et al.*, 1987; Kombrink *et al.*, 1988; Matton and Brisson, 1989; Taylor *et al.*, 1990). Conversely, genes in the pathogen may be activated during growth in the host. The products of these *in planta* induced genes may be necessary for the establishment of the parasite or for the increase of disease severity. Identification of genes of a plant pathogen which are induced in the host may therefore lead to the identification of essential pathogenicity factors.

Differential hybridization can be a useful tool for the identification of *P. infestans* genes whose expression is specifically induced or increased during pathogenesis. Differential screening of cDNA libraries has been frequently and successfully utilized to isolate

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developmentally regulated genes, tissue specific genes or genes which are differentially expressed under distinct physiological conditions (Sargent, 1987; Timberlake, 1980; Timberlake, 1986). Differential hybridization of a cDNA library of *P. infestans*-infected potato tissue will result in the selection of host as well as pathogen-derived cDNAs which cannot readily be distinguished. Therefore our approach for the isolation of putative pathogenicity genes is based on differential screening of a genomic DNA library of *P. infestans*. In this way only genes of fungal origin are selected. Here we describe this non-biased strategy for the isolation of putative pathogenicity genes. Using this strategy, several *in planta* induced *P. infestans* genes were isolated and their expression during growth of the fungus in the host was studied. Characterization of the corresponding gene products and their function during pathogenesis will increase the understanding of the molecular and cellular processes involved in the interaction between *P. infestans* and its host plant.

Materials and Methods

Plant and fungal material. P. infestans strain 88069 (A1 mating type, race 1.3.4.7) which was used throughout this study, was maintained on rye-agar medium containing 2% (w/v) sucrose (Caten and Jinks, 1968). Zoospores were obtained from 2-week-old cultures on 9.4 cm Petri dishes by adding 10 ml of water to the culture and incubating at 10 °C. After a 3 h incubation, typically 10^6 zoospores ml^{-1} were released into the water which was collected and used as inoculum to initiate liquid cultures. To obtain large amounts of mycelium, zoospores were inoculated into rye-sucrose medium or Henniger's synthetic medium (Henniger, 1959) to give a final concentration of 1×10^5 zoospores ml^{-1} of culture medium. After incubation at 18°C for 3 days, the mycelium was harvested, frozen in liquid nitrogen and stored at -80°C until RNA or DNA extractions were performed.

Potato cv. Ajax which contains the R3 gene for late blight resistance but is susceptible to *P. infestans* strain 88069 (race 1.3.4.7) was used as host plant. Leaves were inoculated with sporangia collected from 2-week-old rye-agar cultures. The leaves were first inserted into florist's foam oasis and then inoculated by spraying the upper surfaces with a sporangial suspension (5×10^5 sporangia ml^{-1}) or by spotting 10 μl of the sporangial suspension on the upper surface of the leaves. Leaves were incubated at 18°C and 100% RH under cool fluorescent light (16 h day^{-1}). As a control, leaves sprayed with water were treated similarly to the inoculated leaves.

Differential screening of the P. infestans *genomic library.* A genomic library of *P. infestans* DNA was constructed in λEMBL3 as described previously (Pieterse *et al.*, 1991). For differential screening of the genomic library, 8×10^4 individual recombinant bacteriophages were plated with *Escherichia coli* strain MB406 and incubated as described by Sambrook *et al.* (1989). Of the resulting plaques, four replicas were made on Hybond-N$^+$ membranes (Amersham) according to the manufacturers instructions. [α-^{32}P]dATP labeled first strand

cDNA probes, with a specific activity of $1-2 \times 10^8$ ct min^{-1} μg^{-1}, were synthesized as described by Sargent (1987) using M-MLV reverse transcriptase (Gibco-BRL) and oligo(dT) to prime the synthesis reaction. Three templates for cDNA synthesis were used; (i) 1 μg poly(A)$^+$ RNA isolated from 3-day-old mycelium grown on Henniger's synthetic medium (fungal cDNA probe), (ii) 1 μg poly(A)$^+$ RNA isolated from a zone of 1 cm in width at the outer edge of lesions which developed around the infection site, 3 days after spot-inoculation (interaction cDNA probe), and (iii) 1 μg poly(A)$^+$ RNA isolated from non-infected potato leaves (plant cDNA probe). Duplicate membranes were hybridized with equal amounts (10^7 ct min^{-1}) of the interaction and fungal cDNA probe in a hybridization mix containing $5 \times$SSC (750 mM NaCl, 75 mM sodium citrate), $5 \times$Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin (fraction V)), 0.5% (w/v) SDS and 100 μg ml^{-1} calf thymus DNA under high stringency conditions at 65°C for 16 h. The filters were washed in $2 \times$SSC, 0.5% (w/v) SDS at 65°C and exposed to Kodak X-OMAT S film for 2-7 days using an intensifying screen. As a control, the interaction cDNA probe was removed from the filters which were then rehybridized with the plant cDNA probe (10^7 ct min^{-1}).

Putative positive bacteriophages were selected, plated and screened by a second round of differential hybridization as described above. DNA from the selected phages was isolated using Qiagen columns (Diagen, Düsseldorf, Germany) according to the manufacturers instructions and singly and doubly digested with SalI and SstI. Following electrophoresis on a 0.8% (w/v) agarose gel, the DNA was blotted onto Hybond-N$^+$ membranes by capillary transfer and hybridized with the interaction cDNA probe. Hybridizing DNA fragments were identified and after digestion with the appropriate restriction enzyme(s) and electrophoresis, these fragments were isolated from a 0.8% (w/v) agarose gel. The fragments were labeled by random primer labeling (Feinberg and Vogelstein, 1983) and subsequently used as probes for northern blot hybridizations.

Isolation of RNA, poly(A)$^+$ RNA and northern blot analyses. RNA was isolated from non-inoculated potato leaves, leaves inoculated with *P. infestans* (interaction RNA) and *P. infestans* mycelium grown *in vitro* using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). On the inoculated leaves, symptoms developed in time from small lesions, 24 h after inoculation, into completely colonized, water soaked leaves, 3 days after inoculation. Poly(A)$^+$ RNA was obtained by affinity chromatography on oligo(dT)-cellulose as described by Sambrook *et al.* (1989). For northern blot analyses, 15 μg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond-N$^+$ membranes by capillary transfer as described by the manufacturers instructions. Hybridization of northern blots was performed at 65°C in the hybridization mix described above.
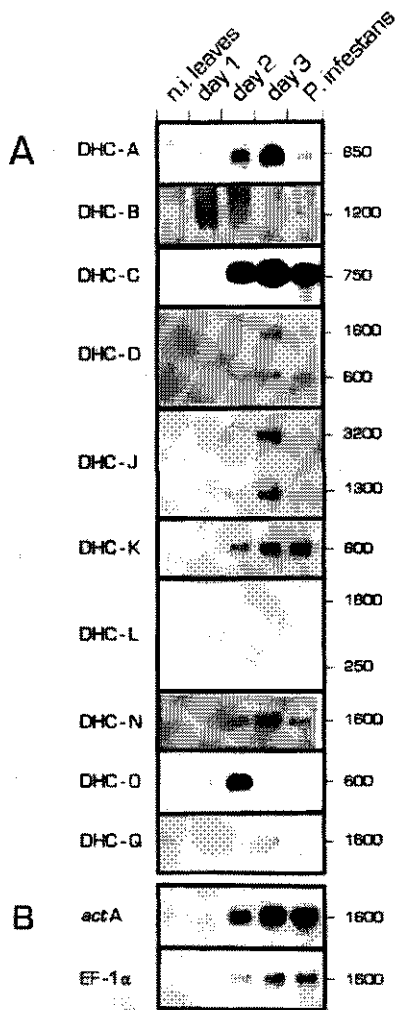
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A

DHC-A — 850

DHC-B — 1200

DHC-C — 750

DHC-D — 1600 / 600

DHC-J — 3200 / 1300

DHC-K — 800

DHC-L — 1800 / 250

DHC-N — 1600

DHC-O — 600

DHC-Q — 1600

B

actA — 1600

EF-1α — 1600

Figure 1. Autoradiographs of northern blots containing total RNA isolated from non-inoculated potato leaves, from leaves colonized by *P. infestans* at different time points after inoculation, and from *P. infestans* mycelium grown *in vitro*. n.i. leaves, RNA isolated from control, non-inoculated leaves; day 1, 2, and 3, RNA isolated from infected leaves 1, 2, and 3 days after inoculation; P. infestans, RNA isolated from 3-day-old mycelium grown on Henniger's synthetic medium. Northern blots were hybridized with the following probes: (A) DHC-A: *SstI/NcoI*-insert from pPIN1 (CMJP, unpublished) containing the first 754 bp of a gene specific 5' non-coding region of the *ubi3R* gene (Pieterse *et al.*, 1991); DHC-B: 1.8 kb *SstI*-fragment from DHC-B (hybridization with the 1.7 kb *SstI*-fragment from DHC-B gave an identical pattern); DHC-C, DHC-D, DHC-J, DHC-K, DHC-L, DHC-O, DHC-Q: restriction fragments indicated in Table 1; DHC-N: 1.1 kb *PstI*-fragment located within the 4.4 kb *SstI*-fragment from DHC-N at a distance of 0.75 kb downstream from the stop codon of the EF-1α gene. (B) *actA*: 2.8 kb *PstI*-insert from pSTA31 containing the complete *P. infestans actA* gene (Unkles *et al.*, 1991); EF-1α: 0.35 kb *EcoRI/XhoI*-insert from pPi119 which is a partial cDNA clone of the *P. infestans* EF-1α mRNA (CMJP, unpublished). Transcript lengths are indicated in nt. The figure shown in panel DHC-A has been published previously by Pieterse *et al.* (1991).

leaves, the fungal biomass increases and in consequence the ratio of fungal to plant RNA increases. When studying the induction of gene expression in plant-fungus interactions by means of northern blot analysis, the mRNA level of a constitutively expressed fungal gene can be used as an internal standard since the increase in signal obtained with a probe of such a marker gene should reflect the relative increase in the proportion of fungal RNA in the interaction RNA mixture. Genes encoding actin and the translation elongation factor 1α (EF-1α) have been shown to be useful marker genes (Mahe *et al.*, 1992). In *P. infestans* the actin gene *actA* (isolated by Unkles *et al.*, 1991) as well as the EF-1α gene (isolated by CMJP, unpublished) are constitutively expressed during colonization of potato leaves. Furthermore, the expression of both genes remains at the same level during growth of *P. infestans* on Henniger's synthetic medium and rye-sucrose medium, during non-sporulating

as well as during sporulating conditions (Pieterse *et al.*, 1993). The *actA* gene has also been shown to be constitutively expressed in zoospores, sporangia and germinating cysts of *P. infestans* (S.E. Unkles, personal communication). In our experiments we used the *actA* and the EF-1α mRNA levels as internal markers to determine the proportion of fungal RNA in the interaction RNA mixture. The specificity of the *actA* and EF-1α probes was analyzed on Southern blots containing potato and *P. infestans* DNA and on northern blots containing RNA isolated from healthy potato leaves and from *P. infestans* grown *in vitro*. Under the hybridization conditions used, the *actA* as well as the EF-1α probe hybridized only to *P. infestans* derived nucleic acids and not to potato RNA or DNA (Fig. 1; Southern blot data not shown).

In order to determine whether the transcripts visualized by northern blot hybridization (Fig. 1) were derived from genes induced *in planta*, the relative amounts of the various mRNA transcripts during pathogenesis were quantified and compared to the mRNA levels detectable during growth of the fungus *in vitro*. To do this, autoradiographs of the northern blots were subjected to densitometric scanning using the Cybertech Image Processing Software, version 1.20 (Cybertech, Berlin). Quantification of the hybridization signals obtained with probes of the marker genes *actA* and EF-1α showed that the proportion of fungal RNA in the interaction RNA mixture isolated from infected leaves increased from 5% 1 day after inoculation to 70% by 3 days after inoculation. In order to determine the expression levels of the putative *in planta* induced genes during pathogenesis, the mRNA levels were normalized to actual fungal RNA levels and the real increase in mRNA levels during growth of the fungus *in planta* was calculated (Table 2).

The densitometric scanning of autoradiographs of the northern blots showed that eight of the ten selected DHCs (DHC-A, DHC-B, DHC-C, DHC-D, DHC-J, DHC-N, DHC-O and DHC-Q) contain one or two genes of which the mRNA levels are significantly higher during pathogenesis compared to the mRNA levels during growth of the fungus *in vitro*. DHC-A, DHC-B, DHC-C, DHC-D, DHC-N, DHC-O and DHC-Q each contain one *in planta* induced gene and these genes were designated *ipiA*, *ipiB*, *ipiC*, *ipiD*, *ipiN*, *ipiO* and *ipiQ*, respectively. From DHC-B, two non-overlapping *Sst*I-fragments of 1.7 and 1.8 kb in length were used as probes. Both fragments hybridized to mRNA transcripts of 1200 nt with equal hybridization patterns (Fig. 1). Presumably, the *ipiB* gene spans the two fragments although it can not be excluded that the two fragments contain two or more genes of a gene cluster. DHC-J contains two distinct *in planta* induced genes which were termed *ipiJ1* (corresponding to the 3200 nt transcript) and *ipiJ2* (corresponding to the 1300 nt transcript). On northern blots, the mixture of the two co-migrating 1.7 kb *Sst*I-fragments from DHC-J hybridizes to the *ipiJ1* and *ipiJ2* transcripts (Fig. 1). Apparently, the *ipiJ1* and *ipiJ2* transcripts are derived from two distinct genes of which each is at least partially located on one of the 1.7 kb *Sst*I-fragments. This is supported by the fact that two *Sal*I-fragments (8.0 and 5.1 kb) and two *Sal*I/*Sst*I-fragments (1.4 and 1.2 kb) from DHC-J hybridize to the interaction cDNA probe.

Table 2. mRNA levels of *Phytophthora infestans* ipi- and pig-genes produced during colonization of potato leaves, normalized on the mRNA levels produced during growth *in vitro*.

gene	location	transcript length (nt)	normalized mRNA levels [1]				
			n.i.[2]	day 1[2]	day 2[2]	day 3[2]	P. infestans[2]
ipiA (*ubi3R*)	DHC-A	850	-	-	10	10	1
ipiB	DHC-B	1200	-	>100	1	1	1
ipiC (*calA*)	DHC-C	750	-	-	5	4	1
ipiD	DHC-D	1600	-	-	10	10	1
pigD	DHC-D	600	-	-	1	1	1
ipiJ1	DHC-J	3200	-	-	10	8	1
ipiJ2	DHC-J	1300	-	-	9	9	1
pigK	DHC-K	800	-	-	1	1	1
pigL	DHC-L	1800	-	-	-	1	1
		250	#	#	-	-	-
ipiN	DHC-N	1600	-	-	5	5	1
ipiO	DHC-O	600	-	35[3]	35[3]	1[3]	-
ipiQ	DHC-Q	1600	-	-	5	9	1

[1] mRNA levels were measured by densitometric scanning of autoradiographs and normalized to actual fungal RNA levels using the signals obtained with probes derived from the constitutively expressed marker genes *actA* and EF-1α. Densitometric scanning of the *actA* and EF-1α signals shown in Fig. 1B revealed that interaction RNAs isolated at day 1, day 2 and day 3 post-inoculation contained 5, 25 and 70% fungal RNA, respectively. All values obtained by densitometric scanning of the hybridization signals shown in Fig. 1A were adjusted to the equivalent of 100% fungal RNA and subsequently compared to those values obtained with RNA isolated from *P. infestans* grown *in vitro*. Numbers indicate the increase in actual mRNA levels in comparison to the mRNA levels in *P. infestans* grown *in vitro* which is set at 1.

[2] see legend Figure 1.

[3] In *P. infestans* grown *in vitro*, *ipiO* mRNA is not detectable. Therefore, signals obtained in lanes containing interaction RNA are compared with each other.

\- no mRNA detectable.

\# mRNA of plant origin.

Two of the nine selected *in planta* induced genes, *ipiB* and *ipiO*, show a transient expression pattern during the infection process. The highest *ipiB* mRNA level is detectable 1 day after inoculation and is more than 100-fold higher than the level during growth of the fungus *in vitro*. Two and 3 days after inoculation, the *ipiB* mRNA level is much lower (Table 2). *IpiO* is highly expressed up to 2 days after inoculation, but by the third day the *ipiO* mRNA level is only 3% of that on days 1 and 2 after inoculation. The *ipiO* mRNA transcript was not

detectable in the fungus grown in culture. The transient expression pattern of the *ipiB* and *ipiO* genes suggests a role for their gene products in the early stages of infection. In comparison to mRNA levels found during growth of the fungus *in vitro*, mRNA levels of the other seven *ipi*-genes were consistently higher during pathogenesis (Table 2). It may be that high levels of their gene products are required during the pathogenic interaction. Expression of the *ipi*-genes in *P. infestans* was also studied during growth on rye-sucrose medium and Henniger's synthetic medium. The mRNA levels of all nine *ipi*-genes were the same on both media (data not shown), indicating that the large differences in chemical composition between these two media did not affect the expression level of the genes.

In addition to the *ipiD* transcript of 1600 nt, the 5.2 kb *SstI*-fragment from DHC-D hybridizes to another transcript of 600 nt (Fig. 1) but the mRNA level of the corresponding *P. infestans* gene, designated *pigD*, does not change during pathogenesis. In all three digests of DHC-D DNA, a single restriction fragment hybridizes to the interaction cDNA probe. The hybridizing fragment in the *SalI/SstI* digest is 1.1 kb in length which makes it unlikely that both *ipiD* and *pigD* are located in this clone. Most likely, *ipiD* and *pigD* are homologous genes and either *ipiD* or *pigD* is located on the 1.1 kb *SalI/SstI*-fragment. DHC-K and DHC-L each contain a single transcriptionally active gene of which the expression level is not increased during pathogenesis. The genes are named *pigK* and *pigL* respectively. In addition to the 1800 nt *pigL* transcript, the 7.0 kb *SalI*-fragment of DHC-L hybridizes to a 250 nt mRNA. In RNA isolated from non-infected leaves the same 250 nt mRNA transcript is present indicating that this transcript is of plant origin. Apparently, there is a substantial sequence homology between the 7.0 kb *SalI*-fragment from DHC-L and the 250 nt plant mRNA. The reason why DHC-L as well as DHC-K hybridized differentially to the fungal and the interaction cDNA probe in the initial screening is not clear.

Discussion

In this paper we have shown that differential screening of a genomic library can be used to select *P. infestans* genes whose expression is significantly increased during growth in the host plant potato relative to their expression during growth of the fungus in a synthetic medium. Differential hybridization of a genomic library has not often been utilized for the selection of differentially expressed genes. In contrast to cDNA libraries, genomic libraries have large vector inserts and high amounts of non-coding and repetitive DNA. In addition, selected clones may have multiple genes on a single vector insert. In combination with the low specificity of the cDNA probes it is more difficult to isolate the genes of interest. In our case the use of a genomic library of *P. infestans* DNA was preferable since this approach yields fungal genes exclusively. The use of a cDNA library representing mRNA from potato infected with *P. infestans*, will yield host- as well as pathogen-derived cDNAs and these cannot be distinguished directly.

In the first differential hybridization twenty DHCs were selected. Ten DHCs still showed a differential hybridization pattern in a dot blot experiment and were studied in more

detail by means of northern blot analysis. In total nine genes whose expression is induced *in planta* were identified. The number of positive genomic clones obtained with this procedure does not reflect the real number of *P. infestans* genes induced in the host. When a genomic clone contains a constitutively expressed gene in the proximity of a differentially regulated one, the latter may not be detectable in the differential screening procedure since the signal of the constitutively expressed gene might overshadow that of the differentially regulated gene. Even if only one transcriptionally active gene is located on a genomic clone, the gene could escape identification if its level of expression was too low for detection by this procedure. It is also unlikely that this procedure allows identification of all possible pathogenicity genes since only those pathogenicity genes whose expression is specifically induced or significantly increased during growth of the pathogen in the host would be identified.

After the isolation of the *ipi*-genes, the next step is the determination of the nucleotide sequence of the coding regions of the isolated genes. So far, the nucleotide sequence of *ipiA*, *ipiB*, *ipiC* and *ipiO* have been determined. *IpiA*, which has been renamed *ubi3R*, appeared to encode polyubiquitin (Pieterse *et al.*, 1991), a protein which 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). *IpiC*, now renamed *calA*, appeared to code for calmodulin (Pieterse *et al.*, 1993), 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). The nucleotide sequence of the transiently expressed genes *ipiB* and *ipiO* have no significant similarity with sequences present in any data library (CMJP, unpublished). Further studies to examine the regulation *ipiB* and *ipiO* gene expression and the function of their gene products during pathogenesis are in progress.

The differential screening is a non-biased approach which should enable the identification of *P. infestans* genes whose role in pathogenicity cannot be predicted by other methods. The study of differentially expressed genes of *P. infestans* during pathogenesis could therefore lead to the identification of important pathogenicity genes. Due to the choice of cDNA probes in the differential screening it is possible that some of the selected *P. infestans ipi*-genes are involved in metabolic adjustments of the pathogen in response to the change in nutrient environment which occurs during infection. Growth conditions *in vitro* are artificial, hence alterations in metabolism may occur when the fungus is transferred from a synthetic medium to the host plant. Nevertheless, changes in the nutrient environment encountered as the parasite invades the host may be an important trigger for the induction of genes involved in pathogenesis, and so, genes in this category may be of interest as well. At present, the molecular and cellular processes which are involved in the interaction of *P. infestans* with its host are poorly understood. Molecular cloning and characterization of *in planta* induced *P. infestans* genes will lead to a better understanding of these processes. Knowledge of the regulation and expression of these putative pathogenicity genes could allow the development of specific control methods for potato late blight.

References

Bodenmann, J., Heiniger, U. and Hohl, H.R. (1985). Extracellular enzymes of *Phytophthora infestans*: endo-cellulase, ß-glucosidases, and 1,3-ß-glucanases. *Canadian Journal of Microbiology* 31: 75-82.

Caten, C.E. and Jinks, J.L. (1968). Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Canadian Journal of Botany* 46: 329-347.

Cheung, W.Y. (1980). Calmodulin - an introduction. *In*: Cheung W.Y. (Ed.), Calcium and Cell Function, Vol. 1. Calmodulin, pp. 2-12. Academic Press, New York.

Feinberg, A.P. and Vogelstein, G. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* 132: 6-13.

Fritzemeier, K.-H., Cretin, C., Kombrink, E., Rohwer, F., Taylor, J., Scheel, D. and Hahlbrock, K. (1987). Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. *Plant Physiology* 85: 34-41.

Henniger, H. (1959). Versuche zur kultur verschiedener rassen von *Phytophthora infestans* (Mont) de By. auf künstlichen Nährböden. *Phytopathologische Zeitschrift* 34: 285-306.

Jarvis, M.C., Threlfall, D.R. and Friend, J. (1981). Potato cell wall polysaccharides: degradation with enzymes from *Phytophthora infestans*. *Journal of Experimental Botany* 32: 1309-1319.

Kombrink, E., Schröder, M. and Hahlbrock, K. (1988). Several "pathogenesis-related" proteins in potato are 1,3-ß-glucanases and chitinases. *Proceedings of the National Academy of Sciences USA* 85: 782-786.

Logemann, J., Schell, J. and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* 163: 16-20.

Mahe, A., Grisvard, J. and Dron, M. (1992). Fungal- and plant-specific gene markers to follow the bean anthracnose infection process and normalize a bean chitinase mRNA induction. *Molecular Plant-Microbe Interactions* 5: 242-248.

Matton, D.P. and Brisson, N. (1989). Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Molecular Plant-Microbe Interactions* 2: 325-331.

Monia, B.P., Ecker, D.J. Crooke, S.T. (1990). New perspectives on the structure and function of ubiquitin. *Bio/technology* 8: 209-215.

Pieterse, C.M.J., Risseeuw, E.P. and Davidse, L.C. (1991). An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Molecular Biology* 17: 799-811.

Pieterse, C.M.J., Verbakel, H.M., Hoek Spaans, J., Davidse, L.C. and Govers, F. (1993). Increased expression of the calmodulin gene of the late blight fungus *Phytophthora infestans* during pathogenesis on potato. *Molecular Plant-Microbe Interactions* 6: 164-172.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual, 2nd edition*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sargent, T.D. (1987). Isolation of differentially expressed genes. *In*: Berger, S.L. and Kimmel, A.R. (Eds.), Methods in Enzymology, Vol. 152. Guide to Molecular Cloning Techniques, pp 423-432. Academic Press Inc., San Diego.

Taylor, J.L., Fritzemeier, K.-H., Häuser, I., Kombrink, E., Rohwer, F., Schröder, M., Strittmatter, G. and Hahlbrock, K. (1990). Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. *Molecular Plant-Microbe Interactions* 3: 72-77.

Timberlake, W.E. (1980). Developmental gene regulation in *Aspergillus nidulans*. *Developmental Biology* 78: 497-510.

Timberlake, W.E. (1986). Isolation of stage- and cell-specific genes from fungi. *In*: Bailey, J.A. (Ed.), NATO ASI Series, Series H; Cell Biology, Vol. 1. Biology and Molecular Biology of Plant-Pathogen Interactions, pp. 343-357. Springer Verlag, Berlin.

Tooley, P.W. and Therriën, C.D. (1987). Cytophotometric determination of the nuclear DNA content of 23 mexican and 18 non-mexican isolates of *Phytophthora infestans*. *Experimental Mycology* 11: 19-26.

Unkles, S.E., Moon, R.P., Hawkins, A.R., Duncan, J.M. and Kinghorn, J.R. (1991). Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* 100: 105-112.

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chapter 4

An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin

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Abstract. An *in planta* induced gene of *Phytophthora infestans* (the causal organism of potato late blight) was selected from a genomic library by differential hybridization using labeled cDNA derived from poly(A)$^+$ RNA of *P. infestans* grown *in vitro* and labeled cDNA made from potato-*P. infestans* interaction poly(A)$^+$ RNA as probes. Sequence analysis showed that the gene codes for ubiquitin, a highly conserved protein which plays an important role in several cellular processes. The structure of the polyubiquitin gene (designated *ubi3R*) is consistent with the structure of other known polyubiquitin genes. It consists of three repeats in a head-to-tail arrangement without intervening sequences, each encoding a ubiquitin unit of 76 amino acids in length. The last ubiquitin unit is followed by an extra asparagine residue at the carboxy-terminal end. Northern and Southern blot analyses revealed that the polyubiquitin gene is a member of a multigene family of which all genes show induced expression *in planta*.

Introduction

Potato late blight caused by the fungus *Phytophthora infestans* (Mont.) de Bary (Oomycetes) is one of the most important diseases of potato. Leaves and tubers of susceptible cultivars become readily infected by this pathogen. The fungus spreads rapidly through the plant tissue causing a destructive necrosis. Resistance to late blight in potato can either be single or multiple gene based. In general single gene based resistance, governed by the so called R-genes, is characterized by a hypersensitive response resulting in a rapid cell death of invaded cells. Multiple gene based resistance is characterized by a low infection efficiency, slow tissue colonization and low sporulation rate. R-gene mediated resistance is commercially not attractive because it becomes rapidly ineffective due to the appearance of new virulent strains of the fungus. Breeding efforts, therefore, aim at introducing durable multiple gene based resistance into commercial potato cultivars. The physiological basis of this type of resistance is hardly understood and as a consequence breeders have to rely on empirical methods.

It can be assumed that the establishment of a pathogenic relation between the potato plant and *P. infestans* involves the mutual interference in cellular processes of each partner. Defence responses of host tissue being colonized (e.g. accumulation of pathogenesis related (PR) proteins and induction of enzymes of the phenylpropanoid pathway) are relatively well studied (Coolbear and Threlfall, 1985; Cuypers *et al.*, 1988; Fritzemeier *et al.*, 1987; Kombrink *et al.*, 1988), but nothing is known about changes in metabolism of the pathogen in response to the host. In other plant-microbe interactions, pathogenic (e.g. dicots-*Agrobacterium tumefaciens* and pea-*Fusarium solani* f.sp. *pisi*) as well as symbiotic interactions (legumes-*Rhizobium* spp.), signal molecules originating from the host have been characterized which induce expression of genes involved in establishing the interaction (Innes *et al.*, 1985; Stachel *et al.*, 1986; Woloshuk and Kolattukudy, 1986). It is therefore tempting to suggest also in the potato-*P. infestans* interaction that host factors induce physiological responses in the pathogen which are necessary for pathogenesis and which are mediated by pathogenicity genes. In view of this, both defence responses of the host and the degree to which pathogenicity genes are activated in the pathogen, ultimately determine speed and

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efficiency of the infection and colonization process and the intensity of sporulation. The result of these processes is reflected in the degree of durable resistance. The identification and characterization of *in planta* induced genes of *P. infestans* therefore will lead to a better understanding of the molecular basis of both pathogenicity of the fungus and durable resistance of the host.

The present study concerns the characterization of a *P. infestans* gene which was isolated from a genomic library by differential screening. The probes used were cDNA made from poly(A)$^+$ RNA of *P. infestans* grown *in vitro* and cDNA synthesized on poly(A)$^+$ RNA isolated from potato leaves infected with a compatible *P. infestans* race. This gene, designated *ubi3R*, codes for ubiquitin, one of the most conserved proteins known to date. The significance of the *in planta* induced expression of this gene will be discussed.

Materials and Methods

Culturing of Phytophthora infestans *and inoculation of potato leaves*. Strain 88069 of *P. infestans* (Mont.) de Bary (A1-mating type, race 1.3.4.7) was isolated from a naturally infected tomato plant in the summer of 1988 and was maintained on rye-agar medium containing 2% sucrose (Caten and Jinks, 1968). Liquid cultures in 25 ml Henniger synthetic medium (Henniger, 1959) were initiated from zoöspores obtained from 2-week-old cultures on rye-sucrose agar at a concentration of $2x10^4$ zoöspores/ml. Cultures were incubated for 14 days at 20 °C to obtain mycelium for DNA and RNA extractions.

Leaves of the potato cultivar "Ajax", which carries the R3-gene for late blight resistance, were inserted in florist's foam oases saturated with water and inoculated with *P. infestans* by spraying a sporangial suspension ($5x10^5$ sporangia/ml) on the axial side of the leaflets. After inoculation, the leaves were incubated at 18 °C at 100% RH in plastic boxes with a transparent lid under cool fluorescent light for 16 hours per day. As control, non-inoculated leaves were treated in the same way. Under these conditions the symptoms of the late blight disease develop from small necrotic lesions, which are first visible 24 hours after inoculation, to completely "water soaked" leaves on day 3. During this period leaves were collected for RNA isolation. Four days after inoculation the fungus sporulates extensively. Secondary infections by necrotrophic bacteria prohibit the isolation of good quality RNA in this stage.

Isolation of genomic DNA and construction of a genomic library. Genomic DNA of *P. infestans* was isolated from mycelium grown in liquid culture. Mycelium (10 g fresh weight) was ground in liquid nitrogen to a fine powder and mixed in 5 ml of extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 50 mM EGTA, 0.8% (w/v) tri-isopropylnaphtalene sulfonic acid (TNS) and 0.48% (w/v) 4-aminosalicylic acid (PAS)) per gram of mycelium and incubated at 55 °C for 5 minutes. The mixture was extracted once with 0.6 volume of water saturated phenol (55 °C). The waterphase was then extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with an equal

volume of chloroform/isoamyl alcohol (24:1). The DNA was precipitated with 0.6 volume of 2-propanol, dissolved in $T_{10}E_1$ (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and further purified by CsCl gradient centrifugation (Maniatis *et al.*, 1982).

A genomic library was constructed in the replacement vector λEMBL3 (Frischauf *et al.*, 1983), according to Frischauf (1987) with minor modifications. High molecular weight DNA was partially digested with *Sau*3AI and fragments of 15 to 23 kb (kilobase pairs) were isolated after centrifugation on a 10-40% sucrose gradient. Three hundred ng of these fragments were ligated to 250 ng of λEMBL3 *Bam*HI-arms (Promega). Packaging of the ligated DNA, using the Packagene *in vitro* packaging system of Promega, was performed according to the manufacturers instructions.

Isolation of RNA and poly(A)⁺ RNA and preparation of labeled cDNA probes. Total RNA was prepared from mycelium of *P. infestans* grown in liquid cultures and from colonized leaflets (interaction RNA) using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). To check for integrity of RNA, the RNA was electrophoresed in a 1.5% agarose-TBE (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA) gel containing 0.5 µg/ml ethidium bromide. Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography as described by Maniatis *et al.* (1982). First strand cDNA with a specific activity of approximately $1\text{-}2\text{x}10^8$ cpm/µg was prepared from 1 µg poly(A)⁺ RNA as described by Sargent (1987) using M-MLV reverse transcriptase (Gibco BRL) and oligo(dT) to prime the synthesis reaction.

Differential screening, subcloning and sequencing. The genomic library was plated on a recombinant deficient *E. coli* host, strain MB406 (*supE*, *recB21*, *recC22*, *sbcB15*, *hfl*A, *hfl*B, *hsd*R⁻), which enables the propagation of a non-biased genomic library (Wyman *et al.*, 1986). Four replica filters of the genomic library were made on Hybond-N⁺ membrane (Amersham) according to the instructions of the manufacturer. The library was differentially hybridized in duplicate at high stringency in hybridization mix containing 5x SSC (750 mM NaCl and 75 mM Na₃-citrate), 5x Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone and 0.1% (w/v) BSA (fraction V)), 0.5% SDS and 100 µg/ml calf thymus DNA at 65 °C for 16 hours using as probes labeled cDNA (10^7 cpm) derived from poly(A)⁺ RNA of the fungus grown *in vitro*, and labeled cDNA (10^7 cpm) derived from interaction poly(A)⁺ RNA (3 days after inoculation). The filters were washed in 2x SSC/0.5% SDS at 65 °C and exposed to Kodak X-Omat S film for 2-7 days at -80 °C. The selected plaques were purified by a second round of differential hybridization as described above.

DNA from the selected lambda clone was digested with several restriction enzymes and separated on a 0.8% agarose-TBE gel. Following electrophoresis, the DNA was transferred to Hybond-N⁺ membrane (Amersham) by capillary transfer (Maniatis *et al.*, 1982) and hybridized at high stringency (65 °C) as described above using ³²P-labeled interaction cDNA as probe. The membrane was washed in 2x SSC/0.5% SDS at 65 °C and exposed to Kodak

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X-AR film for 24 hours at -80 °C. The hybridizing *Sst*I-fragment was subcloned into pTZ19U using standard techniques (Maniatis *et al.*, 1982) which resulted in the plasmid pUB-S (Fig. 1B). Both strands of overlapping subclones of pUB-S were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using a sequencing system (Promega) for sequencing on double-stranded DNA. For analyzing the sequence data and to screen the EMBL Data Library (Hamm and Cameron, 1986) and the GenBank databank (Bilofsky *et al.*, 1986), the Sequence Analysis Software Package, Version 6.0, of the Genetics Computer Group (GCG) of the University of Wisconsin was used.

Southern blot analysis of genomic Phytophthora infestans *DNA.* Genomic DNA (10 μg) of *P. infestans* was digested with *Kpn*I, *Eco*RI, *Bam*HI, *Hind*III, and *Sst*I and size separated on a 0.7% agarose-TBE gel. Following electrophoresis, the DNA was transferred to Hybond-N$^+$ membrane (Amersham) by capillary transfer and hybridized at high stringency (65 °C) as described above to the two ^{32}P-labeled 228 bp (base pairs) *Pvu*II-fragments from the *ubi3R* coding region (Fig. 1). Probe was made by random primer labeling using Promega's Prime-a-Gene labeling kit. The blot was washed in 0.5x SSC/0.1% SDS at 65 °C and exposed to Kodak X-Omat S film for 16 hours at -80 °C.

Northern blot analysis and primer extension of poly(A)$^+$ RNA. For northern blot analysis, 15 μg of total RNA was denatured and electrophoresed on a 1.5% agarose-formaldehyde denaturing gel as described by Maniatis *et al.* (1982). Following electrophoresis, the RNA was transferred to Hybond-N$^+$ membrane (Amersham) by capillary transfer and hybridized at high stringency (65 °C) as described above using the two ^{32}P-labeled 228 bp *Pvu*II-fragments from the coding region of *ubi3R* as probe. The blot was washed in 0.5x SSC/0.1% SDS at 65 °C and exposed to Kodak X-Omat S film for 2 days at -80 °C. Probe was then removed by immersing the membrane in a solution of boiling 0.1% SDS for 5 minutes. The blot was rehybridized as described above using a gene-specific probe from the upstream region of the *ubi3R* gene. The gene-specific upstream DNA fragment was generated from pUB-S by PCR using a primer (5'-GGTTGCCTCGGTTTATG-3') complementary to the sequence at position -2 to -19 relative to the ATG start codon in the *ubi3R* gene and the pUC/M13 sequencing primer (5'-GTTTTCCCAGTCACGAC-3') complementary to a sequence in the vector. The reaction mixture (100 μl) contained 10 ng pUB-S, 120 ng of each primer, 0.2 mM dNTP's and 2 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus) in Taq polymerase buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.001 % gelatin). Amplification was performed in a DNA Thermal Cycler in 18 cycles of 30 seconds at 94°C, 30 seconds at 45°C and 90 seconds at 72°C. The 799 bp PCR product was labeled with ^{32}P-dATP by random primer labeling as described above. After hybridization and exposure, the blot was again deprobed and rehybridized, now using as probe the 2.8 kb *Pst*I-insert from pSTA31 containing the constitutively expressed actin (*act*A) gene of *P. infestans* (Unkles *et al.*, 1991).

To map the 5' end of the *ubi3R* transcript, an oligonucleotide (5'-GTTGCCTCGGTTTATGAG-3') complementary to the sequence at position -2 to -19 relative to the ATG start codon was labeled with ^{32}P at its 5'-end using T4 polynucleotide kinase (Promega) according to the manufacturers instructions. Labeled primer (5 ng) was annealed to 3 μg of poly(A)$^+$ RNA isolated from *in vitro* grown *P. infestans* mycelium and from non-inoculated potato leaves as a control. Extension of the primer was performed in a reaction mixture (25 μl) containing 400 units M-MLV reverse transcriptase (Gibco BRL), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DDT, 3 mM MgCl$_2$, 0.1 μg/μl nuclease-free BSA and 200 μM of each dNTP at 37°C for 45 minutes. The primer extension products were analyzed by electrophoresis on a polyacrylamide gel (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, 8 M urea, 5.7% acrylamide and 0.3% bis-acrylamide) and detected by autoradiography.

