THE ROLE OF HETEROTRIMERIC G-PROTEINS IN DEVELOPMENT AND VIRULENCE OF *PHYTOPHTHORA INFESTANS*.

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The role of heterotrimeric G-proteins in development and virulence of *Phytophthora infestans*.
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Cover image: light micrograph showing sporangia, cleaving sporangia, zoospores, cysts, germinating cysts and germinating cysts forming appressoria, of *P. infestans* strain 88069. Magnification 1000x

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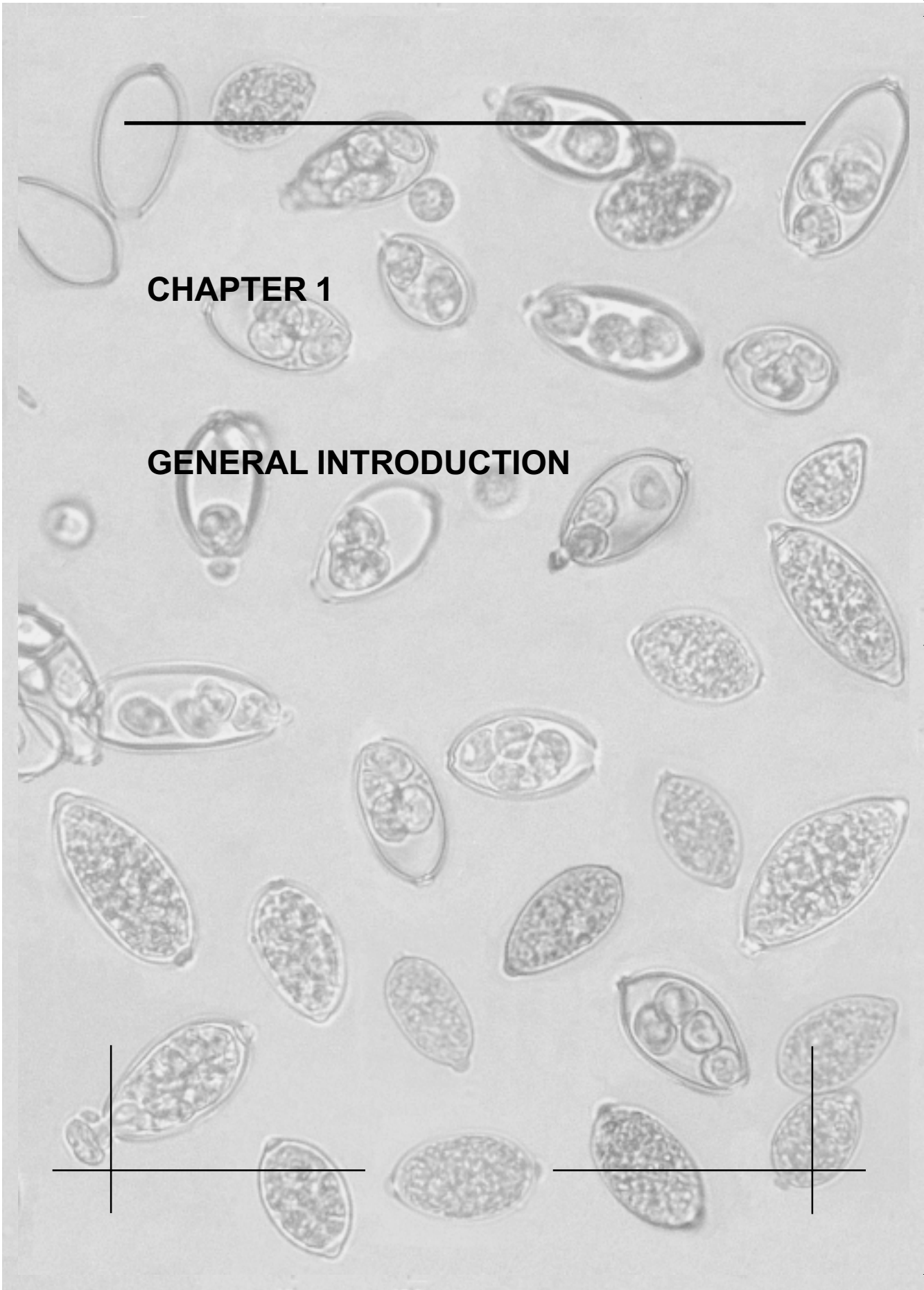
'Almost all aspects of life are engineered at the molecular level, and without understanding molecules we can only have a very sketchy understanding of life itself.'

Francis Crick

contents



CHAPTER 1

GENERAL INTRODUCTION

PHYTOPHTHORA INFESTANS

Potato Late Blight

The first reports of late blight epidemics date from the 1840s. At that time, the disease spread from Mexico to the Northeastern United States and to Europe, where it wiped out entire potato crops in a few successive years (1845-1846) (reviewed in Ristaino, 2002). Because potato was the staple crop in these days, 1.5 million people died of hunger, especially in Ireland, and a large proportion of the Irish population left the country to try their luck elsewhere. This disaster is recorded in history as the Irish potato famine. Although several people before him had proposed that late blight was caused by a microbe, Anton de Bary (1876) was the first to show conclusively that a fungus, which he named *Phytophthora infestans*, was the causal agent of the disease, hence the official name *Phytophthora infestans* (Mont.) de Bary. More than a hundred years later it became clear that *Phytophthora* is not a true fungus but instead belongs to a class that is evolutionary unrelated to fungi (see below).

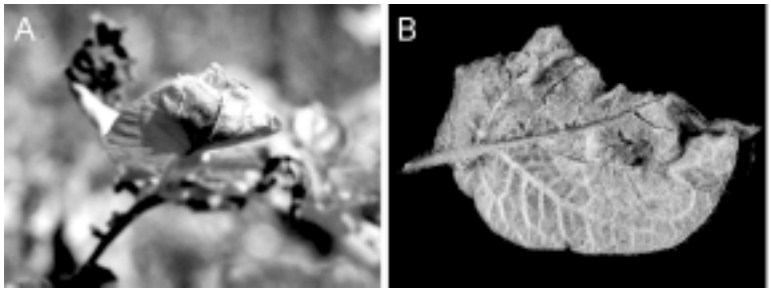


Figure 1.
Symptoms of late blight on potato. (**A**) Leaves of a blighted potato plant turn black and shrivel up. (**B**) A closer look at a lesion on a single potato leaflet. This lesion is covered with a white layer of asexual spores that emerge from the mycelium.

Until the 1980s, only the A1 mating type of *P. infestans* was detected outside Mexico and it is thought that until that time the global population of *P. infestans* outside Central and South America was derived from one single clonal lineage (Goodwin et al., 1994). Since then, the genetic variation within *P. infestans* populations has increased significantly in most parts of the world (Spielman et al., 1991; Fry et al., 1993). This was due to the spreading of the A2 mating type throughout the world in the early 1980s, giving rise to new recombinant types (Drenth et al., 1994; Sujkowsky et al., 1994). The newer genotypes of *P. infestans* are even more aggressive than the old isolates (Day and Shattock, 1997). For that reason, late blight has stayed in the top five of the economically most important plant diseases, causing billions of dollars of crop losses and costs for control measures annually (Schwinn and Staub, 1999).

In the first half of the twentieth century, the wild *Solanum* species *S. demissum* was used as a source of late blight resistance genes. A series of 11 resistance genes was successively crossed into *S. tuberosum*. These genes confer resistance to *P. infestans* based on the gene-for-gene interaction (Keen, 1990). However, each of the genes succumbed to attack by *P. infestans* within a few years. It was only in 2002 that the cloning of the first of these resistance genes, the *R1* gene, conferring resistance to *P. infestans* strains carrying the avirulence gene *Avr1,* was reported (Ballvora et al., 2002). This gene encodes a protein of the NBS-LRR type (Dangl and Jones, 2001). Breeding programs subsequently focused on developing cultivars with resistance based on multiple genes, often referred to as horizontal resistance. However, the idea that horizontal resistance and single-gene resistance are truly distinct is being challenged (Vleeshouwers et al., 2000; Kamoun et al., 1999). In the late 19th century a chemical based on copper sulphate, known as Bordeaux mixture, was introduced to control late blight. The mixture was in use for decades and is still occasionally applied. Metalaxyl and the carbamate fungicides are nowadays the most commonly used fungicides (Schwinn and Staub, 1999).

The disease and life cycle of *Phytophthora infestans*

The host range of *P. infestans* is limited to potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*). A few other *Solanum* species can be infected to a limited extent (Erwin and Ribeiro, 1996). *P. infestans* infection generally starts in the foliage and occasionally on stems. The disease spreads optimally under cool, moist weather conditions. Leaf symptoms consist of water-soaked, dark lesions that expand rapidly resulting in total destruction of the plant in a few days (Figure 1). From the foliage, the disease spreads to the tubers and causes a wet rot before or after harvest (Erwin and Ribeiro, 1996).

Sporangia, the vegetative spores, are spread by wind or in water drops. When they land on a host they either germinate directly or undergo cytoplasmic cleavage to form 7 to 8 swimming zoospores, depending on environmental conditions (Figure 2). Zoospores are attracted to the host where they halt, retract their flagella and secrete materials to form a cell wall. This process is referred to as encystment. A sporangium or cyst germinates and the germ tube can differentiate into an appressorium at the tip. An appressorium is a swelling of the germ tube from which a penetration peg emerges to breach the plant cuticle and cell wall. After having passed the epidermis, the hyphae grow mainly intercellularly and form haustorium-like feeding structures that protrude into mesophyll cells. In later stages, the pathogen adopts a necrotrophic lifestyle: it kills plant cells and feeds on dead tissue. Once in this phase, sporangia are formed on branched hyphae called sporangiophores that stick out to the lesion surface (Vleeshouwers et al., 2000).

P. infestans is heterothallic, meaning that two strains from different mating types are required to complete the sexual cycle. The male gametangial hyphal tip develops into an antheridium and the female gametangium into an oogonium. Meiosis occurs and the male haploid nuclei migrate into the oogonium, which then develops into a so-called oospore. The nuclei fuse during maturation of the oospore. Oospores are able to overwinter in soil (Erwin and Ribeiro, 1996).

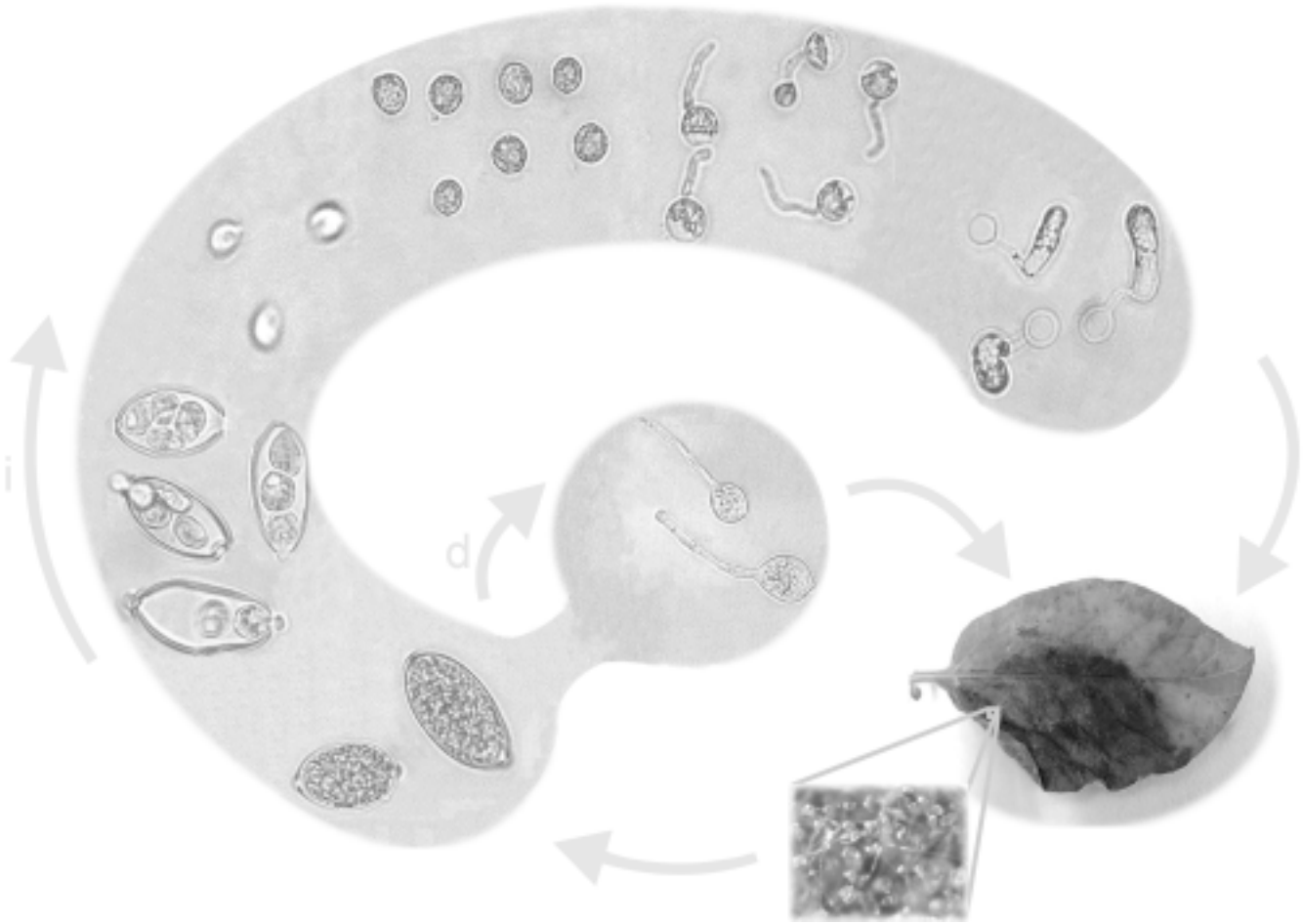


Figure 2.
The asexual lifecycle of *Phytophthora infestans*. Mycelium growing in the diseased leaf produces asexual spores named sporangia. Depending on the temperature, sporangia can either germinate directly (d) or indirectly by formation of zoospores (i). Once zoospores have reached the host, they retract their flagella and form a cell wall (encystment). Cysts germinate and the tip of the cyst germ tube develops into an appressorium that is required for penetration of leaf tissue.

Oomycetes

Phytophthora and related genera such as *Pythium* were traditionally classified as oomycetes within the kingdom Fungi (Erwin and Ribeiro, 1996). This is not surprising considering the fact that they grow as fungal-like mycelium and reproduce sexually and asexually by formation of spores. However, some features of *Phytophthora* and other oomycetes were not in agreement with their classification in the kingdom Fungi. For example, vegetative tissue of oomycetes is diploid whereas most fungi are haploid almost throughout their complete life cycle. Moreover, the cell wall compositions of oomycetes and fungi differ (Erwin and Ribeiro, 1996). In the mid-1980s, Cavalier-Smith was the first to place the oomycetes in a new kingdom which he designated the Chromista (Cavalier-Smith, 1986). Although the exact delimitation of the kingdom to which the oomycetes belong is still unclear, taxonomists nowadays agree on the principal feature of the classification: the presence of so-called heterokont flagella (two different flagella, one of which has two rows of lateral hair-like structures). They have placed the organisms possessing these flagella or organisms of which the ancestors possessed them, in the taxonomic group ' stramenopiles' (Dick, 1990; van de Peer and de Wachter, 1997; Margulis and Schwartz, 2000). In the meanwhile, molecular phylogenetic analyses, including DNA sequence comparisons, have confirmed that oomycetes and fungi are evolutionary unrelated (Figure 3). Differences and similarities between fungi and oomycetes are reviewed in more detail in chapter seven of this thesis.

The oomycetes include saprophytic species and pathogens of plants, animals and insects. Most plant pathogens are found in the order Peronosporales, including, among others, *Phytophthora, Pythium* and *Peronospora* as important genera. There are both aquatic and terrestrial species among the oomycetes. As many oomycetes form swimming zoospores it is hypothesized that they are derived from an aquatic ancestor (Dick, 2001). *Phytophthora* (Greek for 'destroyer of plants') is the best studied oomycete genus since it encompasses species causing severe diseases on economically important crops such as soybean, cocoa and potato and on valuable tree species such as Californian oak (Knight, 2002), Australian jarrah trees (Podger, 1972) and European alder trees (Brasier et al., 1999). Together, over 60 *Phytophthora* species have been identified and the total host range of the genus is expected to include thousands of plant species (Erwin and Ribeiro, 1996).

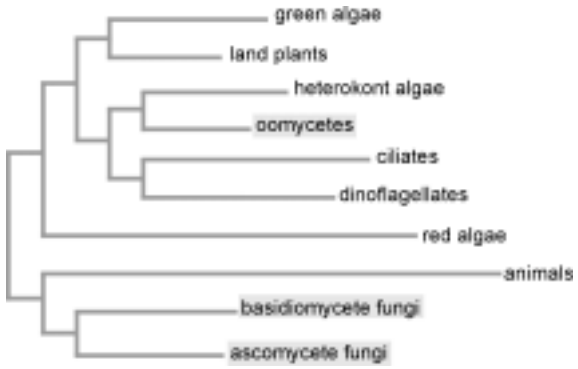


Figure 3.
Phylogenetic tree showing the evolutionary relationships between the major eukaryotic groups (adapted from van de Peer and de Wachter, 1997; Kamoun et al., 1999). Shaded in grey are the groups containing plant pathogens discussed in this thesis: the oomycetes and the ascomycetous and basidiomycetous fungi. Note the evolutionary distance between the oomycetes and fungi.

THE HETEROTRIMERIC G-PROTEIN SIGNALING PATHWAY

Heterotrimeric G-proteins and signal transduction

Cells have developed ways to transduce environmental signals from the outside to the appropriate locations inside the cell. Signals are transported by a series of proteins, signal transduction components, which regulate each other's activities, often through phosphorylation or dephosphorylation. Some signal transduction components produce small molecules called second messengers that also play a role in relaying the signal. Together, signal transduction components and second messengers form signal transduction pathways. The signal transduction pathway involving heterotrimeric G-proteins is probably the most ubiquitous and best studied among eukaryotes.

The first element of the heterotrimeric G-protein pathway is a receptor, located in the plasma membrane. Upon sensing the extracellular signal the receptor undergoes a conformational change. G-protein coupled receptors typically have seven trans-membrane (7TM) domains. In the activated conformation, the receptor binds the heterotrimeric G-protein, consisting of an α, β and γ subunit, and activates it by replacing Gα-bound GDP by GTP. The G-protein subsequently dissociates into the α subunit and the βγ dimer. Gα and Gβγ both activate or inactivate subsequent links in the pathway (Figure 4). The Gα subunit possesses a GTPase domain. It hydrolyzes GTP to form GDP resulting in inactivation and re-association of the three subunits (Gilman, 1987; Neer, 1995).

G-protein α subunits are ca. 40 kD proteins that are highly conserved in animals, fungi, plants and lower eukaryotes such as *Dictyostelium discoidium*. Most Gα subunits are palmitoylated and some are acetylated and/or myristoylated, and are attached to the inside of the plasma membrane. The first domain of the Gα subunit is the GTPase domain consisting of five α helices surrounded by six strands of β sheets. This domain contains the sites for receptor binding as well as for effector and Gβγ binding. The second domain, of which the function is not known, consists entirely of α helices

Figure 4.
Heterotrimeric G-protein activation: the paradigm. Binding of a ligand results in activation of the G-protein-coupled (seven-transmembrane) receptor. The receptor, in turn, activates the G-protein trimer by exchanging the nucleotide GDP for GTP on the G-protein's α subunit. The trimer subsequently dissociates in Gα and Gβγ. Inactivation follows after hydrolysis of GTP by Gα and re-association of Gα and Gβγ.

(reviewed in Neer, 1995).

The β subunits are well-conserved 36 kD proteins. The most conspicuous feature of β subunits is the presence of a domain composed of seven WD (tryptophan/aspartic acid) repeats. This domain has a propeller-like structure and is known to be involved in protein-protein interactions. Gγ subunits are far less conserved than the Gα and Gβ subunits. They are small proteins (6-9 kD) binding to one end of the propeller structure of the β subunits. An isoprenyl residue bound to Gγ attaches the βγ dimer to the membrane (Hamm and Gilchrist, 1996).

Effectors of heterotrimeric G-proteins

Heterotrimeric G-proteins are the switches between (G-protein-coupled) receptors and effector proteins. Receptors are highly specific, generally binding only a single type of ligand. G-proteins, in contrast, are non-specific. They interact with numerous different types of receptors and activate a wide range of effector proteins. How the required specificity is precisely achieved is not yet understood (Albert and Robillard, 2002).

Animals possess large numbers of different G-protein subunits. The scheme showing the signaling pathways downstream of G-proteins in animals has become a real spiderweb over the years (Figure 5). Numerous different routes were discovered, all influencing each other's activities (reviewed in Hamm and Gilchrist, 1996; Dhanasekaran et al., 1998). The most important signaling modules regulated by G-proteins include the cyclic adenosine monophosphate (cAMP) pathway, phospholipid signaling and MAP kinase signaling. Phospholipid signaling will be described in more detail in the next paragraph. cAMP is a second messenger produced by the enzyme adenylyl cyclase, which can be activated or inactivated by the Gα subunit or the Gβγ dimer. The level of cAMP regulates the activity of cAMP-dependent protein kinase (PKA), which consists of two identical regulatory and two identical catalytic subunits. Binding of cAMP to the regulatory subunits leads to dissocation of the regulatory and catalytic subunits. The monomeric form of the catalytic subunit is the active form, phosphorylating, among others, the transcription factor CREB (Alberts et al., 2002). This illustrates how G-protein signaling eventually alters gene expression.

The MAP kinase route forms a cascade of three kinases phosphorylating and thereby activating each other. This pathway is indirectly activated by G-proteins through regulation of protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) and/or the small GTPase Ras (Dhanasekaran et al., 1998). MAPKKK is the generic name for the first kinase in the cascade, MAPKK the second and MAPK is the last kinase in the cascade. Like PKA, MAPKs change the affinity of transcription factors for their promoter target sites (Ligterink and Hirt, 2001). Ion channels transporting calcium and potassium are also under control of G-proteins in animals. These channels play a role e.g. in the transmission of signals in neurons involved in taste, olfaction or vision (Brown and Birnbaumer, 1990).

Plants have only one or two different Gα subunits and the numbers of Gβ and Gγ subunit genes are also limited. The plant hormones auxin, gibberellin (GA) and abcisic acid (ABA) signal through G-proteins, activating the phospholipases A2, D and, possibly, phospholipase C, as well as cation and anion channels (reviewed in Assmann et al., 2002).

Fungi generally possess single Gβ and Gγ subunit genes and between 2 and 4 different Gα subunits (Bölker, 1998). In the budding yeast, *Saccharomyces cerevisiae* the G-protein heterotrimer, consisting of GPA1 (α) and STE4/STE18 (βγ), controls mating and dimorphic growth and is activated by pheromone receptors of the 7TM type. The Gα protein GPA2 is involved in nutrient sensing and is activated by a 7TM receptor that binds glucose (Kraakman et al., 1999). STE4/STE18 activates the protein kinase STE20, which in turn activates a MAP kinase cascade, whereas GPA2 feeds into the cAMP pathway (Lengeler et al., 2000).

Dictyostelium discoidium, a cellular slime mold, is a haploid organism and very amenable to molecular genetic manipulation. Twelve different Gα subunits and only a single Gβ and Gγ subunit have been identified in this organism (Zhang et al., 2001; Jin et al., 1998; P. van Haastert, personal communication). When starved for nutrients, the cells aggregate and form fruiting bodies. This aggregation is based on chemotaxis towards a cAMP gradient, a second messenger secreted by the cells themselves (Parent and Devreotes, 1996). 7TM receptors sensing the cAMP concentration in the environment couple to G-proteins, which in turn activate, amongst others, adenylyl and guanylyl cyclase (reviewed in Van Haastert and Kuwayama, 1997). Activation of G-proteins also results in activation of PI3K and translocation to the membrane of a PH-domain-containing protein called CRAC. This results in actin polymerization and other processes required to establish cell polarity and chemotaxis (reviewed in Weiner, 2002; Aubry and Firtel, 1999) (see also chapter 8).

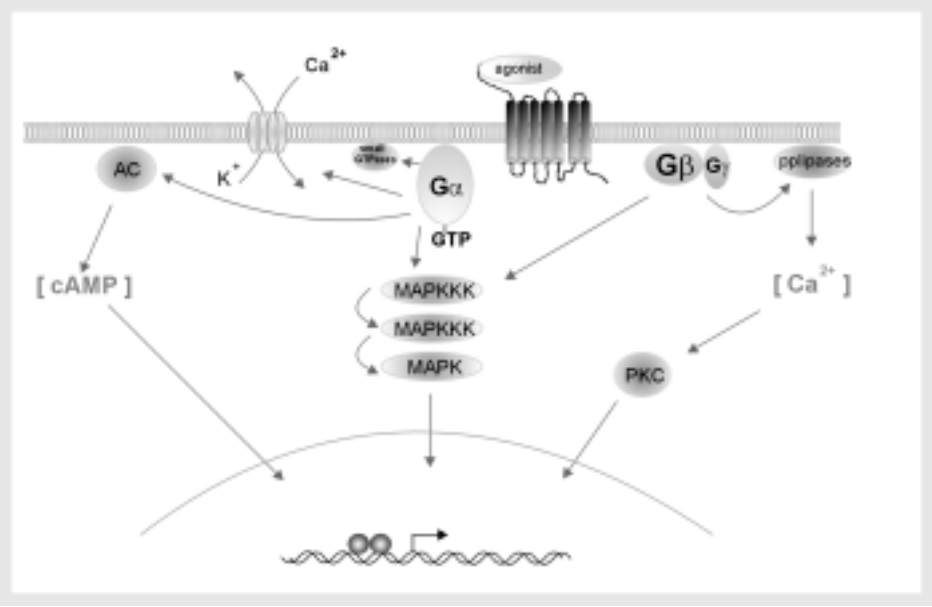


Figure 5.
Downstream effectors of Gα and Gβγ. This figure shows a simplified scheme of G-protein signaling in animals. Most targets can be activated both by Gα and Gβγ. Among the G-protein effectors are adenylyl cyclase (AC) and the cAMP pathway, several small GTPases, the MAPK cascade, phospholipid- and Ca^{2+} signaling and ion channels.

Phospholipase D

Cell membranes and lipid-modifying enzymes play important roles in signal transduction. Three types of phospholipases, phospholipase A2, C and D, have been characterized in detail. They hydrolyze phospholipids and generate second messengers. Phospholipase D (PLD) hydrolyzes structural phospholipids such as phosphatidylcholine, producing the second messenger phosphatidic acid (PA) and choline (Munnik et al., 1998) (Figure 6). In animal cells, PLD is involved in vesicle trafficking and is activated by the small GTPases ARF and RhoA in response to activation by receptor tyrosine kinases (Liscovitch et al., 2000). There are indications that PLD can be activated by heterotrimeric G-proteins in animals (Jones et al., 1999). Plant PLDs are implicated in responses to stress conditions including osmotic stress, drought, wounding and pathogen attack, but also in ABA and ethylene signaling (reviewed in Munnik and Musgrave, 2001). There is pharmacological evidence that PLD is located downstream of heterotrimeric G-proteins in plants (Munnik et al., 1995; Munnik et al., 1998). PLD has not been studied in much detail in fungi, except for the yeasts *S. cerevisiae* and *Candida albicans*. The *S. cerevisiae* PLD SPO14 is required for the formation of new membranes during sporulation. This may also reflect a role for SPO14 in vesicle transport (Rose et al., 1995; Rudge et al., 1998).

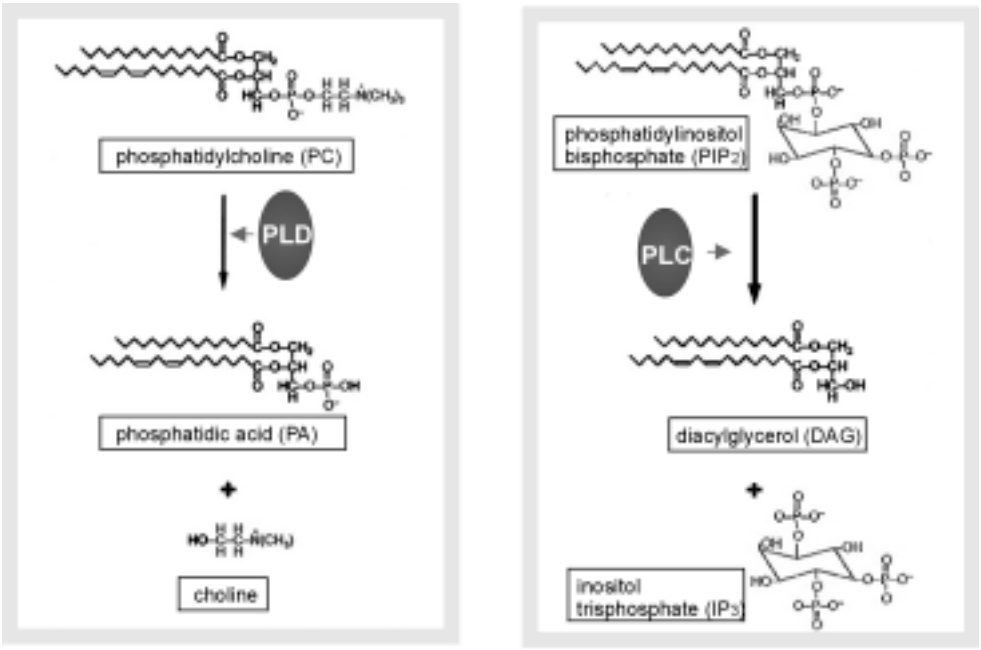


Figure 6.
Phospholipase signaling. Structural phospholipids such as phosphatidylcholine (PC) are hydrolyzed by Phospholipase D (PLD) (left panel). The two products are phosphatidic acid (PA), which is known to function as second messenger, and the headgroup choline. Phospholipase C (PLC) hydrolyzes phosphoinositol 4,5-bisphosphate (PIP_2) (right panel). Both products, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) function as second messengers (adapted from Assmann, 2002)

Table 1. Genes encoding Gα-protein subunits in plant pathogenic fungi*

organism	name of gene	biological function	reference
Magnaporthe grisea	*magA*	sexual development	Liu and Dean, 1997
	magB	conidiation, appressorium development	
	magC	conidiation, sexual development	
	mgb1 (Gβ)	conidiation, vegetative growth, appressorium formation	Nishimura et al., 2003**
Cryphonectria parasitica	*cpg1*	conidiation, pigmentation, female fertility	Choi et al., 1995
	cpg2	growth rate, conidiation	
	cpg3	unknown	Segers et al., 2003**
	cpgb1 (Gβ)	conidiation, pigmentation, female fertility, vegetative growth	Kasahara and Nuss, 1997
Ustilago maydis	*gpa1*	unknown	Regenfelder et al., 1997
	gpa2	unknown	
	gpa3	mating, dimorphic growth	
	gpa4	unknown	
Ustilago hordei	*fil1*	dimorphic switching	Lichter and Mills, 1997
Colletotrichum trifolii	*cgt1*	conidial germination, appressorium development	Truesdell et al., 2000
Botrytis cinerea	*bcg1*	vegetative growth, pigmentation, protease secretion	Gronover et al., 2001
	bcg2	virulence	
Cochliobolus heterostrophus	*cga1*	appressorium development, female fertility, ascospore viability	Horwitz et al., 1999
Fusarium oxysporum	*fga1*	conidiation, heat resistance	Jain et al., 2002
	fgb1	production of virulence factors, hyphal growth, virulence	Delgado et al., 2003**

* no genes encoding Gγ subunits have been cloned from plant-pathogenic fungi so far
** reported at the 22nd Fungal Genetics Conference, Asilomar CA, 18-23 March 2003 (Fungal Genet. Newsl. 50 (suppl.) pp 34; 84; 118 resp).