Results

Selection of in planta *induced* Phytophthora infestans *genes from a genomic library and mapping of one of these genes on the selected lambda clone.* As calculated from microfluorometric determinations of nuclear DNA content (Tooley and Therriën, 1987), the haploid genome size of *P. infestans* is approximately 2.7×10^8 bp. In order to achieve a 99% probability of having any DNA sequence represented in the library, 80,000 recombinant plaques were screened. Differential screening of the genomic library with fungal and interaction cDNA is performed under non-saturating conditions. Under these conditions the intensity of each obtained signal corresponds with the abundancy of a particular cDNA in the probe. A stronger signal obtained after hybridization with interaction cDNA as compared to the signal obtained after hybridization with fungal cDNA indicates a higher abundancy of those cDNA's which are complementary to the DNA in the hybridizing lambda clone. Since the filters are hybridized with equal amounts of labeled cDNA, the procedure even underestimates the difference in abundancy because the quantity of fungal derived cDNA present in the interaction cDNA probe is much less than in the fungal cDNA probe. Thirteen plaques gave a relatively strong signal after hybridization with labeled interaction cDNA and a relatively weak or no signal after hybridization with labeled cDNA of the fungus grown *in vitro*. Approximately fifty additional plaques gave comparable signals after hybridization with both cDNA probes. One of the strongly differential hybridizing plaques (DHC-A) was purified after a second round of differential hybridization. Southern analysis of various restriction fragments of DHC-A with interaction cDNA as probe revealed a strongly hybridizing *Sst*I-fragment of 3.2 kb in length. This fragment containing a putative *in planta* induced gene was subcloned in pTZ19U and from the obtained plasmid pUB-S a restriction map was constructed. The approximate location of the coding region of the differentially expressed gene (closed bar in Fig. 1) was determined by Southern blot analysis using interaction cDNA as probe.
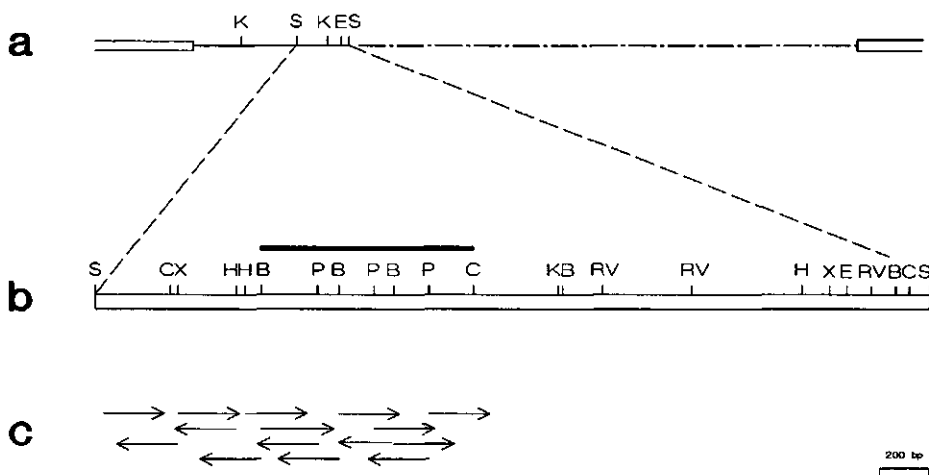
Figure 1. **a.** Lambda clone DHC-A (incomplete restriction map). **b.** Restriction map of the 3.2 kb *Sst*I insert of pUB-S. The closed bar indicates the approximate location of the coding region of the differentially expressed gene as determined by Southern hybridization using labeled interaction cDNA as probe. **c.** The sequencing strategy is indicated by arrows. B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pvu*II; S, *Sst*I; X, *Xho*I; RV, *Eco*RV.

Differentially expressed gene codes for polyubiquitin. Several fragments of the 3.2 kb *Sst*I-insert of pUB-S were subcloned and the sequence of 1632 nt (nucleotides) was determined by dideoxy sequencing on both strands of various overlapping clones (Fig. 2). The sequencing strategy is summarized in Fig. 1. An open-reading frame of 687 nt containing three almost identical 228 nt repeats was found in the 1632 nt sequence (Fig. 2). Comparison of the 687 nt sequence with the sequence databank revealed that the sequence codes for polyubiquitin. The coding region of the polyubiquitin gene encodes three ubiquitin units with a length of 76 amino acids in a head-to-tail arrangement followed by an extra asparagine residue at the carboxy-terminal end. Although the ubiquitin-coding repeats within the gene differ up to 8 out of 228 bp, they code for identical amino acid sequences (Fig. 2). Only 4 or 5 amino acids of the 76 amino acid ubiquitin sequence differ from the sequence of barley, human, chicken and yeast ubiquitin (Fig. 3). Because of the three ubiquitin repeated units we have designated the gene *ubi3R*.

In order to map the 5' end of the *ubi3R* transcript, primer extension was performed on poly(A)$^+$ RNA isolated from the fungus grown *in vitro* and from non-infected potato leaves using an oligonucleotide complementary to the *ubi3R* sequence at positions -2 to -19 relative to the ATG start codon to prime the synthesis reaction. The primer extension products were electrophoresed next to the products of a sequencing reaction using the same oligonucleotide as primer and pUB-S as template DNA. Fig. 4 shows a primer extension product of 51 nucleotides indicating that the transcription initiation site of *ubi3R* is located at position -52 relative to the ATG start codon.

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         -750                 -730                 -710                 -690
GAGCTCCTTCATACGCCCGCGAAGACGGCTCACAAAGTCACCAATGTCGCCCTAGACTCTGCTCGTCCCGGAAAGTTTCGGT
         -670                 -650                 -630                 -610
TGATGAACTCGCGCGCATCGAACTCAGGTCGGTCCAACACGTCGCTCCGAGGGCAAAACCTGTACATGAGACGTTGGTGAG
         -590                 -570                 -550                 -530
ACCTGGCAGCCAGCTTGAAACAGGACTGCAACACGAGGAAGATACTTTGGCTAGCGTGTCGTCCACGTTCTGAGGGAGCT
         -510                 -490                 -470                 -450
TCAATGCGCTTCCATCGCCGTCGCTGTACTCGCTGGCTCTGGCCGACGACGAGCCACGACGCAGTAATGGATCCATTTTC
         -430                 -410                 -390                 -370
GACTCTTTTGAAGCTCGAGGATGACATATGATGGGATATTTGATTTATGTTAGAAGATAGGACTCTCGAAATATTGTAAC
         -350                 -330                 -310                 -290
TTACAAAGATTTACAGAATCTTCGTATAAAAGAGTAGAAAGTAATAAAAATAAGAACACCCCGTAAAAATTGCAGTCGAT
         -270                 -250                 -230                 -210
TTCTTACAATCTGGATTCAGCTTGTGATACCGGTAGCAAGGGGGGTATCACCTGCTGTTTATTTATTTCGCAAATTTTAT
         -190                 -170                 -150                 -130
TCTGTGCGGGGAAGCTTTCGGCTGATAATCCTCTTTTCTCAAATCGAGAAGCTTCGTTTCAGTATGCCTACATCAACCTG
         -110                 -90                  -70                  -50
AGAACAAGGGTGGCCTTATCCCTTCCCCTCGGTAAGATCTATCGCTTCGAGCGCCTCCTTTGCTCATTTTCCATTTTGAG
         -30                  -10        +1         10                   30
CGGAAACACTCAAGCACTCTCATAAACCGAGGCAACTATGCAGATTTTCGTGAAGACTTTGACCGGCAAGACGATCACGC
                                          M1  Q   I   F   V   K   T   L   T   G   K   T   I   T   L
         50                   70                   90                   110
TGGACGTGGAGCCTTCGGACTCGATCGACAACGTCAAGCAAAAAATTCAGGACAAGGAAGGCATCCCTCCTGACCAGCAG
     D   V   E   P   S   D   S   I   D   N   V   K   Q   K   I   Q   D   K   E   G   I   P   P   D   Q   Q
         130                  150                  170                  190
CGTCTGATCTTCGCTGGTAAGCAGCTGGAGGACGGCCGCACGCTGAGCGACTACAACATCCAGAAGGAGTCTACGCTTCA
     R   L   I   F   A   G   K   Q   L   E   D   G   R   T   L   S   D   Y   N   I   Q   K   E   S   T   L   H
         210                  230                  250                  270
CCTGGTGCTTCGCCTGCGTGGTGGCATGCAGATCTTTGTGAAGACTTTGACCGGCAAGACGATCACGCTGGACGTGGAGC
     L   V   L   R   L   R   G   G   M2  Q   I   F   V   K   T   L   T   G   K   T   I   T   L   D   V   E   P
         290                  310                  330                  350
CTTCAGACTCGATCGACAACGTCAAGCAAAAAATTCAGGACAAGGAAGGCATCCCTCCTGACCAACAGCGTCTGATCTTC
     S   D   S   I   D   N   V   K   Q   K   I   Q   D   K   E   G   I   P   P   D   Q   Q   R   L   I   F
         370                  390                  410                  430
GCTGGTAAGGAGCTGGAGGACGGCCGTACGCTGAGCGACTACAACATCCAGAAGGAGTCAACGCTTCATCTGGTGCTTCG
     A   G   K   Q   L   E   D   G   R   T   L   S   D   Y   N   I   Q   K   E   S   T   L   H   L   V   L   R
         450                  470                  490                  510
CCTGCGTGGTGGCATGCAGATCTTTGTGAAGACTTTAACTGGCAAGACGATCACGCTGGACGTGGAGCCTTCAGACTCGA
     L   R   G   G   M3  Q   I   F   V   K   T   L   T   G   K   T   I   T   L   D   V   E   P   S   D   S   I
         530                  550                  570                  590
TCGACAACGTCAAGCAAAAAATTCAGGACAAGGAAGGCATCCCTCCTGACCAGCAGCGTCTGATCTTCGCTGGTAAGCAG
     D   N   V   K   Q   K   I   Q   D   K   E   G   I   P   P   D   Q   Q   R   L   I   F   A   G   K   Q
         610                  630                  650                  670
CTGGAGGACGGCCGCACGCTGAGCGACTACAACATCCAGAAGGAGTCAACGCTTCACCTGGTGCTTCGCCTGCGTGGCGG
     L   E   D   G   R   T   L   S   D   Y   N   I   Q   K   E   S   T   L   H   L   V   L   R   L   R   G   G
         690                  710                  730                  750
TAACTAAATTGGTTCAACCGTAAGGTCGTTTTTTCTTGCCTCGGACCAGTTTGTTTCGCTACTTAATGCTACTTTAAAGA
     N   *
         770                  790                  _____810                 830
AGTTTTAGTAGTATGAAGTGCAATCGATTACTCAATTAAAATGTTTCGACTTCAGAGCTGAATACTACTGGTAGTTATAT
         850                  870
CAATTTTGTGACATCGGCATCCTACGATGGAT
```

Figure 2. Nucleotide sequence of the *Phytophthora infestans* polyubiquitin (*ubi3R*) gene and corresponding amino acid sequence of the encoded protein. The transcription start site as determined by primer extension is indicated by an asterisk. Numbered methionine residues in the amino acid sequence represent the start of the three ubiquitin units. Underlined nucleotides in the 5' non-coding region match the consensus sequence for heatshock promotor elements (CNNGAANNTTCNNG; Bienz and Pelham, 1987). Overlined DNA sequence (AATTAAAA) in the 3'-flanking region of the polyubiquitin gene represents a possible polyadenylation signal. The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X55717.

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Phytophthora infestans   M Q I F V K T L T G K T I T L D V E P S D S I D N V
Yeast                                                      E     S     T
Barley                                                     E     S     T
Chicken                                                    E           T   E
Human                                                      E           T   E


Phytophthora infestans   K Q K I Q D K E G I P P D Q Q R L I F A G K Q L E D
Yeast                    S
Barley                   A
Chicken                  A
Human                    A


Phytophthora infestans   G R T L S D Y N I Q K E S T L H L V L R L R G G N *
Yeast                                                                      *
Barley                               A                                  K *
Chicken                                                                 Y *
Human                                                                   C *
```

Figure 3. Amino acid sequence comparison between the last ubiquitin encoding unit (77 amino acids) of *Phytophthora infestans* polyubiquitin and the last ubiquitin encoding unit of polyubiquitin of yeast (Ozkaynak *et al.*, 1987), barley (Gausing and Barkardottir, 1986), chicken (Bond and Schlesinger, 1986) and human (Baker and Board, 1987). Only amino acid residues which differ from the *P. infestans* sequence are shown in the yeast, barley, chicken and human amino acid sequence.

As in most fungal genes, no typical "TATAA" or "CAAT" boxes are present in the promoter region. Several CT-rich regions, commonly found in the vicinity of the initiation codons of fungal genes, are present upstream of the ATG start codon. Similarities matching the consensus sequence for heat shock promotor elements (Bienz and Pelham, 1987) which have been shown to be present in polyubiquitin genes from a number of organisms, were also found in the 5' non-coding region of the *ubi3R* gene (underlined in Fig. 2). In the 3'-flanking region of the gene, a possible polyadenylation recognition sequence (AATTAAAA) was found at position +797 to +804, 107 nucleotides downstream of the TAA stop codon (overlined in Fig. 2).

The identified ubiquitin gene is a member of a multigene family. Northern blot analysis of RNA isolated from the *in vitro* grown mycelium, using the two 228 bp *Pvu*II-fragments from the coding region of *ubi3R* as probe, shows four bands of approximately 850, 1100, 1350 and 2350 nucleotides in length (Fig. 5A, lane 5). Longer exposure reveals a fifth band of circa 1850 nucleotides just above the plant ubiquitin mRNA. This suggests that the genome of *P. infestans* contains multiple copies of ubiquitin encoding genes of different lengths. Using the gene-specific probe from the *ubi3R* promoter region, it was shown that the 850 nucleotide transcript corresponds to the *ubi3R* gene (Fig. 5B). Southern blot analysis of digested genomic DNA in which the two 228 bp *Pvu*II-fragments from the *ubi3R* coding region were used as a probe (Fig. 6), confirmed that the identified ubiquitin gene belongs to a multigene family. In each digest analyzed, multiple bands hybridize. Among those a

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Figure 4. Primer extension of 3 μg of poly(A)⁺ RNA isolated from *in vitro* grown *Phytophthora infestans* mycelium (lane 1) and non-infected potato leaves (lane 2) using a primer (5'-GTTGCCTCGGTTTATGAG-3')complementary to the *ubi3R* sequence at position -2 to -19.
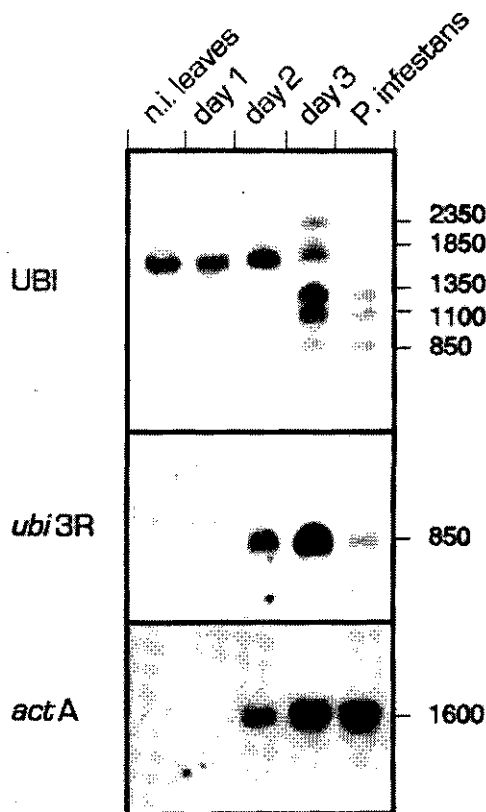
Figure 5. Northern blot analysis of 15 μg of total RNA isolated from non-infected potato leaves (lane 1), colonized potato leaves, 1 day after inoculation (lane 2), 2 days after inoculation (lane 3) and 3 days after inoculation (lane 4) and from *Phytophthora infestans* grown *in vitro* (lane 5). The two 228 bp PvuII-fragments from the *ubi3R* coding region (A), the *ubi3R* gene-specific probe from the promoter region of *ubi3R* (B) and the 2.8 kb PstI-insert from pSTA31 containing the *P. infestans* actin (*actA*) gene (C) were used as probes.

2.3 kb HindIII-, a 3.2 kb SstI- and a 5.3 kb KpnI-band correspond to identical restriction fragments in the DNA of phage DHC-A (Fig. 1). The EcoRI- and BamHI-fragment on the Southern blot differ in size with those in the phage DHC-A because only a part of these fragments is linked to vector DNA. Since the coding region of *ubi3R* does not have any internal KpnI, EcoRI, BamHI, HindIII, and SstI sites and no introns it is evident that the *P. infestans* genome contains multiple copies of ubiquitin encoding genes.

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In planta *induced expression of* Phytophthora infestans *ubiquitin genes.* The differential expression of the characterized ubiquitin gene was confirmed in two ways. In the first procedure interaction RNA, i.e. RNA isolated from inoculated leaves, and RNA isolated from mycelium grown *in vitro* was subjected to northern blot analysis (Fig. 5). Equal amounts of interaction RNA and fungal RNA were applied to the gel. The hybridization signals of the ubiquitin probe with interaction RNA (Fig. 5A, lane 4, 3 days after inoculation) are 2-3 fold stronger in comparison with the signals obtained with RNA isolated from the fungus grown *in vitro* (Fig. 5A, lane 5) indicating that the relative amounts of all five fungal ubiquitin transcripts is higher in interaction RNA. The 1800 nt transcript present in lanes 1-4 of Fig. 5A is a potato ubiquitin messenger. Due to the highly conserved sequence of ubiquitin, the potato ubiquitin

Figure 6. Southern blot analysis of *Phytophthora infestans* DNA (10 μg/lane) digested with the restriction enzymes indicated. The two 228 bp *Pvu*II-fragments from the coding region of *ubi3R* were used as probe.

transcript is cross-hybridizing with the fungal ubiquitin probe. To follow the progression of fungal growth during the development of the disease, the proportion of fungal RNA in the total RNA population isolated from infected leaves was determined. To this end a probe of the constitutively expressed actin (*act*A) gene of *P. infestans* (Unkles *et al.*, 1991) was used for hybridization of the northern blot (Fig. 5C). One day after inoculation the proportion of fungal RNA in the interaction RNA is very low. Due to the increasing amount of fungal biomass during colonization of the leaf tissue, the proportion of fungal RNA in the interaction RNA mixture increases rapidly to approximately 50%, three days after inoculation. Taking this into consideration, the results of the northern blot analyses shows that the expression of the ubiquitin encoding genes during growth of the fungus *in planta* increases 4-6 fold. Using a *ubi3R* gene-specific probe, the induction of expression of the *ubi3R* gene seems to be even higher (Fig. 5B).

In the second procedure the difference in relative abundance of *P. infestans* ubiquitin mRNA's *in planta* and *in vitro* was analyzed by comparitive hybridization of four replica filters of the *P. infestans* genomic library with (1) the random primer labeled 228 bp

Figure 7. Comparative hybridization of four replica filters of the *Phytophthora infestans* genomic library. Filters were hybridized with equal amounts of labeled interaction cDNA derived from poly(A)$^+$ RNA of the interaction, 3 days after inoculation (I), labeled cDNA derived from poly(A)$^+$ RNA of *in vitro* grown *P. infestans* mycelium (F), labeled cDNA derived from poly(A)$^+$ RNA of non-infected potato leaves (P) and the two 228 bp *Pvu*II-fragments from the *ubi3R* coding region labeled by random primer labeling (U).

*Pvu*II-fragments from the *ubi3R* coding region (Fig. 7-U), (2) labeled interaction cDNA derived from poly(A)$^+$ RNA of the interaction, 3 days after inoculation (Fig. 7-I), (3) labeled fungal cDNA made from poly(A)$^+$ RNA of the fungus grown *in vitro* (Fig. 7-F) and (4) labeled cDNA derived from poly(A)$^+$ RNA of non-infected potato leaves (Fig. 7-P) as a control. Lambda clones containing ubiquitin encoding sequences (indicated by arrows in Fig. 7) gave rise to a relatively strong signal when hybridized with labeled interaction cDNA (Fig. 7-I). Hybridization of a control replica filter with labeled cDNA derived from poly(A)$^+$ RNA of non-infected potato leaves (Fig. 7-P) shows very weak or no signals indicating that the signals in Fig. 7-I are predominantly due to hybridization with fungal cDNA. Since the intensity of the signal is positively correlated with the abundance of the corresponding mRNA's, the ubiquitin mRNA's can be classified as highly abundant in the *P. infestans* mRNA population of the interaction. Hybridization of a replica filter with labeled fungal cDNA (Fig. 7-F) shows a relatively weak signal of the same clones indicating that ubiquitin mRNA's are several fold less abundant in the mRNA population of *in vitro* grown mycelium than in the mRNA population of *in planta* produced mycelium. This procedure even underestimates the difference in abundancy of the ubiquitin mRNA's because the filters were hybridized with equal amounts of labeled cDNA whereas the proportion of fungal cDNA in the interaction cDNA probe is approximately half of that in the fungal cDNA probe.

Discussion

Differential hybridization of cDNA libraries has been proven to be a useful tool for the isolation of genes which are differentially expressed under distinct physiological conditions and in particular for the isolation of developmentally regulated genes (Timberlake, 1980; 1986). Differential hybridization of genomic libraries is not often utilized since large vector inserts and low specificity of the labeled cDNA probes make it difficult to map and isolate the genes of interest. Isolation of *in planta* induced genes from *P. infestans* can only be done by differential screening of a genomic library since the use of a cDNA library made from interaction poly(A)$^+$ RNA will yield both differentially expressed plant and fungal genes which cannot directly be distinguished. In developing the procedure using a genomic library it was of utmost importance to use cDNA probes with a high specific activity ($> 10^8$ cpm/μg cDNA) to be able to isolate putative *in planta* induced genes from the *P. infestans* genomic library.

Using the procedure described above a differentially expressed *P. infestans* polyubiquitin gene was isolated from a genomic library. The structure of the selected *ubi3R* gene is completely consistent with the unique structure of known polyubiquitin genes from other organisms (Hershko and Ciechanover, 1986; Monia *et al.*, 1990). The characterized gene consists of three repeats each coding for the 76 amino acid ubiquitin peptide. Southern and northern blot analyses showed that the characterized polyubiquitin gene is a member of a multigene family. The *ubi3R* gene codes for the 850 nt transcript as shown in Fig. 5B. The sizes of the other transcripts (1100, 1350, 1850 and 2350 nt) indicate that they may code for polyubiquitin genes containing up to 4, 5, 7 and 9 ubiquitin units which is a common feature for polyubiquitin gene families.

Ubiquitin is one of the most conserved proteins known to date (for reviews see; Finley and Varshavsky, 1985; Hershko, 1988; Hershko and Ciechanover, 1986; Monia *et al.*, 1990; Rechsteiner, 1987). The 76 amino acid protein occurs in all eukaryotic cells, either free or covalently attached to proteins in the cytosol, plasma membrane or to chromosomal histones. Ubiquitin has been shown to play a key role in several important cellular processes such as the selective degradation of intra-cellular proteins (Hershko, 1988; Hershko and Ciechanover, 1986; Rechsteiner, 1987), maintenance of chromatin structure (Matsui *et al.*, 1979; Mueller *et al.*, 1985), regulation of gene expression (Barsoum and Vashavsky, 1985; Goldknopf *et al.*, 1980) and modification of cell surface receptors (Leung *et al.*, 1987; Siegelman *et al.*, 1986; Yardin *et al.*, 1986). Genes encoding ubiquitin have been characterized for a variety of organisms such as yeast (Ozkaynak *et al.*, 1987), man (Wiborg *et al.*, 1985), chicken (Bond and Schlesinger, 1986), *Xenopus laevis* (Dworkin-Rastl *et al.*, 1984), *Drosophila melanogaster* (Arribas *et al.*, 1986), *Tripanosoma cruzi* (Swindle *et al.*, 1988), and *Caenorhabditis elegans* (Graham *et al.*, 1988). In each case, ubiquitin is encoded by one or more polyubiquitin genes which consist of direct repeats of the 76 amino acid coding units. The last repeat at the 3' end of the polyubiquitin gene is usually followed by an extra amino acid residue which is not conserved among different species. In the *P. infestans ubi3R* gene

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this appears to be an asparagine. Although the unique structure of polyubiquitin genes has been conserved in evolution, considerable variation exists in the number of repeats within each polyubiquitin gene and the number of polyubiquitin encoding loci in the genome.

Recently another class of genes which contain the ubiquitin coding sequence has been identified. In these genes the ubiquitin coding region is fused in frame to the 5'-end of a coding sequence of an unrelated polypeptide (Muller-Taubenberger et al., 1988b; Ozkaynak et al., 1987; Salveson et al., 1987). Their products are called ubiquitin carboxyl extension proteins (UbCEP's) and have been found in several eukaryotic organisms. The carboxyl extension proteins (CEP's) are reported to be ribosomal proteins whose fusion to ubiquitin is shown to facilitate ribosomal biogenesis (Finley et al., 1989). Similar to the ubiquitin encoding sequence, the CEP encoding sequence of these natural gene fusions is conserved among species.

The expression of both classes of ubiquitin genes is found to be differentially regulated under conditions of stress, rapid cell growth or during development (Gausing and Barkardottir, 1986; Ohmachi et al., 1989). Induced expression of polyubiquitin genes by heatshock or other types of stress has been observed in a number of organisms (Bond and Schlesinger, 1985; Muller-Taubenberger et al., 1988a; Ozkaynak et al., 1987). This facilitates an increased production of ubiquitin monomers for the ubiquitin-mediated degradation of abnormal proteins which arise during stress. The expression of UbCEP encoding genes has been demonstrated to be induced under conditions of rapid cell growth and cell division (Monia et al., 1990; Ozkaynak et al., 1987; Swindle et al., 1988). As protein synthesis is a major cellular process during cell growth and since CEP's are identified as ribosomal proteins, the significance of induced expression of UbCEP encoding genes is obvious.

The induced expression of ubiquitin encoding genes in *P. infestans* during colonization of potato leaves may reflect the highly-active metabolic state of the mycelium in the host tissue. During exponential growth *in vitro* on synthetic Henniger medium and on rich rye-sucrose agar medium however, the mycelium is also in a highly-active metabolic state but the ubiquitin encoding genes show a 4-6 fold lower level of expression. This implies that the host environment specifically induces the expression of the ubiquitin encoding genes. Which factors or conditions are due to the *in planta* induced expression of the *P. infestans* ubiquitin genes needs to be investigated. Also the significance of induced expression of the ubiquitin encoding genes during pathogenesis remains te be resolved. In view of the regulatory function of ubiquitin in gene expression (Barsoum and Varshavsky, 1985; Goldknopf et al., 1980) it is tempting to speculate that induction of the characterized *ubi3R* gene may have an effect on the regulation of the expression of genes involved in pathogenicity. The other genes containing the ubiquitin encoding sequence may code for polyubiquitin as well and may have a similar function, but it cannot be excluded that they encode UbCEP's and are involved in biogenesis of ribosomes.

Expression studies should reveal whether the ubiquitin genes are inducible *in vitro* by heatshock and stress or by particular plant factors in order to gain more insight in the

significance of the induced expression of the *P. infestans* ubiquitin genes *in planta*. The *ubi3R* gene is one of the first genes isolated from *P. infestans* and is highly expressed *in vitro*. The promoter region of the gene will be used for the construction of vectors for transformation of *P. infestans*, a necessary tool for the accomplishment of our goal, the isolation and characterization of pathogenicity genes of *P. infestans*.

References

Arribas, C., Sampedro, J. and Izquierdo, M. (1986). The ubiquitin genes in *D. melanogaster*: Transcription and polymorphism. *Biochemica Biophysica Acta* **868**: 119-127.

Baker, R.T. and Board, P.G. (1987). The human ubiquitin gene family: structure of a gene and pseudogenes from the Ub B subfamily. *Nucleic Acids Research* **15**: 443-463.

Barsoum, J. and Varshavsky, A. (1985). Preferential localization of variant nucleosomes near the 5'-end of the mouse dihydrofolate reductase gene. *Journal of Biological Chemistry* **260**: 7688-7697.

Bienz, M. and Pelham, H.R.B. (1987). Mechanisms of heat-shock gene activation in higher eukaryotes. *Advanced Genetics* **24**: 31-72.

Bilofsky, H.S., Burks, C., Fickett, J.W., Goad, W.B., Lewitter, F.I., Rindone, W.P., Swindell, C.D. and Tung, C.S. (1986). The GeneBank genetic sequence data bank. *Nucleic Acids Research* **14**: 1-4.

Bond, U. and Schlesinger, M.J. (1985). Ubiquitin is a heat shock protein in chicken embryo fibroblasts. *Molecular and Cellular Biololgy* **5**: 949-959.

Bond, U. and Schlesinger, M.J. (1986). The chicken ubiquitin gene contains a heat shock promoter and expresses an unstable mRNA in heat-shocked cells. *Molecular and Cellular Biology* **6**: 4602-4610.

Caten, C.E. and Jinks, J.L. (1968). Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Canadian Journal of Botany* **46**: 329-347.

Coolbear, T. and Threlfall, D.R. (1985). A comparison of the sites of phytoalexin accumulation and of biosynthetic activity in potato tuber tissue inoculated with biotic elicitors. *Phytochemistry* **24**: 2219-2224.

Cuypers, B., Schmelzer, E. and Hahlbrock, K. (1988). *In situ* localization of rapidly accumulated phenylalanine ammonia-lyase mRNA around penetration sites of *Phytophthora infestans* in potato leaves. *Molecular Plant-Microbe Interactions* **1**: 157-160.

Dworkin-Rastl, E., Shrutkowski, A. and Dworkin, M.B. (1984). Multiple ubiquitin mRNAs during *Xenopus laevis* development contain tandem repeats of the 76 amino acid coding sequence. *Cell* **39**: 321-325.

Finley, D., Barte, B. and Varshavsky, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* **338**: 394-401.

Finley, D. and Varshavsky, A. (1985). The ubiquitin system: Functions and mechanisms. *Trends in Biochemical Sciences* **10**: 343-347.

Frischauf, A.-M. (1987). Construction and characterization of a genomic library in λ. *In*: Berger, S.L. and Kimmel, A.R. (Eds.), Methods in Enzymology vol 152: Guide to Molecular Cloning Techniques, pp. 190-199. Academic Press Inc., San Diego.

Frischauf, A.-M., Lehrach, H., Poustka, A. and Murray, N. (1983). Lambda replacement vectors carrying polylinker sequences. *Journal of Molecular Biology* **170**: 827-842.

Fritzemeier, K.H., Cretin, C., Kombrink. E., Rohwer, F., Taylor, J., Scheel, D. and Hahlbrock, K. (1987). Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent and avirulent races of *Phytophthora infestans*. *Plant Physiology* **85**: 34-41.

Gausing, K. and Barkardottir, R. (1986). Structure and expression of ubiquitin genes in higher plants. *European Journal of Biochemistry* **158**: 57-62.

Goldknopf, I.R., Wilson, G., Ballal, N.R. and Busch, H. (1980). Chromatin conjugate protein A24 is

56

cleaved and ubiquitin is lost during chicken erythropoiesis. *Journal of Biological Chemistry* **255**: 10555-10558.

Graham, R.W., van Doren, K., Bektesh, S. and Candido, E.P.M. (1988). Maturation of the major ubiquitin gene transcript in *Caenorhabditis elegans* involves the acquisition of a trans-spliced leader. *Journal of Biological Chemistry* **263**: 10415-10419.

Hamm, G.H. and Cameron, G.N. (1986). The EMBL data library. *Nucleic Acids Research* **14**: 5-10.

Henniger, H. (1959). Versuche zur kultur verschiedener rassen von *Phytophthora infestans* (Mont) de By. auf künstlichen Nährböden. *Phytopathologische Zeitschrift* **34**: 285-306.

Hershko, A. (1988). Ubiquitin-mediated protein degradation. *Journal of Biological Chemistry* **263**: 15237-15240.

Hershko, A. and Ciechanover, A. (1986). The ubiquitin pathway for the degradation of intracellular proteins. *Progress in Nucleic Acid Research and Molecular Biology* **33**: 19-56.

Innes, R.W., Kuempel, P.L., Plazinski, J., Canter-Cremers, H., Rolfe, B.G. and Djordjevic, M.A. (1985). Plant factors induce expression of nodulation and host-range genes in *Rhizobium trifolii*. *Molecular and General Genetics* **201**: 426-432.

Kombrink, E., Schröder, M. and Hahlbrock, K. (1988). Several "pathogenesis-related" proteins in potato are 1,3-ß-glucanase and chitinase. *Proceedings of the National Academy of Sciences USA* **85**: 782-786.

Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collings, C., Henzel, W.J., Barnard, R., Waters, M.J. and Wood, W.I. (1987). Growth hormone receptor and serum binding protein: Purification, cloning and expression. *Nature* **330**: 537-543.

Logemann, J., Schell, J. and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* **163**: 16-20.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Matsui, S.I., Seon, B.K. and Sandberg, A.A. (1979). Disappearance of a structural chromatin protein A24 in mitosis: Implications for molecular basis of chromatin condensation. *Proceedings of the National Academy of Sciences USA* **76**: 6386-6390.

Monia, B.P., Ecker, D.J. and Crooke, S.T. (1990). New perspectives on the structure and function of ubiquitin. *Bio/technology* **8**: 209-215.

Mueller, R.D., Yasuda, H., Hatch, C.L., Bonner, W.M. and Bradbury, E.M. (1985). Identification of ubiquitinated histones 2A and 2B in *Physarum polycephalum*: Disappearance of these proteins at metaphase and reappearance at anaphase. *Journal of Biological Chemistry* **260**: 5147-5153.

Muller-Taubenberger, A., Hagmann, J., Noegel, A. and Gerisch, G. (1988a). Ubiquitin gene expression in *Dictyostelium* is induced by heat and cold shock, cadmium, and inhibitors of protein synthesis. *Journal of Cellular Sciences* **90**: 51-58.

Muller-Taubenberger, A., Westphal, M., Jaeger, E., Noegel, A. and Gerisch, G. (1988b). Complete cDNA sequence of a *Dictyostelium* ubiquitin with a carboxy-terminal tail and identification of the protein using an anti-peptide antibody. *FEBS Letters* **229**: 273-278.

Ohmachi, T., Giorda, R., Shaw, D.R. and Ennis, H.L. (1989). Molecular organization of developmentally regulated *Dictyostelium discoideum* ubiquitin cDNAs. *Biochemistry* **28**: 5226-5231.

Ozkaynak, E., Finley, D., Solomon, M.-J. and Varshavsky, A. (1987). The yeast ubiquitin genes: A family of natural gene fusions. *The EMBO Journal* **6**: 1429-1439.

Rechsteiner, M. (1987). Ubiquitin-mediated pathways for intracellular proteolysis. *Annual Review of Cell Biology* **3**: 1-30.

Salveson, G., Lloyd, C. and Farley, D. (1987). cDNA encoding a human homolog of yeast ubiquitin 1. *Nucleic Acids Research* **15**: 5485-5486.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**: 5463-5467.

Sargent, T.D. (1987). Isolation of differentially expressed genes. *In*: Berger, S.L. and Kimmel, A.R. (Eds.), Methods in Enzymology vol 152: Guide to Molecular Cloning Techniques, pp. 423-432. Academic Press Inc., San Diego.

Siegelman, M., Bond, M.W., Gallatin, W.M., St. John, T., Smith, H.T., Fried, V.A. and Weissman, I.L. (1986). Cell surface molecule associated with lymphocyte homing is a ubiquitinated branched-chain glycoprotein. *Science* **231**: 823-829.

Stachel, S.E., Nester, E.W. and Zambryski, P.C. (1986). A plant cell factor induces *Agrobacterium tumefaciens* vir gene expression. *Proceedings of the National Academy of Sciences USA* **83**: 379-383.

Swindle, J., Ajioka, J., Eisen, H., Sanwal, B., Jacquemot, C., Browder, Z., Buck, G. (1988). The

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genomic organization and transcription of the ubiquitin genes of *Trypanosoma cruzi*. *The EMBO Journal* **7**: 1121-1127.

Timberlake, W.E. (1980). Developmental gene regulation in *Aspergillus nidulans*. *Developmental Biology* **78**: 497-510.

Timberlake, W.E. (1986). Isolation of stage- and cell-specific genes from fungi. *In*: Bailey, J.A. (Ed.), Biology and Molecular Biology of Plant-Pathogen Interactions, pp. 343-357. Springer-Verlag, Berlin/Heidelberg.

Tooley, P.W. and Therriën, C.D. (1987). Cytophotometric determination of the nuclear DNA content of 23 mexican and 18 non-mexican isolates of *Phytophthora infestans*. *Experimental Mycology* **11**: 19-26.

Unkles, S.E., Moon, R.P., Hawkins, A.R., Duncan, J. and Kinghorn, J.R. (1991). Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* **100**: 105-112.

Wiborg, O., Pedersen, M.S., Wind, A., Berglund, L.E., Marcker, K.A. and Vuust, J. (1985). The human ubiquitin gene family: Some genes contain multiple directly repeated ubiquitin coding sequences. *The EMBO Journal* **4**: 755-759.

Woloshuk, C.P. and Kolattukudy, P.E. (1986). Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of *Fusarium solani* f. sp. *pisi*. *Proceedings of the National Academy of Sciences USA* **83**: 1704-1708.

Wyman, A.R., Wertman, K.F., Barker, D., Helms, C. and Petri, W.H. (1986). Factors which equalize the representation of genome segments in recombinant libraries. *Gene* **49**: 263-271.

Yardin, Y., Escobedo, J.A., Kuang, W.-J., Fang-Ferng, T.L., Daniel, T.O., Tremble, P.M., Cheng, E.Y., Ando, M.E., Harkins, R.N., Francke, U., Fried, V.A., Ullrich, A. and Williams, L.T. (1986). Structure of the receptor for platelet derived growth factor helps define a family of closely related growth factor receptors. *Nature* **323**: 226-232.

chapter 5

Increased expression of the calmodulin gene of the late blight fungus *Phytophthora infestans* during pathogenesis on potato

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Abstract. In order to isolate *in planta* induced genes encoding putative pathogenicity factors of the late blight fungus *Phytophthora infestans*, a genomic library was differentially screened. For the differential hybridization, labelled first strand cDNA synthesized on (i) mRNA isolated from *P. infestans* infected potato leaves and on (ii) mRNA isolated from the fungus grown *in vitro* were used as probes. This screening resulted in the isolation of the *P. infestans* calmodulin gene. The gene, designated *calA*, contains an open reading frame of 447 base pairs without introns and is unique in the *P. infestans* genome. The predicted amino acid sequence is 89.9%-94.6% identical to calmodulins from higher eukaryotes whereas the identity to calmodulins of higher fungi is significantly less (60.8%-85.1%). Expression studies revealed that the *P. infestans calA* gene is constitutively expressed in *in vitro* grown mycelium. However, during pathogenesis on potato the level of *P. infestans* calmodulin mRNA is approximately five-fold increased.