Heterotrimeric G-proteins in plant pathogenic fungi

The chestnut blight fungus *Cryphonectria parasitica*, the corn smut fungus *Ustilago maydis* and the rice blast fungus *Magnaporthe grisea*, have served as models for dissection of signal transduction pathways involved in fungus-plant interactions. They possess multiple Gα subunit genes that were isolated based on homology with the *S. cerevisiae* Gα subunits. In each of these fungi, at least one of the Gα subunits is important for virulence (reviewed in Kronstad, 1997; Bölker, 1998). Like the *S. cerevisiae* Gα subunit GPA1, the *U. maydis* Gα subunit Gpa3 is required for mating and filamentation (Regenfelder et al., 1997). The Gα subunit MagB from *M. grisea* is involved in conidiation, vegetative growth and appressorium formation (Liu and Dean, 1997), whereas CPG1 from *C. parasitica* plays a role in female fertility, conidiation and pigmentation (Choi et al., 1995).. The *C. parasitica* strains in which the Gβ subunit gene *cpgb1* was disrupted are reduced in conidiation and pigmentation, phenotypes reminding of those of the CPG1-deficient strain. In contrast to the CPG1-deficient strains, the Δ*cpgb1* strains showed increased and denser vegetative growth (Kasahara and Nuss, 1997). During the last few years, the functions of heterotrimeric G-proteins, especially of Gα subunits, were explored in many other plant pathogenic fungi (Table 1).

Levels of cAMP were altered in the Gα subunit knockouts of *M. grisea*, *U. maydis*, and *C. parasitica* and addition of cAMP restored normal development in these mutants. This showed that, like in yeast, G-proteins act at least on the cAMP pathway in these fungi. The analysis of knock-out mutants of the catalytic and regulatory subunits of cAMP-dependent protein kinase and of adenylyl cyclase further supported this notion. The cAMP and MAPK pathways are clearly interconnected in *U. maydis*. Together, they control mating and filamentation and they are both required for pathogenesis. In *M. grisea*, a MAPK called PMK1 is involved in appressorium formation (Xu and Hamer, 1996). Homologues of this MAPK have been identified in many other plant pathogenic fungi.

SCOPE OF THIS THESIS

Heterotrimeric G-proteins are crucial for plant pathogenic fungi to complete their developmental program in response to environmental cues (1). *P. infestans* belongs to the oomycetes, a class of organisms that evolved the ability to infect plants independently from true fungi (2). These two facts were both decisively established in the last decade of the twentieth century and form the rationale behind the research described in this thesis. The aim was to find out whether or not the G-protein pathway is present in *P. infestans* and, if so, to reveal the processes that are under control of this pathway. An important tool and prerequisite to carry out this research was developed by van West et al. (1999). They were the first to show that introduction of additional copies of the *inf1* gene resulted in reduced levels or abolished *inf1* expression in up to 20% of the transformants. This important finding opened the way to targeted mutagenesis to study gene function in *P. infestans*. Without this work it would have been impossible to create G-protein-deficient mutants and without G-protein-deficient mutants, it would have been utterly hard, if not impossible, to determine the function of the G-protein subunits in *P. infestans*.

The heterotrimeric G-protein pathway is conserved in *P. infestans*. This was readily settled by the cloning of the genes encoding a G-protein α and β subunit from *P. infestans* (**chapter 2**). The proteins encoded by these genes, PiGPA1 and PiGPB1, are very similar to the corresponding proteins in other eukaryotes. Both genes are differentially expressed, i.e., their expression varies between different stages in the pathogen's life cycle. This work was carried out in collaboration with Dr. Ana Laxalt. **Chapter 3** sheds light on the role of PiGPA1 in development and virulence of *P. infestans*. PiGPA1-deficient strains, obtained using gene silencing, were affected in normal development and severely affected in virulence on potato. In particular, the zoospores of these strains behaved aberrantly: instead of moving in straight lines as wildtype zoospores do, they continuously changed direction and failed to aggregate.

The function of the Gβ subunit PiGPB1 seems to be unrelated to the function of the Gα subunit PiGPA1, as concluded from the characteristic features of PiGPB1-deficient strains. These strains, similarly obtained by gene silencing, showed a defect in sporangium formation and produced more aerial mycelium on rye sucrose agar plates than wildtype (**chapter 4**). The results of this study suggest that PiGPB1 plays a role in induction of sporulation upon starvation.

Gene expression profiling using cDNA-AFLP was used to find genes of which the expression is controlled by PiGPA1 (**chapter 5**). Dr. Wubei Dong was my collaborator in this project. He compared the expression profiles in sporangia of wildtype and *Pigpa1*-silenced mutants. Some cDNA fragments were isolated that were downregulated in the mutants, whereas others were upregulated. These fragments may guide us to genes under control of PiGPA1 that may play a role in the processes that are affected in the *Pigpa1*-silenced mutants.

Under the supervision of Dr. Teun Munnik, PLD activity in *P. infestans* was characterized (**chapter 6**). This was done by biochemical means rather than by the genetic methods applied to study the role of the G-protein subunit genes (chapter 3 and

4). The G-protein agonists mastoparan and alcohol were shown to activate phospholipase D in various stages of the life cycle of *P. infestans*, suggesting that PLD functions downstream of G-proteins in this organism. This work also revealed a possible role for PLD in zoospore encystment.

In the chapters 2-6 it was shown that G-protein signaling plays a role in developmental processes and virulence in *P. infestans*. Strikingly, some of the processes affected in the *Pigpa1* or *Pigpb1*-silenced mutants (e.g. appressorium formation, sporulation) are also controlled by G-proteins in plant pathogenic fungi. This finding prompted us to make a more extensive comparison of infection strategies of plant pathogenic fungi and oomycetes based on known data (**chapter 7**). This overview shows that taxonomically unrelated organisms have independently developed similar structures and strategies to infect plants with few exceptions. Such a comparative study may be helpful in the design of novel methods to control oomycete plant diseases.

Chapters 2-6 are wrapped up in a general discussion presented in **chapter 8**. The experimental data are discussed in a broader context and we touch upon the advantages and disadvantages of applications of gene-silencing to study the functions of genes in *P. infestans*. Moreover, we discuss the impact our research might have on future directions in controlling late blight.

CHAPTER 2

DIFFERENTIAL EXPRESSION OF G-PROTEIN α AND β SUBUNIT GENES DURING DEVELOPMENT OF *PHYTOPHTHORA INFESTANS*

Ana María Laxalt, Maita Latijnhouwers, Marieke van Hulten and Francine Govers

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ABSTRACT

A G-protein α subunit gene (*Pigpa1*) and a G-protein β subunit gene (*Pigpb1*) were isolated from the oomycete *Phytophthora infestans,* the causal agent of potato late blight. Heterotrimeric G-proteins are evolutionary conserved GTP-binding proteins that are composed of α, β, and γ subunits and participate in diverse signal transduction pathways. The deduced amino acid sequence of both, *Pigpa1* and *Pigpb1,* showed the typical conserved motifs present in Gα or Gβ proteins from other eukaryotes. Southern blot analysis revealed no additional copies of Gα or Gβ subunit genes in *P. infestans* suggesting that *Pigpa1* and *Pigpb1* are single copy genes. By cross-hybridization, homologues of *Pigpa1* and *Pigpb1* were detected in other *Phythophthora* species. Expression analyses revealed that both genes are differentially expressed during asexual development with the highest mRNA levels in sporangia. In mycelium, no *Pigpa1* mRNA was detected. Western blot analysis using a polyclonal PiGPA1 antibody confirmed the differential expression of *Pigpa1*. These expression patterns suggest a role for G-protein mediated signaling during formation and germination of asexual spores of *P. infestans*, developmental stages representing the initial steps of the infection process.

INTRODUCTION

Living organisms sense and respond to the external environment through the activation of signaling pathways. In eukaryotes key regulators in many signaling pathways are the evolutionary conserved heterotrimeric GTP-binding proteins or G-proteins. A G-protein complex is composed of an α, β and γ subunit and can be linked to heptahelical transmembrane receptors (Gilman 1987; Simon et al., 1991; Neer, 1995; Borkovich, 1996). Ligand perception by these receptors results in activation of G-proteins and eventually in activation of regulatory cascades that induce changes in gene expression, cellular function, and metabolism (Dohlman et al., 1987). In the inactive form, the G-protein α subunit binds GDP and the Gβγ dimer with high affinity, forming the Gαβγ complex. Activation results in the exchange of GDP for GTP and the dissociation of the G-protein in α-GTP and a βγ heterodimer. Both the α-GTP complex and the free βγ dimer have been shown to interact with, and alter the activity of a diverse set of effector molecules including adenylyl cyclase, ion channels, phospholipases and phosphodiesterase (Clapham and Neer, 1993; Hamm and Gilchrist, 1996). Hydrolysis of GTP to GDP results in reassociation of the α-GDP subunit and the βγ heterodimer into the inactive Gαβγ heterotrimer.

In higher eukaryotes, G-protein signaling has been implicated in mediating complex biological processes such as the perception of taste, smell and light, hormone action, chemotaxis, neurotransmission, and cell proliferation and differentiation (Dohlman et al., 1991). Also in lower eukaryotes many processes involve G-protein signaling. Studies on several ascomycetous and basidiomycetous fungi revealed a role for G-proteins in fungal development and virulence (reviewed by Lengeler et al., 2000). For example, Ivey et al. (1996) showed that targeted disruption of the $G\alpha_i$ subunit gene

gna-1 of the saprophytic fungus *Neurospora crassa*, resulted in multiple phenotypes during the asexual and sexual life cycle. Similarly, in *Aspergillus nidulans* disruption of the Gα subunit gene *fadA* or the Gβ subunit gene *sfaA*, affected sexual development and conidiation (Hicks et al., 1997; Rosén et al., 1999). In the plant pathogenic corn smut fungus *Ustilago maydis* (Regenfelder et al., 1997), disruption of one of the four Gα genes, *gpa-3*, resulted in loss of pheromone responsiveness and virulence. Disruption of Gα subunit genes in the rice blast fungus *Magnaporthe grisea* (Liu and Dean, 1997) demonstrated that in this pathogen G-protein signaling pathways control vegetative growth, conidiation, conidium attachment, appressorium formation, mating and pathogenicity. Similar results were obtained with the chestnut blight fungus *Cryphonectria parasitica* where disruption of the Gα subunit gene *cpg-1* and the Gβ subunit gene *cpgb-1* resulted in mutants defective in virulence and with aberrant vegetative growth behavior (Gao and Nuss, 1996; Kasahara and Nuss, 1997). Furthermore, Gα mutants of the anthracnose causing fungus *Colletotrichum trifolii* failed to germinate (Truesdell et al., 2000) and those of the corn pathogen *Cocchliobolus heterostrophus* had reduced ability to form appressoria and were female sterile (Horwitz et al., 1999).

The subject of our studies is *Phytophthora infestans*, the causal agent of late blight and one of the most devastating pathogens of potato. *P. infestans* belongs to the oomycetes, a unique group of eukaryotes that includes significant pathogens of plants, insects and animals. The genus *Phytophthora* comprises more than sixty species, which are all serious plant pathogens (Erwin and Ribeiro, 1996). *P. infestans* has a limited host range with potato and tomato as the most important hosts. In contrast, other *Phytophthora* species can have a very broad host range; *P. nicotianae* and *P. palmivora*, for example, infect many plant species belonging to different families. Oomycetes have a fungus-like growth morphology but share little taxonomic affinity to filamentous fungi. This implies that oomycetous plant pathogens have evolved the ability to infect plants completely independently from true fungi. Yet, the way fungi and oomycetes infect and colonize plants is very similar. The evidence that G-protein signaling is involved in fungal pathogenicity suggests that also in oomycetes G-proteins may be crucial. As an extension of our studies on pathogenicity of *P. infestans* we now report the cloning and characterization of the first oomycetous Gα and Gβ subunit genes and show that these genes are differentially expressed during the asexual life cycle with the highest expression in sporangia.

RESULTS

Isolation and characterization of *Phytophthora infestans* Gα and Gβ subunit genes

Degenerate primers corresponding to conserved regions in Gα subunit genes were used for amplification using the polymerase chain reaction (PCR) on *P. infestans and P. nicotianae* genomic DNA. Amplification was achieved with *P. nicotianae* DNA but not with *P. infestans* DNA. DNA sequencing revealed that the amplified *P. nicotianae* fragment was 154 base pairs long and that the deduced amino acid sequence was up to 68% identical to the comparable region in G-protein α subunits of other organisms.

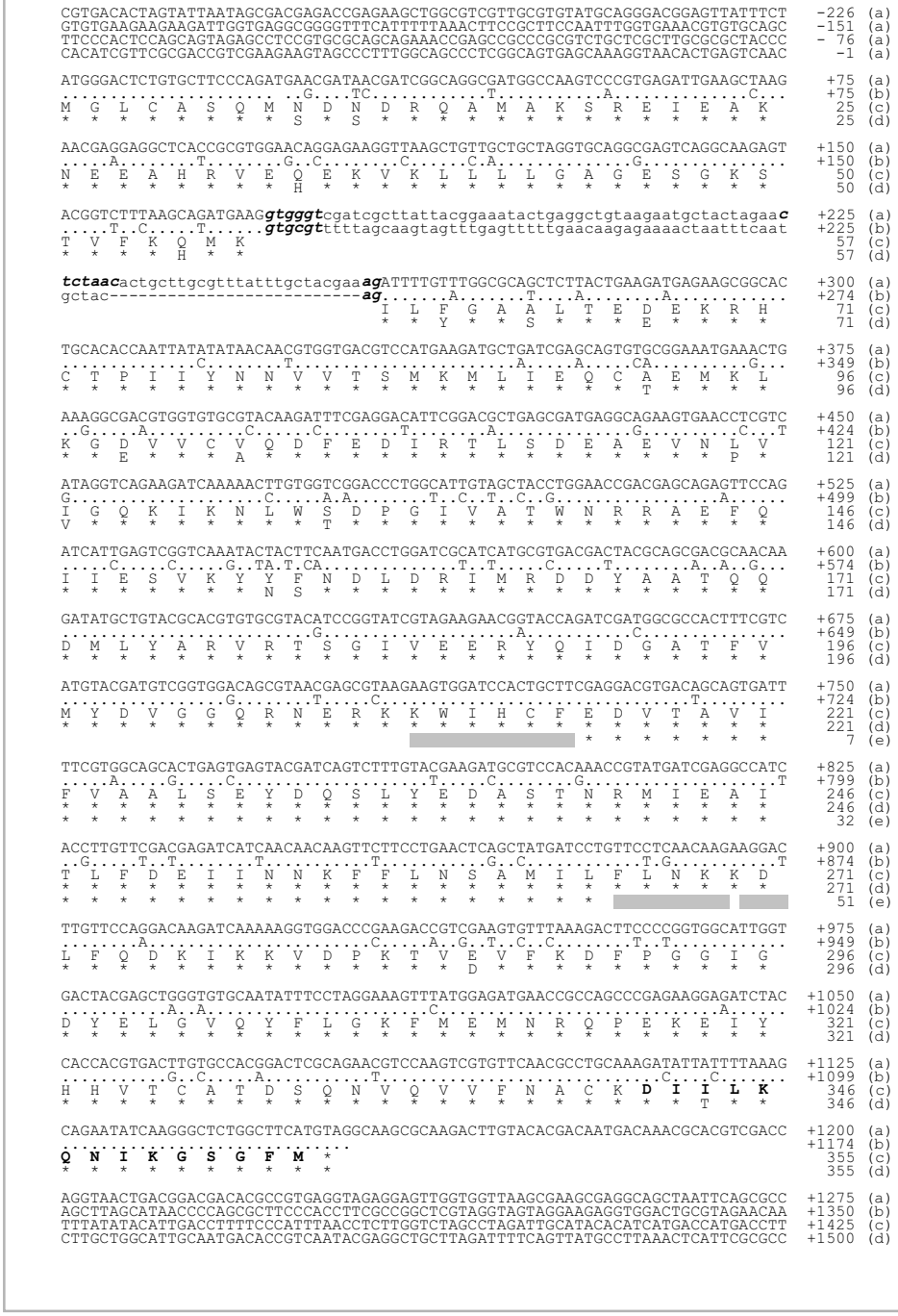
This fragment was used to screen a *P. infestans* EMBL3 genomic library. Subclones from one of the most intensively hybridizing phages (λP8) containing the putative gene were sequenced. The nucleotide sequence of the region containing the gene of interest is shown in Figure 1. The deduced amino acid sequence of the putative gene revealed open reading frames with significant regions of homology to deduced amino acid sequences of several other Gα subunit genes. The gene was designated *Pigpa1* (*Phytophthora infestans* G-protein α-subunit gene; accession number AY050536). Sequence comparison showed that the degenerate reverse primer used for the initial PCR on genomic DNA has three mismatches with the *Pigpa1* sequence and this may explain why we were not successful in amplifying the corresponding fragment on *P. infestans* DNA. Another explanation may lie in the fact that the genome size of *P. infestans* is 3-4 fold larger than that of *P. nicotianae*.

A cDNA clone corresponding to *Pigpa1* was obtained by PCR using cDNA synthesized on sporangia mRNA as template in combination with specific primers that were designed based on the *Pigpa1* sequence (Figure1). Comparison of the cDNA sequence and the genomic sequence revealed that *Pigpa1* contains an intron between position 171 to 258. In *P. infestans* genes introns are rare. The first 6 and the last 2 nt of the intron match consensus sequences for 5′ and 3′ intron splice sites in filamentous fungi (GTPuNGPy and PyAG, respectively; Unkles 1992) and at 27 bp from the 3′ splice junction the intron contains a 7 bp sequence, CTCTAAC, which has 5 out of 7 nucleotides identical to the yeast branch point consensus sequence TACTAAC that is necessary for intron splicing (Unkles, 1992). The intron position in *Pigpa1* is not identical with intron positions in other Gα protein subunit genes. Finally, *Pigpa1* lacks the consensus sequence that surrounds the transcription start site of several *Phytophthora* genes identified to date (Govers et al., 1997).

By PCR on genomic DNA of *Phytophthora palmivora* with 5′ and 3′ specific *Pigpa1* primers a *P. palmivora* homologue of *Pigpa1* was obtained and designated *Ppgpa1* (accession number AY050537). At the amino acid acid level PiGPA1 and PpGPA1 are 95% identical (Figure 1). The intron in *Ppgpa1* is at the same position but it is shorter, 61 instead of 87 bp, and has a completely different sequence than the intron in *Pigpa1*. The 5′ and 3′ intron splice sites again match the consensus but an obvious yeast branch point consensus sequence is lacking.

One EST with high homology to known Gβ subunit genes was found among 1000 partial cDNA sequences derived from genes expressed in mycelium (Kamoun et al., 1999). The full length gene corresponding to EST MY-09-H-10 was isolated from a BAC library and sequenced (accession number AY050538), and was designated *Pigpb1*. The open reading frame is 1032 bp without introns. In the cDNA clone the ORF is followed by a 78 bp 3′ untranslated region preceding the polyA tail. As *Pigpa1, Pigpb1* lacks the consensus sequence at the transcription start site.

Various organisms contain more than one Gα and Gβ subunit gene (Simon et al., 1991). To determine if also *P. infestans* has multiple Gα and Gβ subunit genes Southern blot hybridizations were performed using a *Bam*HI-*Sal*I fragment containing the 3′ part of the coding region of *Pigpa1* and the insert of the *Pigpb1* cDNA (EST MY-09-H-10) as probes. Under low stringent conditions, single hybridizing DNA fragments were detected on Southern blots containing genomic *P. infestans* DNA digested with *Hin*dIII



(Figure 5) and various other restriction enzymes (data not shown). The sizes of the fragments correspond to fragments present in phage λP8 (for *Pigpa1*) or in the BACs (for *Pigpb1*). This suggests that *P. infestans* does not contain additional Gα and Gβ subunit genes that are highly homologous to *Pigpa1* and *Pigpb1*, respectively.

Sequence comparison of PiGPA1 with other Gα subunits

In order to compare the predicted amino acid sequence of the *P. infestans* Gα subunit PiGPA1 with amino acid sequences of other Gα subunits, sequences were aligned using ClustalW (European Bioinformatics Institute - www2.ebi.ac.uk) (Figure 2). The size of PiGPA1, 355 amino acids, does not differ from that of other Gα subunits. The highest identity and similarity (43% and 73%, respectively) were found with the human $G\alpha_{olf}$ subunit (GB01) whereas the identity with Gα subunits of *Caenorhabiditis elegans* (GB01) and the slime mold *Dictyostelium discoideum* (GBA1) was somewhat lower (40 and 42%, respectively). The identity with CPG1 from the ascomycetous fungus *Cryphonectria parasitica* was even lower, 38%. PiGPA1 has key structural motifs typical of members of the Gα subunit family (Gilman, 1987, Simon et al., 1991). These include 5 GTPase domains (G1-G5), a glycine residue at position 2 within a N-terminal consensus myristoylation site (MGXXXS) which increases the affinity of the Gα subunit for the Gβγ subunit, and an arginine residue at position 177 within the G2 domain, which is an established ADP ribosylation site for cholera toxin-protein (Fig 2). PiGPA1 does not have a pertussis toxin-labeling site (CXXX) at its C terminus, suggesting that PiGPA1 is not a member of the Gα inhibitory subfamily (West et al., 1985).

Sequence comparison of PiGPB1 with other Gβ subunits

ClustalW was also used to align the predicted amino acid sequence of the *P. infestans* Gβ subunit PiGPB1 with other Gβ subunits (Figure 3). PiGPB1 is 344 amino acids in size, similar to other Gβ subunits. The highest identity and similarity (57% and 81%, respectively) were found with the *D. discoideum* Gβ subunit GBB1 whereas the identities with Gβ subunits from *H. sapiens* (GBB1), *C. elegans* (GBB1) and *C. parasitica* (CPGB1) were in the same range (55-56%). Gβ subunit proteins belong to an ancient regulatory-

Figure 1.
Nucleotide sequences of *Phytophthora* Gα subunit genes and their deduced amino-acid sequences. (a) Nucleotide sequence of *P. infestans Pigpa1*; position +1 is the first nucleotide of the ATG start codon. (b) Nucleotide sequence of *P. palmivora Ppgpa1*; nucleotides identical to those in *Pigpa1* are indicated by dots; dashes (-) are introduced for optimal alignment. (c) Deduced amino acid sequence PiGPA1. (d) Deduced amino acid sequence PpGPA1; amino acid residues identical to those in PiGPA1 are indicated by asterisks. (e) Deduced amino acid sequence of the 154 bp PCR fragment amplified on genomic *P. nicotianae* DNA. Lower case letters in (a) and (b) indicate the 87-bp intron in *Pigpa1* and the 61 bp intron in *Ppgpa1* respectively, with the intron boundary nucleotides and the branch point consensus in bold italics. The amino acids indicating the conserved regions that were chosen to design degenerate primers for PCR amplification are shaded in gray in (e). The gap in the gray shading indicates a different amino acid in PiGPA1 (K) than expected (I) based on the codon usage in the degenerate reverse primer. The primer sequences used to isolate *Pigpa1* cDNA from sporangia and the *P. palmivora* homologue *Ppgpa1* are underlined in (a). The first 15 and last 4 nucleotides of *Ppgpa1* and the first five amino acids and the last amino acid of PpGPA1 (indicated with dotted lines in (b) and (d) respectively) are derived from the primer sequence and have not been verified by sequencing. The sequence of the peptide used to raise PiGPA1 antiserum is in bold in (c). The nucleotide sequence data are deposited under accession numbers AY050536 (*Pigpa1*) and AY050537 (*Ppgpa1*).

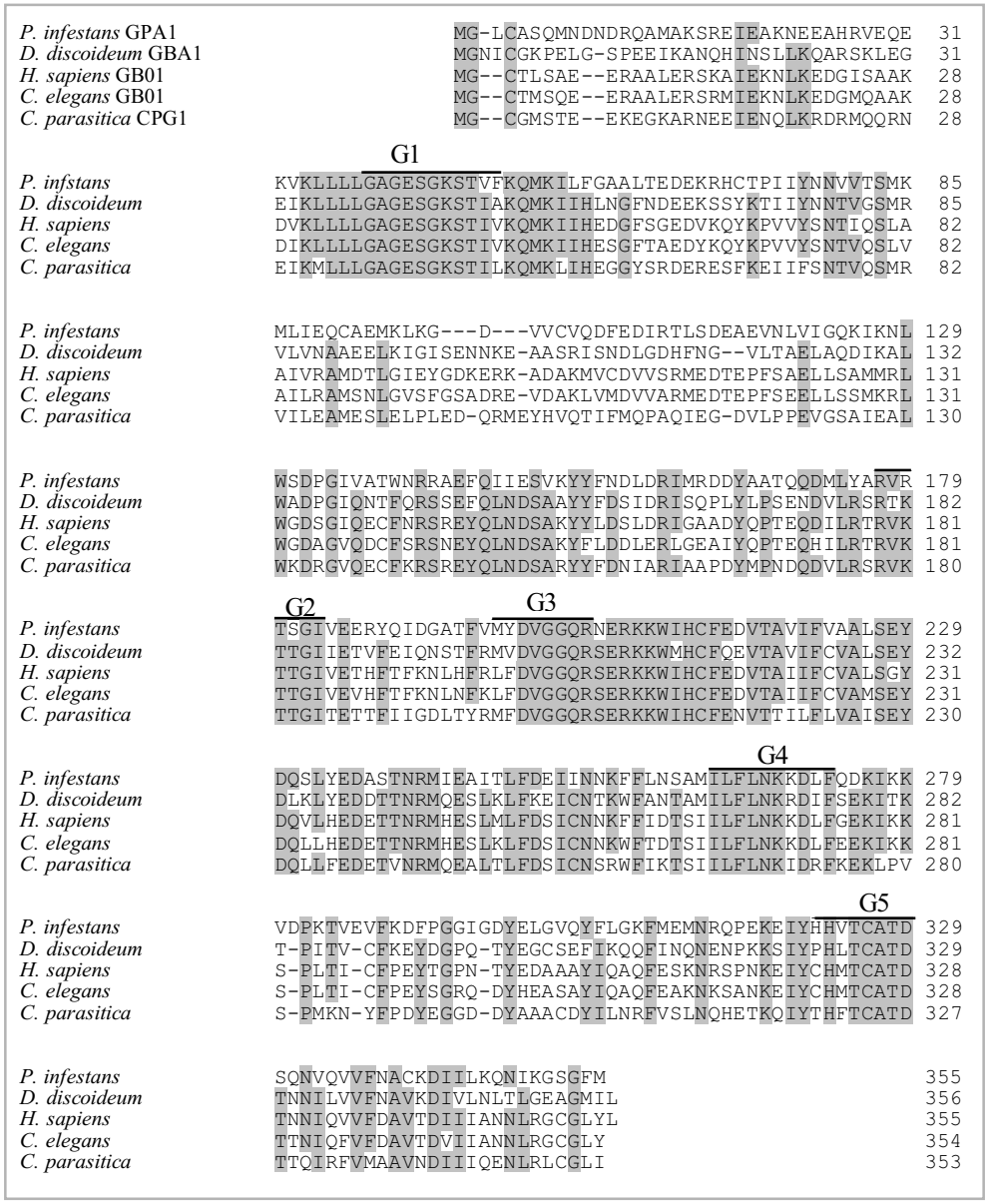


Figure 2.
Comparison of the predicted amino-acid sequence of the *P. infestans* Gα protein subunit PiGPA1 with the Gα protein subunits of *Dictyostelium discoideum* (GBA1, accession no. P16894); *Homo sapiens* (GB01, accession no. P09471), *Caenorabditis elegans* (GBO1, accession no. AAA28059) and *Cryphonectria parasitica* (CPG1, accession no. L32176). Sequences of the Gα proteins were obtained from GenBank. Amino acids that are identical in four or five of the five homologues are shaded in gray. Spaces introduced for optimal alignment are indicated by a dash (-). The deduced locations of the G1 to G5 regions of the GTPase domain (Gilman 1987) are indicated by single lines above the alignment.

protein family characterized by the occurrence of so-called WD repeats, highly conserved repeating units that usually end with Trp-Asp (WD) (Neer et al., 1994). As all other Gβ subunit proteins PiGPB1 has seven such repeats that, apart from 44 amino acids at the N-terminus, span the whole protein. The WD-repeats determine its three dimensional structure: a seven bladed β-propeller (Sondek et al., 1996). Amino acid residues identified by crystallographic analysis as interacting with Gα subunits were found to be highly conserved among five Gβ homologues while those residues identified as interacting with the Gγ subunit varied considerably.

Expression of *Pigpa1* and *Pigpb1* during the life cycle of *P. infestans*

In the asexual lifecycle of *P. infestans* various distinct developmental stages can be recognized. When mycelium starts to sporulate upright branched sporangiophores are formed that produce sporangia as they grow. Sporangia are the asexual propagules and under natural conditions, sporangia are easily dispersed by wind and rain. When sporangia find the appropriate conditions, for example the moist surface of a leaf, they can either act as spores, now called sporangiospores, and germinate directly or develop into zoosporangia forming motile biflagellate zoospores that germinate after encystment. The tip of the germ tube develops into a specialized infection structure, the appressorium, from which a penetration hypha emerges that directly penetrates the leaf surface and grows into the epidermis. Here an infection vesicle is formed. Subsequently, hyphae emerging from the vesicle colonize the mesophyll cells thereby causing rapidly expanding water soaked lesions. After a few days the center of the lesion becomes necrotic and this is soon followed by a complete collapse of the infected leaf. During lesion development numerous sporangiophores emerge from stomata creating new infectious spores.