Introduction

The fungal plant pathogen *Phytophthora infestans* (Mont.) de Bary (Oomycetes), the causal agent of potato late blight, is one of the most devastating diseases of potato (*Solanum tuberosum* L.). Infection of potato leaves and tubers by a virulent strain of this hemibiotrophic fungus initially causes a rapidly spreading lesion followed by necrosis which results in complete destruction of the infected tissue. The specialization of *P. infestans* on solanaceous plants such as potato and tomato implies a mechanism for recognition which most likely requires the exchange of signals between host and pathogen. On the one hand, infection of the host by *P. infestans* induces specific expression of a particular set of host genes, some of which are thought to be involved in defence (Fritzemeier *et al.*, 1987; Taylor *et al.*, 1990; Matton and Brisson, 1989). On the other hand, growth of the fungus on its host plant can lead to induced expression of genes in the pathogen (Pieterse *et al.*, 1992). The products of the *in planta* induced *P. infestans* genes might be involved in the establishment and maintenance of a compatible interaction between the pathogen and its host plant. Our approach to identify factors which determine the pathogenic abilities of *P. infestans* and factors which are involved in the signalling between host and pathogen is to isolate genes of *P. infestans* that show induced or increased expression during pathogenesis. Identification of the proteins encoded by these genes might elucidate some of the molecular and cellular events involved in plant-pathogen interactions.

To isolate *P. infestans* genes whose expression is specifically induced or increased during colonization of the host plant, we screened a genomic library of *P. infestans* by differential hybridization using labelled cDNA probes synthesized on (i) mRNA isolated from *P. infestans*-infected potato leaves and on (ii) mRNA isolated from the fungus grown *in vitro*. This resulted in the isolation of several fungal genes of which the expression is induced during growth of the fungus *in planta*. Characterization of one of the clones led to the identification of an *in planta* induced gene of *P. infestans* coding for

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polyubiquitin (Pieterse *et al.*, 1991). Here we describe the characterization of another *in planta* induced gene of *P. infestans* which appeared to encode calmodulin, a calcium-binding protein which has an essential function in various cellular processes.

Calmodulin, which is highly conserved in all eukaryotic organisms, is one of the major intracellular calcium receptors responsible for mediating cellular responses to the calcium fluxes which are generated by a variety of processes. Upon binding of Ca^{2+}, calmodulin undergoes a conformational change, which enables the Ca^{2+}-calmodulin complex to bind to, and in that way to regulate the activity of a large number of enzymes (Cheung, 1980; Roberts *et al.*, 1986). It is evident that both the Ca^{2+} and the calmodulin concentration play an essential role in this process. An important class of calmodulin-regulated enzymes are protein kinases whose action is recognized as a primary mechanism for the transduction of extracellular stimuli into intracellular events (Hepler and Wayne, 1985; Roberts *et al.*, 1986). Thus through binding of Ca^{2+}, calmodulin can have a regulatory effect on a diverse array of cellular processes. In plants, calmodulin and Ca^{2+} are thought to be involved in the regulation of mitosis, polarized cell growth and cytoplasmic streaming (Marmé and Dieter, 1983; Hepler and Wayne, 1985). In some fungi, e.g. *Metarhizium anisopliae* (St Leger *et al.*, 1989), *Ceratocystis ulmi* (Muthumukar and Nickerson, 1984) and *Candida albicans* (Paranjape *et al.*, 1990), and in the slime molds *Dictyostelium discoideum* (Lydan and O'Day, 1988) and *Physarum polycephalum* (Uyeda and Furuya, 1986), the Ca^{2+}-calmodulin complex has been reported to be involved in growth and differentiation.

In this study we describe the isolation and characterization of the *P. infestans* calmodulin gene (*calA*) and show that the expression level of this gene increases several fold during pathogenesis. The significance of the *in planta* induced expression of the *P. infestans calA* gene will be discussed.

Materials and Methods

Culturing of **Phytophthora infestans** *and inoculation of potato leaves. P. infestans* strain 88069 (A1 mating type, race 1.3.4.7), maintained on rye-agar medium containing 2% (w/v) sucrose (Caten and Jinks, 1968), was used throughout this study. For inoculations of liquid cultures, zoospores were produced by incubating two-week-old rye-agar cultures (in 94 mm Petri dishes) for 3 hours at 10°C with a layer of 10 ml water on top of the mycelium. Liquid cultures were initiated from a zoospore suspension in either Henniger synthetic medium (Henniger, 1959) or rich rye-sucrose medium (1×10^5 zoospores per milliliter) and after incubation at 18°C for 3, 7 and 14 days mycelium was harvested. For inoculation of plants, sporangia were collected from two-week-old rye-agar cultures and concentrated to 5×10^5 sporangia/ml. Leaves were inoculated by spraying the axial side of the leaves with the sporangial suspension. After inoculation, leaves were inserted in florist's foam oasis and incubated at 18°C and 100% relative humidity under

cool fluorescent light (16 hours/day). The partially resistant potato cultivar (cv.) 'Pimpernel' and the moderately susceptible potato cv. 'Ajax' were used as host plants.

Differential screening, subcloning and sequencing. A genomic library of *P. infestans* DNA was constructed in λEMBL3 and screened as described previously (Pieterse *et al.*, 1991). For the differential screening, [α-³²P]dATP labelled first strand cDNA probes with a specific activity of 1-2×10^8 cpm/µg were synthesized on (i) poly(A)⁺ RNA isolated from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium and (ii) poly(A)⁺ RNA isolated from infected leaflets of potato cv. 'Ajax' (3 days post-inoculation) as described by Sargent (1987). Hybridization of filters containing recombinant bacteriophage DNA of the *P. infestans* genomic library was performed at 65°C in hybridization mix containing 5×SSC (750 mM NaCl, 75 mM sodium citrate), 5×Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA (fraction V)), 0.5% (w/v) sodium dodecyl sulphate (SDS) and 100 µg/ml calf thymus DNA. After hybridization, the filters were washed in 2×SSC, 0.5% (w/v) SDS at 65°C and exposed to Kodak X-AR film.

The location of the *in planta* induced genes on the DNA inserts present in the selected genomic λ clones was determined by differential hybridization of Southern blots containing digested DNA of the selected λ clones. A single differentially hybridizing 5.0 kilobase (kb) *Sst*I-fragment from one of the selected λ clones, i.e. DHC-C, was subcloned in the vector pTZ19U resulting in the plasmid named pPiCM-S. Of pPiCM-S, a detailed restriction map was made and several subclones were constructed. All DNA manipulations were performed essentially according to Sambrook *et al.* (1989). To generate overlapping deletion clones, pPiCM-S was partially digested with *Sau*3AI and electrophoresed on a 0.7% (w/v) agarose gel along with linearized pPiCM-S as marker. Singly cut, linearized DNA was isolated from the gel and digested to completion with *Bam*HI. DNA fragments were then circularized by ligation and transformed to *E. coli* cells. DNA sequencing of overlapping subclones and deletion clones was performed on double-stranded DNA by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Multiwell Microtitre Plate Sequencing System (Amersham) and [α-³⁵S]dATP as a label. For the analysis of sequence data and screening of sequence data libraries, the Sequencing Analysis Software Package, version 6.0, of the Genetics Computer Group (GCG) of the University of Wisconsin was used (Devereux *et al.*, 1984).

Isolation of RNA and poly(A)⁺ RNA and northern blot analyses. RNA was isolated from *P. infestans* mycelium, non-inoculated potato leaves and colonized potato leaflets (interaction RNA) using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). Poly(A)⁺ RNA was obtained by affinity chromatography on oligo(dT)-cellulose as described by Sambrook *et al.* (1989). For northern blot analysis, 15 µg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond-N⁺ membranes (Amersham) by capillary transfer as described by the

manufacturer. Northern blots were hybridized at 65°C in the hybridization mix as described above.

Isolation of genomic DNA and Southern blot analyses. Genomic DNA of *P. infestans* and potato was isolated from *in vitro* grown mycelium and from leaves of potato cv. 'Ajax' as has been described previously (Pieterse *et al.*, 1991). Genomic DNA was digested with various restriction enzymes and size-separated on a 0.7% (w/v) agarose gel. Following electrophoresis, the DNA was transferred to Hybond-N$^+$ membrane (Amersham) by capillary transfer. Hybridization of Southern blots was performed at 65°C in the hybridization mix as described above.

DNA probes. The calmodulin probe was derived from the coding region of the *P. infestans calA* gene and consists of a 342 basepair (bp) *Pvu*II/*Hind*III-fragment from pPiCM-S. The 2.8 kb *Pst*I-insert from pSTA31 containing the complete *P. infestans actA* gene (Unkles *et al.*, 1991) was used as actin probe and the 0.35 kb *Eco*RI/*Xho*I-insert from pPi119 which is a partial cDNA clone of the *P. infestans* translation elongation factor 1α (EF-1α) mRNA (C.M.J. Pieterse, unpublished), was used as EF-1α probe. DNA probes were labelled by the random primer labelling technique (Feinberg and Vogelstein, 1983).

Primer extension of poly(A)$^+$ RNA. The oligonucleotide (5'-GCTCTTCGGTCAGCTGG-3') complementary to the sequence at position 9 to 25 relative to the ATG start codon of the *calA* gene was 5'-end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega) according to the manufacturer's instructions. Five ng of labelled primer were annealed to 3 μg of poly(A)$^+$ RNA and extended using M-MLV reverse transcriptase as described by the manufacturer (Gibco BRL). The primer extension products were analyzed by electrophoresis on a 6% (w/v) polyacrylamide gel and visualized by autoradiography.

Results and Discussion

An in planta *induced gene of* P. infestans *codes for calmodulin.* In order to achieve a 99% probability of having any DNA sequence represented, 80,000 recombinant bacteriophages of the *P. infestans* genomic library were differentially screened. This resulted in the selection of 20 recombinant phage clones containing putative *in planta* induced genes of the fungus. The number of positive clones does not provide information on the number of *in planta* induced *P. infestans* genes. One recombinant phage can easily contain a constitutively expressed gene in the proximity of a differentially regulated gene, the latter will not be detectable in the differential screening procedure. In a dot blot experiment, DNA of ten clones hybridized relatively strongly to the cDNA probe derived

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Figure 1. Restriction map and sequencing strategy of the *Phytophthora infestans* calA gene. a. Partial restriction map of the genomic λ clone DHC-C. b. Restriction map of the 5.0 kb SstI-insert of pPiCM-S. C, ClaI; E, EcoRI; H, HindIII; P, PstI; Pv, PvuII; S, SalI; Ss, SstI; and X, XbaI. BamHI does not cut in the 5 kb insert. The closed bar indicates the approximate location of the coding region of the calA gene as determined by differential hybridization experiments. The open bar indicates the coding region of the calA gene as determined by dideoxy sequencing. The hatched area in this bar represents the 342 bp PvuII/HindIII-fragment which was used as probe for northern and Southern blot hybridizations. c. Sequencing strategy for calA. Arrows indicate the direction and the extent of sequencing.

from mRNA isolated from infected leaves whereas hybridization to the cDNA probe synthesized on mRNA isolated from *in vitro* grown mycelium gave relatively weak signals. When the differentially hybridizing DNA fragments from these clones were used as probes on northern blots, it appeared that eight of these ten clones contain a gene of which the expression is significantly increased during growth of the fungus *in planta* (data not shown). There was no cross-hybridization between these eight clones indicating that they contain different genes.

In one of the eight positive clones, DHC-C, the coding region of the gene responsible for the differential hybridization was mapped on a single 5.0 kb SstI-fragment which was subsequently subcloned into pTZ19U. The other SstI-fragments from DHC-C did not hybridize to the cDNA probes. In Figure 1, the restriction map of the resulting plasmid, designated pPiCM-S, is depicted. Additional differential hybridization experiments indicated that the gene is located on two linked PstI-fragments of 1.2 and 1.5 kb respectively. Dideoxy sequencing of various overlapping subclones within the two PstI-fragments resulted in the nucleotide sequence shown in Figure 2. In the sequenced region, a single open reading frame (ORF) of 447 nucleotides coding for a protein of 149

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        -710                -690                -670                -650                -631
TCGGCAAAAATCAACCAAAGCAGAGTCCCTAATTTTCCCCCCAAAAATGATAAAATAGCTGTTTTACAAACATCAGGACCATTAATACAT
        -620                -600                -580                -560                -541
GTATTAATAACAGATGCTGTAAACAGCTCTGCCACCGACCGCCTCAAATCCCCGAGCAGGAAATAGGCCCTAAGGTTTGAAAAATTAAAAA
        -530                -510                -490                -470                -451
TATACTAAAAATATCTATTGTCGTTGTGGCAACTAAATGCTGGCGCCATCAAATATTAACACGCTAAGAAGCTACCACATCGAGGTGACT
        -440                -420                -400                -380                -361
CTCTCGTCGCTCAAGCCTTTCATGCATTATATCTGACTGGAATGTGATCTTTATGAAAGGTCAATTGCATGTAGCAATCAGTTTTTTTTC
        -350                -330                -310                -290                -271
TGCGATCTTCGCTGTGTCGTTGACTGTTCCGTTCTGTCCACAATCCATCGTTTTGTCAAGCCGTGTGCGCCACACCAATGTGCAAAACCA
        -260                -240                -220                -200                -181
AAAATATTTGTACGCACCACATCATGGTCGTACATGATATGGTCGTTCATCCTTGCTCTTTTTAATAATTATGATTTATTGGTGCGGACA
        -170                -150                -130                -110                -91
GACATATTTTAACCAATCGCATATTGCCCACAAATTATCAACTAATCGCAGTACGCCGCTTGGCCGTCCACATTCTTCGTGGTCGTACAA
        -80                 -60                  ▼  -40                -20                 -1
ATCAGGGCCGTCAAAGGCGCCGGTCTCATTTTGGATGGGATCATTGTTGGATTTCCCTCGACACCCCCAAGTGAAAACCGAAGCACCACA
        10                  30                  50                  70                  90
ATGGCTGACCAGCTGACCGAAGAGCAGATTGCCGAGTTCAAGGAGGCGTTCTCCCTGTTCGACAAGGACGGCGATGGAACCATTACCACC
  M   A   D   Q   L   T   E   E   Q   I   A   E   F   K   E   A   F   S   L   F   D   K   D   G   D   G   T   I   T   T
        100                 120                 140                 160                 180
AAGGAGCTTGGCACGGTCATGCGCTCTCTGGGCCAGAACCCCACGGAGGCCGAGCTGCAGGACATGATCAACGAGGTCGACGCTGACGGA
  K   E   L   G   T   V   M   R   S   L   G   Q   N   P   T   E   A   E   L   Q   D   M   I   N   E   V   D   A   D   G
        190                 210                 230                 250                 270
AACGGCACGATTGACTTCCCCGAATTCCTGACCATGATGGCTCGCAAGATGAAGGACACGGACTCCGAGGAGGAGATCCTGGAGGCCTTC
  N   G   T   I   D   F   P   E   F   L   T   M   M   A   R   K   M   K   D   T   D   S   E   E   E   I   L   E   A   F
        280                 300                 320                 340                 360
AAGGTGTTCGACAAGGACGGCAATGGATTCATCTCGGCTGCCGAGCTGCGTCACATCATGACCAACCTGGGCGAGAAGCTTACCGACGAG
  K   V   F   D   K   D   G   N   G   F   I   S   A   A   E   L   R   H   I   M   T   N   L   G   E   K   L   T   D   E
        370                 390                 410                 430                 450
GAGGTCGACGAGATGATCCGCGAGGCCGACATCGACGGCGATGGACAGATCAACTACGAAGAGTTCGTCAAGATGATGATGTCCAAGTAA
  E   V   D   E   M   I   R   E   A   D   I   D   G   D   G   Q   I   N   Y   E   E   F   V   K   M   M   M   S   K   *
        470                 490                 500                 520                 540
GCGTCGCGTCCCCAACGCTTAGTTCCCGATCTTTTGATCCGAAGTATCTTGTTGCCAACGTTGTAGGCGCGGGCGTTTGCTCCCTCTTCG
        550                 570                 590                 610                 630
TTGTAGCTGGCTGCTCCTATCGATCGTAGCTTAAATAATTTAGAAACAGCGTGAAATTGCTTTTTTTTGCTGATGCTTTTTGCGTGGTC
TTTAGTAC
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Figure 2. Nucleotide sequence of the *Phytophthora infestans* *calA* gene and corresponding amino acid sequence of the encoded calmodulin protein. The transcription start site as determined by primer extension is indicated by a closed arrowhead (▼), the TCATT sequence surrounding the transcription initiation site is underlined with a dotted line. The DNA sequence overlined with the dotted line at position -71 to -51 represents the sequence motif which is conserved among calmodulin promoter sequences from several organisms. Underlined DNA sequences depict the CAAT and TATA-like motifs. Overlined DNA sequence (AAATAA) in the 3' flanking region of the calmodulin gene represents a putative polyadenylation signal. The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession number M83535.

amino acids was found. Comparison of the ORF with the nucleotide sequence data library, revealed that the gene codes for a protein which is highly similar to calmodulin, a calcium-binding protein present in presumably all eukaryotes. The deduced amino acid sequence of the gene contains four EF-hand calcium-binding domains (indicated by ★★★ in Fig. 5) which is typical for calmodulins. From these data it was concluded that the *in planta* induced gene present in the recombinant bacteriophage DHC-C encodes calmodulin. Therefore, the gene was designated *calA*.

Analysis of the calA *gene locus.* On a Southern blot containing genomic *P. infestans* DNA digested with various restriction enzymes, a probe derived from the coding region of the *calA* gene (a 342 bp *Pvu*II/*Hin*dIII-fragment from pPiCM-S, see Fig. 1) hybridized to single *Bam*HI- and *Sst*I-fragments of 6.0 kb and 5.0 kb respectively (Fig. 3). Sequencing data revealed that the coding region of the *calA* gene contains an *Eco*RI and a *Pst*I restriction site, and as a result, the *calA* probe hybridizes to two *Eco*RI- (2.6 and 17 kb) and two *Pst*I-fragments (1.2 and 1.5 kb). The length of the hybridizing *Sst*I-fragment is in agreement with the length of the 5.0 kb *Sst*I-fragment found in the recombinant bacteriophage DHC-C. The same holds for the two *Pst*I-fragments and the 2.6 kb *Eco*RI-fragment which are present in pPiCM-S (Fig. 1). The sizes of the other hybridizing fragments could not be verified as they overlap the 5.0 kb *Sst*I-fragment on either one side (17 kb *Eco*RI-fragment) or both sides (6.0 kb *Bam*HI-fragment). From these data it can be concluded that the genome of *P. infestans* contains a single copy of the calmodulin gene. The occurrence of one calmodulin gene per haploid genome is common in most organisms but in a few cases also calmodulin gene families have been reported (Chung and Swindle, 1990; Chien and Dawid, 1984; Fischer *et al.*, 1988). Each member of these calmodulin gene families may be regulated differently (Fischer *et al.*, 1988) but within one organism all calmodulin genes encode identical calmodulin proteins.

Figure 3. Southern blot analysis of *Phytophthora infestans* genomic DNA (Pi) digested with *Bam*HI, *Eco*RI, *Pst*I, and *Sst*I and potato genomic DNA (St) digested with *Eco*RI (10 μg/lane). The 342 bp *Pvu*II/*Hin*dIII-fragment from the coding region of the *calA* gene was used as probe. The size of the markers is indicated in kilobases.

Further characterization of the calA *gene.* The nucleotide sequence of the *calA* gene as shown in Figure 2 includes 720 bp upstream of the translation initiation site and 188 bp downstream of the stop codon. The coding region of the *calA* gene is not interrupted by introns. The putative transcription initiation site of the *calA* gene was determined by primer extension using an oligonucleotide complementary to the *calA* sequence at position 9 to 25 relative to the ATG start codon. The oligonucleotide was annealed to and extended on poly(A)$^+$ RNA isolated from 3-day-old *P. infestans* mycelium grown on

Henniger synthetic medium. A single primer extension product of 71 nucleotides was found indicating that the *calA* transcription initiation site is located at the T at position -46 relative to the ATG start codon (Fig. 4). The oligonucleotide used in this primer extension experiment is 82% homologous to the corresponding region in potato calmodulin mRNA (Jena *et al.*, 1989). To check whether the oligonucleotide hybridized to potato calmodulin mRNA, the primer extension experiment was also performed on poly(A)$^+$ RNA isolated from the potato-*P. infestans* interaction (potato cv. 'Ajax', 3 days post-inoculation). Again, only one primer extension product was detected which is identical in length to the one produced on poly(A)$^+$ RNA isolated from *in vitro* grown mycelium (Fig. 4). Apparently the same *calA* transcription initiation site at position -46 is functional during growth *in vitro* and *in planta*. Potato calmodulin mRNA has a leader sequence of at least 81 nucleotides (Jena *et al.*, 1989). Thus the expression level of the potato calmodulin gene is either

Figure 4. Primer extension analysis of the *calA* transcript. A [γ-^{32}P]ATP 5'-end-labeled oligonucleotide (5'-GCTCTTCGGTCAGCTGG-3'), complementary to the *calA* sequence at position 9 to 25, was annealed to 3 µg poly(A)$^+$ RNA and extended with M-MLV reverse transcriptase. Primer extension products were electrophoresed on a denaturing 6% (w/v) polyacrylamide gel next to the products of a dideoxy sequencing reaction using the same oligonucleotide as primer and pPiCM-S as template DNA (not shown). The extension products synthesized on poly(A)$^+$ RNA isolated from the potato-*Phytophthora infestans* interaction (potato cv. 'Ajax', 3 days post-inoculation) are shown in panel A, the extension products synthesized on poly(A)$^+$ RNA isolated from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium in panel B.

too low to be detected or the oligonucleotide does not hybridize to potato calmodulin mRNA under the hybridization conditions used.

The transcription initiation site of the *calA* gene is preceded by the sequence TCAT. Interestingly, four other *P. infestans* genes, i.e. the polyubiquitin gene *ubi3R* (Pieterse *et al.*, 1991), the actin gene *actA* (Unkles *et al.*, 1991) and the *in planta* induced genes *ipiO1* and *ipiO2* (C.M.J. Pieterse, unpublished) as well as one *Phytophthora megasperma*

gene, i.e. the actin gene (Dudler, 1990), have a TCATT sequence in which the single transcription initiation site is located. One other *Phytophthora* gene in which the site of transcription initiation has been determined is the actin gene *actB* of *P. infestans* (Unkles *et al.*, 1991). However, this gene contains several transcription initiation sites and none of these are located in a sequence motif TCATT. The significance of the conserved TCATT sequence in transcription initiation of *Phytophthora* genes is not clear.

In the promoter region of the *calA* gene, a TATA-like motif (TACAAA) is located 49 nucleotides upstream of the *calA* transcription initiation site at position -95 and a CAAT motif is present at position -167, 121 nucleotides upstream of the transcription initiation site. In the majority of genes identified from higher eukaryotes, the consensus 'core promoter' sequences TATAAA and CAAT are found around 30 bp and 70-90 bp respectively upstream of the major transcription initiation site. However, these motifs are not very common in the promoters of filamentous fungi (Gurr *et al.*, 1987). Whether the sequence motifs present in the *calA* gene are functional transcription signals is unknown. Just upstream of the transcription initiation site, between positions -71 and -51, there is a 20 nucleotide sequence motif which is conserved in calmodulin genes of several organisms (Zimmer *et al.*, 1988). The conservation of this sequence motif, which is also found in some other unrelated eukaryotic genes (Zimmer *et al.*, 1988), ranges between 60 and 80% homology. In the calmodulin genes it is always located within 150 nucleotides upstream of the transcription initiation site. The significance of this motif for transcription or regulation of gene expression is unknown. The 3' untranslated region of the *calA* gene contains a putative polyadenylation signal (AAATAA), 123 nucleotides downstream of the TAA stop codon.

Like most oomycetous genes studied so far but in contrast to the majority of the known calmodulin genes, the *P. infestans calA* gene has no introns. The few other intron-less calmodulin genes are the calmodulin genes of *Achlya klebsiana* (LéJohn, 1989) which is another Oomycete, of *Saccharomyces cerevisiae* (Davis *et al.*, 1986) and of *Trypanosoma cruzi* (Chung and Swindle, 1990). Most calmodulin genes contain up to five introns, some of which occur at more or less conserved positions in the gene (see references mentioned in the legend of Figure 5).

Comparison of P. infestans *calmodulin with calmodulins of other organisms.* The predicted amino acid sequence of the *calA* gene product is highly homologous to calmodulins from other eukaryotic organisms (Fig. 5). The highest identity is found with the calmodulin sequence of the Oomycete *Achlya klebsiana* (96.6%). There are only five amino acid substitutions which are all located outside the four EF-hand calcium-binding domains. The isoleucine residue at position 109 is unique for *P. infestans* calmodulin. *P. infestans* calmodulin is remarkably similar, 90% identity or more, to calmodulins of several vertebrates, invertebrates and plants. Also the similarity with calmodulins of lower eukaryotes such as *Trypanosoma cruzi* (91.9%) and *Chlamydomonas reinhardtii*

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              10        20        30        40        50        60        70        80
               .     .* * * * *. *    .                   .       * * .* * *  * .         .
P. infestans    ----MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVNRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEE
A. klebsiana    ----.................G....................V................................
Human           ----...........................................................................
Chicken         ----...........................................................................
Eel             ----..................................................................K........
D. melongaster  ----...........................................................................
Potato          ---..E.................C......................S.A..Q.........NL...............
C. reinhardtii  MAANTE.............A...........................S...............ML......E..H.D.
T. cruzi        ----.....SN...S...............................Q..S...........L.....Q.S.....
A. nidulans     ----...S.....VS.Y.......Q.................S.S............N...............
C. albicans     ----..EK.S.Q.........S..K.............S.S..T.......VMSD.S................A.
S. pombe        ---MTTRN..D......R....R.Q..N..SN...V....S..A...............T............N...
S. cerevisiae   ----..SSN............A.....MM.S.SSS..A.......LS.S...VN.LM..I.V...HQ.E.S...AL.S.QL.SM...Q.
```

```
               90       100       110       120       130       140       149          % identity
                . * * * * *  .              .              * * * * * .*
P. infestans    ILEAFRVFDKDGNGFISAAELRHIMTNLGEKLTDEEVDEMIREADIDGDGQINYEEFVKMMMSK*------          100.0
A. klebsiana    .....QG............M............................................*------           96.6
Human           .R...R........Y........V.......................V.......Q..TA.*------            94.6
Chicken         .R...R........Y........V.......................V.......Q..TA.*------            94.6
Eel             .R...R........Y........V.......................V.......Q..TA.*------            93.9
D. melongaster  .R...R...............V.........................V.......T..T.*------             95.9
Potato          LK.......Q...........V.........................V.......R..LA.*------            89.9
C. reinhardtii  LR...................V......SE.................V.....V.....R..T.GATDDKDKKGHK*      86.6
T. cruzi        .K...R...............V.........................V..................*------        91.9
A. nidulans     .R.......R.M.........V..SI......D.........Q...R.D.N...QL..Q.*------             85.1
C. albicans     .A.......RN.D.K........LL.SI....S.AD..Q..X...TNN..E.DIQ..TLLLAA.*------         71.6
S. pombe        VR...........Y.TVE..T.VL.S...R.SQ...AD......T...V.....SRVIS..*------            74.5
S. cerevisiae   L.........N.D.L......K.VL.SI......A...D.L..VSA..S.E..IQQ.AALLA..*------         60.8
```

Figure 5. Comparison of the predicted amino acid sequence of *Phytophthora infestans* calmodulin with the sequences of calmodulins from: *Achlya klebsiana* (LéJohn, 1989); man (Fischer *et al.*, 1988); chicken (Putkey *et al.*, 1983); eel (Lagacé *et al.*, 1983); *Drosophila melanogaster* (Yamanaka *et al.*, 1987); potato (Jena *et al.*, 1989); *Chlamydomonas reinhardtii* (Zimmer *et al.*, 1988); *Trypanosoma cruzi* (Chung and Swindle, 1990); *Aspergillus nidulans* (Rasmussen *et al.*, 1990); *Candida albicans* (Saporito and Sypherd, 1991); *Schizosaccharomyces pombe* (Takeda and Yamamoto, 1987); and *Saccharomyces cerevisiae* (Davis *et al.*, 1986). Identical sequences are marked by a dot. The Δ symbol indicates a one residue gap introduced into the sequence to give optimal alignment with the other proteins. Expected Ca^{2+}-binding residues, located in the four EF-hand Ca^{2+}-binding domains as proposed by Watterson *et al.* (1980), are indicated by a *.

(86.6%), is relatively high. Interestingly, calmodulins of higher fungi such as *Aspergillus nidulans* and especially the yeasts are less homologous to *P. infestans* calmodulin (less than 85% identity). Here, the amino acid substitutions are randomly distributedthroughout the sequence. The sequence comparisons suggest that evolutionarily, *P. infestans* is less closely related to higher fungi than to some other eukaryotes. This supports the hypothesis that oomycetous fungi evolved from ancestors other than higher fungi (Barr, 1981; Cavalier-Smith, 1987; Karlovsky and Prell, 1991).

Expression of the **P.** infestans *calmodulin gene during pathogenesis on potato.* The *P. infestans calA* gene was isolated from a genomic library by a procedure which was aimed at the selection of *in planta* induced genes of this fungus. Subsequently, northern blot analyses were performed to confirm that the *calA* gene is indeed a gene which shows induced expression during pathogenesis. When studying gene expression in a plant-fungal interaction by means of northern blot analysis, it is important to take into account the

changing ratios of fungal and plant RNA due to the increase of fungal biomass during colonization of the leaves. By using a constitutively expressed fungal gene as probe for the hybridization of northern blots, the increase in the proportion of fungal RNA in the interaction RNA mixtures can be determined. Genes encoding actin and translation elongation factor 1α (EF-1α) have been shown to be very useful constitutively expressed marker genes for assessing the expression of differentially regulated genes during plant-pathogen interactions (Mahe *et al.*, 1992). In our experiments we used the *P. infestans* actin (*actA*) gene (Unkles *et al.*, 1991) as well as the *P. infestans* EF-1α gene (C.M.J. Pieterse, unpublished) as constitutively expressed marker genes. The specificity of the *calA*, *actA*, and EF-1α probes for *P. infestans* was shown by hybridization of the probes to Southern blots containing genomic *P. infestans* and potato DNA. Under the high stringency conditions used, the probes hybridized only to *P. infestans* DNA and not to potato DNA (*calA* probe: see Fig. 3; *actA* and EF-1α probe: data not shown). Also on northern blots the three probes do not hybridize to potato RNA isolated from non-inoculated leaves (Fig. 6A).

The expression of the *calA* gene was studied during growth of the fungus *in planta* on two different potato cultivars and during growth of the fungus *in vitro*. Leaves of the partially resistant potato cv. 'Pimpernel' and the moderately susceptible potato cv. 'Ajax', were inoculated with isolate 88069 of *P. infestans*. On potato cv. 'Ajax', the first symptoms are visible 24 hours after inoculation. They develop from small lesions into completely "water soaked" areas 3 days post-inoculation. In this period the fungus grows and sporulates at the advancing edges of developing lesions. The centres of the lesions become necrotic and start to decay due to secondary infections by saprophytic microorganisms. Compared to potato cv. 'Ajax', symptom development on potato cv. 'Pimpernel' was delayed for approximately one day. Infected leaves of potato cvs. 'Ajax' and 'Pimpernel' were harvested 1, 2 and 3, and 1, 2, 3 and 4 days post-inoculation respectively. Non-inoculated control leaves were sprayed with water and treated similarly to the inoculated leaves. *In vitro* grown mycelium was harvested from non-sporulating cultures (3-day-old), moderately sporulating cultures (7-day-old) and heavily sporulating cultures (14-day-old) which were grown on both Henniger synthetic medium and rich rye-sucrose medium.

On northern blots hybridized with the *calA* probe, a single transcript can be detected in the lanes containing RNA from *in vitro* grown mycelium and in some of the lanes containing RNA isolated from inoculated leaves (Fig. 6). There is no hybridization with RNA isolated from non-inoculated leaves. The length of the calmodulin mRNA is about 750 nucleotides which is in agreement with the length calculated from the nucleotide sequence. In inoculated leaves of potato cv. 'Ajax' as well as those of potato cv. 'Pimpernel', the *P. infestans* calmodulin mRNA level increases in time. In order to be able to normalize calmodulin mRNA levels to a constant amount of fungal RNA, northern blots were deprobed and rehybridized with, successively, the *actA* probe and the EF-1α

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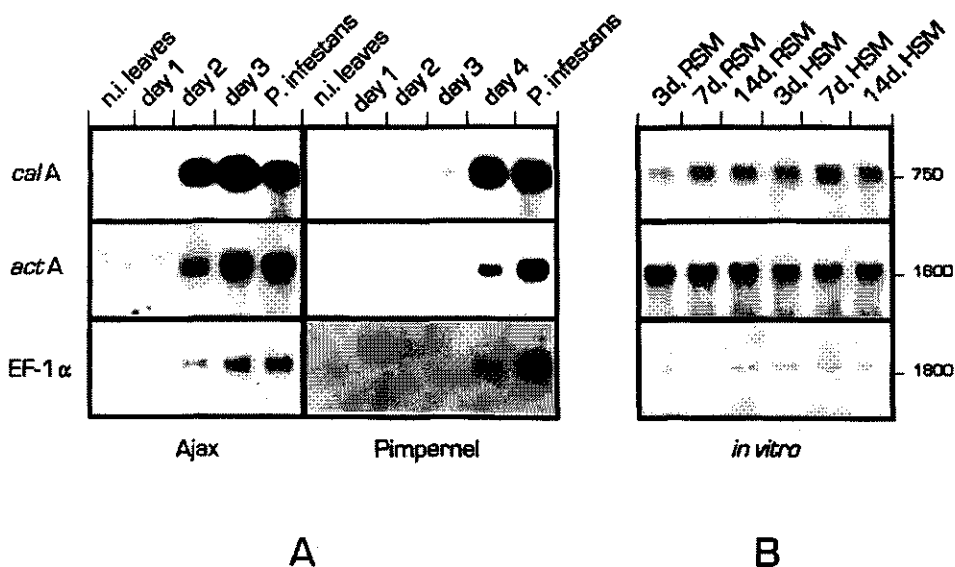
Figure 6. A. Autoradiographs of northern blots containing total RNA isolated from non-inoculated leaves of potato cvs. 'Ajax' and 'Pimpernel' (n.i. leaves), from colonized leaves of both potato cultivars at different time points post-inoculation (day 1-3 and day 1-4 respectively) and from 3-day-old *Phytophthora infestans* mycelium grown on Henniger synthetic medium (P. infestans). B. Autoradiographs of northern blots containing RNA isolated from non-sporulating 3-day-old mycelium (3d), from 7-day-old moderately sporulating mycelium (7d) and from 14-day-old heavily sporulating mycelium (14d) grown *in vitro* on both Henniger synthetic medium (HSM) and rich rye-sucrose medium (RSM). Northern blots were hybridized with probes derived from i) the *P. infestans* calmodulin gene (*calA*), ii) the *P. infestans* actin gene (*actA*), and iii) the *P. infestans* translation elongation factor 1α gene (EF-1α).

probe. During the interaction of *P. infestans* with potato cv. 'Ajax', actin and EF-1α transcripts are first detectable 2 days post-inoculation and the amounts increase in time due to the expanding fungal biomass. During the interaction of *P. infestans* with potato cv. 'Pimpernel' both transcripts are first detectable four days post-inoculation indicating that the colonization of leaves of the partially resistant potato cv. 'Pimpernel' is significantly slower than the colonization of leaves of the moderately susceptible potato cv. 'Ajax'.

The calmodulin, actin and EF-1α mRNA levels during growth of the fungus *in vitro* and *in planta* were quantified by densitometric scanning of the autoradiographs using the Cybertech Image Processing Software, version 1.20 (Cybertech, Berlin). It appeared that the increase in actin mRNA levels due to shifting fungal/plant RNA ratios in the interaction RNA mixtures is comparable to the increase in EF-1α mRNA levels. Hence, the proportion of fungal RNA in the interaction RNA mixture is quite accurately reflected

by the mRNA amounts transcribed from the two constitutively expressed marker genes *actA* and EF-1α. In addition, the densitometric scanning showed that after normalization to actual fungal RNA levels, the amount of *calA* mRNA in the lanes containing interaction RNA ('Ajax', day 2 and 3 and 'Pimpernel', day 4) is approximately five times higher compared to that in the lanes containing RNA from the *in vitro* grown fungus. Under the *in vitro* growth conditions tested, there is no change in the mRNA levels indicating that the *calA*, *actA* and EF-1α genes are constitutively expressed during growth of the fungus *in vitro*. The higher level of *calA* mRNA during growth of the fungus *in planta* is also demonstrated in the primer extension experiment described above. Equal amounts of primer were annealed to and extended on an identical quantity of poly(A)$^+$ RNA isolated from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium and from the Ajax-*P. infestans* interaction, 3 days post-inoculation. The amount of calmodulin mRNA, indirectly visualized by the primer extension products, is several fold higher in the potato-*P. infestans* interaction compared to *P. infestans* grown *in vitro*. These results strongly suggest that the expression of the *P. infestans calA* gene is significantly increased during colonization of potato. Apparently, growth conditions during pathogenesis, directly or indirectly influence the expression level of the *calA* gene.

The role of calmodulin in pathogenesis. Calmodulin plays an essential role in the regulation of a diverse array of cellular processes. In every eukaryotic cell a basic level of calmodulin is required for regular cell functions. In this paper we have shown that during colonization of potato by *P. infestans*, the calmodulin mRNA level in the pathogen increases significantly (approximately five-fold).

In several organisms, including fungi, differential expression of calmodulin genes has been observed. In potato for example, increased levels of calmodulin mRNA are found during tuberization (Jena *et al.*, 1989) and in *Drosophila melanogaster* the calmodulin gene is differentially regulated in the various developmental stages (Yamanaka *et al.*, 1987). In *Aspergillus nidulans*, calmodulin gene expression changes during the cell cycle (Rasmussen *et al.*, 1990). In the oomycetous water molds *Achlya ambisexualis* and *Achlya klebsiana* increased calmodulin gene expression and synthesis are associated with the sporulation process (Suryanarayana and Thomas, 1986; LéJohn, 1989). To investigate whether the five-fold increase in calmodulin mRNA levels which is detectable during pathogenesis, is associated with sporulation in the infected tissue, we studied the *calA* gene expression in *in vitro* grown mycelium under sporulating and non-sporulating conditions by northern blot analysis (Fig. 6B). Although the rate of sporulation differs dramatically in the analyzed mycelia, the amount of *calA* mRNA in non-sporulating, moderately sporulating and heavily sporulating mycelium grown on both synthetic Henniger and rich rye-sucrose medium is similar. This indicates that during growth of the fungus *in vitro*, the expression level of the *calA* gene is not influenced by sporulation nor by the composition of the medium. It can therefore be concluded that induction of calmodulin gene expression during sporulation is not a general phenomena in Oomycetes.

Pathogenesis is a complex process and in fact very little is known about the properties of *P. infestans* which determine its pathogenic character. Elucidation of the precise reason for increased calmodulin mRNA levels in *P. infestans* during pathogenesis is, at this stage, rather difficult. It has been shown in several organisms that via Ca^{2+}-calmodulin complexes, calmodulin is involved in the transduction of extracellular stimuli into intracellular events (Hepler and Wayne, 1985; Roberts *et al.*, 1986). In view of this, Ca^{2+}-calmodulin complexes might be involved in the communication between the two organisms of a plant-microbe interaction. To test the requirement for increased calmodulin levels during pathogenesis, one can think of ways to reduce calmodulin synthesis or calmodulin activity, e.g. by means of anti-sense RNA or via calmodulin inhibitors. Subsequent analyses of the effect of reduced calmodulin synthesis or activity on the pathogenic properties of *P. infestans* can shed more light on the role of calmodulin during pathogenesis. The major drawback of these experiments is the fact that also other calmodulin dependent processes will be influenced, thus complicating the interpretation of such studies.

The *P. infestans calA* gene was not cloned by the use of heterologous calmodulin probes but was selected via a differential hybridization procedure aimed at the isolation of *in planta* induced *P. infestans* genes. To our knowledge this is the first observation that a pathogenic plant-microbe interaction is associated with increased calmodulin mRNA levels in the pathogen. However, at this moment the precise role of calmodulin in the pathogenic process, if any, is still unclear.

References

Barr, D.J.S. (1981). The phytogenetic and taxonomic implications of flagellar rootlet morphology among zoosporic fungi. *Biosystems* **14**: 359-370.

Caten, C.E. and Jinks, J.L. (1968). Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Canadian Jounal of Botany* **46**: 329-347.

Cavalier-Smith, T. (1987). The origin of fungi and pseudofungi. *In*: Rayner, A.D.M., Brassier, C.M. and D. Moore (Eds.), Evolutionary Biology of the Fungi, pp. 339-354. Press Syndicate of the University of Cambridge.

Cheung, W.Y. (Ed.) (1980). Calcium and Cell Function, vol. I. Calmodulin. Academic Press, New York.

Chien, Y.-H. and Dawid, I.B. (1984). Isolation and characterization of calmodulin genes from *Xenopus laevis*. *Molecular and Cellular Biology* **4**: 507-513.

Chung, S.-H. and Swindle, J. (1990). Linkage of the calmodulin and ubiquitin loci in *Trypanosoma cruzi*. *Nucleic Acids Research* **18**: 4561-4569.

Davis, T.N., Urdea, M.S., Masiarz, F.R. and Thorner, J. (1986). Isolation of the yeast calmodulin gene: Calmodulin is an essential protein. *Cell* **47**: 423-431.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* 12: 387-395.

Dudler, R. (1990). The single-copy actin gene of *Phytophthora megasperma* encodes a protein considerably diverged from any other known actin. *Plant Molecular Biology* 14: 415-422.

Feinberg, A.P. and Vogelstein, G. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* 132: 6-13.

Fischer, R., Koller, M., Flura, M., Mathews, S., Strehler-Page, M.-A., Krebs, J., Penniston, J.T., Carafoli, E. and Strehler, E.E. (1988). Multiple divergent mRNAs code for a single human calmodulin. *Journal of Biological Chemistry* 263: 17055-17062.

Fritzemeier, K.-H., Cretin, C., Kombrink, E., Rohwer, F., Taylor, J., Scheel, D. and Hahlbrock, K. (1987). Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. *Plant Physiology* 85: 34-41.

Gurr, S.J., Unkles, S.E. and Kinghorn, J.R. (1987). The structure and organization of nuclear genes of filamentous fungi. *In*: Kinghorn, J.R. (Ed.), Gene Structure in Eukaryotic Microbes, pp. 93-139. IRL Press, Oxford.

Henniger, H. (1959). Versuche zur kultur verschiedener rassen von *Phytophthora infestans* (Mont.) de By. auf künstlichen Nährböden. *Phytopathologische Zeitschrift* 34: 285-306.

Hepler, P.K. and Wayne, R.O. (1985). Calcium and plant development. *Annual Review of Plant Physiology* 36: 397-439.

Jena, P.K., Reddy, A.S.N. and Poovaiah, B.W. (1989). Molecular cloning and sequencing of a cDNA for plant calmodulin: Signal-induced changes in the expression of calmodulin. *Proceedings of the National Academy of Sciences USA* 86: 3644-3648.

Karlovsky, P. and Prell, H.H. (1991). The TRP1 gene of *Phytophthora parasitica* encoding Indole-3-Glycerolphosphate Synthase-N-(5'-Phosphoribosyl) Anthranilate Isomerase. Structure and evolutionary distance from homologous fungal genes. *Gene* 109: 161-165.

Lagacé, L., Chandra, T., Woo, S.L.C. and Means, A.R. (1983). Identification of multiple species of calmodulin messenger RNA using a full length complementary DNA. *Journal of Biological Chemistry* 258: 1684-1688.

LéJohn, H.B. (1989). Structure and expression of fungal calmodulin gene. *Journal of Biological Chemistry* 264: 19366-19372.

Logemann J., Schell J. and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* 163: 16-20.

Lydan, M.A. and O'Day, D.H. (1988). Different developmental functions for calmodulin in *Dictyostelium*: trifluoperazine and R24571 both inhibit cell and pronuclear fusion but enhance gamete formation. *Experimental Cell Research* 178: 51-63.

Mahe, A., Grisvard, J. and Dron, M. (1992). Fungal- and plant-specific gene markers to follow the bean anthracnose infection process and normalize a bean chitinase mRNA induction. *Molecular Plant-Microbe Interactions* 5: 242-248.

Marmé, D. and Dieter, P. (1983). Role of Ca^{2+} and calmodulin in plants. Cheung, W.Y. (Ed.), Calcium and Cell Function, vol. IV., pp. 263-311. Academic Press, New York.

Matton, D.P. and Brisson, N. (1989). Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Molecular Plant-Microbe Interactions* 2: 325-331.

Muthumukar, G. and Nickerson, K.W. (1984). Ca(II)-calmodulin regulation of fungal dimorphism in *Ceratocystis ulmi*. *Journal of Bacteriology* 159: 390-392.

Paranjape, V., Gupta Roy, B. and Datta, A. (1990). Involvement of calcium, calmodulin and protein phosphorylation in morphogenesis of *Candida albicans*. *Journal of General Microbiology* 136: 2149-2154.

Pieterse, C.M.J., Risseeuw, E.P. and Davidse, L.C. (1991). An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Molecular Biology* 17: 799-811.

Pieterse, C.M.J., de Wit, P.J.G.M. and Govers, F. (1992). Molecular aspects of the potato-*Phytophthora infestans* interaction. *Netherlands Journal of Plant Pathology* 98 supplement 2: 85-92.

Putkey, J.A., Ts'ui, K.F., Tanaka, T., Lagacé, L., Stein, J.P., Lai, E.C. and Means, A.R. (1983). Chicken calmodulin genes: A species comparison of cDNA sequences and isolation of a genomic clone. *Journal of Biological Chemistry* 258: 11864-11870.

Rasmussen, C.D., Means, R.L., Lu, K.P., May, G.S. and Means, A.R. (1990). Characterization and expression of the unique calmodulin gene of *Aspergillus nidulans*. *Journal of Biological Chemistry* **265**: 13767-13775.

Roberts, D.M., Lukas, T.J. and Watterson, D.M. (1986). Structure, function, and mechanism of action of calmodulin. *CRC Critical Reviews of Plant Sciences* **4**: 311-339.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (Eds.) (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**: 5463-5467.

Saporito, S.M. and Sypherd, P.S. (1991). The isolation and characterization of a calmodulin-encoding gene (CMD1) from the dimorphic fungus *Candida albicans*. *Gene* **106**: 43-49.

Sargent, T.D. (1987). Isolation of differentially expressed genes. *In*: Berger, S.L. and A.R. Kimmel (Eds.), Methods in Enzymology, vol. 152: Guide to Molecular Cloning Techniques, pp. 423-432. Academic Press, San Diego.

St Leger, R.J., Roberts, D.M. and Staples, R.C. (1989). Calcium- and calmodulin-mediated protein synthesis and protein phosphorylation during germination, growth and protease production by *Metarhizium anisopliae*. *Journal of General Microbiology* **135**: 2141-2154.

Suryanarayana, K. and Thomas, D. des S. (1986). Asexual spore release from saprolegniaceous watermoulds: Involvement of calmodulin. *Journal of General Microbiology* **132**: 593-598.

Takeda, T. and Yamamoto, M. (1987). Analysis and *in vivo* disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. *Proceedings of the National Academy of Sciences USA* **84**: 3580-3584.

Taylor, J.L., Fritzemeier, K.-H., Häuser, I., Kombrink, E., Rohwer, F., Schröder, M., Strittmatter, G. and Hahlbrock, K. (1990). Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. *Molecular Plant-Microbe Interactions* **3**: 72-77.

Unkles, S.E., Moon, R.P., Hawkins, A.R., Duncan, J.M. and Kinghorn, J.R. (1991). Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* **100**: 105-112.

Uyeda, T.Q.P. and Furuya, M. (1986). Effects of low temperature and calcium on microfilament structure in flagellates of *Physarum polycephalum*. *Experimental Cell Research* **165**: 461-472.

Watterson, D.M., Sharief, F. and Vanaman, T.C. (1980). The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain. *Journal of Biological Chemistry* **255**: 962-975.

Yamanaka, M.K., Saugstad, J.A., Hanson-Painton, O., McCarthy, B.J. and Tobin, S.L. (1987). Structure and expression of the *Drosophila* calmodulin gene. *Nucleic Acids Research* **15**: 3335-3348.

Zimmer, W.E., Schloss, J.A., Silflow, C.D., Youngblom, J. and Watterson, D.M. (1988). Structural organization, DNA sequence and expression of the calmodulin gene. *Journal of Biological Chemistry* **263**: 19370-19383.

chapter 6

Structure and genomic organization of the *ipiB* and *ipiO* gene clusters of *Phytophthora infestans*

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Abstract. Two *in planta* induced (*ipi*) genes, designated *ipiB* and *ipiO*, of the potato late blight fungus *Phytophthora infestans* (Mont.) de Bary, were isolated from a genomic library by a differential hybridization 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 (nt) sequence and genomic organization of the *ipiB* and *ipiO* genes. 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 (aa), 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, respectively. The *ipiO* genes code for two almost identical 152 aa proteins which do not have any homology with sequences present in data libraries. The IPI-B as well as the IPI-O proteins contain a putative signal peptide of 20 and 21 aa respectively, suggesting that they are transported out of the cytoplasm. In the promoter regions of the *ipiB* and *ipiO* genes, a 16 nt sequence motif, matching the core sequence GCTCATTYYNCA(A/T)TTT, was found. This sequence motif appears to be present around the transcription start point (*tsp*) of seven out of eight oomycetous genes of which the *tsp* has been determined, suggesting that oomycetes have a sequence preference for transcription initiation.

Introduction

The oomycetous fungus *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 (Choi *et al.*, 1992; Fritzemeier *et al.*, 1987; Hahlbrock *et al.*, 1989; Martini *et al.*, 1993; Schröder *et al.*, 1992; Taylor *et al.*, 1990). 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. Characterization 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 synthesized 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 characterization 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 the fungus *in vitro* but during pathogenesis on potato, the expression levels are consistently 5-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 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 characterization and genomic organization of the members of the *ipiB* and *ipiO* gene clusters.

Results and Discussion

Isolation and genomic organization 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 hybridizing clones (DHCs), DHC-B and DHC-O, contain *in planta* induced genes which are highly expressed in 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 hybridized with a labeled first strand cDNA probe which was synthesized 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 bar in Fig. 1A and 1C). These fragments were subcloned and a detailed restriction endonuclease profile of the DNA surrounding the *ipiB* and *ipiO* genes was determined (Fig. 1B and 1D). In both cases, repetition of specific endonuclease profiles was observed. Cross-hybridization experiments showed that these repeated areas are highly homologous suggesting the presence of a cluster of similar genes.

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 hybridized using labeled interaction cDNA as probe. Of the DHC-B subclones, one 0.9 kb *Sst*I-*Hinc*II fragment and two 0.98 kb *Sst*I-*Pst*I fragments hybridized with the interaction cDNA probe whereas of the DHC-O subclones, two 0.63 kb *Sst*I-*Xba*I fragments hybridized (indicated with dotted lines in Fig. 1B and 1D). This indicates that the coding regions of the *ipiB* and *ipiO* genes are constrained within these respective DNA fragments. On northern blots

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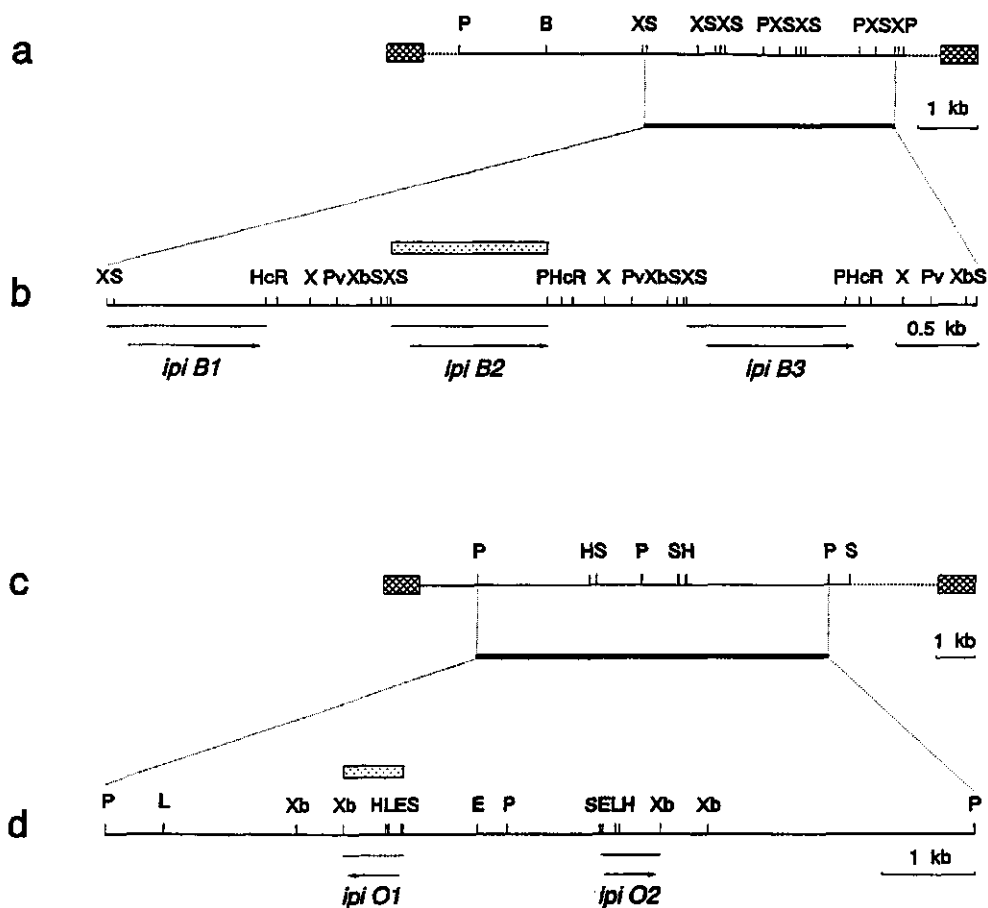
Figure 1. Organization of the *P. infestans ipiB* and *ipiO* genes. a and c; partial restriction map of λEMBL3 recombinant phages DHC-B (a) and DHC-O (c) which were isolated from a genomic library of *P. infestans* DNA by differential hybridization as described by Pieterse *et al.* (1993a). Hatched bars represent λEMBL3 arms. Discontinuous lines represent DNA regions of unknown length and restriction endonuclease profile. Closed bars 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 cDNA synthesized 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 and 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, hybridized with labeled interaction cDNA as probe. Arrows indicate positions and directions of the coding sequences of the *ipiB* and *ipiO* genes as assessed by dideoxy sequencing. Dotted bars show the DNA fragments which were used as probes for hybridization of genomic Southern blots and for the isolation of the *ipiO1* cDNA clone from the λZAP cDNA library. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Hc, *Hinc*II; L, *Sal*I; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RV; S, *Sst*I; X, *Xho*I; Xb, *Xba*I.

containing RNA isolated from *P. infestans*-infected potato leaves, probes derived from all three DHC-B fragments hybridized to a mRNA transcript of 1200 nt in length (Pieterse *et al.*, 1993a). Probes derived from the two DHC-O fragments both hybridized to a mRNA transcript of 600 nt in length. Considering the repetitive restriction endonuclease profile, the cross-hybridization, and the size of the DNA fragments in comparison to the length of the hybridizing 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* (Fig. 1B), the ones on DHC-O *ipiO1* and *ipiO2*, respectively (Fig. 1D). There is no cross-hybridization between the *ipiB* and *ipiO* genes.

Southern blot analyses of genomic **P. infestans** *DNA.* 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 hybridized 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 Fig. 1B and 1D). The *ipiB2* probe hybridized to approximately ten *Pst*I, *Xho*I and *Sst*I fragments (Fig. 2A). 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 hybridized 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 (Fig. 2B). 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 hybridization conditions used, the *ipiB* nor the *ipiO* probe hybridized to potato DNA (Fig. 2) indicating that there are no highly similar sequences present in the potato genome.

Nucleotide sequence of the **ipiB** *genes.* The DNA sequence of the 5424-bp *Xho*I-*Sst*I fragment (Fig. 1B), comprising the coding regions of *ipiB1*, *ipiB2* and *ipiB3*, was determined by dideoxy sequencing and is shown in Fig. 3. Three highly homologous open reading frames (ORFs) of 903, 1029 and 1041 nt were found at positions where the coding regions of the *ipiB* genes were predicted (Fig. 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 hybridize to the *ipiB2* probe (data not shown)
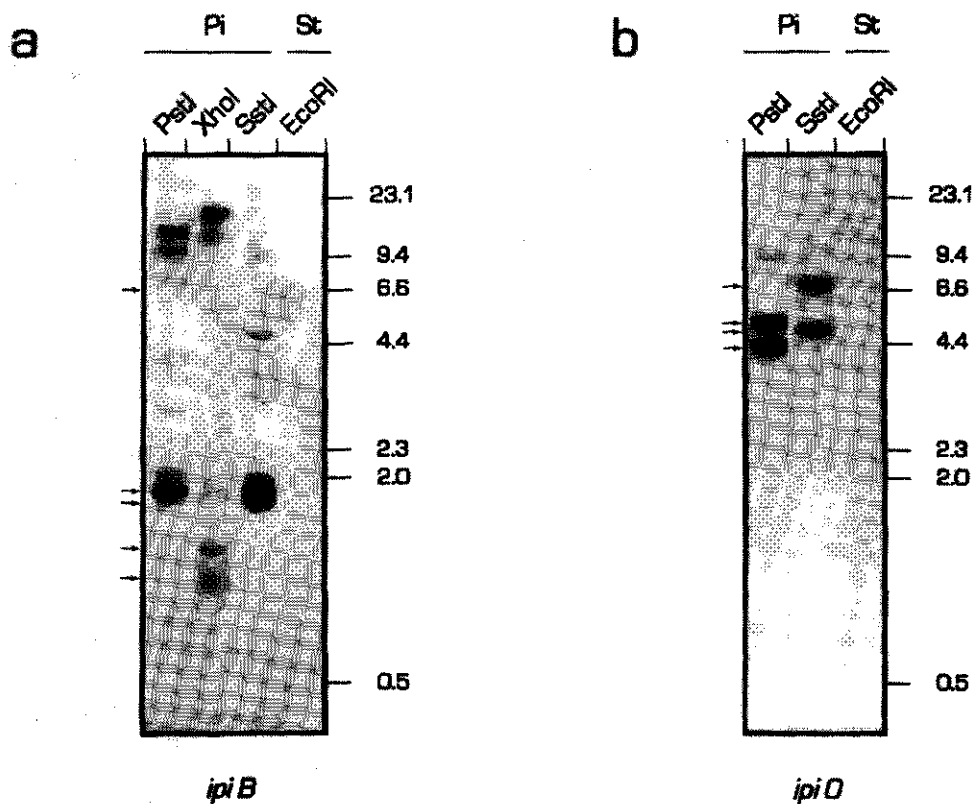
Figure 2. Autoradiographs of Southern blots containing genomic DNA of (Pi) *P. infestans* (strain 88069) and (St) potato (cultivar 'Ajax'), hybridized with the *ipiB* and *ipiO* probes. Genomic DNA was isolated as described by Pieterse *et al.* (1991). Ten μg of genomic DNA was digested with restriction endonucleases *Eco*RI, *Pst*I, *Sst*I or *Xho*I. After electrophoresis on a 0.7% agarose gel, Southern blotting was performed on Hybond-N⁺ membranes (Amersham) according to the manufacturers instructions. Blots were hybridized with probes derived from the 0.98 kb *Sst*I-*Pst*I fragment of DHC-B (a) and the 0.63 kb *Sst*I-*Xba*I fragment of DHC-O (b) as indicated by the dotted bars in Fig. 1B and 1D, respectively. Probes were labeled by random primer labeling (Feinberg and Vogelstein, 1983). Hybridization 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 0.2×SSC, 0.1% SDS at 65°C and exposed to Kodak X-OMAT S film. Molecular size markers are indicated in kb. Arrows indicate hybridizing DNA fragments present in DHC-B and DHC-O, respectively.

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-hybridization with a probe derived from the 0.82 kb intergenic region suggesting that the 5' regulatory sequences of the *ipiB1* gene are similar to those of *ipiB2* and *ipiB3* (data not shown).

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        XhoI SstI
        CTCGAGGTGACATCAAACAGCTCTCGTGCATTCGAAGGCTGTCCAAGAACACGCAAGCCCAACATGTTCAGCAGTAGCAAGATCGCCGCC        90
                                        M  F  S  S  S  S  K  I  A  A      9 (a)
        GTGTGCCTCGCCGTCGTGGCCGCTAACCAACACCGCGTATGCTGAGAAGCAGGCCGCCCAGACGTTCGGACTGCTTGGCGGTGGTCTGCAC       180
        V  C  L  A  V  V  A  V  C  L  A  V  V  A  L  T  N  S  T  A  Y  A  E  K  E  A  A  Q  T  F  G  L  L  G  A  G  L  H       39 (a)
        GGTGGTGCCGGACTCTATGGAGCTGGCGGCGTGCCGGTCTTCGACTGGTCGAGCCCGGCGTTGGTGGTCGTGTACGGTCGAGGTCCCGCCTAT     270
        G  G  A  G  L  Y  G  A  G  A  A  V  G  L  H  G  G  A  G  V  G  A  G  L  Y  G  R  G  A  G  Y       69 (a)
        GCTGGTGCTGGTCGGTCGTCTACGGTCGAGGTGCTCGGTGGGCGTTGGTGGTCGGCTGGGCTTGTACGGTCGAGGCGGCCGGATATGGTGGTGGCTGGT     360
        G  G  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  V  G       99 (a)
        GCTGGGCTGTTTGGTCGGGCAGGCGGCTACGGTGGCGGCTGGTGCTGGCGTCGGAGCTGGCGTTGGCGGTGCGGCCGCGTCGGTAACGGGATAC     450
        A  G  L  F  C  R  A  A  C  G  V  G  V  G  V  G  L  Y  G  R  G  A  G  V  G  L  Y  G  R  G  A  G  V  G  G  N  G  Y      129 (a)
        GGCGGTGTTGGTGTCGGAGGAGTTACGGGTGTCGGTGCTGGTGTCGCAGGAACTACCGGTGTCGGTGCTGGTGCCGGTGTGGAGGTGGT      540
        G  C  V  C  V  G  G  V  T  V  G  V  G  V  G  V  T  V  G  G  V  G  V  G  A  V  G  V  G  V  S      159 (a)
        GTGAGTGGTAACGTCGCTGTTGGCGTGGCGCGTGGCGGTGTAATCACTCGTCGTCGGCACGTTGGCGTGGCGGGGCTGCGAAACGGTGGAGTGAGT      630
        V  S  G  N  V  G  V  G  V  G  V  G  V  N  A  A  V  G  V  G  V  G  V  G  V  G  V  S      189 (a)
        GCCAATGCTGGTGCTCGGCCGCCGGAGCTGAGTGCGGAAACACTGGTGCTCGGCCTCGGAGGCTGGAGCGGAGTGGAGGTGCTAACGGTGGAGTGAGC     720
        A  N  A  G  V  A  G  V  S  G  N  T  G  A  G  V  G  G  A  G  G  A  N  G  C  V  S      219 (a)
        GCCAATGCTGGTGTCGGAGCTGGTCTCGCCGGCATCGGCCCGGTGTCGGCGGATCGGTCGGTGTGGAGGAGCGGCAAGTACTGGTGCGGGT      810
        A  N  A  G  V  G  A  G  V  G  V  G  V  G  A  A  S  T  C  A  G       249 (a)
        GGAGCTACGGACAACGACGGCGACGGCCAGAACTAGCACATCTACGTCGCAGAACGGCGGGCCCAAGGCAACTGCAACTACCACGACTGCAAGCA     900
        G  A  T  T  T  T  D  G  R  T  S  T  S  T  S  Q  N  G  G  P  R  Q  L  Q  L  P  R  L  Q  A      279 (a)
        CGACAAGGCACAGCCGGCACGGCGTGCTTCGACGAAGCAGACAGGTTATCGCATGCTGCGGTGGCGGTGAGCCGGAGGAATAGTGTCTTGTT     990
        R  Q  R  T  A  G  T  G  Y  R  M  L  R  S  Q  *      301 (a)
        HincII                                                   EcoRV
        GACAACTTCTCCTGGCTACGTTGATCAGCTCTTAGCTCATCTCGCTCAAAGAGGCGATCTGCCGATATCTGTCTATAATTTTTAATACCG    1080

        TCGTGTAGTAAGAAAATAAATGTGCTGTTGTTTCTCATTGCTGTCGGGTTTTGGCTTCTTTATGCGTCGCTTGCAGTTAGTAACCATCGTA   1170
        TGAATTTAATTTGCTGAATGTTTGGTCCGTAGAGCACTTTTTGATTCGGAATTATACTATGTTTAGTAACTGTGATATTGAAGTTGCAGT   1260
        XhoI
        CGAGAAAGGCCAACTTGCTGCTTTTGTGAGTACATGAGCAAAAGAAAACATCGAGTAGCTTAGCTCAGGATTCTAGCGCACTCGTTGCGTGC   1350
                                                                PvuII
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        TTGCAGGGACATCTTACTTAGACGTTTCGCCTTTGCAAGAACCTACAAATGTAAGCATACCGGTACATATGGGCCTCGGCATACTAGCAAAA   1530
        TGCTGGTCATCATGGCGTCGAAAAAGACCTTCTCCCTGTCGATTGTGTGTCATAGGCAAGGTAGACTCCTCGGCACCGCAGTGTGCTCTCTTA   1620
                                                XbaI                                      SstI
        TACCACCTACCTGCCTTGTACACATGATCTAGACGACCCGTCAATGCGGGACCGACGGAAAAGGTTGTCGAGTGGCCGCCGTCCAGAGCTCA   1710
        XhoI SstI
        TTTCCCATTCTCCTCCCTCGAGCTCACATCAAACAGCTCTCGTGCATTCGAAGGCTGTCCAAGAACACGCAAGCCCAACATGTTCAGCAG   1800
                                                                    M  F  S  S          (b)
        TAGCAAGATCGCCGCCGTCTGCCTCGCCGTCGTGGCCGCTAACCAACACCGCGTATGCTGAGAAGCAGGCCGCCCAGACGTTCGGGACTGCT   1890
        S  K  I  A  A  V  C  L  A  V  V  A  V  A  L  T  N  S  T  A  Y  A  E  K  E  A  A  Q  T  F  G  L  L       34 (b)
        TGCCGCTCGCTGTCTCCACGGTCGTGCGGGACTCTATGGAGCTGGCGGCGTGCCGGTCTTCATGGTGGAGCCGGGGTTGGTGCTGGTCTGTACGG   1980
        G  A  G  L  H  G  G  A  G  V  G  A  G  L  Y  G  R  G  A  G  Y  G  G  V  G  A  G  L  Y  G       64 (b)
        TCGAGTCGCCGGCTATGGTGGTCGTGGTGCTGGTCTGTACGGTCGAGGTGCTGGTGGCGTTGGTGCTGGGGCTTGTACGGTCGAGGTGCCGG   2070
        R  G  A  G  Y  G  G  A  G  A  G  L  Y  G  R  G  A  G  V  G  A  G  L  Y  G  R  G  A  G       94 (b)
        ATATGCTGGCCGCTGGTGCTGGTCGTGTACGGTCGAGGTGCTGGTGGCGTTGGTGCTGGGGCGTGTACGGTCGAGGTGCCGGATATGGTGGCGGC   2160
        Y  G  G  A  G  A  G  L  Y  G  R  G  A  G  Y  G  G  V  G  A  G  L  Y  G  R  G  A      124 (b)
        TGCTGCTGTCTCTACGGTCGAGGTGCTGGTGGCGTTGGTGCTGGGGCTGTACGGTCGAGGCGGCCGCATATGGTGGTCGTGCTGCTGGGCT   2250
        G  A  G  L  Y  G  R  G  A  G  Y  G  G  V  G  A  G  L  Y  G  R  G  A  G  Y  G  G  V      154 (b)
        CTTTGGTCGCGAAACCCGGCTACGGAGGCGGCTGGCGTCGGGCGTCGGGGCTCGCTTGCCGGGCGGGGCCTCGGTAACGGATACGGCGGGTGT   2340
        F  G  R  E  A  G  Y  G  G  A  G  V  G  V  G  A  G  L  G  N  G  Y  G  G  V      184 (b)
        TGGTGTGGAGGAACTACGGGTGTCGGTGCTGGTGCTGCCGGTGTCGGAGGTGGTGAGCTGGTAACGTCGGTGCTGGCGTGGCGTGGGCCTGGGCCGG   2430
        G  V  G  G  T  T  G  V  G  A  G  V  G  G  G  V  G  V  G  V  N  V  G  A  G  V  G  V  G       214 (b)
        TAATGCTGCTGTCGGCGGCTGGTGTGGGGGGGGCTGCGAACGGTGGACTCGGTGCCAATGCTGGTGTCGGCCGGGGAGTGAGTGGGAACGC   2520
        N  A  A  V  G  A  G  V  G  G  A  A  N  G  G  V  G  A  G  V  G  V  S  G  N  T      244 (b)
        TGGTGCTGGGCGTCGGAGGTGGAGCGGAGTGAGTGCTAACGGTGGAGTGAGCCGCAATGCTGGTGTCGGGAGGTGGTGTCGGGGGATCGGGC   2610
        G  A  C  V  G  G  A  S  G  G  A  N  G  V  S  A  N  A  G  V  G  G  V  C  S  A      274 (b)
        CGGTGTCGGCGGATCGGTCGGTGTCGGGAGGACGGCCAAGTACTGGTGCGGGTGGAGCTACGACAACGACGGACGGCAGAACTAGCACATC   2700
        G  V  G  S  V  G  G  A  A  S  T  G  A  G  G  A  T  T  T  T  D  G  R  T  S  T  S      304 (b)
                                                    PstI
        TACGTCGCAGAACGGCGGGCCAAGGCAACTGCAACTACCACGACTGCAGGCACGACCACGCACAGCCGGCACGGGTGCTTCGACGAAGCA   2790
        T  S  Q  N  G  G  P  R  Q  L  Q  L  P  R  L  Q  A  R  P  R  T  A  G  T  G  A  S  T  K  Q      334 (b)
                                            HincII
        GACAGGTTATCGCATGCTGCGGTGGCAGTGAGCCGGAGGAATAGTGTCTTGTTGTTGAGTAGTTGTCCTGGGTACGTTGATCAGCTCTTAGCTG   2880
        T  G  Y  R  M  L  R  S  Q  *      343 (b)
                EcoRV
        ATCTCGCTCAAAGAGGCGGATCTGCCGATATCTGTCTATAATTTTTAATACCGTCGTCTAGTAAGAAAATAAATCTGCTCGTTCGTTTCTCAT   2970
        TGCTGTCGGGTTTTGCTTCTTTATGCGTCGCTTGCAGTTAGTAAGCATCGTATCAATGTAATTTGCTGAATGTTTAGGTGCGTAGAGCACT   3060
                                        XhoI
        TTTTGATTCGGAATTATACTATGTTTAGTAACTGTGATATTGAAGTTGCACTCGAGAAAGGCCAACTTGCTGCTTTTGTGAGTACATGAAGC   3150
        AAAAGAAAACATCGAGTAGCTTAGCTCAGGACTCTAGCGCACTCGTTGCGTGCTGGCTGCATTATTGACCAGCCTTCGCCGTCTGCACCGG   3240
                                                        PvuII
        GGCCGTATCGCATTTAACGAGCAGTCATGACCGTGTGAGGTGGCGTGACGGTCCATTGGAGTTCACATGTTAGTTAGTTTTGGCCTTGCAAGA   3330
        ATCCACAATGTAAGCATACCGGTACATATGGGCCGTCGGGCATACTAGCAAAATGCTGGTCATCATGGCGTCGCAAAAAGACCTTCTCCCTGT   3420
                                                    XbaI
        CGATTCTGTGTGTCTATACCAAGTAGACTCCTCGGCACGCAGTGTGCTCTCTTATACCACCTACCTGCCTTGTACACATGATCTAGACGACCG   3510
                                SstI                    XhoI SstI
        TCAATGCGGGACCGACGGAAAAGGTTGTCGAACGTGCCGCCGTCCAGAGCTCATTTCCCATTCTCCTCCCTCGAGCTCACATCAAACAGCTC   3600
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                                M  F  S  S  K  I  A  A  V  C  L  A  V  V  A  L       17 (c)
        AACCAACAGCCGCGTATGCTGAGAAGGAGGCCGCCCAGACGTTCGGGACTGCTTGGCGGTGGTCTGCACGGTGGTGCGGGGACTCTATGGAGC   3780
        T  N  S  T  A  Y  A  E  K  E  A  A  Q  T  F  G  L  L  G  A  G  L  H  G  G  A  G  L  Y  G  A       47 (c)
        TGGCGCTGCCGATCTTCATGGTGGAGCCAGCGGTTGGTGCTGGTGTTGACGGTGCGAGGTGCGGGCTATGGTGGCGGCTGGTGCTGGTCGTGGTCGTA   3870
        G  A  A  D  L  H  G  G  A  S  V  G  A  G  L  Y  G  R  G  A  G  Y  G  G  A  G  A  G  L  Y       77 (c)
        CGCCGGCATATGGTGGCGCTGGTGCTGTCGGTCGTACGGTCGAGGTGCTGGTGGCGCTGCCTGCTGGGCGTGTACGGTCGAGGTGCGGGCTATGG   3960
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        TGGTGCTGGTGTCGCTGGGCTTGTACGGTCGAGGTGCGGGCTATGGTGGCGGCTGGTGCTGCCGGGGCTGTACGGTCGAGGTGCGGGCTATGG   4050
        G  A  G  A  G  L  Y  G  R  G  A  G  Y  G  G  A  G  A  G       137 (c)
        TCTGTATGCTGCGAGCTGCTGTGCGGTTGCTGCTGGGCTTGTACGGTCGAGGCGGCCGCGCATATGGTGGTGCTGGTGCTGGGCAGCTGTTTGGTCG   4140
        L  Y  G  R  G  A  G  Y  G  G  V  G  A  G  L  G  F  G  R      167 (c)
        GGAAGCCGGCTTCGGAGGCGGCTGGCGCTGGGCGGTCGGCAGCTGCCGGTTGCCCCGCGGCGGGCCTCGGTAACGGCATAGGGGGGTGTTGGTGTCGG   4230
        E  A  C  F  G  A  G  A  G  V  A  A  G  V  P  A  G  L  G  N  A  Y  G  G  V  G  G       197 (c)
        G  T  T  G  V  G  A  G  A  G  V  G  G  N  G  V  S  G  N  V  G  V  G  G  N  A  A       227 (c)
        TCTCCCGCGCTCGTGTCGGCGGGCGTCGGGAACGGTGGACTGAGTGCCAAATGCTGGTGTCGGCGGCGGGAGTGAGTCGGGAACACTCGTTGCGTGCGT   4410
        V  G  A  G  V  G  G  A  A  N  G  G  V  S  A  N  A  G  V  G  V  S  G  N  T  G  A  G       257 (c)
        CGTCGGAGGTGGAGCGGAGTGAGCGTGCTAACGGTGCAGTGAGCGCCAATGCTGGTGTCGGGAGGTGGTGTCGGCGGGATCGGCCCGTGTGGG   4500
        V  G  G  A  S  G  G  A  N  G  V  S  A  N  A  G  V  G  G  V  G  C  G       287 (c)
        AGGAGCGGCAAGTACTGGTGTGGGTGGACCTACCAAAACGACGGACGGCAAAACTAGCACATCTACGTCGCAGAACGGCGGGCCAAGGCA   4590
        G  A  A  S  T  C  V  G  G  A  T  K  T  T  D  G  K  T  S  T  S  T  S  Q  N  G  G  P  R  Q      317 (c)
                                                            PstI
        ACTGCAACTACCACCACGTGGAGCCACGACCACGCACGACAGCCGGCACGGGTGCTTCGACGAAGCAGACAGGTTATCGCATGCTGCGGTCGCA   4680
        L  Q  L  P  R  L  Q  A  R  P  R  T  A  G  T  G  A  S  T  K  Q  T  G  Y  R  M  L  R  S  Q      347 (c)
                    HincII                                                                    EcoRV
        GTGAGCCGAGGAATAGTGTCTTGTTGAGTACTTCTCCTGGCTACGTTGATCAGCTCTTAGCTCATCTCGCTCAAAGAGGCGATCTGCCGA   4770
        *
        TATCTGTGTATAATTTTTAATACCGTCGTCTAGTAAGAAAATAAATGTGCTGTTGTTTGTTTCTCATTGCTGTCGGGGTTTTGGCTTTTATGCGG   4860
        TCGCTTGCAGTTAGTAAGCATCGTATCAATGTAATTTGCTGAATGTTTGGTGCGTAGGAGCACTTTTTGATTCGGAATTATACTATGTTTA   4950
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        GTAACTGTGATATTGAAGTTGCACTCGAGAAAGGCAACTTGCTGCTTTTGTGAGTACATGAGCAAAAGAAAACATCGAGTAGCTTAGCTC   5040
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                PvuII
        GACCCGTCTGAGCTGCCTGACGGTCATTGGAGGCACATGTTACTTAGAGTTTGGCCTTTGCAAGAACCTAGAACTGTAAGCATACCGGTACA   5220
        TATGGGGCCTCGGCATACTAGCAAAATGCTGGTCATCATGGCGTCCAAAAAGACCTTCTCCCTGTCGATTGTGTGTCTATACCAAGTAGAC   5310
                                                        XbaI
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                SstI
        GCCGAGTGGCCGCCGTCCAGAGCTC   5424
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Figure 3. Nucleotide sequence of the *P. infestans ipiB* gene cluster. The nt sequence starts at the most left *Xho*I site shown in Fig. 1B and extends up to the most right *Sst*I site depicted in the same figure. Deduced aa 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 GCTCATTYYNCA(A/T)TTT (underlined); CT-rich regions (overlined); putative signal sequence cleavage sites (⇑); and potential polyadenylation signals AATAAA (overlined). For sequencing, overlapping subclones clones 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 nt and aa sequences as shown in Figures 3, 4, 5, 6 and 7 were performed using the Sequence Analysis Software Package, version 7.1 of the Genetics Computer Group (GCG) of the University of Wisconsin (Devereux *et al.*, 1984).

Nucleotide sequence of the **ipiO** *genes.* The DNA sequence of the 3440-bp *Xba*I fragment, comprising the coding regions of the *ipiO1* and *ipiO2* gene was determined by dideoxy sequencing (Fig. 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 (Fig. 1D). In Figure 4, lines indicated by (a) show the nt sequence of the *Xba*I-*Pst*I fragment on which the *ipiO1* gene is located. Lines indicated by (b) show the nt sequence of the adjacent *Pst*I-*Xba*I fragment containing the *ipiO2* gene. Two ORFs of 456 nt were found at positions where the coding regions of the *ipiO* genes were predicted (Fig. 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 (Fig. 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 (Fig. 1D). The nt sequences of the *ipiO1* and *ipiO2* genes 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-hybridizing 0.6 kb *Xba*I fragment downstream of their coding regions (Fig. 1D). In the 5' regions, the DNA sequences diverge upstream of positions -637 resulting in a unique 950 nt intergenic DNA region.

Structural features of the **ipiB** *and* **ipiO** *genes.* The *tsp* of the *ipiO* genes was determined by primer extension. A 5' end labeled oligonucleotide complementary to nt +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 *tsp* corresponds to the A at position -26 relative to the ATG start codon (marked by a ▼ in Fig. 4). A direct comparison of the nt sequence surrounding the major *tsp* of eight distinct oomycetous genes in which the *tsp* have been determined, revealed that these oomycetous genes have a sequence preference for transcription initiation. In seven out of eight genes, the major *tsp* is located within the sequence motif GCTCATTYYNCA(A/T)TTT (Table 1) which is invariably situated within the first 100 nt

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```
PstI
CTGCAGTGCAGCCGCCTTTATCAAAAATATCCGGAGTTGCGAGAACATTTTTCATGCTGTGCTGTTGTAAATTGTGGGATTCG   -1136 (a)

TGCTGTCGTGTCGTAATCAAAAAGAACATGCAGTCGATTACCTAATAGGAAACTGTAGTACCGTATGGAACGACTCGTTCCCA   -1053 (a)
                                                            PstI
ATGAGCATTCAACAAGAAACTTCCTCGCTATTAATCGCGTCCAGTAGCATCAATAATGCGATTCAAGCCCACTCTGGTACTGT    -970 (a)
                                          CTGCAGC.GCGCAGAGGGACG.GGTGAAA.C.AGAACA         -969 (b)
                                          EcoRI
TCATCGGGGGTTTCCTCTGGAGCTGTATATATTTCACAGACGGCCGAGAATTCTCTTCTCGTCAACAGCGGCCGGCCGTCAAAC    -887 (a)
AAT.AAAAC.CAAAAAAAAACAAGC..CCCTGAC..AGTGATG....C.GATG.G.....CAGT.GCA..AAGCC..CGCGG.       -886 (b)

GTTAAACTTATCGTAAACAAGAATCGGCCGTCTCAGGGGAGCGCAAAATCAACATGAAGAAGAGAGGCACGTTGGGATATCGAA    -804 (a)
.CGTT....GCGAAGGTTGGCCCGACG.CGAG.TTTCGCAGCG.GCCAAGCAT..C.CTCTCC...ACG.GAAATC.T.TT.C      -803 (b)

TGTCTCTCGATTCATCACAGACACCGGGTAAATACAAGGCTCTAGTCAAGTTTCTAGTGGTGTACTGGATGCTAGTTGCAGGCG    -721 (a)
...GA..GTC.TTA.GAC.G..AGC..ACG.A.TTCTAAAAAGT.G..A.GA.ATCC.TCC.C.GT.TCTT..CA.TGT.AT.       -720 (b)

TCTTTGGCGTTTTCAAGATTAAGCAAAGGGGCTCCAGTGACAACGAACCTACAACAAGCTGCGCACACGGCCGTTCGCTTAGG    -638 (a)
GTGA..AGCAA.A.CG.TAATCTTGG.TTAA.AGA.AAT.T.ATAT.ATCGTTTATCCTCATTTTGGGC..ACGAGCAGC.C.       -637 (b)

AAGCGGTGATTACGACCCAGCAGACCGAAGGATAAGCTGCCTGTTTGAATAAATATTTGTTTTTTCCTTGCACTCAAATCGTTA    -555 (a)
....A....................................A.......T..........G..................         -554 (b)

CAATTGTAATCGGATATGCTGCTGTTCAGTTGCGTACTTTCTTGTACTTTCTTGCGCAAATCCATGTGGTTTTCGAAATTCCC    -472 (a)
...................T.......................................................            -471 (b)

CAATCAAATGACCGCCTCAAAGGCAACCCGTTTCCCGTAAATCTGGGGGCATCTTAAAAATAGTCTTGTAGCGCAAAAACGAC    -389 (a)
...............................................-.........................             -389 (b)

TGCGAATCAGCTAATTCTGACCCCATTTTTGAAATCTACGCCCGATTTTTACCCCTTATATCTAGCTGTTGCTATAGGATTTGG    -306 (a)
.............................................................................          -306 (b)

GATGCTGAATCTTCATAACAGATGGCTACATTTTGGTCCAAGGACTTGTGACGAGTACAATAATAAGATGATGTATCAACGGT    -223 (a)
..........................................................................            -223 (b)

AAGACCAGCTTTACCAATAGTTTATTAATATATCCTGATCTAAAACTTTTTGATTTTCGTGGCCACTTGCAGACTGAAAGTGG    -140 (a)
...........................T...............C...................................        -140 (b)

GGAGCAACTGCAAGTTGCAGGTCGCCTCATCCTGAGATGGTGGATCAGTAGGAGGACTGGCTACGACACATCTGCTCCTTTAC     -57 (a)
.............................................................................           -57 (b)
                    SstI  ▼      EcoRI              +1
TTAAGGCTACGACATGTCGCGCTAAGAGGCTGATTGTGAATTCATTTCTTTCCGGCAATGCGTTCGCTCCTGTTGACCGTGCTT    +27 (a)
.....................G.......................................................          +27 (b)
                                                    M  R  S  L  L  L  T  V  L            9 (c)
                                                    *  *  *  *  *  *  *  *  *            9 (d)
                                                                                     →
TTGAACCTGGTCGTGCTTCTAGCAACCACTGGGGCAGTTTCATCCAATCTCAACACCGCCGTGAATTACGCTTCCACATCCAA   +110 (a)
.......................................................A...................            +110 (b)
L  N  L  V  V  L  L  A  T  T  G  A.V  S  S  N  L  N  T  A  V  N  Y  A  S  T  S  K      37 (c)
*  *  *  *  *  *  *  *  *  *  *  *ⁱ*  *  *  *  *  *  *  *  *  *  *  *  *  *  *          37 (d)
           SalI                            HindIII
GATTCGCTTTCTGTCGACTGAGTACAACGCCGATGAAAAAGAAGCTTGCGGAGGTGACTACAACAATGAGGTCACAAAAGAGC   +193 (a)
.............................................................................           +193 (b)
I  R  F  L  S  T  E  Y  N  A  D  E  K  R  S  L  R  G  D  Y  N  N  E  V  T  K  E  P     65 (c)
*  *  *  *  *  *  *  *  *  *  *  *  *  *  *  ‗‗‗‗‗‗‗  *  *  *  *  *  *  *  *  *          65 (d)

CCAACACGTCTGACGAAGAGCGGGCGTTTTCTATCTCAAAGTCTGCGGAATACGTGAAGATGGTACTTTATGGATTCAAACTT   +276 (a)
.............................................................................           +276 (b)
N  T  S  D  E  E  R  A  F  S  I  S  K  S  A  E  Y  V  K  M  V  L  Y  G  F  K  L        92 (c)
*  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *             92 (d)

GGATTTTCTCCTCGCACTCAGTCCAAGACGGTGTTGCGATACGAAGATAAACTGTTTACGGCTCTCTATAAATCCGGAGAGAC   +359 (a)
.....................................................................C.........TA..     +359 (b)
G  F  S  P  R  T  Q  S  K  T  V  L  R  Y  E  D  K  L  F  T  A  L  Y  K  S  G  E  T    120 (c)
*  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  L  *  *  *            120 (d)

GCCGAGAAGCCTAAGGACCAAGCATCTCGATAAGGCTTCCGCTAGCGTATTTTTCAACAGATTCAAAAAATGGTACGATAAAA   +442 (a)
.....T.....................................G................................C......     +442 (b)
P  R  S  L  R  T  K  H  L  D  K  A  S  V  F  F  N  R  F  K  K  W  Y  D  K  N          148 (c)
*  I  *  *  *  *  *  *  *  *  *  *  G  *  *  *  *  *  *  N  *  *  *  *                 148 (d)

ACGTTGGCCCTAGCTAGCTGGTGCCGGTTAAGACTGTCATGCTATGACATCGTATAGCAACCATGCAGACTTCAAATAATTCG   +525 (a)
.............................................................................          +525 (b)
V  G  P  S                                                                            152 (c)
*  *  *  *                                                                            152 (d)
                                                         *                      XbaI
AGACAGCCGAAATTTAATGCAGTCATCCTTTACTGCACCGGTGTTTTTGAAGCCGTCCACGTGTCTTTTTCTAATTCTCTAGA   +608 (a)
................T................................A...........................          +608 (b)
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Figure 4. Nucleotide sequence of the *P. infestans ipiO* gene cluster and deduced aa sequences of the IPI-O1 and IPI-O2 proteins. (a) 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* (Fig. 1D). (b) nucleotide sequence of *ipiO2* starting from the same *Pst*I site and extending to the *Xba*I site downstream of *ipiO2* (Fig. 1D). Nucleotides identical to those in *ipiO1* are indicated by dots. To allow optimal alignment, one dash (-) is introduced at position -427 in the *ipiO2* nt sequence; (c) Deduced aa sequence of the IPI-O1 protein; (d) Deduced aa 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 GCTCATTYYNCA(A/T)TTT (single underline);

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tsp (▼); CT-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 (closed bar); putative N-glycosylation site (double underline). 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 linearized master subclone DNA as marker. Singly digested, linearized DNA was isolated from the gel and digested to completion with *Bam*HI. DNA fragments were then circularized 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) 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 μl of a suspension of sporangia from *P. infestans* strain 88069 (5×10^5 sporangia/ml).

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 *tsp* 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 *tsp* of the *ipiB* genes were not determined. The *P. infestans* genome may contain more *ipiB* genes in addition to the ones characterized 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 *tsp* of oomycetous genes suggests that the motif is important for transcription initiation. Since this motif is not conserved in genes of higher fungi, plants or animals, the GCTCATTYYNCA(A/T)TTT sequence motif can be considered to be a consensus sequence for transcription initiation in oomycetous 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. However, 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 the *ipiB2* and *ipiB3* gene there are no typical TATAAA-like motifs. A CAAT motif is present at positions -139 to -136 relative to the ATG start codon (overlined in Fig. 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 CT-rich region directly downstream of the transcription initiation consensus sequence (overlined in Fig. 3 and 4). CT-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 nt sequence surrounding the translation start codons of the *ipiB* and *ipiO* genes (CCAAC<u>ATG</u>TT and CGGCA<u>ATG</u>CG, respectively) follow the Kozak consensus sequence for translation initiation (Kozak, 1984), the most conserved nt 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 Fig. 4). A putative polyadenylation signal AAATAA (consensus

Table 1. Alignment of the DNA regions surrounding the conserved sequence motif GCTCATTYYNCA(A/T)TTT which is present within the first 100 nt upstream of the ATG start codon of eight oomycetous genes [a].

gene	DNA sequence surrounding the conserved GCTCATTYYNCA(A/T)TTT 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* (Fig. 3), *ipiO1* and *ipiO2* (Fig. 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 and Michelmore, 1990) and *hsp70* (Judelson and Michelmore, 1989).

[b] Numbers refer to the position of the first and last nt of the depicted sequence relative to the ATG start codon of the respective gene. Nucleotides marked with an asterisk match with the consensus sequence GCTCATTYYNCA(A/T)TTT. 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 *tsp*.

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

[d] *tsp* has not been determined.

[e] The nt 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 *tsp* 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).

AATAAA) was found 48 nt upstream of the poly(A) tail in the cDNA sequence (overlined in Fig. 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 Fig. 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 oomycetous genes studied to date predominantly lack introns. Among all oomycetous genes reported so far (Dudler, 1990; Judelson and Michelmore, 1989; Judelson and Michelmore, 1990; LéJohn, 1989; Moon *et al.*, 1992; Pieterse *et al.*, 1991; Pieterse *et al.*, 1993b; Unkles *et al.*, 1991) 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 and 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 nt sequence of the *P. infestans niaA* gene encoding nitrate reductase (C.M.J. Pieterse, unpublished) suggests the presence of an intron in this gene as well.

Analysis of the IPI-B amino acid sequences. The IPI-B1 (301 aa), IPI-B2 (343 aa) and IPI-B3 (347 aa) 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 (Fig. 5). The IPI-B proteins have a high content of glycine residues and their glycine-rich domains show up to 47% identity to the glycine rich domains of several plant glycine-rich proteins. Most plant glycine-rich proteins are characterized by their repetitive primary structure consisting of up to 70% glycine residues which are arranged in short aa repeats. Furthermore, they usually have an amino-terminal signal sequence for transport out of the cytoplasm (Showalter, 1993). Analysis of the predicted aa sequences revealed that the IPI-B proteins share these characteristics with the plant glycine-rich proteins. The IPI-B
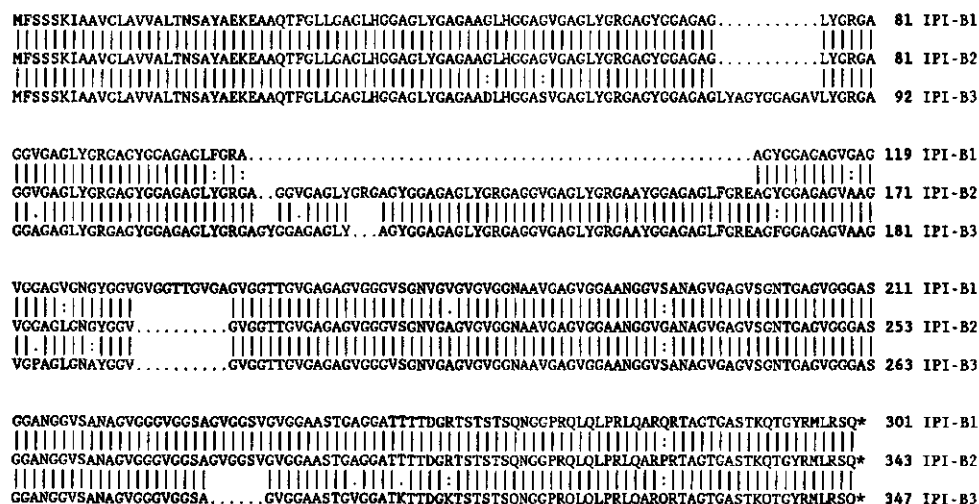
Figure 5. Comparison of the predicted aa sequences of the IPI-B proteins. Gaps are introduced in the sequence to obtain optimal alignment. Numbers indicate position of last aa.

proteins are composed of four domains. The first 20 aa at the amino-terminal end (domain I) comprise a putative signal sequence for secretion, which can be recognized by a hydrophobic region in the hydropathy plot shown in Fig. 6A. The signal sequence cleavage site, predicted according to Von Heijne (1986), is located between a serine (aa 20) and an alanine residue (aa 21). The hydropathy plot shows that the putative signal sequence is followed by a short hydrophilic region of 10 aa (domain II). Domain III, comprising 74-80% of the protein, consists of a large glycine-rich region with up to 47% glycine residues (IPI-B1 43%; IPI-B2 47%; IPI-B3 46%). The primary structure of the glycine-rich domain is highly repetitive with two typical repeats (Fig. 7). The first repeat is characterized 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 carboxyl termini of the predicted IPI-B proteins are composed of a 46 aa hydrophilic region (domain IV).

To our knowledge, this is the first report on genes encoding fungal glycine-rich proteins. Many plant genes encoding glycine-rich proteins have been characterized (Condit and Keller, 1990; Showalter, 1993) and most of them have been implicated to be cell wall proteins (Condit *et al.*, 1990; Keller *et al.*, 1988; 1989a; 1989b). Whether the *P. infestans* IPI-B proteins are structural proteins associated with the fungal cell wall needs to be investigated. Since the *ipiB* mRNA is detectable at high levels in early stages of infection, it is tempting to speculate that the IPI-B proteins are involved in the development of infection structures.
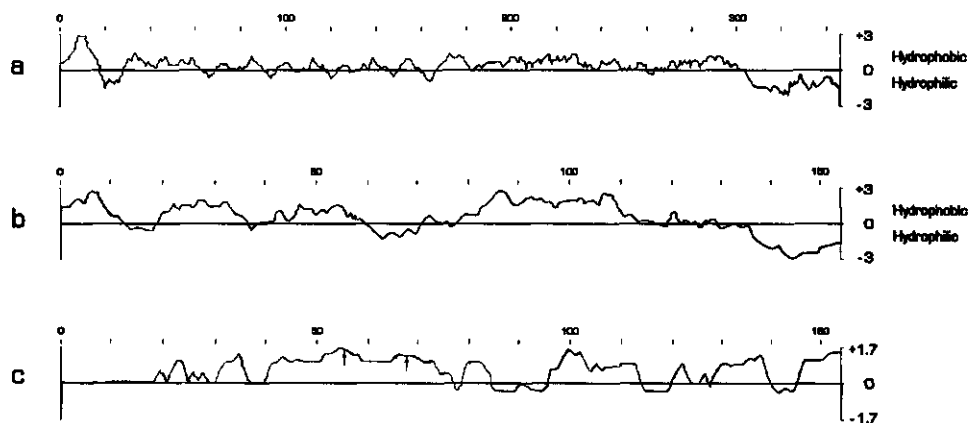
Figure 6. Hydropathy plot of the deduced aa sequence of IPI-B2 (A) and IPI-O1 (B), and antigenic index of the predicted aa sequence of IPI-O1 (C). Hydrophobicity was determined by the method of Kyte and Doolittle (1982). Along the coordinate, aa positions in the proteins are indicated. In (A) and (B), 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 and 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.

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domain I   (hydrophobic)   MFSSSKIAAVCLAVVALTNS   20

domain II (hyrophilic)   AYAEKEAAQT   30

domain III (hydrophobic)
```
                    F G L L                                 34
                  G A G L H G                               40
                  G A G L Y G                               46
                A GAA G L H G                               57
                V G A G L Y G R G A G Y G G                 71
                A G A G L Y G R G A       G G               83
                V G A G L Y G R G A G Y G G                 97
                A G A G L Y G R G A       G G              109
                V G A G L Y G R G A G Y G G                123
                A G A G L Y G R G A       G G              135
                V G A G L Y G R G A A Y G G                149
                A G A G L F G R E A G Y G G                163
              A G A G V       A A G V G G                  174
                  A G L       G N G Y G G                  183
                              V G V G G TT                 190
                        G V G A                            194
                        G A G V G G                        200
                              G V S G N                    205
                  VGA G V G V G G NAA                      217
                    V G A C V G G AAN                      227
                        G   G V G   ANA                    234
                  GV G A G V S G NT                        244
                        G A G V G G                        250
                        G A S   G G AN                     257
                        G     G V S   AN                   263
                            A G V G G                      268
                              G V G G                      272
                        S A G V G G                        278
                        S V G V G G AA                     286
                        S T G A G G A                      293
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domain IV (hydrophilic)   TTTTDGRTSTSTSQNGGPRQLQLPRL   319
                            QARPRTAGTGASTKQTGYRMLRSQ*   343