To determine the expression patterns of the *P. infestans* Gα and Gβ subunit genes in these distinct development stages, levels of *Pigpa1* and *Pigpb1* mRNA were analyzed by northern blot hybridization. Total RNA isolated from different developmental stages was denatured, separated by electrophoresis and transferred to nylon membrane, and subsequently hybridized to a *Pigpa1* and a *Pigpb1* probe. As shown in Figure 4A a major *Pigpa1* transcript of approximately 1100 nt was detected. The highest level of *Pigpa1* mRNA was found in sporangia. Also zoospores, cysts and germinating cysts contain *Pigpa1* mRNA but, in contrast, no *Pigpa1* mRNA could be detected in mycelium. A *Pigpb1* transcript of approximately 1200 nt in length was found in all developmental stages but one: it was not detectable in zoospores. Similar to *Pigpa1* the highest amount of *Pigpb1* mRNA was found in sporangia. To exclude the possibility that the differential expression of *Pigpa1* and *Pigpb1* is strain specific, we analyzed the mRNA levels in another *P. infestans* strain. Also in strain 90128 the highest levels were found in sporangia (data not shown). During *in planta* growth *Pigpa1* mRNA was first detectable five days after inoculation, three days after the appearance of actin mRNA (data not shown). Since five days after inoculation, sporulation is abundant the observed accumulation of *Pigpa1* mRNA is probably due to an increase in the relative amount of sporangium mRNA in the total pool of mRNA isolated from infected plant tissue.

To determine if the different *Pigpa1* mRNA levels detected in the various

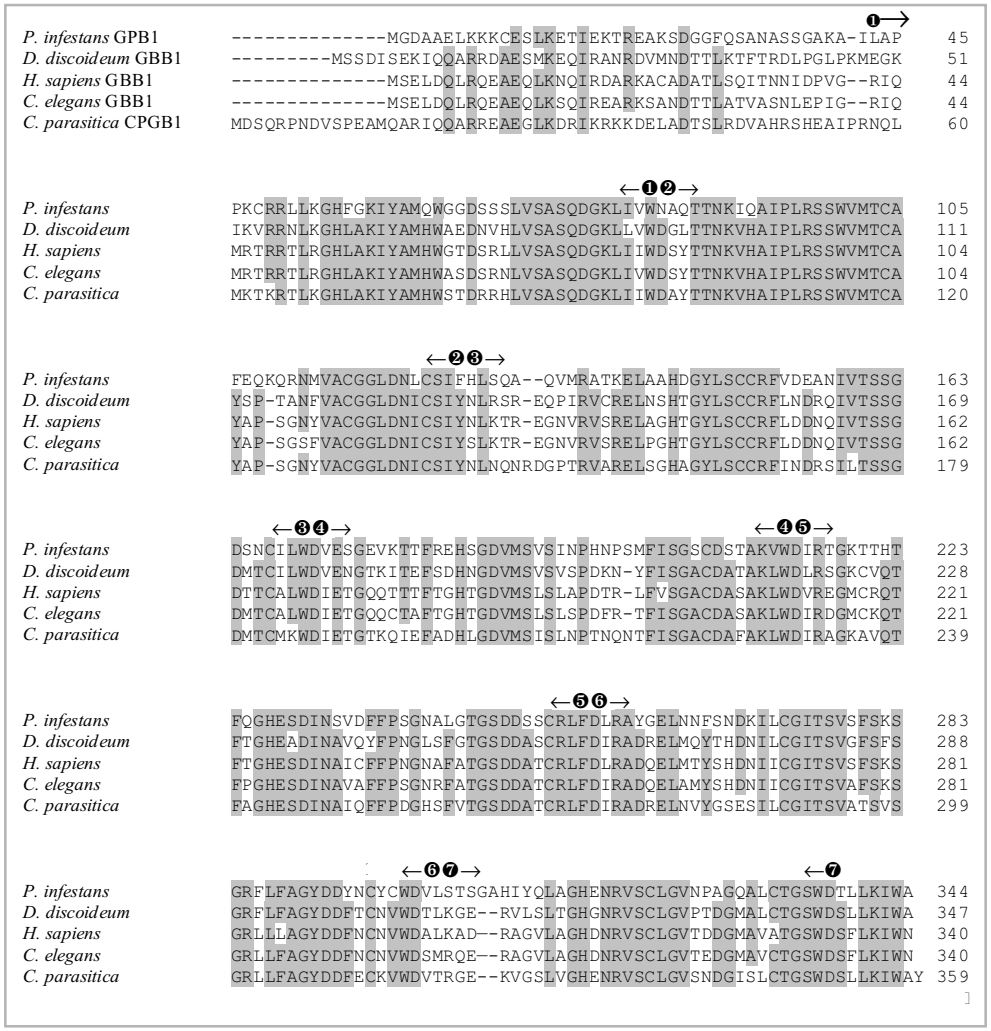


Figure 3.
Comparison of the predicted amino-acid sequence of the *P. infestans* Gβ protein subunit PiGPB1 with the Gβ protein subunits of *Dictyostelium discoideum* (GBB1, accession no. P36408); *Homo sapiens* (GBB1, accession no. P04901), *Caenorabditis elegans* (GBB1, accession no. P17343) and *Cryphonectria parasitica* (CPGB1, accession no. O14435). Sequences of the Gβ proteins were obtained from GenBank. Amino acids that are identical in four or five of the five homologues are shaded in gray. Spaces introduced for optimal alignment are indicated by a dash (-). Numbers and arrows above the sequences delineate each of the seven WD-repeats (Sondek *et al*., 1996).

developmental stages correlate with PiGPA1 protein levels we analyzed the presence of PiGPA1 by western blot analyses. Polyclonal antibodies recognizing PiGPA1 were raised against a synthetic peptide identical to 14 amino acids at the C-terminus of PiGPA1 (Figure 1). The antiserum specifically recognized a FLAG-PiGPA1 fusion protein produced in *E. coli* upon expression of *Pigpa1* cDNA cloned in a pFLAG-ATS expression vector (data not shown) demonstrating that the antiserum recognizes PiGPA1. On

western blots containing proteins extracted from zoospores, cysts, germinating cysts and sporangia a protein was detected with the PiGPA1 antiserum and the size of 43 kD corresponded to size expected for PiGPA1 (Figure 4B). With pre-immune serum the band was not detected and also in the lane containing protein extracts from mycelium the band was absent. Since the protein has the expected molecular weight and is present in those cell types that also contain *Pigpa1* mRNA it is very likely that this protein is PiGPA1. To confirm this we incubated a Western blot containing protein extracts from sporangia with the PiGPA1 antiserum in the presence of the peptide that was used as an antigen for generating the PiGPA1 antiserum. As shown in Figure 4C the 43 KDa band is absent when the peptide is present thereby demonstrating that the peptide competes away the primary antibodies and that the 43 KDa protein is indeed PiGPA1.

In summary, these results show that different developmental stages of *P. infestans* contain different levels of *Pigpa1* and *Pigpb1* mRNA. Moreover, in those cell types that contain *Pigpa1* mRNA the transcript is translated into protein. We conclude that *Pigpa1* and *Pigpb1* have differential but different expression profiles in the various developmental stages of *P. infesta*ns. Yet, the two genes both have the highest expression levels in sporangia.

Sequences homologous to *Pigpa1* and *Pigpb1* in other Phytophthora species

To determine whether other oomycetes have sequences homologous to *Pigpa1* and *Pigpb1* we performed Southern blot analyses and hybridized *Hin*dIII digested genomic DNA isolated from various *Phytophthora* species and two *Pythium* species with probes from *Pigpa1* and *Pigpb1* at low stringent conditions. In almost all lanes at least one fragment was found that hybridized specifically to the *Pigpa1* and *Pigpb1* probe, respectively (Figure 5A and 5B). One exception was *Pythium sylvaticum,* in which we could not detect a fragment that is homologous to *Pigpa1*. In contrast, a *Pigpb1* homologue was present in this species. These results demonstrate that *Pigpa1* and *Pigpb1* are highly conserved within the genus *Phytophthora* and to some extent within the Pythiaceae family. In addition, in most species the homologues seem to be single copy genes.

DISCUSSION

In this paper we describe the characterization of two novel *P. infestans* genes that encode two of the three subunits that constitute heterotrimeric G-proteins. The *Pigpa1* gene encodes an α subunit and *Pigpb1* a β subunit. To our knowledge these are the first cloned heterotrimeric G-protein genes of an organism that belongs to the class of oomycetes. For cloning the Gα subunit gene we exploited the highly conserved nature of Gα subunits found in many eukaryotes and used a degenerate primer PCR approach. The Gβ subunit gene was selected from a database containing a limited number of *P. infestans* ESTs obtained from a cDNA library from mycelium (Kamoun et al., 1999). We cloned the full-length genes, studied expression of both *Pigpa1* and *Pigpb1* during the life cycle of *P. infestans* and analyzed various *Phytophthora* species for the presence

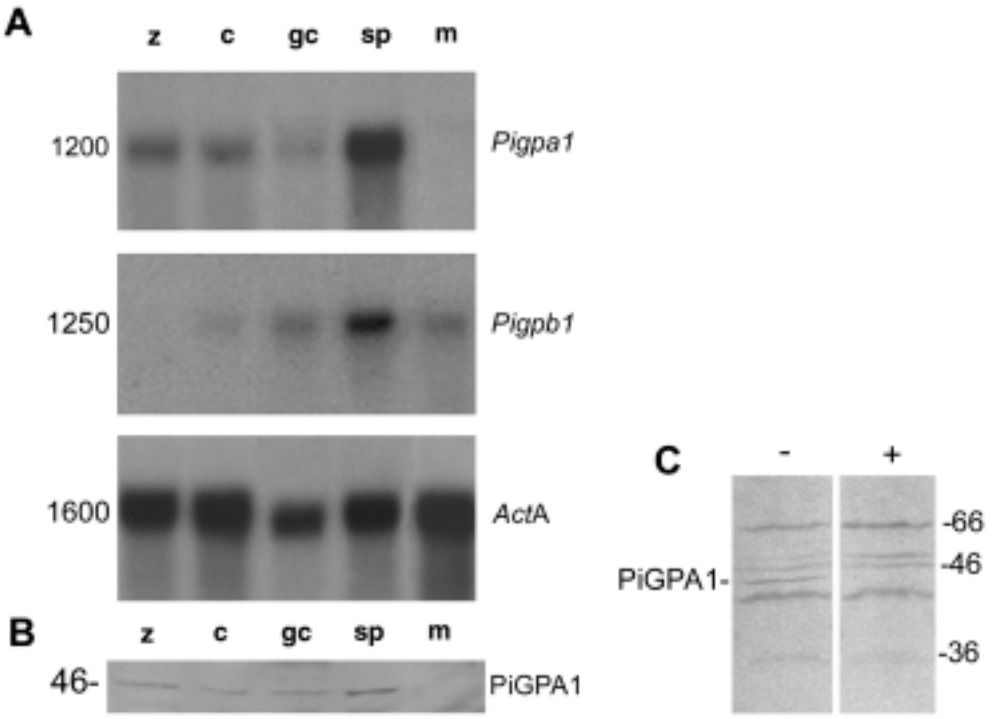


Figure 4.
Expression of *P. infestans Pigpa1* and *Pigpb1* in different development stages.
Autoradiographs of a (**A**) northern blot and (**B** and **C**) western blots containing total RNA or protein extracts from *P. infestans* strain 88069 zoospores (z), cysts (c), cysts germinating for 2.5 hours in water (gc), sporangia (sp) and mycelium (m). The northern blot was sequentially hybridized with *Pigpa1*, *Pigpb1* and *actA* probes. The *Act*A probe, derived from a constitutively expressed *P. infestans* actin gene (Unkles *et al.*, 1991), was used to check the amounts of RNA loaded on the gel. The approximate sizes of the transcripts in nucleotides are shown on the left. The western blot was incubated with PiGPA1 antiserum. Only the region with the 43 KDa PiGPA1 protein is shown. In (C) two identical western blots containing protein extracts from sporangia were incubated with PiGPA1 antiserum in the absence (-) or presence (+) of 40 µg/ml of the peptide used as an antigen for the generation of PiGPA1 antiserum. Molecular weight markers are indicated on the right.

of *Pigpa1* and *Pigpb1* homologues.

The predicted amino acid sequence of *Pigpa1* shows a high degree of similarity to known Gα subunits and all conserved characteristic key structural motifs necessary for GTPase activity are present. Intensive attempts to clone additional Gα subunit genes from *P. infestans* were so far unsuccessful. It is possible that a single gene produces all Gα subunit molecules required for signaling in *P. infestans*. Also in the basidiomycetous fungus *Ustilago hordei* (Lichter and Mills, 1997) and the ascomycetes *Cochliobolus heterostrophus* (Horwitz et al., 1999) and *Colletotrichum trifolii* (Truesdell et al., 2000) only a single copy of a Gα subunit gene was detected but in contrast, several other fungi contain more than one Gα subunit gene and each of these seems to function in a different signaling pathway. Examples are *Saccharomyces cerevisiae* (Nakafuku et

al., 1988), *S. pombe* (Isshiki et al., 1992), *Neurospora crassa* (Ivey et al., 1996), *Ustilago maydis* (Regenfelder et al., 1997), *Magnaporthe grisea* (Mitchell and Dean, 1995) and *Cryphonectria parasitica* (Gao and Nuss, 1996).

As *Pigpa1*, *Pigpb1* seems to be a single copy gene in *P. infestans*. Gβ subunit proteins belong to the ancient regulatory protein family of WD-repeat proteins and eukaryotic organisms contain a whole range of these proteins. In the current *P. infestans* EST database we found at least four different cDNA clones with homology to WD repeat proteins. However, the non-redundant EST we selected for isolating *Pigpb1* is the only true candidate for a Gβ subunit gene among a total of 2400 ESTs. It is highly homologous to known Gβ subunits with many of the conserved amino acids at sites that interact with Gα. In several organisms only a single copy of the Gβ subunit gene is found. Examples are *Arabidopsis* and *S. cerevisiae*. In *S. cerevisiae* the only Gβ subunit is the pheromone activated STE4 that is quite different in its primary structure when compared to all other Gβ subunits known so far. *C. elegans* has two Gβ subunits genes of which only GBB1 is essential (van der Linden et al., 2001).

RNA blot analysis showed that different cell types contain different levels of *Pigpa1* and *Pigpb1* mRNA demonstrating differential expression in the various developmental stages of *P. infestans* with the highest mRNA accumulation in sporangia. In mycelium neither PiGPA1 nor *Pigpa1* mRNA was detectable. This is consistent with the fact that the current EST database from the mycelial cDNA library with over 2400 sequences, does not contain a *Pigpa1* sequence. With only one Gα subunit gene and a universal role for heterotrimeric G-proteins in many cellular signaling processes one would expect to find PiGPA1 in every cell type. It may well be that the amounts of PiGPA1 and *Pigpa1* mRNA in mycelium are below the detection levels but the possibility that PiGPA1 is dispensable in mycelium can not be excluded. Recent findings in yeast suggest that some G-protein subunits participate in signaling independent from other subunits (Lengeler et al., 2000). Signaling pathways involving only one of the G-protein subunits may also occur in *P. infestans* and may explain why the expression patterns of the two single copy genes are different.

The finding that both genes are expressed in pre-infection stages, albeit at different levels, suggests that in those stages Gα and Gβ, together with a yet unkown Gγ subunit, form a heterotrimeric G-protein that is activated by extracellular signals binding to heptahelical transmembrane receptors. For pathogens it is important to sense the environment and to find the appropriate hosts for extracting nutrients. For several fungal plant pathogens, both plant and human, it has been demonstrated that G-protein signaling is crucial in the pre-infection stage and for the subsequent invasion of host tissue (Lengeler et al., 2000). Despite the fact that fungi and oomycetes are taxonomically very divergent they have many features in common and it is very likely that also oomycete pathogens rely on G-protein signaling to invade and colonize host tissue. With the availability of *P. infestans* genes encoding subunits of heterotrimeric G-proteins it is now possible to analyze the role of G-protein mediating signaling in late blight pathogenesis in more detail. The diploid nature of oomycetes hampers 'knock-out' mutagenesis in these organisms but via targeted gene silencing (van West et al., 1999) it will be possible to obtain PiGPA1 and PiGPB1 deficient *P. infestans* strains. These mutants will help to elucidate the function of PiGPA1 and PiGPB1 and to unravel

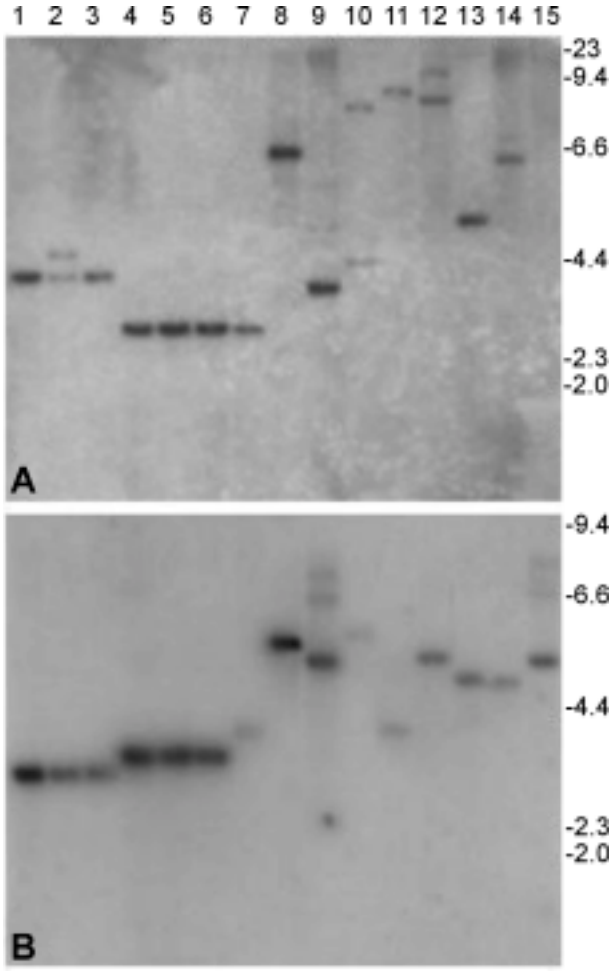


Figure 5.
Southern blot analysis of genomic DNA from various *Phytophthora* and *Pythium* species and hybridised at low stringency with (**A**) the 0.5-kb *Bam*H1-*Sal*I fragment from the coding region of *Pigpa1* gene and (**B**) with the insert of the *Pigpb1* cDNA clone MY-09-H-10. The DNA was digested with *Hin*dIII and isolated from *P. infestans* strains 88069 (lane 1), 90128 (2) and IPO-0 (3), *P. mirabilis* strains CBS678.85 (4), CBS-136.86 (5) and CBS150.88 (6), *P. phaseoli* strain CBS556.68 (7), *P. nicotianae* strain USA1751 (8), *P. cactorum* strain 0436 (9), *P. palmivora* strain PD 93/56 (10), *P. porri* strain HH (11), *P. cinnamomi* strain 2 (12), *P. vignae* strain 20853 (13), *Pythium aphanidermatum* strain 933 (14) and *Pythium sylvaticum* strain 28 (15). Molecular-size markers in kb are indicated on the right.

signal transduction pathways in *P. infestans* that may be required for pathogenicity. Interference of such pathways may form the basis for new strategies to control late blight.

MATERIALS AND METHODS

Strains and culture conditions

P. infestans strains 88069 (A1 mating type, race 1.3.4.7) and 90128 (A2 mating type, race 1.3.4.6.7.8. 10.11), from the Netherlands were used throughout this study. The isolates were routinely grown in the dark on rye agar medium supplemented with 2% (w/v) sucrose (RS-medium) (Caten and Jinks, 1968) or modified Plich medium (Van der Lee et al., 1997) at 18° C. Mycelium for isolation of genomic

DNA was obtained by growing the strains in liquid RS-medium for five to ten days. Mycelium, sporangia, zoospores, cysts and germinating cysts for isolation of RNA and protein were obtained as described by Van West et al. (1998). Other *Phytophthora* and *Pythium* species used in this study are listed in the legend of Figure 5.

PCR amplification, cloning and sequencing

Genomic DNA from *P. nicotianae* strain H1111 was used as template in a PCR reaction. The sequences of the degenerate primers that correspond to conserved regions of G-protein α subunit genes were 5′-CGGATCCAA(A/G)TGGAT(T/C/A)CA(T/C)TG(T/C)TT-3′and 5′GGAATTC(A/G)TC(T/A/G)AT(T/C)TT(G/A)TT(T/A/C)AG(A/G)AA-3′. The underlined sequences correspond to a *Bam*HI and *Eco*RI restriction site, respectively. The primers, which were used previously to clone Gα subunit genes from a filamentous fungus (Choi et al. 1995), correspond to amino acid residues KWIHCF and FLNKID, respectively (see Figure 1). The PCR was performed under the following conditions: 90 s 94°C; 90 s 37°C; 90 s 72°C for 35 cycles. The PCR product was cloned in pBluescriptKS+ resulting in plasmid pPngpa. The insert of pPngpa was used as a probe for screening a λEMBL3 genomic library of *P. infestans* strain 88069 (Pieterse et al., 1991). Filters containing plaques that covered four times the *P. infestans* genome were screened. Initially 14 lambda clones were selected of which 11 remained positive in the second screening. Positive plaques were purified and after mapping of restriction sites on the phage DNA, fragments from phage lP8 containing a putative Gα subunit gene were subcloned in pBluescriptII (SK) according to standard procedures (Sambrook et al., 1989) and sequenced.

In order to isolate a cDNA clone of the Gα subunit gene, cDNA was synthesized with reverse transcriptase on mRNA isolated from sporangia (Sambrook et al., 1989). This cDNA was used as template in a PCR reaction with primers corresponding to the 5′ and 3′ end of the open reading frame of the Gα subunit gene (gpa1: 5′-CCCTCGAGATGGGACTCTGTGCT-3′ and gpa2: 5′-GGGGTACCTTGCGCTTGCCTACAT-3′). The underlined regions correspond to a *Xho*I and *Kpn*I restriction site, respectively. PCR conditions were 30 s 95°C; 30 s 68°C; 1 min 72°C for 30 cycles. The PCR products were cloned in pGEM-T and sequenced. The same primers were used to amplify a fragment on genomic DNA isolated from *Phytophthora palmivora* strain P6390. The fragment was cloned into pCR4-Topo (Invitrogen) and sequenced.

An expressed sequence tag (EST) database containing around 1000 partial cDNA sequences from genes expressed in mycelium from *P. infestans* strain DDR7602 (Kamoun et al., 1999) was searched for the presence of Gβ subunit cDNA clones. Only one EST, MY-09-H-10, showed high homology; several others showed weak homology. The insert of MY-09-H-10, a 734 bp *Not*I-*Sal*I fragment in vector pSPORT1, was sequenced and used as a probe to screen a *P. infestans* BAC library. This genomic library is from strain H30P04, a F1 progeny from cross 71 (Whisson et al., 2001). The number of BAC colonies that was screened covered approximately three genome equivalents. Three BAC clones hybridized. Restriction mapping showed that the three BACs fit in one contig. Restriction fragments containing the Gβ subunit gene were subcloned in pBluescriptII (SK) and sequenced.

Dideoxy chain-termination sequencing was carried out using an AmpliCycle sequencing kit (Perkin Elmer, Foster City, CA). Sequence analysis was performed using software DNA Strider 1.0 (C. Marck Institute de Recherche Fondamentale, France) and DNAstar. Database homology searches were performed using BLAST software package as available through the Internet (Altschul et al., 1990).

DNA probes

As DNA templates for probe synthesis the following fragments were used: the 154 bp insert from pPngpa containing part of the coding region of a *P. nicotianae* Gα subunit gene, the 484 bp *Sal*I-*Bam*HI fragment from *Pigpa1*, the 734 bp *Not*I-*Sal*I insert from EST MY-09-H-10, and the 796-bp *Hin*dIII fragment from pSTA31 containing the coding region of the *P. infestans* actin gene, *actA* (Unkles et al., 1991). All DNA templates were purified with Qiaex II Agarose Gel Extraction kit (Qiagen GmbH, Hilden, Germany). The probes were radiolabeled with [α-^{32}P]dATP using the Random Primers DNA Labeling System (Gibco BRL, Gaithersburg, MD). To remove unincorporated nucleotides, the Qiaquick Nucleotide Removal kit (Qiagen GmbH) was used.

Southern and northern blot analyses

Genomic DNA of *P. infestans* and related species was isolated from mycelia as described by Pieterse et al. (1991). DNA was digested with restriction enzymes and size-separated on 0.7% agarose/TBE gels. Following electrophoresis, DNA was blotted onto Hybond-N+ membranes (Amersham) by capillary transfer (Sambrook et al., 1989) and hybridized. Total RNA from *P. infestans* sporangia, zoospores, cysts, germinating cysts and mycelium was isolated using the guanidine hydrochloride extraction method (Logemann et al., 1987). For northern blot analyses, 10 to 15 µg of total RNA was denatured in DMSO/glyoxal, electrophoresed according to Sambrook et al. (1989) and transferred to Hybond N+ membranes according to the manufacturer (Amersham). Hybridizations with radiolabeled probes were performed at 65°C or 55 °C in 0.5 M sodium phosphate buffer, 7% SDS, and 1 mM EDTA (pH 7.2). The filters were washed in 0.5X SSC (75 mM NaCl and 7.5 mM sodium citrate) and 0.1% SDS at 65°C or 55°C and exposed to Kodak X-OMAT AR films (Eastman Kodak Co., Rochester, NY).

Western blot analyses

Protein extracts were prepared by suspending sporangia, zoospores, cysts or germinated cysts collected from one 14.5 cm rye agar plate in 300 µl extraction buffer composed of 20 mM Tris pH 7.5, 10 mM dithiothreitol, 5% glycerol, 1% SDS, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, and modified from Choi et al. (1995). For extracting proteins from mycelium, mycelium grown in liquid medium was frozen in liquid nitrogen and ground to powder. 200 µl powder was mixed with 300 µl extraction buffer. Spores and cysts were disrupted by grinding with sand and disruption was checked under the microscope. Samples were centrifuged for 5 min at 14 000 rpm to remove cell debris. The protein content was determined using an assay developed by Bradford (1976) and according to the protocol provided by Biorad. 50 µg protein of each sample was separated by SDS/PAGE and blotted onto a nitrocellulose membrane (Schleicher and Schuell). PiGPA1 antiserum was raised against a peptide corresponding to 14 amino acids at the C-terminal end of PiGPA1 (in bold in Figure 1) and supplemented with a cysteine at the N-terminus (Eurogentec, Belgium). The specificity of the PiGPA1 antiserum was tested in a competition experiment in which 40 µg/ml of the peptide was added during incubation of a western blot containing protein extracts from sporangia with the antiserum. Western blots were incubated with PiGPA1 antiserum at a 1:500 dilution and with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham) at a 1:2500 dilution. Proteins recognized by the antiserum were visualized using a chemiluminescence detection kit (Amersham).

ACKNOWLEDGEMENTS

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CHAPTER 3

A Gα SUBUNIT CONTROLS ZOOSPORE MOTILITY, APPRESSORIUM FORMATION AND VIRULENCE IN THE POTATO LATE BLIGHT PATHOGEN *PHYTOPHTHORA INFESTANS*

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ABSTRACT

The heterotrimeric G-protein pathway is a ubiquitous eukaryotic signaling module that is known to regulate growth and differentiation in many plant pathogens. We previously identified *Pigpa1*, a gene encoding a G-protein α subunit from the potato late blight pathogen *Phytophthora infestans*. *P. infestans* belongs to the class oomycetes, a group of organisms that has not yet been studied at this level. To elucidate the function of *Pigpa1*, PiGPA1-deficient mutants were obtained by homology-dependent gene silencing. The *Pigpa1*-silenced mutants produced zoospores that turned 6-8 times more frequently, causing them to swim relatively short distances compared to wildtype. Attraction to the surface, a phenomenon known as negative geotaxis, was impaired in the mutant zoospores, as well as autoaggregation and chemotaxis towards glutamic and aspartic acid. Zoospore production was reduced by 20% to 45% in different *Pigpa1*-silenced mutants and the mutants developed fewer and often deformed appressoria. Infection efficiencies on potato leaves ranged from 2% to 17% in the *Pigpa1*-silenced mutants, compared to 85% in wildtype, showing that virulence is severely impaired. In contrast, transformants expressing mutated forms of PiGPA1, containing amino acid substitutions (R177H and Q203L) predicted to result in constitutively active forms of the protein, were found to cause lesions with higher growth rates than the wildtype, suggesting that virulence was enhanced in these mutants. The results prove that *Pigpa1* is crucial for pathogenicity of an important oomycete plant pathogen.

INTRODUCTION

Phytophthora infestans is the potato pathogen responsible for the notorious late blight disease that causes yearly losses of billions of dollars and which is a threat to potato production all over the world. During the last decade, the aggressiveness of the pathogen population increased significantly (Fry and Goodwin, 1997) and current control methods have become inadequate. We aim at elucidating signal transduction pathways that govern this pathogen's development and pathogenicity. Insight in these pathways will eventually be useful in designing novel control strategies.

Phytophthora infestans belongs to the oomycetes, a class of organisms that is evolutionarily related to the golden-brown and heterokont algae (Margulis and Schwartz, 2000). In the vegetative stage, *P. infestans* grows as coenocytic mycelium. The asexual propagules, called sporangia, are formed on branched sporangiophores that emerge from hyphae. Sporangia of *P. infestans* are wind- and water-dispersed and germinate either directly or indirectly, depending on the temperature. At ambient temperatures above 12 °C, sporangia germinate directly. At temperatures below 12 °C, sporangia undergo cytoplasmic cleavage followed by membrane synthesis, resulting in the formation of zoospores that possess two flagella. These wall-less spores are thought to swim towards the host, but the underlying mechanism remains subject of discussion (Cameron and Carlile, 1978; Deacon and Donaldson, 1993; Gow et al., 1999; Morris et al., 1998; van West et al., 2002; Zentmyer, 1961). Once the zoospores have reached the host, they differentiate into walled cysts, which subsequently

germinate. The germ tube develops into an appressorium from which a penetration peg emerges that enters epidermal cells.