Figure 7. The four domains in the IPI-B2 protein. In domain III, the glycine-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 aa.

Analysis of the IPI-O amino acid sequences. The *ipiO* genes encode a 152 aa protein (Fig. 4) with a predicted molecular weight of 17.2 kDa and an isoelectric point of 10.5. The deduced aa sequences of IPI-O1 and IPI-O2 are 97% homologous. They differ in only 4 aa residues at positions 117, 122, 134 and 143. The predicted IPI-O1 and IPI-O2 aa sequences show no significant similarity with any sequence present in data libraries. The proteins contain a putative amino-terminal signal sequence which suggests that they are secreted (see Fig. 6B for hydropathy plot). According to the rules of Von Heijne (1986), the signal sequence cleavage site was predicted between an alanine (aa 21) and a valine residue (aa 22). A putative cell attachment sequence, recognized 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 and Wolf, 1988) it appears that the RGD tripeptide as well as the NTSD sequence are located on potentially exposed surface peaks (Fig. 6C), indicating that their function as cell attachment sequence or N-glycosylation site, respectively, is not hindered.

The RGD motif was first identified in the animal cell-substratum adhesion proteins fibronectin, fibrinogen, vitronectin and the Von Willebrand coagulation factor (Ruoslathi and

Pierschbacher, 1986), and was found to be crucial for the interaction with their cell surface receptors, the so-called integrins (D'Souza et al., 1988; Hynes, 1987). These specific ligand-receptor interactions mediate adhesion of cells to the extracellular matrix and are essential for normal development of animal cells. Also in lower eukaryotes and bacteria, cell adhesion molecules with a functional RGD tripeptide have been found, e.g. discoidin I which plays a role in aggregation of the slime mold *Dictyostelium discoideum* (Gabius et al., 1985). Moreover, in pathogenicity factors of some bacterial human pathogens, a functional RGD tripeptide was found to interact with integrins on the surface of host cells, thereby mediating uptake into the host cells (Finlay, 1990; Relman et al., 1990; Russell and Wright, 1988). The RGD tripeptide is also included in the attachment site of the foot-and-mouth disease virus (Fox et al., 1989). In plants, RGD-dependent cell wall-cell membrane adhesions have been demonstrated to occur (Schindler et al., 1989; Zhu et al., 1993) and proteins immunologically related to human vitronectin and its receptor have been detected (Quatrano et al., 1991; Sanders et al., 1991; Schindler et al., 1989). Whether the *P. infestans* IPI-O proteins have cell adhesion properties is currently under investigation.

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 only 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 the eight distinct oomycetous genes in which the *tsp* have been determined, transcription initiation occurs in the conserved sequence motif GCTCATTYYNCA(A/T)TTT.

(3) The coding regions of the *ipiB* and the *ipiO* genes are not interrupted by introns, a feature which is observed in most oomycetous genes studied so far.

(4) The members of the *ipiB* gene cluster encode three novel, highly homologous glycine-rich proteins. The IPI-B proteins have a putative signal sequence for transport out of the cytoplasm and a highly repetitive glycine-rich domain, both features which are often found in plant cell wall glycine-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 amino-terminal signal sequence. In addition, they contain a RGD motif which might function as a cell attachment sequence, and a putative N-glycosylation site.

References

Cheung, W.Y. (Ed.) (1980). Calcium and Cell Function, vol. I. Calmodulin. Academic Press, New York.

Choi, D., Ward, B.L. and Bostock, R.M. (1992). Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. *The Plant Cell* 4: 1333-1344.

Condit, C.M. and Keller, B. (1990). The glycine-rich cell wall proteins of higher plants. *In*: Adair, W.S. and Mecham, R.P. (Eds.), Organization and Assembly of Plant and Animal Extracellular Matrix, pp. 119-135. Academic Press, New York.

Condit, C.M., McClean, B.G. and Meagher, R.B. (1990). Characterization of the expression of the petunia glycine-rich protein-1 gene product. *Plant Physiology* 93: 596-602.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* 12: 387-395.

D'Souza, S.E., Ginsberg, M.H., Burke, T.A., Lam, S.C.-T. and Plow, E.F. (1988). Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science* 242: 91-93.

Dudler, R. (1990). The single-copy actin gene of *Phytophthora megasperma* encodes a protein considerably diverged from any other known actin. *Plant Molecular Biology* 14: 415-422.

Feinberg, A.P. and Vogelstein, G. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* 132: 6-13.

Finlay, B.B. (1990). Cell adhesion and invasion mechanisms in microbial pathogens. *Current Opinions in Cell Biology* 2: 815-820.

Fox, G., Parry, N.R., Varnett, P.V., McGinn, B., Rowlands, D.J. and Brown, F. (1989). The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). *Journal of General Virology* 70: 625-637.

Fritzemeier, K.-H., Cretin, C., Kombrink, E., Rohwer, F., Taylor, J., Scheel, D. and Hahlbrock, K. (1987). Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. *Plant Physiology* 85: 34-41.

Gabius, H.-J., Springer, W.R. and Barondes, S.H. (1985). Receptor for the cell binding site of Discoidin I. *Cell* 42: 449-456.

Gurr, S.J., Unkles, S.E. and Kinghorn, J.R. (1987). The structure and organization of nuclear genes of filamentous fungi. *In*: Kinghorn, J.R. (Ed.), Gene Structure in Eukaryotic Microbes, pp. 93-139. IRL Press, Oxford.

Hahlbrock, K., Arabatzis, N., Becker-André, M., Joos, H.-J., Kombrink, E., Schröder, M., Strittmatter, G. and Taylor, J. (1989). Local and systemic gene activation in fungus-infected potato leaves. *In*: Lugtenberg, B.J.J. (Ed.), Signal Molecules in Plants and Plant-Microbe Interactions, NATO ASI Series, volume H36, pp. 241-249. Springer Verlag, Berlin, Heidelberg.

Hynes, R.O. (1987). Integrins, a family of cell surface receptors. *Cell* 48: 549-554.

Jameson, B.A. and Wolf, H. (1988). The antigenic index: a novel algorithm for predicting antigenic determinants. *CABIOS* 4: 181-186.

Judelson, H.S. and Michelmore, R.W. (1989). Structure and expression of a gene encoding heat-shock protein *Hsp70* from the oomycete fungus *Bremia lactucae*. *Gene* 79: 207-217.

Judelson, H.S. and Michelmore, R.W. (1990). Highly abundant and stage-specific mRNAs in the obligate pathogen *Bremia lactucae*. *Molecular Plant-Microbe Interactions* 3: 225-232.

Karlovsky, P. and Prell, H.H. (1991). The *TRP1* gene of *Phytophthora parasitica* encoding indole-3-glycerolphosphatesynthase-N-(5'-phosphoribosyl)anthranilateisomerase: structure and evolutionary distance from homologous fungal genes. *Gene* 109: 161-165.

Keller, B., Sauer, N. and Lamb, C.J. (1988). Glycine-rich cell wall proteins in bean: Gene structure and association of the protein with the vascular system. *The EMBO Journal* 7: 3625-3633.

Keller, B., Schmid, J. and Lamb, C.J. (1989a). Vascular expression of a bean cell wall glycine rich protein-ß-glucuronidase gene fusion in transgenic tobacco. *The EMBO Journal* 8: 1309-1314.

Keller, B., Templeton, M.D. and Lamb, C.J. (1989b). Specific localization of a plant cell wall glycine-rich protein in protoxylem cells of the vascular system. *Proceedings of the National Academy of Sciences USA* 86: 1529-1533.

Kozak, M. (1984). Composition and analysis of sequences upstream from the translational start site in eukayotic mRNA. *Nucleic Acids Research* 12: 857-872.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic charcter of a protein.

Journal of Molecular Biology **157**: 105-132.

LéJohn, H.B. (1989). Structure and expression of fungal calmodulin gene. *Journal of Biological Chemistry* **264**: 19366-19372.

Martini, N., Egen, M., Rüntz, I. and Strittmatter, G. (1993). Promoter sequences of a potato pathogenesis-related gene mediate transcriptional activity selectively upon fungal infection. *Molecular and General Genetics* **236**: 179-186.

Monia, B.P., Ecker, D.J. and Crooke, S.T. (1990). New perspectives on the structure and function of ubiquitin. *Bio/technology* **8**: 209-215.

Moon, R.P., Unkles, S.E., Duncan, J.M., Hawkins, A.R. and Kinghorn, J.R. (1992). Sequence of the *Phytophthora infestans* glyceraldehyde-3-phosphate dehydrogenase-encoding gene (*gpdA*). *Plant Molecular Biology* **18**: 1209-1211.

Pieterse, C.M.J., Risseeuw, E.P., and Davidse, L.C. (1991). An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Molecular Biology* **17**: 799-811.

Pieterse, C.M.J., de Wit, P.J.G.M. and Govers, F. (1992). Molecular aspects of the potato-*Phytophthora infestans* interaction. *Netherlands Journal of Plant Pathology* **98** supplement 2: 85-92.

Pieterse, C.M.J., Riach, M.B.R., Bleker, T., van den Berg-Velthuis, G.C.M. and Govers, F. (1993a). Isolation of putative pathogenicity genes of the potato late blight fungus *Phytophthora infestans* by differential hybridization of a genomic library. *Physiological and Molecular Plant Pathology*, in press.

Pieterse, C.M.J., Verbakel, H.M., Hoek Spaans, J., Davidse, L.C. and Govers, F. (1993b). Increased expression of the calmodulin gene of the late blight fungus *Phytophthora infestans* during pathogenesis on potato. *Molecular Plant-Microbe Interactions* **6**: 164-172.

Quatrano, R.S., Brian, L., Aldridge, J. and Schultz, T. (1991). Polar axis fixation in *Fucus* zygotes: components of the cytoskeleton and extracellular matrix. *Development* Supplement **1**: 11-16.

Relman, D., Tuomanen, E., Falkow, S. Golenbock, D.T., Saukkonen, K. and Wright, S.D. (1990). Recognition of a bacterial adhesin by an integrin: macrophage CR3 ($\alpha_M\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. *Cell* **61**: 1375-1382.

Ruoslathi, E. and Pierschbacher, M.D. (1986). Arg-Gly-Asp: A versatile cell recognition signal. *Cell* **44**: 517-518.

Russell, D.G. and Wright, S.D. (1988). Complement receptor type 3 (CR3) binds to an Arg-Gly-Asp-containing region of the major surface glycoprotein, gp63, of *Leishmania* promastigotes. *Journal of Experimental Medicine* **168**: 279-292.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (Eds.) (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanders, L.C., Wang, C.-S., Walling, L.L. and Lord, E.M. (1991). A homolog of the substrate adhesion molecule vitronectin occurs in four species of flowering plants. *The Plant Cell* **3**: 629-635.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**: 5463-5467.

Schindler, M., Meiners, S. and Cheresh, D.A. (1989). RGD-dependent linkage between plant cell wall and plasma membrane: Consequences for growth. *Journal of Cell Biology* **108**: 1955-1965.

Schröder, M., Hahlbrock, K. and Kombrink, E. (1992). Temporal and spatial patterns of 1,3-ß-glucanase and chitinase induction in potato leaves infected by *Phytophthora infestans*. *The Plant Journal* **2**: 161-172.

Showalter, A.W. (1993). Structure and function of plant cell wall proteins. *The Plant Cell* **5**: 9-23.

Taylor, J.L., Fritzemeier, K.-H., Häuser, I., Kombrink, E., Rohwer, F., Schröder, M., Strittmatter, G. and Hahlbrock, K. (1990). Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. *Molecular Plant-Microbe Interactions* **3**: 72-77.

Unkles, S.E. (1992). Gene organization in industrial filamentous fungi. *In*: Kinghorn, J.R. and Turner, G. (Eds.), Applied Molecular Genetics of Filamentous Fungi, pp. 28-53. Blackie Academic & Professional, Glasgow.

Unkles, S.E., Moon, R.P., Hawkins, A.R., Duncan, J.M. and Kinghorn, J.R. (1991). Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* **100**: 105-112.

Von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic acids Research* **14**: 4683-4690.

Zhu, J.-K., Shi, J. Singh, U., Wyatt, S.E., Bressan, R.A., Hasegawa, P.M. and Carpita, N.C. (1993). Enrichment of vitronectin- and fibronectin-like proteins in NaCl-adapted plant cells and evidence for their involvement in plasma membrane-cell wall adhesion. *The Plant Journal* **3**: 637-646.

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chapter 7

Expression of the putative pathogenicity genes *ipiB* and *ipiO* of *Phytophthora infestans in planta* and *in vitro*

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Submitted

Abstract. The *ipiB* and the *ipiO* genes of the potato late blight fungus *Phytophthora infestans* (Mont.) de Bary encode putative pathogenicity factors. Expression of these genes was studied during pathogenesis on different host tissues and different host plants, some of which have specific resistance against *P. infestans* infection. During pathogenesis on leaves and tubers of the fully susceptible potato cv. Ajax and on leaves of the fully susceptible tomato cv. Moneymaker, the *P. infestans ipiB* and *ipiO* genes show a transient expression pattern with the highest mRNA levels in early stages of infection. During the interaction with leaves of the partially resistant potato cv. Pimpernel, the expression is also transient but accumulation and dispersion of the mRNAs is delayed. Also in *P. infestans* inoculated onto a race-specific resistant potato cultivar and onto the nonhost *Solanum nigrum ipiB* and *ipiO* mRNA is detectable during the initial stages of infection. Apparently, the expression of the *ipiB* and the *ipiO* genes is activated in compatible, incompatible and nonhost interactions. In encysted zoospores, *ipiB* and *ipiO* mRNA accumulation was not detectable but during cyst germination and appressorium formation on an artificial surface, the genes are highly expressed. Expression studies in *in vitro* grown mycelium revealed that during nutrient starvation the expression of the *ipiB* and the *ipiO* genes is induced. For *ipiO* gene expression, carbon deprivation appeared to be sufficient. The *ipiO* gene promoters contain a sequence motif which functions as a glucuse repression element in yeast and this motif might be involved in the regulation of *ipiO* gene expression.

Introduction

The filamentous oomycete *Phytophthora infestans* (Mont.) de Bary is the causal agent of the late blight disease on potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.). *P. infestans* is a hemibiotrophic pathogen with a rather narrow host range, all host plants being *Solanaceae*. On potato, the fungus infects both foliage and tubers and is capable to develop and spread rapidly through host tissue causing a destructive necrosis.

The disease cycle starts when sporangia of *P. infestans* come into contact with a moist leaf surface. The sporangia germinate directly or form motile biflagellate zoospores which germinate after encystment. At the tip of the germ tubes, appressoria are formed and infection tubes emerging from these appressoria penetrate the epidermal cells (Pristou and Gallegly, 1954). In a fully compatible interaction, hyphal structures grow from the epidermis into the mesophyll cell layer, occasionally forming haustorium-like feeding structures (Coffey and Wilson, 1983). Soon after colonization, sporangiophores emerge from the stomata forming new inoculum which can infect neighbouring plants. In a race-specific resistant host, the fungus is arrested in growth at a very early stage of infection. This is due to a hypersensitive response (HR) of the host plant which is characterized by rapid cell death of primary invaded host cells and a limited number of cells surrounding the infection site (Tomiyama, 1963). The black nightshade *Solanum nigrum* L., a common weed in western Europe, is considered to be a nonhost for *P. infestans*. After infection of *S. nigrum*, *P. infestans* stops growing just prior to penetration of the palisade mesophyll cell layer and before haustoria are produced (Colon *et al.*, 1993).

Molecular studies on the potato-*P. infestans* interaction have demonstrated that upon infection, the expression of certain genes in the host plant is activated (Choi *et al.*, 1992;

Fritzemeier *et al.*, 1987; Hahlbrock *et al.*, 1989; Martini *et al.*, 1993; Matton and Brisson, 1989; Schröder *et al.*, 1992; Taylor *et al.*, 1990). Several of these genes encode products which might be involved in the inhibition of pathogen development. Also in the pathogen, interaction with the host plant is accompanied by the activation of specific genes (Pieterse *et al.*, 1991; 1993a; 1993c). Gene products of these so-called *in planta* induced genes may be putative pathogenicity factors necessary for establishment and maintenance of basic pathogenicity or for the increase of disease severity. To date hardly anything is known about the molecular basis underlying pathogenicity of *P. infestans*. Potential pathogenicity factors such as cell wall degrading enzymes, which may facilitate pathogen entry or dispersion through the host, have been studied (Bodenmann *et al.*, 1985; Cole, 1970; Förster, 1988; Jarvis *et al.*, 1981) but their involvement in disease development has never been established convincingly. All the subsequent stages in the development of the disease cannot simply be explained by the action of this type of enzymes. In particular, the specificity of *P. infestans* for solanaceous hosts, implies a mechanism for recognition in which an exchange of signals between host and pathogen might be essential. Successful colonization may require the expression of particular pathogenicity genes. It is feasible that signal molecules originating from the host, or environmental conditions in the host, induce the expression of genes in the pathogen which are necessary for the establishment of a successful interaction. Hence, one approach to gain more insight in the molecular processes involved in pathogenesis, is based on the characterization of *P. infestans* genes which show induced expression during the interaction with the host plant. Once these genes are isolated, their products can be identified and their role in pathogenicity can be studied.

Recently, we described the selection of nine *in planta* induced genes by differential hybridization of a genomic library of *P. infestans* (Pieterse *et al.*, 1993a). Two of these, *ubi3R* and *calA*, encode ubiquitin (Pieterse *et al.*, 1991) and calmodulin (Pieterse *et al.*, 1993c), respectively. They are expressed during growth of the fungus *in vitro* but the expression levels increase and are continuously five-fold higher during pathogenesis on potato (Pieterse *et al.*, 1991; Pieterse *et al.*, 1993c). Two other *in planta* induced genes, *ipiB* and *ipiO*, appeared to encode novel proteins with yet unknown functions. They both belong to small gene clusters. The *ipiB* gene cluster consists of three genes, *ipiB1*, *ipiB2* and *ipiB3*, encoding three highly homologous glycine-rich proteins of 302 (IPI-B1), 343 (IPI-B2) and 347 (IPI-B3) amino acids (aa), respectively (Pieterse *et al.*, 1993b). The IPI-B proteins share up to 47% similarity with several plant glycine-rich proteins which have been implicated to be cell wall proteins. The *ipiO* gene cluster comprises two nearly identical genes, *ipiO1* and *ipiO2*, each encoding a 152 aa protein (IPI-O1 and IPI-O2, respectively). The IPI-O proteins do not show homology with sequences present in data libraries (Pieterse *et al.*, 1993b).

In this paper we describe the expression of the *P. infestans ipiB* and *ipiO* genes during the interaction of the pathogen with susceptible hosts, a race-specific resistant host, a partially resistant host, and a nonhost. To gain more insight in the regulatory mechanism of the activation of *ipiB* and *ipiO* expression during growth of the fungus *in planta*, we attempted to induce expression of the genes *in vitro*. It appeared that growth of the fungus under starvation conditions resulted in the activation of *ipiB*, as well as *ipiO* gene expression.

Materials and Methods

Culturing of **Phytophthora infestans.** Two strains of *P. infestans* were used in this study: isolate 88069 (A1 mating type, race 1.3.4.7) and 88177 (A1 mating type, race 1.3.4.7.10.11). These isolates were grown in the dark at 18°C on rye-agar medium containing 2% (w/v) sucrose (Caten and Jinks, 1968). For *in vitro* expression studies, liquid cultures were initiated from sporangia collected from 2-week-old rye-agar cultures in either Henniger synthetic medium (Henniger, 1959) or rich rye-sucrose medium (1×10^5 sporangia/ml; 10 ml per 94 mm Petri dish). After incubation at 18°C for 3 days, mycelia were washed twice with large volumes of media defined in the text, or with milliQ or tap water (milliQ water has been filtered through a Millipore MilliQ purification system). Subsequently mycelia were transferred to 10 ml of milliQ or tap water, or to 10 ml of the defined media and incubated at 18°C for the time periods indicated in text and legends. Mycelia were harvested, frozen in liquid nitrogen and stored at -80°C until RNA extractions were performed.

Production of zoospores, cysts, germinating cysts and appressoria. Zoospores were obtained by adding 10 ml of water to 2-week-old rye-agar cultures (94 mm Petri dishes) followed by incubation at 10°C. After three hours of incubation, typically 10^6 zoospores per ml were released in the water. Cyst formation was initiated by vortexing the zoospore suspension for two minutes. Germinating cysts were obtained by incubating the cysts in water for two hours at 18°C. Appressoria formation was induced *in vitro* by allowing encysted zoospores to germinate on Plastibrand polypropylene bags (Brand, cat. no. 759 05, Germany) at 18°C and 100% relative humidity for two hours (E. Schmelzer, personal communication). Encysted zoospores, germinating cysts and germinated cysts with appressoria were harvested, collected by centrifugation and frozen in liquid nitrogen.

Inoculation of plants. For *in planta* expression studies, the following plants were used: potato cultivar (cv.) Ajax and tomato cv. Moneymaker which are both susceptible to the *P. infestans* strains used; potato cv. Pimpernel which is also susceptible but has a high level of field resistance to *P. infestans*; potato line Black 2424 a(5) (further referred to as potato line R8) which contains the *R8* gene conferring race-specific resistance to *P. infestans* isolates containing the corresponding avirulence gene (e.g. isolate 88069); the black nightshade *Solanum nigrum* which is a nonhost to *P. infestans*. Detached leaves were inoculated by spraying the axial side with a suspension of sporangia collected from 2-week-old rye-agar cultures. A spore suspension of 5×10^5 sporangia/ml was used for inoculation of potato cvs. Ajax and Pimpernel and tomato cv. Moneymaker. For inoculation of potato line R8 and *S. nigrum*, a spore suspension of 1×10^7 sporangia/ml was used. Inoculated leaves, inserted in florist foam oasis, were incubated at 18°C and 100% relative humidity under cool fluorescent light for 16 hours per day. Tubers of potato cv. Ajax were inoculated by spraying one cm thick tuber slices with the sporangia suspension. Tuber slices were placed in Petri dishes and

incubated under the same conditions as inoculated leaves. As controls, leaves and tuber slices were sprayed with water and treated similarly to inoculated leaves and tubers.

RNA isolation and northern blot analysis. RNA was isolated from *P. infestans* encysted zoospores, germinating cysts, mycelium, non-infected plant tissue and infected plant tissue (interaction RNA) using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). For northern blot analyses, 15 μg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond-N$^+$ (Amersham) by capillary transfer as described by Sambrook *et al.* (1989). Northern blots were hybridized with DNA probes at 65°C for 20 hours in hybridization mix containing 0.5 M sodium phosphate buffer (NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.2), 7% SDS and 1 mM EDTA. Subsequently, blots were washed in 0.5×SSC, 0.1% SDS at 65°C and exposed to Kodak X-OMAT AR film.

DNA probes. The *ipiB* probe was derived from a 0.98 kb *SstI-PstI* fragment from the genomic phage clone DHC-B containing the *ipiB2* coding sequence (Pieterse *et al.*, 1993b). The 0.63 kb *SstI-XbaI* fragment from the genomic phage clone DHC-O containing the *ipiO1* coding sequence (Pieterse *et al.*, 1993b) was used as template to synthesize the *ipiO* probe. The 2.8 kb *PstI* insert from pSTA31 containing the complete *P. infestans actA* gene (Unkles *et al.*, 1991) was used for preparation of the actin probe and the 0.35 kb *EcoRI-XhoI* insert from pPi119. A partial cDNA clone of the *P. infestans* translation elongation factor 1α (EF-1α) mRNA (C. Pieterse, unpublished), was used to synthesize the EF-1α probe. Probes were labeled with [α-^{32}P]dATP by random primer labelling (Feinberg and Vogelstein, 1983). On Southern blots, all probes have been shown to be specific for *P. infestans*. Under the hybridization conditions used, there is no cross-hybridization with potato DNA sequences (Pieterse *et al.*, 1993b; Pieterse *et al.*, 1993c). On northern blots, however, the *ipiB* probe cross-hybridizes in some cases to RNA isolated from uninfected potato and *S. nigrum* leaves. In the lanes containing interaction RNA, these RNAs can be distinguished from *P. infestans ipiB* mRNA since they differ in size.

Results

Expression of the ipiB *and* ipiO *genes in compatible interactions.* The differential screening procedure, which resulted in the isolation of the *ipiB* and *ipiO* genes, was aimed at the selection of *in planta* induced genes of *P. infestans*. To confirm that the expression of the *ipiB* and *ipiO* genes was specifically induced or significantly increased during growth of the pathogen in the plant, and to examine their expression pattern during pathogenesis, northern blot analyses were performed. Initially, the expression was analyzed in the interaction from which the cDNA probes for the differential screening were derived, i.e. the interaction between *P. infestans* isolate 88069 and the fully susceptible potato cv. Ajax (Ajax-88069 interaction). On leaves of potato cv. Ajax, first symptoms are visible 24 hours after
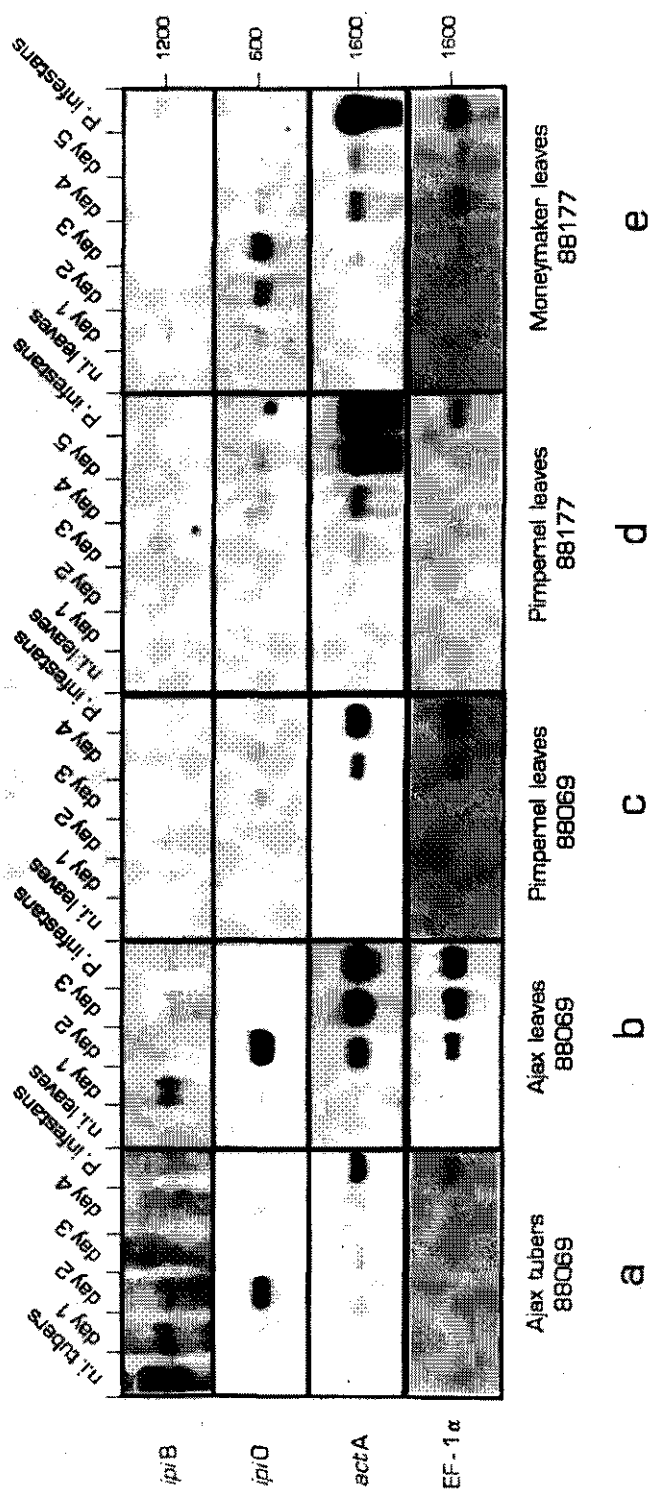
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Figure 1. Autoradiographs of northern blots containing total RNA isolated from non-inoculated tuber slices or leaves of the fully susceptible potato cv. Ajax, the partially resistant potato cv. Pimpernel, and the susceptible tomato cv. Moneymaker. Pimpernel (n.i. tubers and n.i. leaves, respectively), from colonized tuber slices or leaves of these host plants at different time points post-inoculation with *Phytophthora infestans* isolate 88069 or 88177 (day 1-5), and from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium (P. infestans). Northern blots were hybridized with random primer labeled probes derived from *P. infestans* genes *ipiB2*, *ipiO1*, *actA*, and EF-1α. Transcript lengths are indicated as number of nucleotides.

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inoculation. They develop from small lesions into completely "water soaked" areas three days post-inoculation. In this period the fungus grows and sporulates at the advancing edges of lesions. The centres of the lesions become necrotic and start to decay due to secondary infections by saprophytic microorganisms. On 1 cm thick tuber slices, infection results in complete colonization of the tissue over a period of three days. After three days, mycelium appears at the non-inoculated side of the tuber slice.

Total RNA isolated from non-infected tissue, from infected tissue harvested at several time points post-inoculation and from *P. infestans* grown *in vitro* for three days on Henniger synthetic medium was electrophoresed and transferred to membranes. Each lane on the northern blots contains equal amounts of total RNA. RNA extracted from infected tissue (interaction RNA) consists of a mixture of fungal and plant RNA of which the ratio changes during colonization. Signals obtained with probes of differentially regulated genes should therefore be normalized to actual fungal RNA levels. Actin and translation elongation factor 1α (EF-1α) mRNA levels have been shown to be good internal standards for the quantification of fungal RNA in the interaction RNA mixture (Mahe *et al.*, 1992; Pieterse *et al.*, 1993a; Pieterse *et al.*, 1993c). Therefore, the increase of fungal biomass was visualized with probes derived from the constitutively expressed *P. infestans* genes *actA* encoding actin (Unkles *et al.*, 1991) and EF-1α encoding translation elongation factor 1α (C.M.J. Pieterse, unpublished). In the Ajax leaves-88069 interaction, actin and EF-1α transcripts are first detectable two days post-inoculation and the amounts increase in time due to the expanding fungal biomass (Fig. 1b). In Ajax tubers-88069 interaction, a similar actin and EF-1α mRNA accumulation is observed (Fig. 1a). However, 4 days after inoculation the actin and EF-1α mRNA levels decrease. This is most likely due to the fact that in these stages colonization of the tuber tissue is completed. The fungal biomass is not expanding any longer and due to possible death of the fungus in old colonized tissue, the relative amount of actin and EF-1α mRNA declines.

Accumulation of *ipiB* and *ipiO* transcripts was examined by northern blot hybridization using probes derived from *ipiB2* and *ipiO1*. The length of the mRNA hybridizing to the *ipiB* probe is approximately 1200 nt whereas the *ipiO* probe hybridizes to a mRNA of approximately 600 nt. This is in agreement with the lengths of the open reading frames found in the *ipiB* and *ipiO* genes (Pieterse *et al.*, 1993b). By making use of gene-specific oligonucleotides for the *ipiO1* and the *ipiO2* gene, it was determined that during pathogenesis both *ipiO* genes are expressed (data not shown). Therefore, the hybridization signal obtained with the *ipiO* probe represents both *ipiO1* and *ipiO2* mRNA. With regard to the *ipiB* hybridization, it is unknown whether expression of *ipiB1*, *ipiB2*, and/or *ipiB3* is responsible for the hybridization signal. Since the *ipiB* genes belong to a multigene family it is also possible that mRNAs derived from other *ipiB*-like genes contribute to the observed *ipiB* mRNA accumulation.

In the 88069 infected Ajax leaves and Ajax tubers, relatively high levels of *ipiB* mRNA are observed at day one post-inoculation (Fig. 1a and 1b). One to two days later, the *ipiB* mRNA levels are decreased dramatically to a very low or undetectable level. In lanes

containing RNA isolated from non-inoculated tubers an *ipiB* cross-hybridizing band of approximately 1100 nt is visible. Since the *P. infestans ipiB* genes encode glycine-rich proteins (Pieterse *et al.*, 1993b), it is likely that this band represents a mRNA encoding a potato glycine-rich protein. In infected leaves as well as in tubers, *ipiO* transcripts are observed in relatively high amounts during the first two days post-inoculation. In advanced stages of colonization of both tissues, three days post-inoculation, *ipiO* mRNA accumulation declines rapidly. In the fungus grown *in vitro* on Henniger synthetic medium, *ipiB* as well as *ipiO* transcripts are hardly detectable. These data demonstrate that during pathogenesis on both leaves and tubers of a susceptible host the *ipiB* as well as the *ipiO* genes are transiently expressed with the highest expression levels during the early stages of infection.

To determine whether the transient expression patterns of the *ipiB* and *ipiO* genes observed in *P. infestans* isolate 88069 during the interaction with the fully susceptible potato cv. Ajax are similar in compatible interactions with other *P. infestans* isolates and other host plants or cultivars, *ipiB* and *ipiO* expression was analyzed in leaves of the partially resistant potato cv. Pimpernel inoculated with *P. infestans* isolate 88069 (Pimpernel-88069 interaction) and 88177 (Pimpernel-88177 interaction), and in leaves of the susceptible tomato cv. Moneymaker infected with isolate 88177 (Moneymaker-88177 interaction). Pimpernel has a high level of race-nonspecific resistance or field resistance to *P. infestans* which results in lower infection efficiencies, slower tissue colonization and reduced sporulation (Umaerus, 1970). On leaves of potato cv. Pimpernel and tomato cv. Moneymaker, symptom development was similar to that observed on leaves of potato cv. Ajax but was delayed for approximately one to two days. During the Pimpernel-*P. infestans* interactions, the actin and EF-1α transcripts are first detectable three to four days post-inoculation (Fig. 1c and 1d). This supports the observation that colonization of leaves of the partially resistant potato cv. Pimpernel is significantly slower compared to the interaction with the fully susceptible potato cv. Ajax. In the Moneymaker-88177 interaction, actin and EF-1α mRNA levels initially accumulate followed by a decline at the end of the time course (Fig. 1e). This indicates that, in similarity to what happens in the Ajax tubers-88069 interaction, the relative fungal biomass decreases at the end of the infection process.

Although the overall levels of *ipiB* and *ipiO* mRNA are lower in the Pimpernel-88069, the Pimpernel-88177 and the Moneymaker-88177 interactions, the transient expression patterns of the *ipiB* and *ipiO* genes comparable to those found in the Ajax-88069 interactions. However, in comparison to the fully susceptible interactions Ajax-88069 and Moneymaker-88177 (Fig. 1a, 1b and 1e), *ipiB* and *ipiO* mRNA accumulation is slower in the partially resistant Pimpernel leaves (Fig. 1c and 1d). Also the decrease in expression of *ipiB* and *ipiO* starts later. Moreover, the decrease in *ipiO* mRNA is much less dramatic than in the fully susceptible cvs. Ajax and Moneymaker where *ipiO* mRNA disappears almost completely.

Expression of the* ipiB *and* ipiO *genes in an incompatible and a nonhost interaction. In susceptible and resistant potato cvs., the frequency of penetration of epidermal cells by infection hyphae of *P. infestans* is generally the same (Gees and Hohl, 1988). However, in
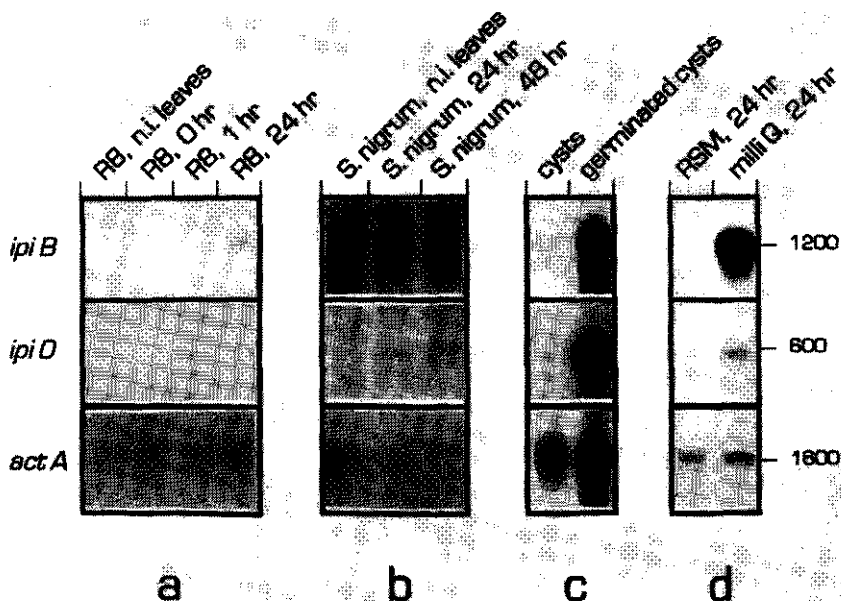
Figure 2. Autoradiographs of northern blots containing total RNA isolated from (a) non-inoculated leaves of potato line R8 (R8, n.i. leaves), inoculated R8 leaves immediately after inoculation with *Phytophthora infestans* isolate 88069 (R8, 0 hr), one hour post-inoculation (R8, 1 hr) and 24 hours post-inoculation (R8, 24 hr), (b) non-inoculated leaves of *Solanum nigrum* (S. nigrum, n.i. leaves) and *S. nigrum* leaves inoculated with *P. infestans* isolate 88069, 24 hours (S. nigrum, 24 hr) and 48 hours post-inoculation (S. nigrum, 48 hr), (c) encysted zoospores of *P. infestans* isolate 88069 (cysts) and cysts which were allowed to germinate for two hours in water (germinated cysts), and (d) 4-day-old *P. infestans* mycelium (isolate 88069) grown for three days on rye-sucrose medium followed by a 24 hour incubation on fresh rye-sucrose medium (RSM, 24 hr) or milliQ water (milliQ, 24 hr). Northern blots were hybridized with probes derived from *P. infestans* genes *ipiB2, ipiO1* and *actA*. Transcript lengths are indicated as number of nucleotides.

incompatible interactions with resistant cvs. carrying race-specific R-genes, fungal growth is confined to the infection site due to the HR of invaded host cells. In the nonhost *S. nigrum*, infection hyphae continue to grow from the epidermal cells into the spongy mesophyll. Growth of the fungus is arrested in this cell layer before haustoria are produced (Colon *et al.*, 1993).

To study the expression of the *ipiB* and *ipiO* genes during an incompatible interaction and a nonhost interaction, leaves of potato line R8 and *S. nigrum* were inoculated with *P. infestans* isolate 88069. Infected R8 leaves were harvested 0, 1, and 24 hours after inoculation. At 24 hours post-inoculation, the invaded host cells were dead and further colonization was prohibited. Infected *S. nigrum* leaves were harvested 24 and 48 hours post-inoculation. Small necrotic lesions were visible 48 hours post-inoculation. Disease symptoms did not develop further indicating that fungal growth was arrested in an early stage.

Northern blots containing RNA isolated from non-inoculated and inoculated leaves were hybridized with the *ipiB*, *ipiO*, actin and EF-1α probe, respectively. Although leaves of potato line R8 and *S. nigrum* were heavily inoculated, litte or no mRNA of the constitutively

expressed actin and EF-1α genes can be detected in lanes containing RNA isolated from inoculated leaves indicating that the proportion of fungal RNA in the interaction RNA mixtures is very low (Fig. 2a and 2b; EF-1α hybridization is not shown but is similar to the actin hybridization). Nevertheless, in infected R8 leaves *ipiB* and *ipiO* mRNA is detectable 24 hours post-inoculation. In lanes containing RNA isolated from R8 leaves immediately after inoculation (Fig. 2a; R8, 0 hr), no hybridization signals can be detected. This indicates that expression of the *ipiB* and *ipiO* genes is activated within 24 hour after inoculation. At 24 and 48 hours after inoculation of the nonhost *S. nigrum* (*S. nigrum*-88069 interaction), accumulation of *ipiB* and *ipiO* mRNA is observed. In addition to the 1200 nt *ipiB* mRNA, extra bands are visible which cross-hybridize with the *ipiB* probe. These extra bands are also visible in lanes containing RNA isolated from non-inoculated *S. nigrum* leaves. It must therefore be concluded that they represent *S. nigrum* mRNAs which are most likely derived from homologous genes encoding glycine-rich proteins. These results demonstrate that also in initial stages of incompatible and nonhost interactions, the *ipiB* and *ipiO* genes are expressed.

Expression of the ipiB *and* ipiO *genes prior to host cell penetration.* In early stages of compatible, incompatible and nonhost interactions *ipiB* and *ipiO* mRNA accumulation is be observed. Immediately after inoculation of the leaves no *ipiB* and *ipiO* mRNA is detectable (Fig 2a; R8, 0 hr). However, this does not exclude the possibility that the genes are expressed but that the relative proportion of fungal RNA in the interaction RNA mixture is too low to detect *ipiB* and *ipiO* mRNA. To assess whether the *ipiB* and *ipiO* genes are transcriptionally activated in stages prior to host cell penetration, their expression was studied in encysted zoospores and in germinating cysts by northern blot analyses. Hybridization with the actin probe (Fig. 2c) and the EF-1α probe (not shown) showed that the RNA amounts in the two lanes are not equal. Hybridization with the *ipiB* and *ipiO* probes revealed that, despite unequal loading, *ipiB* and *ipiO* mRNAs accumulate to high levels in germinating cysts whereas in encysted zoospores, *ipiB* and *ipiO* mRNA is hardly detectable (Fig. 2c). Expression of the *ipiB* and *ipiO* genes was further analyzed in germinated cysts with appressoria formed at the tip of the germ tubes. Appressorium formation can be induced *in vitro* by spreading zoospores on an artificial surface of polypropylene (E. Schmelzer, personal communication). Under the conditions used, approximately 50% of the germ tubes formed an appressorium. Due to the low amount of material which can be obtained with this method, RNA yields are relatively low. Hybridization of a northern blot containing the total amount of RNA isolated from appressoria revealed the presence of *ipiB* and *ipiO* mRNA in this RNA sample (data not shown) whereas actin and EF-1α mRNA could not be detected. It must be concluded that the *ipiB* and *ipiO* genes are expressed prior to host penetration as soon as the encysted zoospores germinate. The expression continues when the fungus forms appressoria and starts to penetrate the host. Apparently, the very first activation of *ipiB* and *ipiO* gene expression does not require contact with host tissue. Unfortunately, our data do not allow us to draw conclusions on changes in expression levels during appressorium formation and host penetration. Hence, it is not clear whether contact with the host enhances the expression.
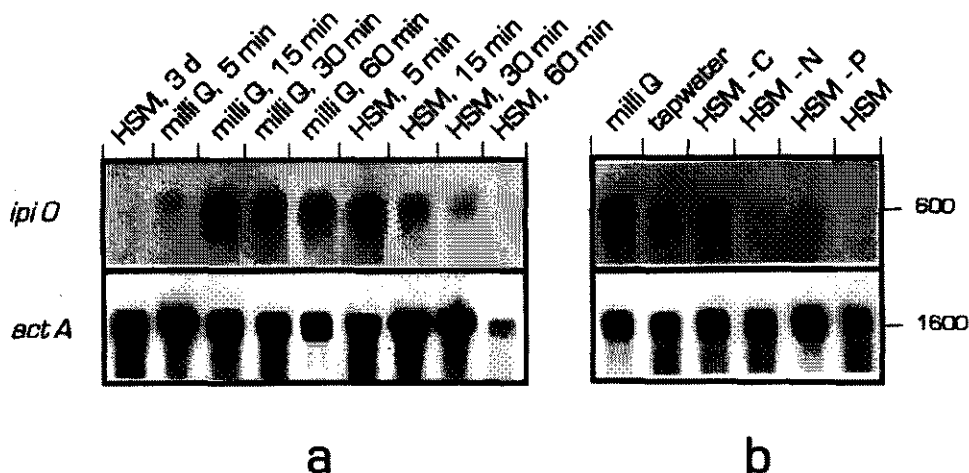
Figure 3. Autoradiographs of northern blots containing total RNA isolated from (a) 3-day-old *P. infestans* mycelium (isolate 88069) grown on Henniger synthetic medium (HSM, 3d), 3-day-old mycelium grown on HSM followed by a 5, 15, 30 and 60 minutes incubation on milliQ water (milliQ, 5, 15, 30, and 60 min), and 3-day-old mycelium grown on HSM followed by a 60 minutes incubation on milliQ and a subsequent incubation of 5, 15, 30 and 60 minutes on fresh HSM (HSM, 5, 15, 30, and 60 min) and (b) 3-day-old *P. infestans* mycelium (isolate 88069) grown on Henniger synthetic medium followed by a one hour incubation on milliQ water (milliQ), tap water (tap water), fresh Henniger synthetic medium (HSM), and HSM deprived of carbon sources (HSM -C), nitrogen sources (HSM -N) or phosphate sources (HSM -P). Northern blots were hybridized with probes derived from *P. infestans* genes *ipiO2* and *actA*. Transcript lengths are indicated as number of nucleotides.

Induction of ipiB *and* ipiO *gene expression by nutrient deprivation.* Germinated cysts, in which the *ipiB* and *ipiO* genes are expressed at high levels, were obtained by incubating encysted zoospores in water for three hours. Under these conditions the fungus is in fact exposed to starvation stress. To get more insight in the influence of growth conditions on transcriptional regulation of the *ipiB* and *ipiO* genes, we attempted to induce *ipiB* and *ipiO* gene expression by nutrient deprivation of *in vitro* grown mycelium. Rich rye-sucrose medium (RSM) was inoculated with spores. After three days the mycelia were transferred to either milliQ water or fresh RSM and were allowed to grow for another day. Northern blots containing RNA isolated from these mycelia were hybridized with the *ipiB*, *ipiO* and actin probes. In RNA isolated from mycelium which was grown for an additional 24 hours on fresh RSM, *ipiB* and *ipiO* transcripts are hardly detectable (Fig. 2d). However, 24 hours after transfer of the mycelium to milliQ water, both *ipiB* and *ipiO* transcripts accumulated to high levels indicating that *ipiB* and *ipiO* gene expression is induced during nutrient deprivation.

Transcriptional activation of the *ipiO* genes by nutrient deprivation was studied in more

detail. The accumulation of *ipiO* mRNA was assessed in mycelium which was grown in defined Henniger synthetic medium (HSM) for three days and subsequently transferred to milliQ water. Mycelium was harvested 5, 15, 30 and 60 minutes after transfer to milliQ water. After 60 minutes on milliQ water, the mycelium was transferred back to fresh HSM and harvested after 5, 15, 30 and 60 minutes. As soon as five minutes after transfer to milliQ water, *ipiO* mRNA can be detected and it reaches a maximum level within 15 minutes (Fig. 3a). When the mycelium is subsequently transferred from miliQ water to HSM, the accumulated *ipiO* mRNA disappears completely within 60 minutes.

To investigate whether the induction of *ipiO* gene expression *in vitro* is caused by general starvation conditions or whether limitation of specific components in the medium is the inducing factor, the expression of *ipiO* was examined in 3-day-old mycelia grown on HSM which were transferred to milliQ water, tap water or HSM deprived of nitrogen, carbon or phosphate sources. After one hour of incubation on milliQ water or tap water, high levels of *ipiO* mRNA can be detected (Fig. 3b). Similar amounts of *ipiO* mRNA can be detected in mycelium which was transferred to HSM deprived of carbon sources whereas in mycelia which were transferred to HSM without nitrogen or phosphate sources, accumulation of *ipiO* mRNA does not occur. These data suggest that expression of the *ipiO* genes is induced *in vitro* under conditions of carbon deprivation although it can not be excluded that a change in osmolarity causes the induction of *ipiO* gene expression.

The specific starvation conditions for induction of *ipiB* gene expression are currently investigated. Preliminary results indicate that neither carbon, nor nitrogen or phosphate deprivation activates *ipiB* gene expression *in vitro*.

Discussion

Expression of the *ipiB* and *ipiO* genes of *P. infestans* was studied during pathogenesis on different host tissues and different host plants with various types of resistance against *P. infestans*. During pathogenesis on leaves and tubers of the fully susceptible potato cv. Ajax and on leaves of the fully susceptible tomato cv. Moneymaker, the *ipiB* and *ipiO* genes show a transient expression pattern in the two *P. infestans* isolates tested. The highest mRNA levels are observed in early stages of infection suggesting a role for the IPI-B and IPI-O proteins in the onset of the interaction. On leaves of the partially resistant potato cv. Pimpernel, the expression patterns of *ipiB* and *ipiO* genes are also transient but the accumulation and disappearance of the mRNAs is strongly delayed. Hybridization of the northern blots with the actin and EF-1α probes shows that the increase in fungal biomass within leaf tissue of the partially resistant potato cv. Pimpernel is lower than in tissue of fully susceptible plants. This indicates that *ipiB* and *ipiO* gene expression is correlated with the rate of tissue colonization. This can also be observed when comparing the *ipiB* and *ipiO* mRNA accumulation patterns in infected Ajax and Moneymaker leaves. On the latter host, disease development is approximately one day slower than on potato cv. Ajax. The decline in *ipiB* and *ipiO* mRNA levels starts also one day later, indicating that expression of the *ipiB*

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and *ipiO* genes is activated as long as new uninfected tissue is available. As soon as host tissue is completely colonized, the expression of the *ipiB* and *ipiO* genes ceases.

In encysted zoospores, *ipiB* and *ipiO* mRNA accumulation can not be detected. However, during cyst germination and the formation of appressoria, both genes are highly expressed which demonstrates that the expression of the *ipiB* and *ipiO* genes is already induced in stages prior to host penetration and that the very first induction of *ipiB* and *ipiO* gene expression is probably not directly dependent on host factors. Also in the initial stages of the incompatible interactions between the race-specific resistant potato line R8 and the nonhost *S. nigrum*, the *ipiB* and *ipiO* genes are transcriptionally active. Apparently, the regulatory conditions for *ipiB* gene expression, as well as those for *ipiO* gene expression, are similar during pathogenesis on hosts carrying distinct resistance properties. The outcome of the resistance reaction determines the speed of colonization and thus the duration of conditions which support *ipiB* and *ipiO* gene expression. During pathogenesis, the timing of *ipiB* gene activation and repression differs from that of the *ipiO* genes. In addition, *ipiO* gene expression is specifically induced by carbon deprivation whereas this seems not to be the case for the *ipiB* genes. These observations indicate that the regulatory mechanisms involved in *ipiB* and *ipiO* gene expression are different.

Germination of encysted zoospores and sporangia of *P. infestans* occurs naturally on leaf surfaces in a moist environment where they are deprived of nutrients. In initial stages of the interaction, the fungus uses its own food reserves. Once these reserves are exhausted, the host provides all the nutrients essential for growth and development of the fungus (Hohl, 1991). To obtain these nutrients, *P. infestans* has to colonize the host tissue. Hence, the pathogen must activate the machinery required for growth and development in the host environment. Starvation stress might therefore be a potential trigger for the expression of pathogenicity genes. From the *in vitro* experiments it is evident that starvation stress is the inducing environmental condition for transcriptional activation of the *ipiB* and *ipiO* genes. Several other *in planta* induced genes of plant pathogenic microorganisms show induced expression upon starvation stress, e.g. the avirulence gene *avr9* of the tomato leaf mold fungus *Cladosporium fulvum*, which is induced by nitrogen deprivation (van den Ackerveken *et al.*, 1993) and the *MGR1* gene of the rice blast fungus *Magnaporthe grisea* which is transcriptionally activated during nitrogen and glucose starvation (N. Talbot, personal communication). It is tempting to speculate that starvation conditions or changes in nutrient conditions encountered upon infection are general stimuli for the induction of pathogenicity genes. Subsequent production of pathogenicity factors can facilitate development of the fungus resulting in the establishment of a compatible interaction in which the fungus obtains nutrients from the host plant. When fungal spores germinate on a resistant host or a nonhost, the initial stimuli for the activation of pathogenicity genes will be the same as on a susceptible host. However, the resistance response of the resistant host prevents further growth of the fungus. The fact that in *S. nigrum, ipiB* and *ipiO* mRNA is detectable at 48 hours post-inoculation suggests that the fungus is still deprived of nutrients at that stage. Apparently, this nonhost interaction fails to develop into a biotrophic stage.

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gene	DNA region surrounding glucose repression element
SUC2, element A	-482 AGTAATA AAAATGCGCGG AAT -502 ***********
SUC2, element B	-451 TTAGGAA ATTATCCGGGG GCG -431 ***********
GAL1	-200 TTAGCCT TATTTCTGGGG TAA -180 ***********
GAL4	-96 GAAGCTG AAAATCTGGGG AAG -76 ***********
ipiO1	-441 TTTCCCG TAAATCTGGGG GCA -421 ***********
ipiO2	-440 TTTCCCG TAAATCTGGGG CAT -420 ***********

Figure 4. Alignment of glucose repression elements in the *GAL1*, *GAL4*, and *SUC2* gene of *Saccharomyces cerevisiae* (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991) with a homologous sequence in the promoter regions of the *ipiO1* and *ipiO2* genes of *Phytophthora infestans* (Pieterse *et al.*, 1993b). Nucleotides matching the consensus sequence $\frac{AAAA}{TTTT}T\frac{CC}{GT}GGGG$ as proposed by Nehlin *et al.* (1991) are indicated by an asterisk. Numbers indicate positions relative to the ATG start codon.

Carbon deprivation appears to be a specific stimulus for transcriptional activation of the *ipiO* genes. Interestingly, the *ipiO1* and *ipiO2* gene have a sequence motif in their 5' flanking regions (Pieterse *et al.*, 1993b), which is highly homologous to a glucose repression element present in the promoters of the glucose repressed genes *GAL1*, *GAL4*, and *SUC2* (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991) of *Saccharomyces cerevisiae* (Fig. 4). A C_2H_2 zinc-finger DNA binding protein encoded by the *MIG1* gene of *S. cerevisiae* has been shown to be involved in repression of gene expression by binding to the glucose repression element in the promoter regions of these three genes under glucose rich conditions. Whether this sequence motif in the 5' flanking regions of the *ipiO* genes indeed functions as a glucose repression element in *P. infestans* needs to be investigated.

References

Bodenmann, J., Heiniger, U. and Hohl, H.R. (1985). Extracellular enzymes of *Phytophthora infestans*: endo-cellullase, ß-glucosidases, and 1,3-ß-glucanases. *Canadian Journal of Microbiology* 31: 75-82.

Caten, C.E. and Jinks, J.L. (1968). Spontaneous variability of single isolates of *Phytophthora infestans*. I.

Cultural variation. *Canadian Journal of Botany* **46**: 329-347.

Choi, D., Ward, B.L. and Bostock, R.M. (1992). Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. *The Plant Cell* **4**: 1333-1344.

Coffey, M.D. and Wilson, U.E. (1983). Histology and cytology of infection and disease caused by *Phytophthora*. *In*: Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.), *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology, pp. 289-301. American Phytopathological Society, St. Paul, Minnesota.

Cole, A.L.J. (1970). Pectic enzyme activity from *Phytophthora infestans*. *Phytochemistry* **9**: 337-340.

Colon, L.T., Eijlander, R., Budding, D.J., van Ijzendoorn, M.T., Pieters, M.J.J. and Hoogendoorn, J. (1993). Resistance to potato late blight (*Phytophthora infestans* (Mont.) de Bary) in *Solanum nigrum*, *S. villosum* and their sexual hybrids with *S. tuberosum* and *S. demissum*. *Euphytica* **66**: 55-64.

Feinberg, A.P. and Vogelstein, G. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**: 6-13.

Förster, H. (1988). Pectinesterases from *Phytophthora infestans*. *Methods in Enzymology* **16**: 355-361.

Fritzemeier, K-H., Cretin, C., Kombrink, E., Rohwer, F., Taylor, J., Scheel, D. and Hahlbrock, K. (1987). Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. *Plant Physiology* **85**: 34-41.

Gees, R. and Hohl, H.R. (1988). Cytological comparison of specific (R3) and general resistance to late blight in potato leaf tissue. *Phytopathology* **78**: 350-357.

Hahlbrock, K., Arabatzis, N., Becker-André, M., Joos, H.-J., Kombrink, E., Schröder, M., Strittmatter, G. and Taylor, J. (1989). Local and systemic gene activation in fungus-infected potato leaves. *In*: Lugtenberg, B.J.J. (Ed.), Signal Molecules in Plants and Plant-Microbe Interactions, NATO ASI Series, volume H36, pp. 241-249. Springer Verlag, Berlin, Heidelberg.

Henniger, H. (1959). Versuche zur kultur verschiedener rassen von *Phytophthora infestans* (Mont) de By. auf künstlichen Nährböden. *Phytopathologische Zeitschrift* **34**: 285-306.

Hohl, H.R. (1991). Nutrition. *In*: Advances in Plant Pathology, volume 7; *Phytophthora infestans*, the Cause of Late Blight of Potato, pp. 53-84. Academic Press, London, San Diego.

Jarvis, M.C., Threlfall, D.R. and Friend, J. (1981). Potato cell wall polysaccharides: degradation with enzymes from *Phytophthora infestans*. *Journal of Experimental Botany* **32**: 1309-1319.

Kombrink, E., Schröder, M. and Hahlbrock, K. (1988). Several "pathogenesis-related" proteins in potato are 1,3-ß-glucanases and chitinases. *Proceedings of the National Academy of Sciences USA* **85**: 782-786.

Logemann, J., Schell, J. and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* **163**: 16-20.

Mahe, A., Grisvard, J. and Dron, M. (1992). Fungal- and plant-specific gene markers to follow the bean anthracnose infection process and normalize a bean chitinase mRNA induction. *Molecular Plant-Microbe Interactions* **5**: 242-248.

Martini, N., Egen, M., Rüntz, I. and Strittmatter, G. (1993). Promoter sequences of a potato pathogenesis-related gene mediate transcriptional activity selectively upon fungal infection. *Molecular and General Genetics* **236**: 179-186.

Matton, D.P. and Brisson, N. (1989). Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Molecular Plant-Microbe Interactions* **2**: 325-331.

Nehlin, J.O., Carlberg, M. and Ronne, H. (1991). Control of yeast *GAL* genes by MIG1 repressor: a transcriptional cascade in the glucose response. *The EMBO Journal* **10**: 3373-3377.