To study the physiology and development of *P. infestans* at a molecular level, it is of great importance to be able to create strains in which genes, thought to play a role in those processes, are replaced or inactivated. The diploid nature of the vegetative stage of oomycetes makes gene disruption in *P. infestans* difficult to accomplish. Van West et al. (1999), however, described that introduction of extra copies of a target gene in the *P. infestans* genome can result in decreased or loss of expression of both the endogenous and the transgene in a subset of the transformants. This approach, based on homology-dependent gene silencing, was used to obtain *P. infestans* transformants deficient in the elicitin INF1. These mutants were used to demonstrate that INF1 acts as species-specific avirulence factor (Kamoun et al., 1998) and this was an important milestone that opened possibilities for functional analysis in oomycetes.

Heterotrimeric G-proteins function as molecular switches to transduce extracellular signals to intracellular effectors. Signals are received by the extracellular domains of so-called seven membrane-spanning (7TM) receptors, resulting in their activation (Neer, 1995). Upon activation, the cytoplasmic domain of a receptor recruits a heterotrimeric G-protein and brings about the exchange of GDP for GTP on the G-protein's α subunit, leading to dissociation and activation of the Gα and the Gβγ subunits. Ion channels, adenylyl cyclase and phospholipase C are among the effectors of $G\alpha_{GTP}$, whereas Gβγ is known to feed into the MAPK pathway in some organisms. It can also activate PI3Kinase and the phospholipases C and A2 (reviewed in Hamm and Gilchrist, 1996). G-protein activation eventually leads to changes in gene expression, which allows the cells to respond adequately to extracellular signals.

Heterotrimeric G-proteins were first found in mammals, where thousands of different 7TM receptors couple to hundreds of different possible G-protein trimer combinations, transducing an astonishingly wide range of signals (reviewed in Dhanasekaran et al., 1998). In contrast, plants appear to have only few genes encoding G-protein subunits (Arabidopsis genome inititative, 2000; Mason and Botella, 2000, 2001). They were recently shown to be involved in hormone and phytochrome signaling and organ development (Okamoto et al., 2001; Ullah et al., 2001; Wang et al., 2001; Lease et al., 2001; reviewed in Assmann, 2002). *Dictyostelium discoidium* and *Caenorabditis elegans* both possess large numbers of Gα subunits (11 and 20, respectively) that were assigned prominent roles in perception and chemotaxis (reviewed in Parent and Devreotes, 1996; Jansen et al., 1999). Like plants, fungi possess only few G-proteins (Lengeler et al., 2000). In *Saccharomyces cerevisae*, they are implicated in mating, glucose sensing and dimorphic growth (Versele et al., 2001). In a number of fungal pathogens, G-proteins were also shown to affect developmental processes such as asexual sporulation, spore germination, pigmentation and appressorium formation, and virulence (reviewed in Kronstad, 1997; Bölker et al., 1998; Borges-Walmsley and Walmsley, 2000 and Lengeler et al., 2000).

Recently, we described the isolation and characterization of a Gα subunit gene (*Pigpa1*) and a Gβ subunit gene (*Pigpb1*) of *P. infestans* (Laxalt et al., 2002). Both genes are differentially expressed in the different developmental stages of *P. infestans*, with the highest expression in sporangia. To study the function of the Gα subunit gene,

Pigpa1, we developed transformants expressing constitutively activated forms of PiGPA1, and PiGPA1-deficient mutants. Phenotypic characterization of the mutants revealed a clear role for PiGPA1 in zoospore swimming behavior and – taxis. and modest roles in zoospore release and appressorium development. The virulence of the PiGPA1-deficient mutants was severely affected.

RESULTS

Gain-of-function mutations in the Gα subunit PiGPA1

Mutations of a conserved arginine (R201 in human $G\alpha_s$) into a histidine or a cysteine and of a conserved glutamine (Q227 in human $G\alpha_s$) into a leucine are known to disrupt the GTPase activities of the Gα subunit and to lock the Gα protein in its active, GTP-bound state (Landis et al., 1989). The gain-of-function character of the Gα proteins containing either of these mutations has been explored to unravel the role of G-proteins in mammals (Freissmuth and Gilman, 1989; Landis et al., 1989; Wong et al.,1991), *C. elegans* (Mendel et al., 1995) and the fungi *S. cerevisiae* (DeSimone and Kurjan, 1998), *U. maydis* (Regenfelder et al., 1997), *Cryphonectria parasitica* (Segers and Nuss, 2003) and *Neurospora crassa* (Yang and Borkovich, 1999). In these fungi, the mutations were dominant and conferred phenotypes opposite to, or otherwise related to the phenotype of a knock-out of the same gene. To study the effects of gain-of-funtion mutations in PiGPA1 on *P. infestans,* strain 88069 was co-transformed with either of the two alleles of *Pigpa1*, one resulting in the before-mentioned arginine to histidine substitution (R177H) and the other in the glutamine to leucine substitution (Q203L), in combination with pTH209, a plasmid containing the geneticin resistance marker *npt*II (Judelson et al., 1991). The integration of the mutant alleles of *Pigpa1* into the genome of the *P. infestans* was analyzed using Southern blot analysis (Figure 1A). In the mutants, 1 to 3 extra bands hybridized to the *Pigpa1* probe, in addition to the band corresponding to the endogenous copy of *Pigpa1*, indicating that at least 1 – 3 copies of the mutant *Pigpa1* alleles had integrated into the genome. The expression of the mutant alleles of *Pigpa1* was analyzed by RT-PCR on RNA isolated from sporangia, using primers that anneal to the coding region of *Pigpa1,* followed by restriction analysis. The R177H and Q203L mutations create unique restriction sites in *Pigpa1* for the restriction enzymes *Nsp1* and *SnaB1* respectively, that allow distinction of wildtype and mutant *Pigpa1* transcripts after digestion of the RT-PCR products with these enzymes. Figure 1B shows that with the wildtype recipient strain 88069, only undigested RT-PCR product was obtained and that with the four selected transformants, R1 and R2 (R177H) and Q1 and Q2 (Q203L), both undigested and digested RT-PCR products were obtained. This demonstrates that both endogenous *Pigpa1* and the introduced mutant alleles of *Pigpa1* are expressed. Northern blot and RT-PCR analyses showed that the combined expression of endogenous *Pigpa1* and the introduced mutant alleles of *Pigpa1* is similar to wildtype *Pigpa1* expression (Figure 1C and D). R1, R2, Q1 and Q2 were tentatively named 'PiGPA1-gain-of-function mutants'.

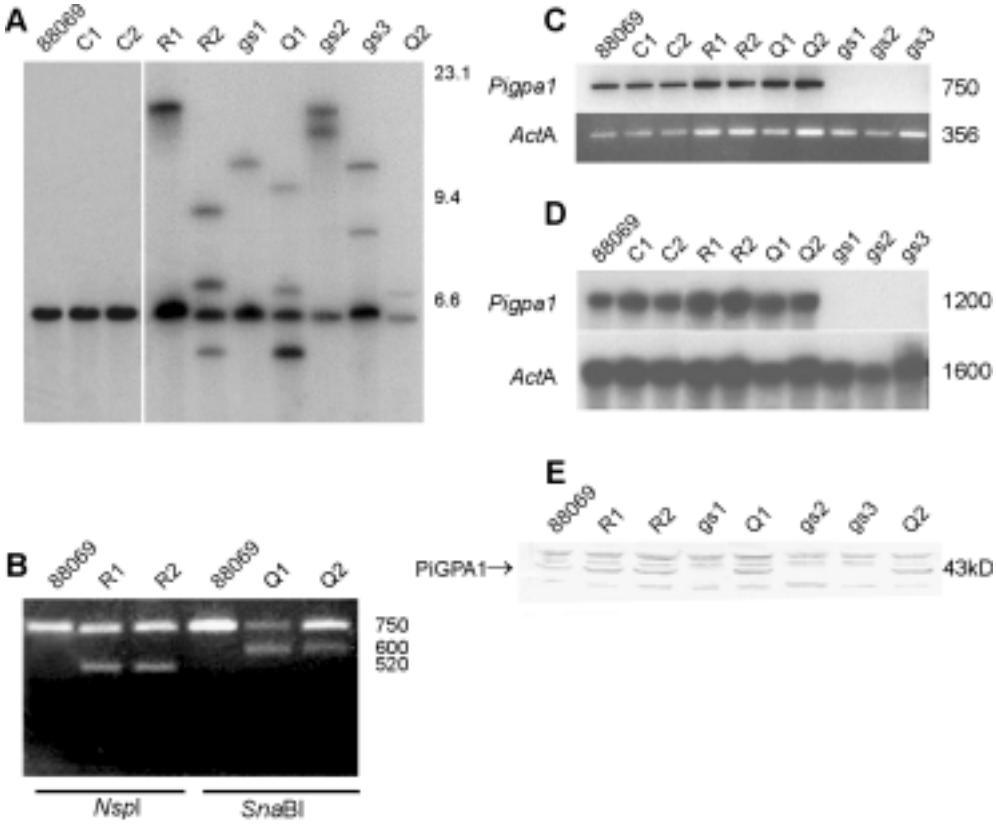

Figure 1. Analysis of *P. infestans* transformants
Analysis of plasmid integrations of pCGR or pCGQ and of *Pigpa1* expression in the wildtype recipient strain (88069), the control transformants that were only transformed with the selection plasmid pTH209 (C1, C2), the PiGPA1-gain-of-function mutants expressing the R177H allele (R1, R2) or the Q203L allele (Q1, Q2) and the *Pigpa1*-silenced mutants (gs1, gs2, gs3).
A. Southern blot analysis of plasmid integrations in the control transformants and the mutants. Genomic DNA was digested with *Eco*R1. The coding region of *Pigpa1* was used as a probe. Markers in kb are indicated on the right.
B. Restriction analysis of RT-PCR products of the PiGPA1-gain-of-function mutants. The transformants in which the R177H allele was introduced (R1 and R2) were digested with *Nsp*I and those with the Q203L allele (Q1 and Q2) with *Sna*B1. The sizes of the restriction fragments (in bp) are indicated on the right.
C. RT-PCR analysis and **D**. northern blot analysis of *Pigpa1* expression. The sizes of the RT-PCR products generated using primers corresponding to *Pigpa1* and to *Act*A (in bp) and of the mRNA products detected with probes derived from *Pigpa1* and *Act*A (in bases) are indicated on the right.
E. Western blot analysis of the PiGPA1-gain-of-function mutants and the *Pigpa1*-silenced mutants. Antiserum was raised against a 14 amino acid peptide representing the C-terminus of PiGPA1 and was shown to be specific for PiGPA1 in a competition experiment (Laxalt et al., 2002). The arrow indicates the position of PiGPA1.

Pigpa1-silenced mutants

To obtain PiGPA1-deficient mutants, we searched for transformants in which the *Pigpa1* gene was silenced. Previous studies in our laboratory demonstrated that, with the *inf1* gene, silencing was achieved by transforming *P. infestans* with various constructs containing the *inf1* coding region under control of different promoters or lacking a promoter (van West et al., 1999). Therefore, transformants that had one or more copies

of the mutated *Pigpa1* alleles integrated into the genome were screened for absence of *Pigpa1* mRNA. RT-PCR on sporangial RNA of one transformant in which the R177H allele of *Pigpa1* was introduced (gs1) and of two transformants in which the Q203L allele was introduced (gs2 and gs3) failed to give products using the normal number of 20 PCR cycles (Figure 1C). gs1, gs2 and gs3 are independent transformants. Using 30 cycles, a faint band was generated on RNA of gs1, whereas with RNA of gs2 and gs3 still no RT-PCR products were detected (results not shown). Southern blot analysis (Figure 1A) revealed that in these silenced transformants similar numbers of integrations had occurred as in the transformants expressing the transgene. The absence of *Pigpa1* mRNA in all three transformants was confirmed by northern analysis (Figure 1D). On a western blot, antiserum recognizing PiGPA1 failed to detect a protein of the size of PiGPA1 in the lanes where protein extract of gs1, gs2 and gs3 was loaded (Figure 1E). These results show that the *Pigpa1* gene is silenced completely in transformants gs2 and gs3, and that silencing of this gene is nearly complete in gs1. The RT-PCR experiments were repeated after subculturing the transformants on Rye Sucrose Agar (RSA) for six months (ca. 12 subcultures) with similar outcome, indicating that the silencing is stable.

Pigpa1-silenced mutants show normal colony morphology but zoospore release is less eficient

As G-protein subunits regulate sporulation and hyphal growth in many fungal pathogens, the *Pigpa1*-silenced mutants and PiGPA1 gain-of-function mutants were cultured on rye sucrose agar (RSA) and their colonies (microscopically) examined. No differences were observed between the wildtype strain 88069 and the mutants, except for a reduction in radial growth rate by 32% of one of the *Pigpa1*-silenced mutants (gs3) (Table 1). The radial growth rates of the two other *Pigpa1*-silenced mutants were initially lower than of the wildtype (approximately 80% of wildtype) but they both grew nearly at the rate of the wildtype after subculturing several times. Oospores were formed abundantly when the mutants were grown in the presence of an A2 mating type strain, suggesting that mating is not affected by silencing of *Pigpa1* or constitutive activation of the corresponding protein.

Sporangia of wildtype strain 88069 and of the *Pigpa1*-silenced and PiGPA1-gain-of-function mutants were induced to release zoospores by incubating them in Petri's solution at 4 °C for 2 h. After 2 h, zoospores had escaped from 91% of the sporangia of 88069. In the four PiGPA1-gain-of-function mutants and in the control transformants, zoospore release was as efficient as in the wildtype (Figure 2A). In contrast, zoospore release in the *Pigpa1*-silenced mutants was significantly less efficient. In gs1, gs2 and gs3 the efficiency was reduced by 37%, 16% and 44% respectively, when compared to wildtype. Incubation for longer than 2 h did not improve the efficiency of zoospore release in the mutants. 11% (gs1), 16% (gs2) and 19% (gs3) of all zoospores of the *Pigpa1*-silenced mutants were unusually large, which is the result of incomplete cleavage of the sporangial cytoplasm. The size of these deformed zoospores, which generally moved at low speed, varied from twice the size of a regular zoospore to the size of an entire sporangium (Figure 2A and B). These large zoospores were only rarely observed in the recipient strain 88069 and in the PiGPA1-gain-of-function mutants (1% - 3%).

Table 1. *Pigpa1* mRNA levels and radial growth rates on RSA of strain 88069, control strains, *Pigpa1*-silenced mutants and PiGPA1-gain-of-function mutants.

Strain/transformant[a]	*Pigpa1* mRNA [b]	radial growth rate per 48h. (% of wildtype) [e]
88069	+	100
C1	+	98 ± 3
C2	+	93 ± 10
gs1	-	83 ± 5
gs2	-	82 ± 10
gs3	-	68 ± 8
R1	+[c]	100 ± 5
R2	+[c]	103 ± 8
Q1	+[d]	97 ± 3
Q2	+[d]	94 ± 10

[a] all transformants were generated using 88069 as the recipient strain
[b] (+) present, (–) absent, see Figure 1C and D
[c] mixed pool of wildtype *Pigpa1* mRNA and mRNA of the R177H allele of *Pigpa1* (Fig. 1B).
[d] mixed pool of wildtype *Pigpa1* mRNA and mRNA of the Q203L allele of *Pigpa1* (Fig 1B).
[e] the means of six independent measurements are presented.