Nehlin, J.O. and Ronne, H. (1990). Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *The EMBO Journal* **9**: 2891-2898.

Pieterse, C.M.J., Risseeuw, E.P. and Davidse, L.C. (1991). An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Molecular Biology* **17**: 799-811.

Pieterse, C.M.J., Riach, M.R., Bleker, T., van den Berg-Velthuis, G.C.M. and Govers, F. (1993a). Isolation of putative pathogenicity genes of the potato late blight fungus *Phytophthora infestans* by differential screening of a genomic library. *Physiological and Molecular Plant Pathology*, in press.

Pieterse, C.M.J., van West, P., Verbakel, H.M., Brassé, P.W.H.M., van den Berg-Velthuis, G.C.M. and Govers, F. (1993b). Structure and genomic organization of the *ipiB* and *ipiO* gene clusters of *Phytophthora infestans*. *Gene*, in press.

Pieterse, C.M.J., Verbakel, H.M., Hoek Spaans, J., Davidse, L.C. and Govers, F. (1993c). Increased expression of the calmodulin gene of the late blight fungus *Phytophthora infestans* during pathogenesis on potato. *Molecular Plant-Microbe Interactions* **6**: 164-172.

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Pristou, R. and Gallegly, M.E. (1954). Leaf penetration by *Phytophthora infestans*. *Phytopathology* **44**: 81-86.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd edition. New York: Cold Spring Harbor Laboratory Press.

Schröder, M., Hahlbrock, K. and Kombrink, E. (1992). Temporal and spatial patterns of 1,3-ß-glucanase and chitinase induction in potato leaves infected by *Phytophthora infestans*. *The Plant Journal* **2**: 161-172.

Taylor, J.L., Fritzemeier, K.-H., Häuser, I., Kombrink, E., Rohwer, F., Schröder, M., Strittmatter, G. and Hahlbrock, K. (1990). Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. *Molecular Plant-Microbe Interactions* **3**: 72-77.

Tomiyama, K. (1963). Physiology and biochemistry of disease resistance of plants. *Annual Review of Phytopathology* **1**: 295-324.

Unkles, S.E., Moon, R.P., Hawkins, A.R., Duncan, J.M. and Kinghorn, J.R. (1991). Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* **100**: 105-112.

van den Ackerveken, G.F.J.M., Dunn, R.M., Cozijnsen, T.J., Vossen, P., van den Broek, H.W.J. and de Wit, P.J.G.M. (1993). *Nitrogen limitation induces expression of the avirulence gene avr9 in Cladosporium fulvum; a reflection of growth conditions in planta*? Submitted.

chapter 8

Expression and antisense inhibition of transgenes in *Phytophthora infestans* is modulated by choice of promoter and position effects

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Gene, in press

Abstract. Procedures were identified for manipulating the expression of genes in the oomycete fungus, *Phytophthora infestans*. The activities of five putative promoter sequences, derived from the 5' regions of oomycete genes, were measured in transient assays performed in protoplasts and in stable transformants. The sequences tested were from the *ham34* and *hsp70* genes of *Bremia lactucae*, actin-encoding genes of *P. infestans* and *P. megasperma*, and a polyubiquitin-encoding gene of *P. infestans*. Experiments using the *GUS* reporter gene (encoding β-glucuronidase) demonstrated that each 5' fragment had promoter activity but that their activities varied over a greater than tenfold range. Major variation was revealed in the level of transgene expression in individual transformants containing the same promoter::*GUS* or promoter::*lacZ* fusion. The level of expression was not simply related to the number of genes present, suggesting that position effects were also influencing expression. Fusions between the *ham34* promoter, and full-length and partial *GUS* genes in the antisense orientation blocked the expression of *GUS* in protoplasts and in stable transformants.

Introduction

The oomycete fungus, *Phytophthora infestans*, causes late blight on potato, tomato, and several other Solanaceous plants. Procedures for introducing genes and manipulating their expression are required for molecular genetic studies of *P. infestans*. The development of these methods has progressed more slowly for oomycetes than for the higher fungi (i.e. ascomycetes and basidiomycetes), in part due to the lack of suitable promoters. Although frequently considered as fungi due to their generally filamentous growth habit, the oomycetes have poor taxonomic affinity with the higher fungi, based on studies of cell wall composition (Bartnicki-Garcia, 1968), steroid metabolism (Warner *et al.*, 1983), and rRNA sequence (Förster *et al.*, 1990). Promoters from genes of non-oomycetes, including ascomycete and basidiomycete fungi, displayed no activity in transient expression assays in several species of oomycetes, while oomycete promoters (from *Bremia lactucae*) were active (Judelson *et al.*, 1992). Fusion of the *B. lactucae* promoters to genes for drug resistance enabled the isolation of stable transformants of *P. infestans* and *P. megasperma* f. sp. *glycinea*; analysis of marker gene transcripts confirmed the recognition of the *B. lactucae* promoters (Judelson *et al.*, 1991, 1993).

The goal of this study was to expand the technologies for manipulating gene expression in *P. infestans*. The expression of *GUS* and *lacZ*, and the inhibition of GUS expression using antisense sequences, were characterized in stable transformants and in transient assays using promoters derived from five genes of oomycetes. A subset of these promoters had previously been tested using *GUS* in transient assays only (Judelson *et al.*, 1992). These data will be crucial to our future studies, in which we will manipulate genes important to the pathogenicity, host-cultivar specificity, and growth of *P. infestans*.

Table 1. Vectors used in this study[a].

Type	Vector	Source of promoter	Coding sequence
GUS expression	pTH207	*hsp70* from *B. lactucae* (590 bp)	*GUS* (1870 bp)
	pHAMT35G	*ham34* from *B. lactucae* (920 bp)	*GUS* (1870 bp)
	pDHAMT35G	*ham34* from *B. lactucae* (400 bp)	*GUS* (1870 bp)
	pPMA-HAM	actin from *P. megasperma* (1000 bp)	*GUS* (1870 bp)
	pPAC-HAM	actin from *P. infestans* (400 bp)	*GUS* (1870 bp)
	pPUB-HAM	ubiquitin from *P. infestans* (757 bp)	*GUS* (1870 bp)
GUS antisense	pHAMT35G-Rev	*ham34* from *B. lactucae* (920 bp)	anti-*GUS* (1870 bp)
	pHAMT35GD-Rev	*ham34* from *B. lactucae* (920 bp)	anti-*GUS* (1870 bp)
lacZ expression	pHAMT35L	*ham34* from *B. lactucae* (920 bp)	*lacZ* (3700 bp)
drug resistance	pTH209	*hsp70* from *B. lactucae* (590 bp)	*nptII*
	pTH210	*hsp70* from *B. lactucae* (590 bp)	*hpt*

[a] All vectors are based in pUC19 and contain transcription fusions with the indicated coding sequences, promoter, and a 550 bp transcriptional terminator fragment from the *ham34* gene. Plasmids pHAMT35G, pPMA-HAM, pTH207, pTH209, and pTH210 have been described (Judelson *et al.*, 1991, 1992). pPUB-HAM and pPAC-HAM were constructed by excising the *ham34* promoter from pHAMT35G, and inserting either a 757 bp or 400 bp promoter fragment, respectively, from the ubiquitin (*ubi3R*) or actin-encoding *(actA)* genes of *P. infestans* (Pieterse *et al.*, 1991; Unkles *et al.*, 1991). The ubiquitin fragment was excised from pPIN1 (C.M.J. Pieterse and F. Govers, unpublished) by digestion with *Eco*RI, treatment with PolIk, and digestion with *Nco*I, and inserted into pHAMT35G digested with *Hind*III, treated with PolIk, and digested with *Nco*I. The actin fragment was excised as a *Hind*III-*Nco*I fragment from pSTA48 (Unkles *et al.*, unpublished) and inserted into pHAMT35G digested with *Hind*III and *Nco*I. pDHAMT35G was constructed by deleting a 520 bp *Hind*III-*Pvu*II fragment from the 5' end of the *ham34* promoter in pHAMT35G. pHAMT35L was constructed by removing the *lacZ* gene from pSVβ-Gal (Promega) by digestion with *Stu*I and *Xba*I, blunt-ending the fragment with PolIk, and inserting it into the *Sma*I site of expression plasmid pHAMT35 (Judelson and Michelmore, 1991). pHAMT35G-Rev and pHAMT35GD-Rev were constructed by inserting *GUS* sequences from pHAMT35G (excised as either the full-length 1870 bp ORF by digestion with *Eco*RI and *Nco*I, or as 550 bp of 5' ORF sequences by digestion with *Eco*RV and *Nco*I) in the antisense orientation into the *Sma*I site of pHAMT35, following treatment of the *GUS* fragments with PolIk.

Results and Discussion

Comparison of promoters in transient assays. Presumed transcriptional regulatory sequences from the upstream regions of five oomycete genes were tested for their abilities to express the *GUS* marker gene in protoplasts of *P. infestans*. For convenience, these sequences are referred to as "promoters", i.e., fragments of DNA determining the initiation of transcription, although other regulators of expression such as enhancers or silencers might be present (Johnson and McKnight, 1989). The promoters were from the *ham34* gene of *B. lactucae*, which encodes a highly expressed, putative structural protein (Judelson and Michelmore, 1990); a *hsp70* gene of *B. lactucae* (Judelson and Michelmore, 1989); actin-

Table 2. Comparison of promoter activities in transient assays.

Vectors [a]	Promoter fused to *GUS*	GUS specific activity (%) [b]	
pTH207	*hsp70* from *B. lactucae*	819±66	(100%)
pHAMT35G	*ham34* from *B. lactucae*	334±18	(41%)
pPMA-HAM	actin from *P. megasperma*	273±32	(33%)
pPAC-HAM	actin from *P. infestans*	134±8	(16%)
pPUB-HAM	ubiquitin from *P. infestans*	76±8	(9.3%)
none		1±0	(0.1%)

[a] As described in Table 1. Equimolar amounts of each vector corresponding to 10 μg pTH207 were introduced into 2×10^6 protoplasts of strain P1306 of *P. infestans* (provided by Michael Coffey, University of California, Riverside) as described (Judelson *et al.*, 1991).
[b] After an overnight incubation of the protoplasts in rye media (Caten and Jinks, 1968) containing 1 M mannitol, an extract was prepared from the protoplasts and assayed for GUS activity using 4-methylumbelliferyl-ß-glucuronide as described (Judelson *et al.*, 1991). Activity is expressed as pmole 4-methylumbelliferone released per minute per mg protein at 37 °C. Values are averages of four independent transformations, ± standard error. Indicated in parentheses are relative activities, compared to pTH207.

encoding genes of *P. megasperma* and *P. infestans* (Dudler, 1990; Unkles *et al.*, 1991), and a polyubiquitin-encoding gene of *P. infestans* (Pieterse *et al.*, 1991). *GUS* expression vectors varying only in their promoter sequences (Table 1) were introduced into protoplasts, and GUS activity was determined after an overnight incubation (Table 2). Each construct enabled the expression of the marker gene, but up to ten-fold differences in the apparent activities of the promoters were observed (Table 2).

Since the 5' regions tested as promoters ranged from 400 to 1000 bp, it was possible that the larger and more active fragments (such as the 920 bp promoter fragment in pHAMT35G) might contain transcriptional enhancers not present in the smaller elements. However, this was not supported by separate experiments with pDHAMT35G, which contained a truncated (400 bp) *ham34* promoter. The levels of transient GUS activity obtained using pDHAMT35G were 93±12% of that obtained with pHAMT35G.

Comparison of promoters in stable transformants. The relative activities of each promoter were tested in stable transformants obtained by cotransformation of the *GUS* plasmids with the G418 [R] plasmid, pTH209. Approximately 80% of the resulting drug-resistant colonies stained positively for GUS activity in histochemical assays. Variation in the intensity of staining was observed, both between colonies transformed with different *GUS* plasmids and those with the same plasmid.

Quantitative measurements of GUS in transformants revealed considerable variation in activity between the promoters, as well as between individuals containing the same promoter-*GUS* fusion (Fig. 1, top). For each promoter tested, the specific activity of GUS was

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B. lactucae ham34

B. lactucae hsp70

P. infestans actin

P. megasperma actin

P. infestans ubiquitin

B. lactucae ham34

INDIVIDUAL GUS OR *LacZ* COTRANSFORMANT OF *P. INFESTANS*

β-GLUCURONIDASE ACTIVITY

β-GALACTOSIDASE ACTIVITY
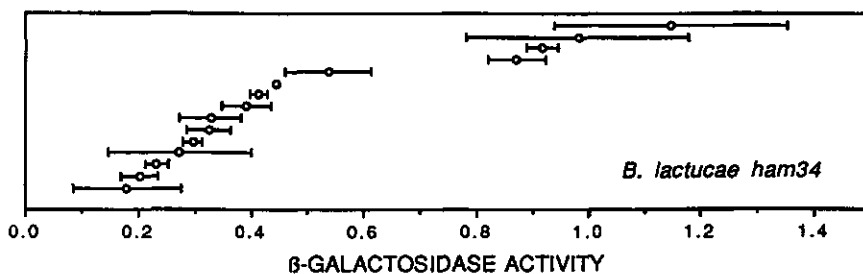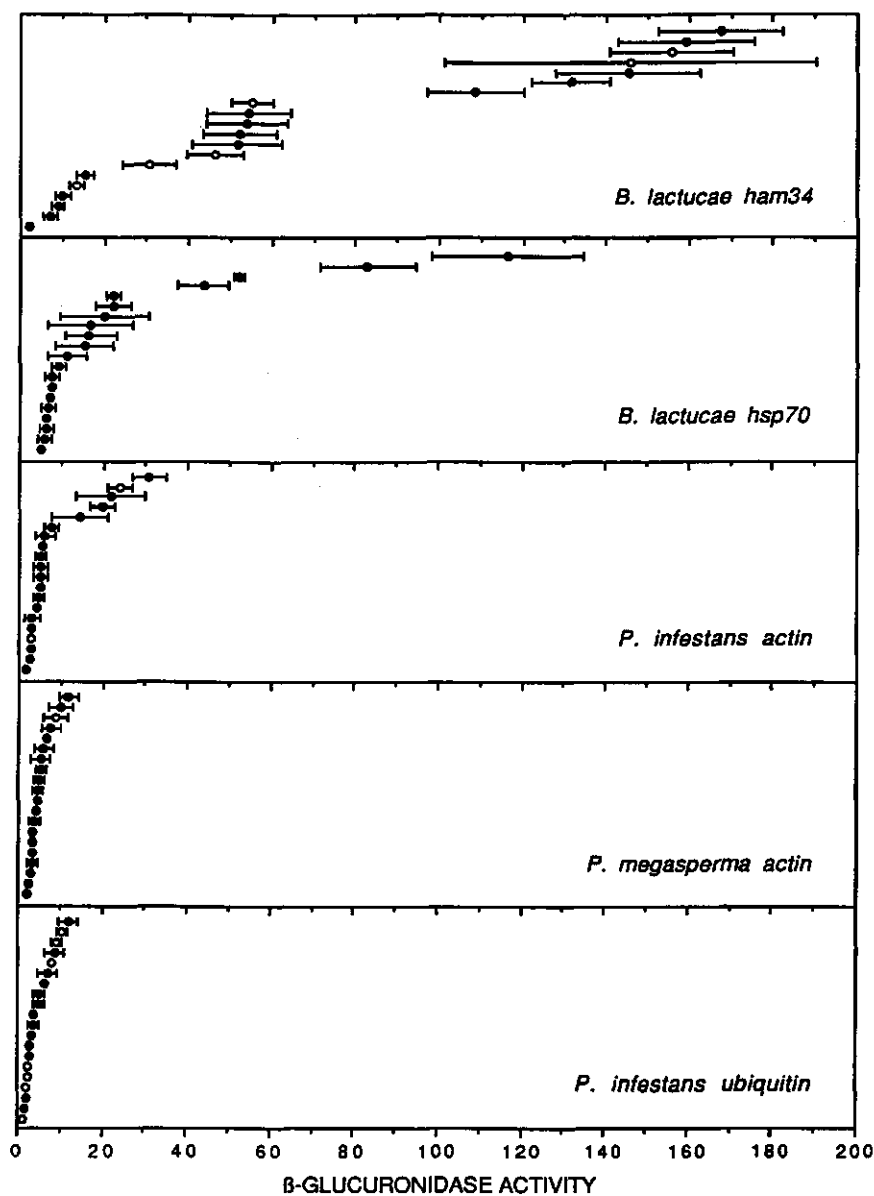
Figure 1. Transgene expression in stable transformants. The specific activities (expressed as nmole *p*-nitrophenol released per min per mg protein at 37 °C) of GUS or ßGal in transformants of *Phytophthora infestans* were determined as described below. The promoter fused to *GUS* or *lacZ* is indicated in each panel. Individual transformants are represented along the vertical axis by each circle. Error bars represent range of values obtained from duplicate cultures, and are omitted when within the symbol.

Methods: Protoplasts were transformed as described (Judelson *et al.*, 1991) using either linear (●) or supercoiled (○) plasmids. Cotransformation was performed using equimolar ratios of the nonselected plasmids (*GUS* or *lacZ*) and G418^R plasmid pTH209 (generally 15 to 30 μg each). For experiments using the linear plasmids, the DNA was digested with *Eco*RI (cleaving 3' of the *ham34* terminator present in each vector) and purified by extraction with phenol and with chloroform, and precipitation with ethanol, prior to transformation. G418^R colonies were assayed histochemically for GUS or ßGal activity approximately 2 weeks after DNA treatment. Samples were stained overnight at 37 °C in 75 μl of assay solutions for GUS (0.05 % 5-bromo-4-chloro-3-indolyl-ß-*D*-glucuronide/10 mM Na$_2$PO$_4$, pH 7.2/5 mM K$_4$Fe(CN)$_6$/5 mM K$_3$Fe(CN)$_6$/0.1 % Triton X-100) or for ßGal (0.05 % 5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside/10 mM Na$_2$PO$_4$, pH 7.2/50 mM NaCl/3.3 mM K$_4$Fe(CN)$_6$/3.3 mM K$_3$Fe(CN)$_6$/0.1 % Triton X-100).

Specific activities were determined by colorimetric assay of extracts of vegetative mycelia. Care was taken to obtain cultures of equal development, due to the coenocytic growth habit of *P. infestans*. Mycelia were obtained by inoculating duplicate 5 ml cultures of clarified rye broth (Caten and Jinks, 1968), in a 16 mm × 100 mm plastic tube, with 2 mm^2 plugs of mycelia excised from the leading edge of an expanding colony. After five to six days at 19 °C the colonies had filled the bottom half of the tubes, remaining submerged. Microscopic examinination of selected cultures confirmed that no sporulation occurred under these conditions. Occasional cultures growing much faster or slower than average were omitted from analysis. Mycelia were harvested by centrifugation and blended for 15 seconds at 4 °C in 0.5 ml GUS extraction buffer (Judelson and Michelmore, 1991). After clarification by centrifugation at 12,000 × *g* for 1 min, the extracts were assayed for activity at 37 °C using 2 mM *p*-nitrophenol-ß-glucuronide in GUS extraction buffer, or 2 mM *p*-nitrophenol-ß-galactoside in GUS extraction buffer plus 1 mM MgCl$_2$. Untransformed background activities of GUS and ßGal of 0.0±0 and 0.08±0.01 u, respectively, were subtracted from the above data.

determined in vegetative mycelia from 20 transformants randomly selected from among the histochemically-staining colonies. Although most transformants were not examined in Southern blots, it is likely that the plasmids had integrated at different genomic sites in different transformants since homologous recombination has not been observed during *P. infestans* transformation. The average relative activities for each promoter in the stable transformants did not precisely parallel the ranking observed in the transient assays. For example, the average activity of the *ham34* promoter increased relative to the other promoters. This may reflect differences in the binding of regulatory proteins to newly introduced versus stably integrated DNA, possibly related to the organization of chromatin. Alternatively, in a transient assay possible negative regulators may have been titrated by the large number of introduced molecules of DNA. Also, the *hsp70* promoter may have been induced to a higher level by factors produced in response to stresses associated with protoplasting in the transient assay, as reported in plants (Ainley and Key, 1990).

Major differences in expression among individual transformants were demonstrated for each promoter. To test if this was unique to *GUS*, cotransformants expressing βGal were obtained using pHAMT35L (*lacZ*) and pTH209 (G418^R). The variation in ßGal activity in 15 randomly selected cotransformants (Fig. 1, bottom) was similar to that of GUS. Several observations suggested that this could be attributed to chromosomal position effects. No straightforward relationship was revealed between GUS activity (in pHAMT35G cotransfor-

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mants) and the relative copy number of *GUS* sequences determined by dot-blot analysis (Fig. 2), although such an assay does not distinguish between intact and partial *GUS* genes. Southern hybridization analysis of nine other transformants containing pHAMT35G also failed to indicate a simple relationship between the relative number of intact *GUS* genes and their expression (not shown). Heterokaryosis was also considered as a cause of the low levels of GUS in some transformants. However, this was not supported by the observation that single-zoospore derivatives (zoospores are typically uninuclear) of two weakly-expressing transformants each expressed low levels of GUS activity similar to those observed in the original transformants (11 and 15 derivatives, respectively). Finally, the variation appeared determined at the level of transcription, as the levels of GUS mRNA detected in Northern blots of RNA from eight transformants expressing pPUB-HAM paralleled enzyme activities (not shown).

Stability of expression. The expression of the *GUS* transgenes was usually mitotically stable, as measured by histochemical assays performed periodically during several months of vegetative culture. Transformants also continued to express the genes during growth in tomato as shown by histochemical staining and RNA hybridization. However, rare instances of a loss of GUS activity were noted involving each promoter. For example, of 57 pHAMT35G transformants that originally demonstrated GUS activity, 53 continued to display similar levels of GUS activity after three months in culture. 21 of the 53 transformants were grown for an additional three months, during which one lost GUS activity.

Five transformants in which the expression of *GUS* had been silenced were studied in more detail. DNA hybridization analysis indicated that two lacked *GUS* sequences, suggesting excision of the gene, sorting out of heterokaryons, or accidental mislabeling of cultures. The other three transformants retained normal pHAMT35G sequences, based on several diagnostic tests with restriction enzymes. No evidence was obtained to support hypermethylation of the silenced *GUS* genes, based on digests using the methylation-sensitive enzymes *Hpa*II, which cuts within the *GUS* ORF, or *Pvu*II, which digests within the promoter and terminator in pHAMT35G. To more definitively determine the integrity of the silenced *GUS* gene, pHAMT35G sequences were recovered from two silenced transformants by digestion of genomic DNA with *Hind*III, ligation, and transformation of *Eschericia coli*; this was possible since tandem arrays of a few vector molecules frequently exist in transformants of *P. infestans* (Judelson *et al.*, 1991). Ampicillin-resistant colonies were recovered that contained plasmids identical in diagnostic restriction digest patterns to pHAMT35G. Two plasmids recovered from each of the two strains were reintroduced into *P. infestans*; all four behaved identically to the original pHAMT35G vector in terms of the frequency, intensity, and stability (over three months) of *GUS* expression. Silencing in at least these two transformants therefore did not appear to involve the mutation or excision of sequences required for *GUS* expression.

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Figure 2. Relationship between GUS activity and copy number. Relative copy numbers of the *GUS* gene were determined by dot-blot hybridization as described below; error bars represent standard accumulated error. GUS activities were determined as described in Fig. 1; error bars indicate the range of GUS specific activities between duplicate cultures.
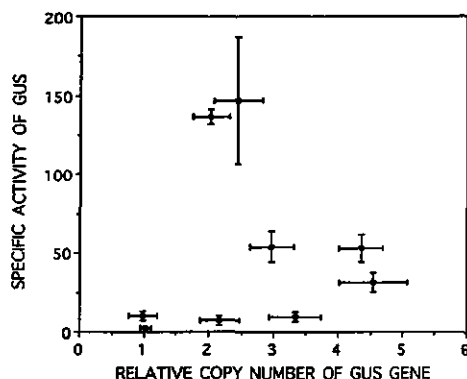
Methods: DNA was extracted from pHAMT35G cotransformants as described (Raeder and Broda, 1985), and concentrations were determined by measurement of optical density. On nylon filters (Hybond N⁺, Amersham) placed in a 96-well vacuum manifold (Bio-Rad), 62.5 ng, 250 ng, 1 μg, and 2 μg of DNA from transformants were spotted (in duplicate), along with a copy-number reconstruction containing pHAMT35G (linearized with *Hind*III) corresponding to 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 copies per haploid genome (in duplicate), relative to 250 ng of genomic DNA and a genome size of 2.5×10^8 bp as determined by microfluorometry (Tooley and Therriën, 1987). Salmon DNA was added to each sample to a total of 2 μg DNA. To apply the DNA to the membrane, the membrane was first wetted with 2×SSC, and then the DNA samples, in 10×SSC, were drawn through the filter using slow vacuum. The filters were rinsed with 0.4 N NaOH, removed from the manifold, washed for 5 min in 0.4 N NaOH, and washed for 2 min in 2×SSC. One filter was hybridized to a probe for the *GUS* ORF (1.87 kb *Nco*I-*Kpn*I fragment of pHAMT35G). A duplicate filter was hybridized to a fragment of low-copy *P. infestans* DNA (PIG-3). Hybridization was performed at 65 °C in 0.5 M Na₂PO₄, pH 7.2/7% SDS/0.25 mM Na₂EDTA using ³²P-labeled probes prepared by the random primer method, and filters were washed twice for 15 min in 1×SSC/0.3% SDS/0.1% sodium pyrophosphate at 65 °C and 15 min in 0.1×SSC/0.3% SDS/0.1% sodium pyrophosphate at 65 °C. Films from autoradiography were analyzed using a computing laser densitometer and Imagequant software (Molecular Dynamics). Copy numbers were determined by comparing the unknowns to the internal pHAMT35G standard curve, using 4 points in the linear range. Adjustments were made for minor variation in DNA loading between samples using the filter hybridized to the PIG-3 genomic probe.

Inhibition of gene expression using antisense RNA. The feasibility of using antisense RNA to inhibit the expression of genes in *P. infestans* was initially tested in transient assays, by treating protoplasts with varying ratios of sense and antisense *GUS* constructs (Fig. 3). A full-length antisense construct (pHAMT35G-Rev) and one containing only 550 nt of 5' coding sequences (pHAMT35GD-Rev) were both effective at reducing GUS activity derived from sense vectors containing the *ham34* promoter (pHAMT35G) or ubiquitin promoter (pPUB-HAM). Increasing amounts of the antisense construct resulted in increased inhibition. Less antisense vector was required to reduce the expression of *GUS* from the ubiquitin promoter, which is weaker than the *ham34* promoter.

The full- and partial-length antisense constructs were also tested in stable transformants, by retransformation of strains J613 and T513. J613 was a GUS⁺, G418ᴿ transformant constructed using pTH209 and pPUB-HAM (GUS specific activity of 11.3±1.6 u). T513 was a GUS⁺, Hygᴿ transformant obtained with pHAMT35G and pTH210 (GUS specific activity of 90±16 u). The antisense constructs were introduced into J613 and T513 by cotransformation with pTH210 or pTH209, respectively.
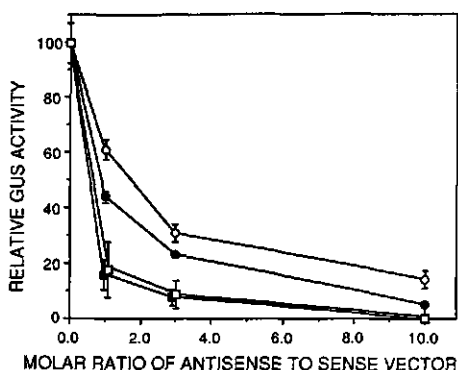
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Figure 3. Antisense inhibition of GUS in protoplasts. Transient assays were performed in protoplasts of *P. infestans* as described in the legend of Table 2, using varying ratios of sense and antisense *GUS* expression plasmids. Values are expressed as per cent expression of GUS, relative to samples treated with *GUS* sense plasmids alone (average GUS specific activities were 68 ± 3 u for pHAMT35G, and 18 ± 1 u for pPUB-HAM); the average of four determinations is presented. Error bars indicate the range of values, and are omitted when within the symbol. Each treatment used 5 μg of pPUB-HAM or pHAMT35G, plus varying amounts of the antisense plasmids as indicated. (●), pHAMT35G and pHAMT35G-Rev; (○) pHAMT35G and pHAMT35GD-Rev; (■) pPUB-HAM and pHAMT35G-Rev; (□) pPUB-HAM and pHAMT35GD-Rev.

Among derivatives of J613, six of 34 colonies resulting from cotransformation with pHAMT35GD-Rev and four of 20 resulting from cotransformation with pHAMT35G-Rev showed little or no GUS staining in a histochemical assay. Of 18 colonies randomly selected from the 54 Hyg[R] transformants, 12 were shown by PCR to contain antisense *GUS* vectors. Therefore, of the 54 colonies assayed for GUS, about 67% would have been expected to contain the antisense gene. Apparently, only a fraction of these expressed levels of antisense RNA adequate to inhibit the accumulation of GUS. In a control treatment executed in parallel, each of 30 colonies recovered by retransformation of J613 using pTH210 alone expressed levels of GUS activity similar to J613.

Attempts to inhibit *GUS* expression in T513, where GUS was expressed using the strong *ham34* promoter, were less successful. Of 47 colonies recovered from the retransformation of T513, only one (treated with pHAMT35G-Rev) showed an obvious reduction of GUS activity based on histochemical staining. Each of 24 control transformants (retransformed with pTH209 only) continued to express *GUS*.

Several data indicated that inhibition of *GUS* expression in the GUS-negative derivatives of J613 was the authentic consequence of antisense inhibition, rather than spontaneous silencing of the *GUS* transgene. Hybridization analysis of RNA from transformants Q22 and Q25 (derivatives of J613 containing pHAMT35GD-Rev) detected GUS mRNA in addition to a much higher level of antisense RNA (Fig. 4). The level of GUS mRNA was much lower than that observed in J613 and Q17, a retransformant of J613 obtained using pTH210 but not an antisense vector. The reduction in sense mRNA levels is similar to that observed in other species (Rothstein *et al.*, 1987), but not as extreme as in some (Robert *et al.*, 1989). Degradation of sense-antisense complexes may account for this reduction. Quantitative assays also revealed low but detectable levels of GUS activity in Q22 and Q25 (0.20 and 0.22 u), above the negligible levels in wild-type *P. infestans*, indicating that 98% inhibition had been achieved.

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Figure 4. Antisense inhibition of *GUS* expression in stable transformants. RNA was resolved on 1.2% agarose-2.2 M formaldehyde gels as described (Judelson *et al.*, 1991), transferred to nylon membranes, and hybridized to ³²P-labeled probes as in Fig. 2. Lane 1, 2 μg RNA from J613; lane 2, 2 μg RNA from Q17 (J613 retransformed with pTH210 only); lanes 3 and 4, 2 μg RNA from Q22 and Q25 (J613 retransformed with pTH210 and pHAMT35GD-Rev). The filter was hybridized to a probe for the 5' end of the *GUS* gene (a 550 bp *Nco*I-*Eco*RV fragment of pHAMT35G) (top panel), and then stripped and rehybridized with a probe for actin (750 bp *Hind*III fragment from *actA*; Unkles *et al.*, 1991). Band sizes (indicated in the left margin) were determined using a RNA ladder (BRL) as a size standard.
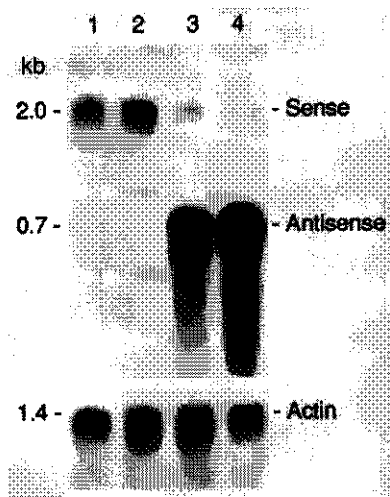
Conclusions

(1) DNA fragments from the upstream regions of five oomycete genes were shown to have promoter activity in protoplasts and in stable transformants of *P. infestans*. The strengths of the promoter fragments varied substantially, indicating opportunities for expressing genes to high or low levels in future experiments. Two sequences shown here and previously (Judelson and Michelmore, 1991) to be active in *P. infestans* came from a related oomycete, *B. lactucae*. All of these promoters may therefore be useful throughout the oomycetes.

(2) Significant variability was observed in the quantitative expression of foreign genes in *P. infestans*, which was not simply correlated with copy number. Similar variation resulting from chromosomal position effects has been widely reported in plants and animals (Al-Shawi *et al.*, 1990; Dean *et al.*, 1988; Spradling and Rubin, 1983). The influence of position effects on transgene expression in higher fungi has been reported, but on average appears less extreme than observed for plants, animals, and *P. infestans* (Bunkers, 1991; Kinsey and Rambosek, 1984; Van Gorcom *et al.*, 1985). Position effects in *P. infestans* may reflect the more complex genomes with extensive repeat sequences in the *Phytophthora* genus, compared to other fungi (Mao and Tyler, 1991).

(3) The expression of transgenes was generally stable, but occasionally their expression ceased. Silencing of the transgene was not associated with a discernible mutation in the vector, indicating that caution must be exercised when using transformation to evaluate the function of genes. Instability of transforming sequences has been described in higher fungi but is commonly associated with the excision of transforming sequences (Tooley *et al.*, 1992; Keller *et al.*, 1991).

(4) Antisense RNA was effective in inhibiting the accumulation of gene products such as GUS, especially when a strong promoter (i.e., from the *ham34* gene) could be used to repress the expression of a gene with a weaker promoter (i.e., from the ubiquitin gene). Demonstration of the antisense technique is particularly meaningful since gene disruption strategies, commonly performed in some higher fungi, are not yet feasible in *P. infestans* since homologous recombination between plasmid and genomic sequences has not been observed. Also, since oomycetes are diploid, two rounds of disruption would be required to silence a native gene.

References

Ainley, W.M. and Key, J.L. (1990). Development of a heat shock inducible expression cassette for plants - characterization of parameters for its use in transient expression assays. *Plant Molecular Biology* 14: 949-967.

Al-Shawi, R., Kinnaird, J., Burke, J. and Bishop, J.O. (1990). Expression of a foreign gene in a line of transgenic mice is modulated by a chromosomal position effect. *Molecular and Cellular Biology* 10: 1192-1198.

Bartnicki-Garcia, S. (1968). Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annual Reviews of Microbiology* 22: 87-108.

Bunkers, G.J. (1991). Expression of the *Escherichia coli* β-glucuronidase gene in *Pseudocercosporella herpotrichoides*. *Applied Environmental Microbiology* 57: 2896-2900.

Caten, C.E. and Jinks J.L. (1968). Spontaneous variability of single isolates of *Phytophthora infestans*, I. Cultural variation. *Canadian Journal of Botany* 46: 329-348.

Dean, C., Jones, J., Favreau, M., Dunsmuir, P. and Bedbrook, J. (1988). Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. *Nucleic Acids Research* 16: 9267-9283.

Dudler, R. (1990). The single copy actin gene of *Phytophthora megasperma* encodes a protein considerably diverged from any other known actin. *Plant Molecular Biology* 14: 415-422.

Förster, H., Coffey, M.D., Elwood, M. and Sogin, M.L. (1990). Sequence analysis of the small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution. *Mycologia* 82: 306-312.

Johnson, P.F. and McKnight, S.L. (1989). Eukaryotic transcriptional regulatory proteins. *Annual Reviews of Biochemistry* 58: 799-839.

Judelson, H.S., Coffey, M.D., Arredondo, F.R. and Tyler, B.M. (1993). Transformation of the soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* occurs by DNA integration into single or multiple chromosomes. *Current Genetics* 23: 211-218.

Judelson, H.S. and Michelmore, R.W. (1989). Structure and expression of a gene encoding heat-shock protein Hsp70 from the oomycete fungus *Bremia lactucae*. *Gene* 79: 207-217.

Judelson, H.S. and Michelmore, R.W. (1990). Highly abundant and stage-specific mRNAs in the obligate pathogen *Bremia lactucae*. *Molecular Plant-Microbe Interactions* 3: 225-232.

Judelson, H.S. and Michelmore, R.W. (1991). Transient expression of foreign genes in the oomycete *Phytophthora infestans* using *Bremia lactucae* regulatory sequences. *Current Genetics* 19: 453-459.

Judelson, H.S., Tyler, B.M. and Michelmore, R.W. (1991). Transformation of the oomycete pathogen, *Phytophthora infestans*. *Molecular Plant-Microbe Interactions* 4: 602-607.

Judelson, H.S., Tyler, B.M. and Michelmore, R.W. (1992). Regulatory sequences for expressing genes in oomycete fungi. *Molecular and General Genetics* 234: 138-146.

Keller, N.P., Bergstrom, G.C. and Yoder, O.C. (1991). Mitotic stability of transforming DNA is determined by its chromosomal configuration in the fungus *Cochliobolus heterostrophus*. *Current Genetics* 19: 227-233.

Kinsey, J.A. and Rambosek, J.A. (1984). Transformation of *Neurospora crassa* with the cloned *am* (glutamate dehydrogenase) gene. *Molecular and Cellular Biology* 4: 117-122.

Mao, Y. and Tyler, B.M. (1991). Genome organization of *Phytophthora megasperma* f. sp. *glycinea*. *Experimental Mycology* 15: 283-291.

Pieterse, C.M.J., Risseeuw, E.P. and Davidse, L.C. (1991). An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Molecular Biology* 17: 799-811.

Raeder, U. and Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Letters of Applied Microbiology* 1: 17-20.

Robert, L.S., Donaldson, P.A., Ladaique, C., Altosaar, I., Arnison, P.G. and Fabijanski, S.F. (1989). Antisense RNA inhibition of β-glucuronidase expression in transgenic tobacco plants. *Plant Molecular Biology* 13: 399-409.

Rothstein, S.J., DiMaio, J., Strand, M. and Rice, D. (1987). Stable and heritable inhibition of the expression of nopaline synthase in tobacco expressing antisense RNA. *Proceedings of the National Academy of Sciences USA* 84: 8439-8443.

Spradling, A.C. and Rubin G.M. (1983). The effect of chromosomal position on the expression of the *Drosophila* xanthine dehydrogenase gene. *Cell* 34: 47-57.

Tooley, P.W., Leung, H. and Leong, S.A. (1992). Meiotic and mitotic stability of transforming DNA in the phytopathogenic fungus *Magnaporthe grisea*. *Current Genetics* 21: 55-60.

Tooley, P.W. and Therriën, C.D. (1987). Cytophotometric determinations of the nuclear DNA content of 23 Mexican and 18 non-Mexican isolates of *Phytophthora infestans*. *Experimental Mycology* 11: 19-26.

Unkles, S.E., Moon, R.P., Hawkins, A.R., Duncan, J.M. and Kinghorn, J.R. (1991). Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* 100: 105-112.

van Gorcom, R.F.N., Pouwels, P.H., Goosen, T., Visser, J., van den Broek, H.W.J., Hamer, J.E., Timberlake, W.E. and van den Hondel, C.A.M.J.J. (1985). Expression of an *Escherichia coli* β-galactosidase fusion gene in *Aspergillus nidulans*. *Gene* 40: 99-106.

Warner, S.A., Sovocool, G.W. and Domnas, A.J. (1983). Sterols of selected species of oomycetes and hypochytridiomycetes. *Mycologia* 75: 285-291.

chapter 9

General discussion

General discussion

Differential gene expression in **Phytophthora** infestans *during pathogenesis on potato.* To gain more insight in the molecular processes involved in pathogenesis of the late blight fungus *P. infestans*, we isolated differentially expressed genes from a genomic library of *P. infestans* (chapter 3). Characterization of genes whose expression is induced or significantly increased during pathogenesis might enable us to identify pathogenicity factors which play a pivotal role in the establishment and maintenance of the pathogen in the host, or which are important for the increase of disease severity. Most avirulence genes and pathogenesis related genes of plant pathogenic fungi known to date, e.g. *avr9, avr4, ecp1* and *ecp2* of the tomato leaf mold fungus *Cladosporium fulvum* (van Kan *et al.*, 1991; Joosten *et al.*, 1993; van den Ackerveken *et al.*, 1993b), *MGR1* of the rice blast fungus *Magnaporthe grisea* (N. Talbot, personal communication), the cutinase gene of the foot rot fungus *Fusarium solani* f.sp. *pisi* (Woloshuk and Kolattukudy, 1986) and the extracellular polygalacturonase gene of the stalk rot fungus *Fusarium moniliforme* (De Lorenzo *et al.*, 1987), show induced expression during pathogenesis. Also in plant pathogenic bacteria, it has been shown that the expression of several pathogenicity genes is induced *in planta* (Willis *et al.*, 1991). This indicates that differential hybridization of DNA libraries is potentially a powerful approach for the isolation of putative pathogenicity genes. Moreover, the approach is non-biased enabling the identification of genes whose role in pathogenicity can not be predicted by other methods. However, the employed differential screening procedure does not exclusively select pathogenicity genes. Also *in planta* induced genes may be isolated of which the products are involved in adaptations to a change in nutrient environment encountered upon invasion of the host may be isolated. Nevertheless, by identifying this type of differentially expressed genes one can acquire a better understanding of the cellular processes which change during pathogenesis. It is very unlikely that the differential screening procedure allows identification of all possible pathogenicity genes since only those pathogenicity genes whose expression is specifically induced or significantly increased during growth of the pathogen in the host will be identified.

In total nine distinct *in planta* induced genes of *P. infestans* have been isolated (chapter 3) four of which were characterized in more detail. Two genes, *ubi3R* (chapter 4) and *calA* (chapter 5), appeared to code for the ubiquitous proteins ubiquitin and calmodulin, respectively. The other characterized *in planta* induced genes, *ipiB* and *ipiO*, both belong to small gene clusters encoding novel proteins with yet unknown functions (chapter 6).

ubi3R *and* **calA.** The proteins encoded by *ubi3R* and *calA*, ubiquitin and calmodulin respectively, are highly conserved in all eukaryotic organisms indicating that they play important roles in basic cellular processes. This is the first time that increased expression of genes encoding ubiquitin and calmodulin has been found in association with fungal pathogenicity. Although these proteins can not be considered as primary pathogenicity factors, they may have important functions in cellular processes which change during

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pathogenesis. Ubiquitin has been shown to play a key role in selective degradation of abnormal proteins which arise during stress, in maintenance of chromatin structure, in modification of cell surface receptors and in regulation of gene expression (Monia *et al.*, 1990), all processes which are likely to proceed during pathogenesis. Failure to regulate these processes correctly, due to insufficient ubiquitin concentrations, may cause the pathogenic process to be unsuccessful. Calmodulin is one of the major intracellular Ca^{2+} receptors and is responsible for mediating cellular responses to Ca^{2+} fluxes which are generated by a variety of processes. It has been shown in several organisms that, via Ca^{2+}-calmodulin complexes, calmodulin is involved in the transduction of signals from extracellular stimuli into intracellular events (Hepler and Wayne, 1985; Roberts *et al.*, 1986). Therefore, it is tempting to speculate that calmodulin is involved in the communication between plant and pathogen. Unfortunately, the involvement of both ubiquitin and calmodulin in various basic cellular processes makes it difficult to study the significance of increased *ubi3R* and *calA* gene expression during pathogenesis. Manipulating the synthesis or activity of ubiquitin or calmodulin will influence all other cellular processes which are dependent on these proteins. As a consequence, a straightforward interpretation of such studies will be impossible.

ipiB. The *ipiB* genes belong to a gene family of which three members, *ipiB1*, *ipiB2* and *ipiB3*, have been characterized. These three highly homologous genes are closely linked and encode glycine-rich proteins. The *ipiB* genes are the first glycine-rich protein encoding genes identified in filamentous fungi. The primary structure of the IPI-B proteins consists of an amino terminal signal peptide for secretion followed by a highly repetitive, glycine-rich domain which comprises most of the protein. The IPI-B proteins share these characteristics with plant glycine-rich proteins of which some have been shown to be associated with the cell wall (Showalter, 1993). In germinating cysts and during early stages of the interaction, *ipiB* mRNA accumulates to very high levels. The possible association of the *ipiB* gene products with the cell wall suggests that they are involved in the development of infection structures like appressoria or primary hyphae. Immunological studies with antisera raised against the *ipiB* proteins could provide more insight in this matter. In more advanced stages of the infection process and during growth of the fungus *in vitro*, *ipiB* mRNA is hardly detectable indicating that for mycelial growth, high levels of IPI-B proteins are not required.

ipiO. The *P. infestans* genome contains two almost identical *ipiO* genes which are closely linked and code for proteins which have no homology with sequences present in any data library. Like the IPI-B proteins, the IPI-O proteins contain an amino terminal signal peptide suggesting that the proteins are secreted. The mature proteins have a putative N-glycosylation site and contain the tripeptide sequence Arg-Gly-Asp (RGD), a conserved motif found in several mammalian extracellular matrix proteins in which it functions as a cell attachment sequence (Ruoslathi and Pierschbacher, 1986). Accumulation of *ipiO* mRNA follows a transient pattern and coincides with colonization of the host cells. Once colonization of host tissue is completed, *ipiO* mRNA level decreases dramatically to low or undetectable levels.

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Also during growth of the fungus *in vitro*, *ipiO* mRNA can not be detected indicating that IPI-O proteins are synthesized exclusively during the biotrophic phase of the pathogenic interaction. Based on the assumption that the IPI-O proteins are extracellular, we speculate that they might have a function in the extracellular matrix where fungal components interact directly with plant cells. In view of this, the RGD sequence motif in the IPI-O proteins might play a role in pathogenicity of *P. infestans*. We hypothesize that through the RGD sequence, IPI-O can either be involved in attachment of *P. infestans* to the plant cells or disturb normal RGD mediated signalling which supposedly occurs in healthy plant tissues.

The RGD tripeptide motif was first identified in several mammalian adhesion proteins, such as fibronectin, vitronectin, fibrinogen, type I collagen and the Von Willebrand coagulation factor, and it was demonstrated to be a cell attachment sequence crucial for cell-substratum adhesion (Dedhar *et al.*, 1987; Pierschbacher and Ruoslathi, 1984; Ruoslathi and Pierschbacher, 1986; Suzuki *et al.*, 1985). The RGD tripeptide is responsible for the interaction of extracellular adhesion proteins with cell surface receptors, the so-called integrins (D'Souza *et al.*, 1988; Hynes, 1987). These specific ligand-receptor interactions mediate adhesion of cells to the extracellular matrix and play an essential role in intercellular communication and development of mammalian cells (Damsky and Bernfield, 1991). Pierschbacher and Ruoslathi (1984) showed that RGD-mediated adhesion can be disrupted specifically by small synthetic RGD-containing peptides which compete with the RGD-containing extracellular adhesion molecules for integrin binding sites. Also in lower eukaryotes, cell adhesion molecules with a functional RGD-tripeptide are found. An example is discoidin I which plays a role in the aggregation of the slime mold *Dictyostelium discoideum* (Gabius *et al.*, 1985). Moreover, it was found that pathogenicity factors of some bacteria pathogenic for humans contain a functional RGD-tripeptide which interacts with integrins on the surface of host cells, thereby mediating uptake into the host cells (Finlay, 1990; Relman *et al.*, 1990; Russell and Wright, 1988). Furthermore, the RGD-containing VP1 protein of the foot-and-mouth disease virus appears to interact with integrin-like molecules on the surface of the host cells in a RGD-dependent manner, suggesting that also viruses utilize RGD-dependent interactions for host invasion (Fox *et al.*, 1989).

If the IPI-O proteins truly function as adhesion molecules in a RGD-dependent fashion, then receptor-ligand interactions, i.e. plasma membrane-cell wall interactions (Roberts, 1990), involving RGD-containing proteins and integrin-like molecules should occur in fungi and plants. Although the walled cells of plants differ from animal cells, there are several indications that RGD-mediated adhesion interactions occur in plants. First, proteins immunologically related to human vitronectin and its integrin receptor have been found in plants and green algae (Quatrano *et al.*, 1991; Sanders *et al.*, 1991; Schindler *et al.*, 1989). Secondly, Schindler *et al.* (1989) showed that the RGD-containing hexapeptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), specifically disrupted cell membrane-cell wall attachment in soybean suspension cells whereas the control hexapeptides Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) and Gly-Asp-Gly-Arg-Ser-Pro (GDGRSP) did not. Thirdly, studies of Zhu *et al.* (1993) demonstrated that protoplasts prepared from tobacco

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cells which are adapted to grow in high salt concentrations, adhere tightly to each other and that this typical tight adhesion is specifically blocked by the GRGDSP hexapeptide. They also found that vitronectin-like proteins are enriched in these high salt adapted cells. Finally, Sanders *et al.* (1991) found that vitronectin-like proteins were enriched on the surfaces of cells lining the stylar duct through which pollen tubes grow to deliver the sperm cells to the ovule. All these observations suggest that RGD-dependent adhesion processes similar to those found in mammals, occur at the surface of the plasma membrane of plant cells. Attachment of plant pathogenic bacteria to the host cells can also be mediated by this type of adhesive interactions, e.g. *Agrobacterium tumefaciens* has been shown to utilize a vitronectin-like protein on the plant cell surface as the receptor for its initial attachment to host cells (Wagner and Matthysse, 1992).

Using plasmolysis techniques, Nozue *et al.* (1979) demonstrated tight adherence of the potato plasmalemma to infecting hyphae of *P. infestans* from the earliest time of penetration. In addition, Hohl (1991) and Gross *et al.* (1993) demonstrated adhesion of hyphae of *Phytophthora megasperma* and *P. infestans* to protoplasts of soybean and parsley, respectively. Based on these findings it can be postulated that fungal hyphae produce extracellular adhesion proteins which facilitate attachment to host cells. When these adhesion molecules function via RGD-dependent ligand-receptor interactions it is also possible that they compete with RGD-containing proteins produced by the host, thereby disturbing the regular RGD-mediated ligand-receptor interactions and creating beneficial conditions for colonization of the host tissue. In view of this, it would be interesting to express the *ipiO* gene in transgenic plants and examine the effect on cell wall-cell membrane interactions. Another function of fungal adhesion molecules may be in the extrahaustorial matrix, a very thin layer of unknown material between the haustorium and the plasmalemma of the host cell. The extrahaustorial matrix is thought to be of fungal origin and may have a function in recognition or in nutrient uptake, either through suppressing a host resistance response, or by activating physiological changes in the host which are beneficial for the maintenance of a compatible interaction between host and pathogen (Coffey and Wilson, 1983). Whether the *P. infestans* IPI-O proteins indeed have cell adhesion properties and operate as hypothesized above, and whether RGD-mediated adhesion interactions in general play a role in fungal pathogenesis, are interesting questions to address in the future. In this respect, the effect of RGD-containing synthetic peptides on the adherence of potato cells to hyphae of *P. infestans* could be studied.

Regulation of ipiB *and* ipiO *gene expression.* The expression of the *P. infestans ipiB* and *ipiO* genes is specifically induced *in planta* whereas during growth of the fungus in a rich culture medium, expression is not detectable. However, during growth of the fungus *in vitro* the expression of both the *ipiB* and *ipiO* genes can be activated by nutrient deprivation (chapter 7). Interestingly, transcriptional activation by nutrient deprivation has been reported for several other *in planta* induced genes of plant pathogens, e.g. the avirulence gene *avr9* of *Cladosporium fulvum* which is specifically expressed when the nitrogen source in the

growth medium is limited (van den Ackerveken *et al.*, 1993a) and the putative pathogenicity gene *MGR1* of *Magnaporthe grisea* of which the expression can be induced *in vitro* by glucose deprivation (N. Talbot, personal communication). It is tempting to speculate that upon infection of a host plant, nutrient deprivation or changes in nutrient conditions trigger the expression of a set of pathogenicity genes of which the gene products facilitate the establishment of a beneficial environment for the pathogen. If transcriptional activation of pathogenicity genes by nutrient deprivation is a general phenomenon, selecting for genes which are specifically expressed in starvation stressed cells can be a useful approach for the isolation of putative pathogenicity genes of a wide range of plant pathogens.

***Further analyses of the function of* ipiB *and* ipiO.** The possible functions of the products of the *ipiB* and *ipiO* genes of *P. infestans* are still hypothetical and further experiments have to be performed to determine the significance of the IPI-B and IPI-O proteins in pathogenesis. *In situ* localization of *ipiB* and *ipiO* mRNA and of the IPI-B and IPI-O proteins could provide more information about the specific site of action. The most direct way to study the function of a gene product is by disrupting the gene of interest and examine the effect on the pathogenic properties of *P. infestans*. In several plant pathogenic fungi, e.g. *Cladosporium fulvum* (Marmeisse *et al.*, 1993), *Fusarium solani* f.sp. *pisi* (Stahl and Schäfer, 1992) and *Magnaporthe grisea* (Sweigard *et al.*, 1992), the possible function of putative pathogenicity genes or avirulence genes has been studied by gene disruption or gene replacement. However, this approach requires a highly efficient DNA transformation system and although the procedure to obtain stable transformants of *P. infestans* is established (Judelson *et al.*, 1991), the efficiency of transformation is still very low. Moreover, *P. infestans* is a heterothallic and diploid organism; some isolates are even tetraploid. Therefore, inactivation of a target gene requires simultaneous disruption of at least two alleles or sexual progeny containing two disrupted alleles have to be obtained via forced self fertilization. Another problem specifically encountered with the *ipiB* genes is the fact that these genes are members of a multigene family. Although the activity of the individual members of the *ipiB* gene family has not been studied so far, it is conceivable that disruption of one *ipiB* gene or even a small *ipiB* gene cluster will not be sufficient to obtain a significant inhibition of IPI-B protein synthesis. Therefore, a better approach to study their function might be reducing or blocking the synthesis of IPI-B or IPI-O by means of anti-sense RNA. The anti-sense technique has been tested in *P. infestans* using sense and anti-sense *GUS* constructs and it was shown to be effective in inhibiting the accumulation of GUS up to 98% (chapter 8).

References

Coffey, M.D. and Wilson, U.E. (1983). Histology and cytology of infection and disease caused by *Phytophthora*. *In*: Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.), *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology, pp. 289-301. American Phytopathological Society, St. Paul,

Minnesota.

Damsky, C.H. and Bernfield, M. (1991). Cell-to-cell contact and extracellular matrix. *Current Opinions in Cell Biology* 2: 813-814.

Dedhar, S., Ruoslathi, E. and Pierschbacher, M.D. (1987). A cell surface receptor complex for collagen type I recognizes the arg-gly-asp sequence. *Journal of Cell Biology* 104: 585-593.

De Lorenzo, G., Salvi, G., Degra, L., D'ovidio, R. and Cervone, F. (1987). Induction of extracellular polygalacturonase and its mRNA in the phytopathogenic fungus *Fusarium moniliforme. Journal of General Microbiology* 133: 3365-3373.

D'Souza, S.E., Ginsberg, M.H., Burke, T.A., Lam, S.C.-T. and Plow, E.F. (1988). Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science* 242: 91-93.

Finlay, B.B. (1990). Cell adhesion and invasion mechanisms in microbial pathogenesis. *Current Opinions in Cell Biology* 2: 815-820.

Fox, G., Parry, N.R., Varnett, P.V., McGinn, B., Rowlands, D.J. and Brown, F. (1989). The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). *Journal of General Virology* 70: 625-637.

Gabius, H.-J., Springer, W.R. and Barondes, S.H. (1985). Receptor for the cell binding site of Discoidin I. *Cell* 42: 449-456.

Gross, P., Julius, C., Schmelzer, E. and Hahlbrock, K. (1993). Translocation of cytoplasm and nucleus to fungal penetration sites is associated with depolymerization of microtubules and defence gene activation in infected, cultured parsley cells. *The EMBO Journal* 12: 1735-1744.

Hepler, P.K. and Wayne, R.O. (1985). Calcium and plant development. *Annual Review of Plant Physiology* 36: 397-439.

Hohl, H.R. (1991). Host-pathogen interactions in *Phytophthora. In*: Lucas, J.A., Shattock, R.C., Shaw, D.S. and Cooke, L.R. (Eds.), *Phytophthora*, pp. 295-311. Cambridge University Press, Cambridge.

Hynes, R.O. (1987). Integrins, a family of cell surface receptors. *Cell* 48: 549-554.

Joosten, M.H.A.J., Cozijnsen, T.J. and de Wit, P.J.G.M. (1993). A fungal tomato pathogen circumvents host plant resistance by a single basepair change in an avirulence gene. Submitted.

Judelson, H.S., Tyler, G.M. and Michelmore, R.W. (1991). Transformation of the oomycete pathogen *Phytophthora infestans. Molecular Plant-Microbe Interactions* 4: 602-607.

Marmeisse, R., van den Ackerveken, G.F.J.M., Goosen, T., de Wit, P.J.G.M. and van den Broek, H.W.J. (1993). Disruption of the avirulence gene *avr9* in two races of the tomato pathogen *Cladosporium fulvum* causes virulence on tomato genotypes with the complementary resistance gene *Cf9. Molecular Plant-Microbe Interactions*, in press.

Monia, B.P., Ecker D.J. and Crooke, S.T. (1990). New perspectives on the structure and function of ubiquitin. *Bio/technology* 8: 209-215.

Nozue, M., Tomiyama, K. and Doke, N. (1979). Evidence for adherence of host plasmalemma to infecting hyphae of both compatible and incompatible races of *Phytophthora infestans. Physiological Plant Pathology* 15: 111-115.

Pierschbacher, M.D. and Ruoslathi, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 309: 30-33.

Quatrano, R.S., Brian, L., Aldridge, J. and Schultz, T. (1991). Polar axis fixation in *Fucus* zygotes: components of the cytoskeleton and extracellular matrix. *Development* Supplement 1, 11-16.

Relman, D., Tuomanen, E., Falkow, S., Golenbock, D.T., Saukkonen, K. and Wright, S.D. (1990). Recognition of a bacterial adhesin by an integrin: macrophage CR3 ($\alpha_M\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis. Cell* 61: 1375-1382.

Roberts, D.M., Lukas, T.J. and Watterson, D.M. (1986). Structure, function, and mechanism of action of calmodulin. *CRC Critical Reviews of Plant Sciences* 4: 311-339.

Roberts, K. (1990). Structures at the plant cell surface. *Current Opinions in Cell Biology* 2: 920-928.

Ruoslathi, E. and Pierschbacher, M.D. (1986). Arg-Gly-Asp: A versatile cell recognition signal. *Cell* 44: 517-518.

Russell, D.G. and Wright, S.D. (1988). Complement receptor type 3 (CR3) binds to an Arg-Gly-Asp-containing region of the major surface glycoprotein, gp63, of *Leishmania* promastigotes. *Journal of Experimental Medicine* 168: 279-292.

Sanders, L.C., Wang, C.-S., Walling, L.L. and Lord, E.M. (1991). A homolog of the substrate adhesion molecule vitronectin occurs in four species of flowering plants. *The Plant Cell* 3: 629-635.

Schindler, M., Meiners, S. and Cheresh, D.A. (1989). RGD-dependent linkage between plant cell wall and plasma membrane: Consequences for growth. *The Journal of Cell Biology* 108: 1955-1965.

Showalter, A.W. (1993). Structure and function of plant cell wall proteins. *The Plant Cell* 5: 9-23.

Stahl, D.J. and Schäfer, W. (1992). Cutinase is not required for fungal pathogenicity on pea. *The Plant Cell* 4: 621-629.

Suzuki, S., Oldberg, A., Hayman, E.G., Pierschbacher, M.D. and Ruoslathi, E. (1985). Complete amino-acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *The EMBO Journal* 4: 2519-2524.

Sweigard, J.A., Chumley, F.G. and Valent, B. (1992). Disruption of a *Magnaporthe grisea* cutinase gene. *Molecular and General Genetics* 232: 183-190.

van den Ackerveken, G.F.J.M., Dunn, R.M., Cozijnsen, T.J., Vossen, P., van den Broek, H.W.J. and de Wit, P.J.G.M. (1993a). Nitrogen limitation induces expression of the avirulence gene *avr9* in *Cladosporium fulvum*; a reflection of growth conditions *in planta*? Submitted.

van den Ackerveken, G.F.J.M., van Kan, J.A.L., Joosten, M.H.A.J., Muisers, J.M., Verbakel, H.M. and de Wit, P.J.G.M. (1993b). Characterization of two putative pathogenicity genes of the fungal tomato pathogen *Cladosporium fulvum*. *Molecular Plant-Microbe Interactions* 6: 210-215.

van Kan, J.A.L., van den Ackerveken, G.F.J.M. and de Wit, P.J.G.M. (1991). Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal tomato pathogen *Cladosporium fulvum*, causal agent of tomato leaf mold. *Molecular Plant-Microbe Interactions* 4: 52-59.

Wagner, V.T. and Matthysse, A.G. (1992). Involvement of a vitronectin-like protein in attachment of *Agrobacterium tumefaciens* to carrot suspension culture cells. *Journal of Bacteriology* 174: 5999-6003.

Willis, D.K., Rich, J.J. and Hrabak, E.M. (1991). *hrp* genes of phytopathogenic bacteria. *Molecular Plant-Microbe Interactions* 4: 132-138.

Woloshuk, C.P. and Kolattukudy, P.E. (1986). Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of *Fusarium solani* f.sp. *pisi*. *Proceedings of the National Academy of Sciences USA* 83: 1704-1708.

Zhu, J.-K., Shi, J., Singh, U., Wyatt, S.E., Bressan, R.A., Hasegawa, P.M. and Carpita, N.C. (1993). Enrichment of vitronectin- and fibronectin-like proteins in NaCl-adapted plant cells and evidence for their involvement in plasma membrane-cell wall adhesion. *The Plant Journal* 3: 637-646.

Summary

The plant pathogenic fungus *Phytophthora infestans* (Mont.) de Bary is the causal agent of potato late blight, the most important and devastating fungal disease of potato. The molecular and cellular processes involved in pathogenicity of this hemibiotrophic fungus are poorly understood. The aim of the research described in this thesis was to gain more insight in these processes. A novel and unbiased approach was explored to isolate *P. infestans* genes which might have a function in pathogenesis. The approach was based on the assumption that the expression of pathogenicity genes of *P. infestans*, i.e. genes which are essential for the establishment and maintenance of basic compatibility or for the increase of disease severity, is specifically induced or significantly increased during pathogenesis. The strategy implies the isolation of *in planta* induced (*ipi*) genes by differential screening of a genomic library of *P. infestans*, subsequent characterization of their gene products, and elucidation of their function in pathogenesis.

By differential screening, nine distinct *in planta* induced genes were isolated, i.e. *ipiA*, *ipiB*, *ipiC*, *ipiD*, *ipiJ1*, *ipiJ2*, *ipiN*, *ipiO* and *ipiQ*. Expression studies revealed that the *ipiB* and *ipiO* genes have a transient expression pattern during pathogenesis with the highest levels in early stages of the interaction. The seven other *ipi*-genes show a continuous 5-10 fold increase in expression level during growth of the fungus in the host.

The *P. infestans* genes *ipiA*, *ipiB*, *ipiC* and *ipiO* were further characterized. Two of these appeared to encode ubiquitous proteins: *ipiA*, which was renamed *ubi3R*, encodes ubiquitin and *ipiC*, renamed *calA*, codes for calmodulin. Both proteins are highly conserved in eukaryotic organisms and have been shown to play important roles in basic cellular processes such as selective degradation of abnormal proteins (ubiquitin) and signal transduction (calmodulin). This is the first time that an increased expression of ubiquitin and calmodulin genes is found in association with pathogenicity of a plant pathogenic fungus. However, the significance of increased ubiquitin and calmodulin levels in *P. infestans* during pathogenesis remains to be resolved. The *ipiB* gene belongs to a gene family consisting of at least three highly homologous genes, *ipiB1*, *ipiB2* and *ipiB3*, which are clustered on the genome in a head-to-tail arrangement. The *ipiB* genes encode novel glycine-rich proteins (GRPs) of 301 (IPI-B1), 343 (IPI-B2), and 347 (IPI-B3) amino acids (aa) respectively. The primary structure of the IPI-B proteins is composed of a N-terminal signal sequence followed by a large, highly repetitive glycine-rich domain. The glycine-rich domain is 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, respectively. The characteristic primary structure is also found in plant GRPs of which some have been shown to be associated with the plant cell wall. The *P. infestans* genome contains two *ipiO* genes, *ipiO1* and *ipiO2*, which are closely linked and arranged in an inverted orientation. The *ipiO* genes encode two nearly identical 152 aa proteins which have no homology with any known sequence. As the IPI-B proteins, the IPI-O proteins contain a N-terminal signal sequence suggesting that they are excreted. In addition, the IPI-O proteins have a putative N-glycosylation site and a typical Arg-Gly-Asp (RGD)

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tripeptide motif. The RGD tripeptide is conserved in several mammalian extracellular adhesion proteins in which it functions as a cell attachment motif. Whether the IPI-O proteins have adhesion properties is unknown.

The four *in planta* induced *P. infestans* genes *ubi3R*, *calA*, *ipiB* and *ipiO*, are among the first of the limited group of oomycetous genes to be isolated and sequenced. As in most known oomycetous genes, the coding sequence of these genes is not interrupted by introns. Within 100 nucleotides upstream of their ATG start codon, a conserved motif matching the consensus sequence GCTCATTYYNCA(A/T)TTT was identified. Comparison of the 5' non-transcribed regions of eight distinct oomycetous genes revealed that this sequence motif surrounds the transcription start point of the majority of these genes, suggesting that oomycetous genes have a GCTCATTYYNCA(A/T)TTT sequence preference for transcription initiation.

The expression of the *ipiB* and *ipiO* genes was studied in more detail. The *ipiB* genes as well as the *ipiO* genes are expressed in germinating cysts prior to host penetration. During pathogenesis both genes are transiently expressed. The highest mRNA levels are found in early stages of infection. Also during initial stages of interactions of *P. infestans* with a race-specific resistant host and the nonhost *Solanum nigrum* L., the expression of the *ipiB* and *ipiO* genes is induced. During growth of the fungus *in vitro*, nutrient deprivation appeared to be a strong stimulus for the induction of *ipiB* and *ipiO* gene expression. For several other pathogenesis related genes characterized in fungal plant pathogens it has been shown that nutrient starvation induces their expression. Whether starvation conditions mimic the nutrient condition encountered upon infection and, as such, function as a general stimulus for the activation of pathogenicity genes remains to be established.

Manipulation of gene expression in *P. infestans* is an important tool to study gene function. The possibility to manipulate gene expression in *P. infestans* by anti-sense RNA was tested. First, the promoters of five oomycetous genes, among which the *P. infestans ubi3R* gene, were fused to the reporter ß-glucuronidase (*GUS*) gene of *E. coli* and the activity of the promoters was determined in transient expression assays as well as in stable transformants. The *hsp70* and *ham34* genes of *Bremia lactucae* appeared to have the highest activity. When the *GUS* gene in the anti-sense orientation fused to the *ham34* promoter, is introduced in transgenic *P. infestans* containing sense *GUS* constructs, the accumulation of GUS is effectively inhibited. This indicates that the anti-sense technique is suitable to study gene function in *P. infestans*.

Samenvatting

De plantepathogene schimmel *Phytophthora infestans* (Mont.) de Bary is de veroorzaker van de aardappelziekte, één van de belangrijkste schimmelziekten van de aardappel. Van moleculaire en cellulaire processen die een rol spelen in de pathogenese van deze hemibiotrofe schimmel is nog maar weinig bekend. Het in dit proefschrift beschreven onderzoek was gericht op het verkrijgen van meer inzicht in deze processen. Er is een nieuwe benadering gebruikt om genen van *P. infestans* te isoleren waarvan de genproducten mogelijk een functie hebben in de pathogenese. De methode is gebaseerd op de aanname dat de expressie van pathogeniteitsgenen, i.e. genen van de schimmel die essentieel zijn voor het aangaan en handhaven van een compatibele interactie met de waardplant, specifiek geïnduceerd wordt of toeneemt tijdens de pathogenese. De strategie omvat de isolatie van *in planta* geïnduceerde genen (zogenaamde *ipi*-genen) door middel van differentiële screening van een genomische bank van *P. infestans*, het karakteriseren van de producten van deze genen en het bestuderen van hun functie tijdens het infectieproces.

Via differentiële screening werden negen verschillende *ipi*-genen geïsoleerd: *ipiA*, *ipiB*, *ipiC*, *ipiD*, *ipiJ1*, *ipiJ2*, *ipiN*, *ipiO* en *ipiQ*. Expressiestudies hebben aangetoond dat de *ipiB* en *ipiO* genen sterk tot expressie komen in het beginstadium van de interactie en niet, of heel zwak, in latere stadia. De expressie van de zeven andere *ipi*-genen vertoont een 5-10 voudige toename gedurende alle stadia van de interactie.

De *P. infestans* genen *ipiA*, *ipiB*, *ipiC* en *ipiO* zijn verder gekarakteriseerd. *IpiA* en *ipiC* coderen voor twee evolutionair sterk geconserveerde eiwitten, ubiquitine en calmoduline. De desbetreffende genen worden respectievelijk *ubi3R* en *calA* genoemd. Beide eiwitten komen voor in alle eukaryote organismen en spelen een belangrijke rol in fundamentele cellulaire processen zoals bijvoorbeeld de selectieve afbraak van abnormale eiwitten (ubiquitine) en signaaltransductie (calmoduline). Het is voor het eerst dat een toename in de expressie van ubiquitine en calmoduline genen gevonden is in associatie met de pathogenese van een plantepathogene schimmel. Waarom de hoeveelheid ubiquitine en calmoduline in *P. infestans* toeneemt tijdens de pathogenese is nog onduidelijk. Het *ipiB* gen behoort tot een genfamilie bestaande uit ten minste drie homologe genen, *ipiB1*, *ipiB2* en *ipiB3*, die op het genoom achter elkaar in een cluster liggen. De *ipiB* genen coderen voor glycine-rijke eiwitten met een lengte van respectievelijk 301 (IPI-B1), 343 (IPI-B2) en 347 (IPI-B3) aminozuren. De primaire structuur van de IPI-B eiwitten bestaat voornamelijk uit een N-terminaal signaalpeptide, wat er op duidt dat deze eiwitten worden uitgescheiden, en een sterk repeterend glycine-rijk domein. Dit glycine-rijk domein heeft twee prominente aminozuur motieven met de basissequenties A/V-G-A-G-L-Y-G-R en G-A-G-Y/V-G-G. Deze karakteristieke primaire structuur is ook gevonden in glycine-rijke eiwitten die voorkomen in planten en waarvan sommigen geassocieerd zijn met de celwand. *P. infestans* heeft twee *ipiO* genen, *ipiO1* en *ipiO2*, die gekoppeld en in een omgekeerde oriëntatie op het genoom liggen. De *ipiO* genen coderen voor twee bijna identieke eiwitten van 152 aminozuren en hebben geen homologie met enig ander bekend eiwit. Net als de IPI-B eiwitten hebben de

IPI-O eiwitten een N-terminaal signaalpeptide wat er op wijst dat deze eiwitten worden uitgescheiden. Daarnaast hebben de IPI-O eiwitten een mogelijke N-glycosyleringsplaats en een typisch Arg-Gly-Asp (RGD) tripeptide motief. Dit RGD tripeptide is een geconserveerd motief in een aantal dierlijke extracellulaire eiwitten die een rol spelen in celadhesie. Voor de celadhesie eigenschappen van deze eiwitten is het RGD motief cruciaal. Het is nog niet bekend of de IPI-O eiwitten celadhesie eigenschappen bezitten.

De vier *in planta* geïnduceerde genen van *P. infestans*, *ubi3R*, *calA*, *ipiB* en *ipiO*, behoren tot de zeer beperkte groep van genen die geïsoleerd en gekarakteriseerd zijn uit schimmels die behoren tot de klasse der Oömyceten. Net als de meeste andere tot nu toe beschreven oömycetengenen, bevatten deze vier genen geen introns. In de eerste 100 nucleotiden stroomopwaarts van de ATG start codons is een geconserveerd motief aanwezig met de consensus sequentie GCTCATTYYNCA(A/T)TTT. Dit motief bleek ook aanwezig te zijn in het promotergebied van zes andere oömycetengenen. Nadere bestudering van de positie van dit motief wees uit dat het in de meeste gevallen rondom de transcriptiestartplaats van de desbetreffende genen gelokaliseerd is. Dit suggereert dat de sequentie GCTCATTYYNCA(A/T)TTT mogelijk een rol speelt bij de initiatie van transcriptie van oömycete genen.

De expressie van de *ipiB* en *ipiO* genen is in meer detail bestudeerd. Zowel de expressie van de *ipiB* genen als dat van de *ipiO* genen wordt geïnduceerd in kiemende cysten voordat de waardplant wordt gepenetreerd. Tijdens de interactie met de waardplant komen beide genen voornamelijk in de vroege stadia van het infectieproces sterk tot expressie. In een later stadium neemt de expressie af tot een laag of niet detecteerbaar nivo. Tijdens de beginstadia van een incompatible interactie en een interactie tussen *P. infestans* en de niet-waardplant *Solanum nigrum* L. komen beide genen ook sterk tot expressie. Verder werd aangetoond dat de expressie van beide genen geïnduceerd wordt als de schimmel tijdens groei *in vitro* wordt blootgesteld aan nutriëntenlimitatie. Ditzelfde fenomeen is ook waargenomen bij pathogenese gerelateerde genen van enkele andere plantepathogene schimmels. Het suggereert dat voedsellimitatie een algemeen voorkomend mechanisme is voor de inductie van de expressie van pathogeniteitsgenen in plantepathogene schimmels.

Manipulatie van genexpressie is een belangrijk hulpmiddel bij de bestudering van de functie van bepaalde genen. De anti-sense RNA techniek, één van de methoden om genexpressie te manipuleren, is onderzocht op toepasbaarheid in *P. infestans*. De activiteit van de promoters van vijf oömycetengenen, waaronder het *P. infestans ubi3R* gen, is getest in protoplasten en in stabiele transformanten. Hierbij is gebruik gemaakt van fusies tussen de desbetreffende promoters en de coderende sequentie van het ß-glucuronidasegen (GUS-gen) van *E. coli*. De promoters van de *Bremia lactucae* genen *hsp70* en *ham34* bleken de hoogste activiteit te vertonen. Transformatie van constructen bestaande uit het GUS-gen in de anti-sense oriëntatie en de *ham34* promoter naar transgene *P. infestans* stammen met daarin een actief GUS-gen resulteerde in een effectieve remming van de GUS activiteit. Hieruit blijkt dat de anti-sense RNA techniek een bruikbare methode is om genexpressie in *P. infestans* te manipuleren.

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Account

The contents of this thesis are based on the following papers:

Judelson, H.S., Dudler, R., Pieterse, C.M.J., Unkles, S.E. and Michelmore, R.W. (1993). Expression and antisense inhibition of transgenes in *Phytophthora infestans* is modulated by choice of promoter and position effects. *Gene*, in press.

Pieterse, C.M.J., Risseeuw, E.P. and Davidse, L.C. (1991). An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Molecular Biology* **17**: 799-811.

Pieterse, C.M.J., Risseeuw, E.P. and Davidse, L.C. (1991). An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *In*: Hennecke, H. and Verma, D.P.S. (Eds.), Advances in Molecular Genetics of Plant-Microbe Interactions, vol. I, pp. 272-275.

Pieterse, C.M.J., de Wit, P.J.G.M. and Govers, F. (1992). Molecular aspects of the potato-*Phytophthora infestans* interaction. *Netherlands Journal of Plant Pathology* **98** Supplement 2: 85-92.

Pieterse, C.M.J., Derksen, A.-M.C.E., Folders, J. and Govers, F. (1993). Expression of the putative pathogenicity genes *ipiB* and *ipiO* of *Phytophthora infestans in planta* and *in vitro*. Submitted.

Pieterse, C.M.J., Riach, M.B.R., Bleker, T., van den Berg-Velthuis, G.C.M. and Govers, F. (1993). Isolation of putative pathogenicity genes of the potato late blight fungus *Phytophthora infestans* by differential hybridization of a genomic library. *Physiological and Molecular Plant Pathology*, in press.

Pieterse, C.M.J., van West, P., Verbakel, H.M., Brassé, P.W.H.M., van den Berg-Velthuis, G.C.M and Govers, F. (1993). Structure and genomic organization of the *ipiB* and *ipiO* gene clusters of *Phytophthora infestans*. *Gene*, in press.

Pieterse, C.M.J., Verbakel, H.M., Hoek Spaans, J., Davidse, L.C. and Govers, F. (1993). Increased expression of the calmodulin gene of the late blight fungus *Phytophthora infestans* during pathogenesis on potato. *Molecular Plant-Microbe Interactions* **6**: 164-172.

Curriculum vitae

Corné Pieterse werd geboren op 11 februari 1964 te Leimuiden. In 1981 behaalde hij het HAVO diploma aan het Ashram College te Alphen aan den Rijn en vervolgens in 1983 het VWO diploma aan ditzelfde College. Aansluitend begon hij met de studie Plantenveredeling aan de toenmalige Landbouwhogeschool te Wageningen alwaar hij in november 1988 *cum laude* afstudeerde. De ingenieursstudie omvatte als afstudeervakken Somatische Celgenetica (dr ir M. Koornneef) en Moleculaire Biologie (dr ir F. Govers). De stage werd doorgebracht bij het MSU-DOE Plant Research Laboratory te East Lansing, USA (dr N.V. Raikhel). In december 1988 begon hij bij de vakgroep Fytopathologie van de Landbouwuniversiteit met het promotieonderzoek waarvan de resultaten staan beschreven in dit proefschrift. Vanaf 1 april 1993 is hij als wetenschappelijk medewerker werkzaam bij de vakgroep Botanische Oecologie en Evolutiebiologie (projectgroep Fytopathologie) van de Universiteit Utrecht.

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