Zoospores of *Pigpa1*-silenced mutants show aberrant swimming patterns

Normally, zoospores of *P. infestans* move in a helical fashion and swim in straight lines. When they hit an object they make a sharp turn. Careful observation of the mode of swimming of the 'normal sized' zoospores of the *Pigpa1*-silenced mutants revealed that they turned with a much higher frequency than wildtype zoospores and that they made sharp turns regardless of whether they hit an object or not. Figure 3A shows the average number of sharp turns of individual zoospores per min and figure 3B depicts the swimming patterns of wildtype and mutant zoospores as observed through the microscope. Wildtype zoospores made turns only once every 7.5 s whereas *Pigpa1*-silenced zoospores turned every 1.1 s. As a result of this, the zoospores of the *Pigpa1*-silenced mutants covered only very small distances compared to wildtype zoospores. During the observations, the mutant zoospores rarely left the microscopic field whereas the wildtype zoospores did so very frequently.

Another difference between zoospores of the wildtype and of the *Pigpa1*-silenced mutants that was detected by microscopical observation was the absence of negative geotaxis in the *Pigpa1*-silenced mutants. The tendency of zoospores to move towards the water surface, was first described by Cameron and Carlile (1977) and can easily be observed in a zoospore suspension in a well of a microtiter plate or in a droplet on a microscope slide. The zoospores of the wildtype strain and of the PiGPA1-gain-of-function mutants showed a clear preference for the water surface. In contrast, zoospores of the *Pigpa1*-silenced mutants showed even distribution over the entire volume of the suspension, indicating that they are defect in negative geotaxis. The aberrant, 'large' zoospores of the *Pigpa1*-silenced mutants were mainly detected at the bottom of the

well or droplet, possibly due to their low speed or relatively high weight.

Zoospores of *Pigpa1*-silenced mutants fail to autoaggregate

When a suspension with a high concentration (equal to or higher than 5×10^5 zoospores per ml) of wildtype *P. infestans* zoospores in water was poured into a petri dish, aggregates of zoospores were visible as white spots within less than 1 minute. Gentle jiggling of the Petri dish made the aggregates disappear and new aggregates were formed instantly, this time in a linear pattern. The lines were thought to be the result of uneven distribution of the zoospores after the jiggling, with areas of higher density functioning as nuclei for formation of new aggregates. A real time movie was made to illustrate the unusually high speed with which these aggregates are formed. In a volume of 1 ml, a minimal zoospore concentration of 2×10^5 per ml was required for formation of visually detectable aggregates to take place. The wildtype, the control strains and the PiGPA1 gain-of-function mutants all aggregated similarly. Under the same conditions, zoospores of the *Pigpa1*-silenced mutants failed to form aggregates (Figure 4). Even when incubation was prolonged up to ten fold the time required for wildtype zoospores to aggregate, the zoospores of the *Pigpa1*-silenced mutants still showed a completely even distribution.

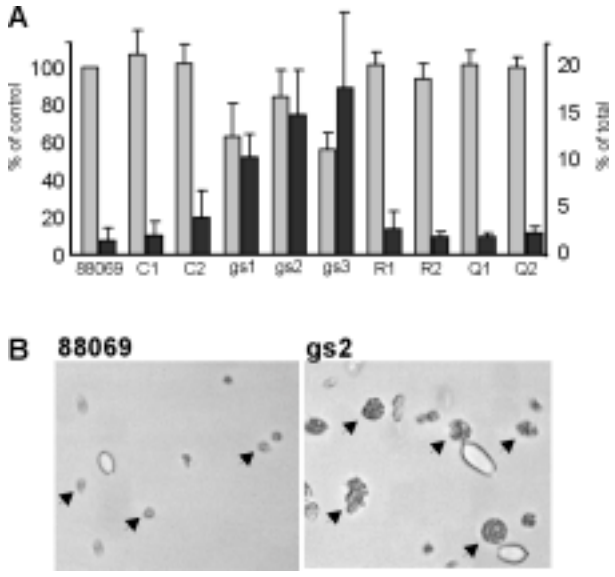

Figure 2. Zoospore release and cytoplasmic cleavage in PiGPA1-gain-of-function mutants and *Pigpa1*-silenced mutants. (**A**) Grey bars show zoospore release as percentage of the control, i.e., the wildtype recipient strain 88069 (left y-axis). Black bars show the number of 'large', aberrant zoospores, as percentage of the total number of zoospores (right y-axis). The average of nine (zoospore release) and six (cytoplasmic cleavage) independent experiments is presented. 88069, wildtype recipient strain; C1, C2, control transformants; gs1, gs2, gs3, *Pigpa1*-silenced mutants; R1, R2, Q1, Q2, PiGPA1-gain-of-function mutants.
(**B**) Sporangia of wildtype strain 88069 and one of the *Pigpa1*-silenced mutants (gs2) were incubated at 4ºC for 2 h to release zoospores. Zoospores were photographed with the bottom of the microtiter plate in focus. Arrows indicate individual zoospores.

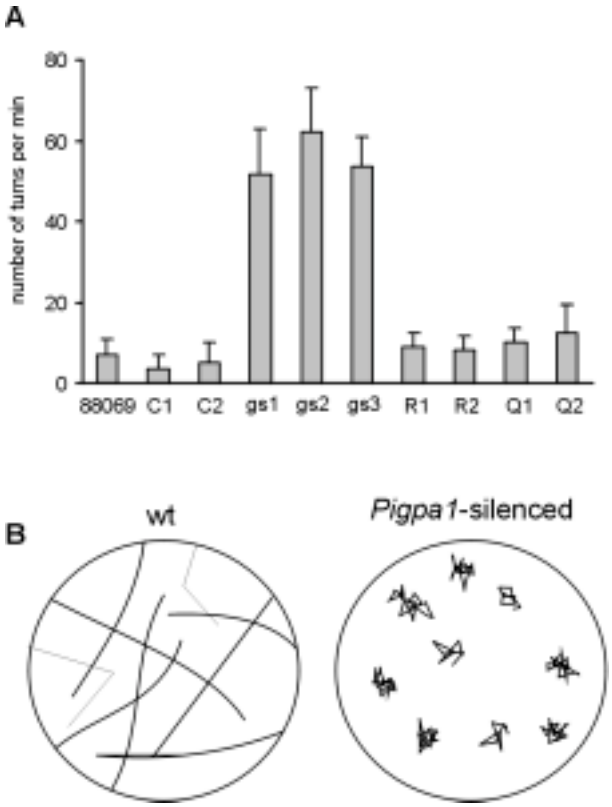

Figure 3. A. Zoospores of *Pigpa1*-silenced mutants have higher turning frequencies. Individual zoospores were monitored for 1 min and the number of turns counted. Presented are the means of multiple zoospores from four independent zoospore isolations. 88069, wildtype recipient strain; C1, C2, control transformants; gs1, gs2, gs3, *Pigpa1*-silenced mutants; R1, R2, Q1, Q2, PiGPA1-gain-of-function mutants. **B**. Swimming patterns of wildtype and mutant zoospores as observed through the microscope. Each line represents the swimming pattern of one zoospore.

Chemotaxis is impaired in zoospores of *Pigpa1*-silenced mutants

It was tested whether chemotaxis was also affected in zoospores of the *Pigpa1*-silenced mutants. To analyze the chemotactic response to glutamic acid, swim-in tests as described by Khew and Zentmyer (1973) were performed with zoospore suspensions of 88069 and of the mutants. In this assay, a microscopic chamber is filled with a zoospore suspension. A glass capillary, filled with a chemotactic compound in 1% water agar, is inserted into the chamber, causing a chemical gradient in the chamber. Glutamic acid, a compound that attracts zoospores of various *Phytophthora* species (Khew and Zentmyer, 1973) was used to test chemotaxis in the *Pigpa1*-silenced mutants. This compound also causes encystment in *P. infestans* at concentrations higher than 25 mM (unpublished observations). Wildtype zoospores and zoospores of the PiGPA1 gain-of-function mutants aggregated and encysted at the mouth of the capillary tubes containing glutamic acid at concentrations of 25 mM and higher (Figure 5). Zoospores of the *Pigpa1*-silenced mutants failed to aggregate at the tip of the capillaries even at 100 mM glutamic acid, which was the highest concentration tested. Like wildtype zoospores, zoospores of the *Pigpa1*-silenced mutants were induced to encyst in the presence of glutamic acid, but the cysts remained uniformly spread over the entire chamber. Similar effects were

observed with aspartic acid as chemotactic compound in the capillaries. The defects in chemotaxis and autotaxis in the *Pigpa1*-silenced mutants suggest that *Pigpa1* plays a role in gradient sensing, directional swimming or both.

Pigpa1-silenced mutants form fewer appressoria

Germ tubes of cysts of *P. infestans* produce appressoria if they are in contact with a hydrophobic surface such as propylene foil or microtiter plate wells. In most cases, these appressoria are formed directly after emergence of the germ tube (Figure 6A). A similar sequence of events was observed when cysts of control transformants or PiGPA1-gain-of-function mutants were allowed to germinate on a hydrophobic surface. No difference in the morphology of their appressoria nor in the efficiency of appressorium formation were observed. In contrast, the *Pigpa1*-silenced mutants rarely produced normal appressoria. Instead, the germ tubes became much longer and the tips only showed small swellings (Figure 6B). This phenotype was most severe in mutant gs3. Mutants gs2 and gs1 occasionally formed normal appressoria (< 20 % of wildtype).

The activity of adenylyl cyclase, the enzyme responsible for synthesis of the second messenger cAMP from ATP is often controlled by heterotrimeric G-proteins (Sunahara et al., 1996; Borges-Walmsley and Walmsley, 2000). The resulting change in the level of cAMP functions as an important intracellular signal in many systems. To investigate if appressorium development in *P. infestans* is mediated through the cAMP pathway, it was tested whether exogenous application of cAMP to germinated cysts of the *Pigpa1*-silenced mutants was able to restore appressorium formation. Cysts were incubated in the presence of cAMP, 8-(4-Chlorophenylthio) cAMP (pCPT-cAMP), a membrane permeable cAMP analogue, or the phosphodiesterase inhibitor 3-Isobutyl-1-methylxanthine (IBMX), all in a concentration range of 0 to 5 mM. None of these compounds had an effect on appressorium development (results not shown), suggesting that there is no direct role for cAMP in this process.

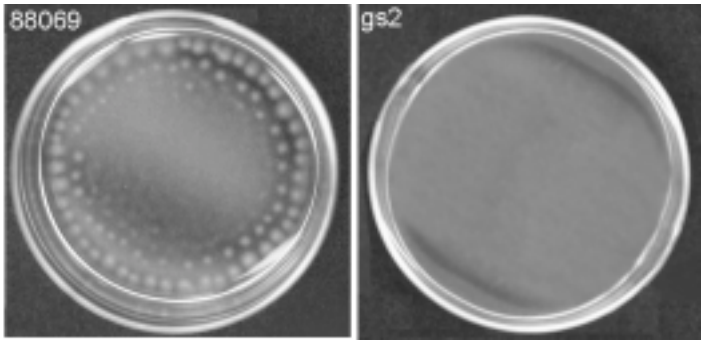

Figure 4. *Pigpa1*-silenced mutants fail to autoaggregate.
Zoospore suspensions of wildtype strain 88069 and of *Pigpa1*-silenced mutant gs2 were poured into a Petri dish (∅5 cm) and allowed to autoaggregate. Pictures were taken after 2 min.

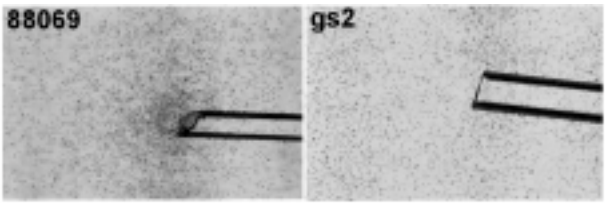

Figure 5. Zoospores of the *Pigpa1*-silenced mutants do not show chemotaxis to glutamic acid
Microscopic chambers, in which capillaries with 50 mM glutamic acid were inserted, were filled with equal numbers of zoospores of wildtype 88069 or of *Pigpa1*-silenced mutant gs2. Photographs were taken after 30 min incubation at room temperature. The results with gs1 and gs3, the other two *Pigpa1*-silenced mutants, were similar to gs2. Magnification, 50x

The virulence of the *Pigpa1*-silenced mutants is severely impaired

To determine the effect of PiGPA1 deficiency on virulence, leaves of potato cultivar Nicola, a variety that is moderately susceptible to late blight, were spot-inoculated with zoospores. Lesion sizes were measured three, four and five days post inoculation (dpi). In Table 2, the infection efficiencies (IE), and the average lesion growth rates (LGR) of the successful infections, as observed in three independent experiments, are presented. At five dpi, 85% of the leaves inoculated with the wildtype strain 88069 showed large, sporulating lesions (Figure 7). In contrast, most leaves inoculated with the *Pigpa1*-silenced mutants showed spots consisting of a few necrotic cells at the site of inoculation. Only a small percentage of the inoculations with gs1, gs2 and gs3, resulted in expanding lesions (infection efficiencies of 13%, 17% and 2%, respectively). The growth rate of the expanding lesions of the *Pigpa1*-silenced mutants was lower than of the lesions caused by 88069 (70%, 57% and 78% of wildtype, respectively) and they generally showed limited sporulation. In contrast, the growth rates of the lesions caused by three out of four of the PiGPA1-gain-of-function mutants were significantly higher than the growth rates of the lesions caused by 88069, i.e. 127%, 148%, and 134% for R1, R2 and Q1, respectively. This suggests that the presence of a constitutively active form of PiGPA1 enhances virulence of *P. infestans*. The virulence of the two control transformants C1 and C2 was unaltered compared to the wildtype strain and the three silenced strains (results not shown).

To confirm that the silencing was maintained during *in planta* growth, the pathogen was re-isolated from twelve of the expanding lesions caused by inoculations with *Pigpa1*-silenced mutants. Phenotypic analysis showed that the growth rate and zoospore behavior were still similar to those of the primary mutants, thus indicating that *Pigpa1* was still silenced in all twelve isolates.

To investigate if the aberrant zoospore swimming behavior of the *Pigpa1*-silenced mutants had a large effect on virulence, leaves were inoculated with cysts instead of zoospores. Identical numbers of cysts and zoospores were used and the ratio between the infection efficiencies of cysts and of zoospores was determined. For wildtype 88069, inoculation with cysts resulted in a 10% reduction in infection efficiency compared to inoculation with zoospores. In contrast, the infection efficiencies of cysts of the *Pigpa1*-silenced mutants were higher than the infection efficiencies of their zoospores. The

increase in infection efficiency ranged from 10% to 88% of the efficiency with zoospores (results not shown). There was much variation among experiments, due to the low number of successful infections in the mutant strains. These results show that the aberrant swimming pattern and lack of aggregation of zoospores of the *Pigpa1*-silenced mutants indeed affected virulence. However, inoculation with cysts by far did not restore full virulence in the *Pigpa1*-silenced mutants, indicating that other defects such as the reduced appressorium development and also negatively affected virulence.

Apart from the three independent inoculation experiments performed on cultivar Nicola, the experiment was repeated on two very susceptible potato varieties, Bintje and G254. The outcome of this test confirmed the reduction in virulence of the *Pigpa1*-silenced mutants and the higher lesion growth rate of three of the PiGPA1-gain-of-function mutants (results not shown).

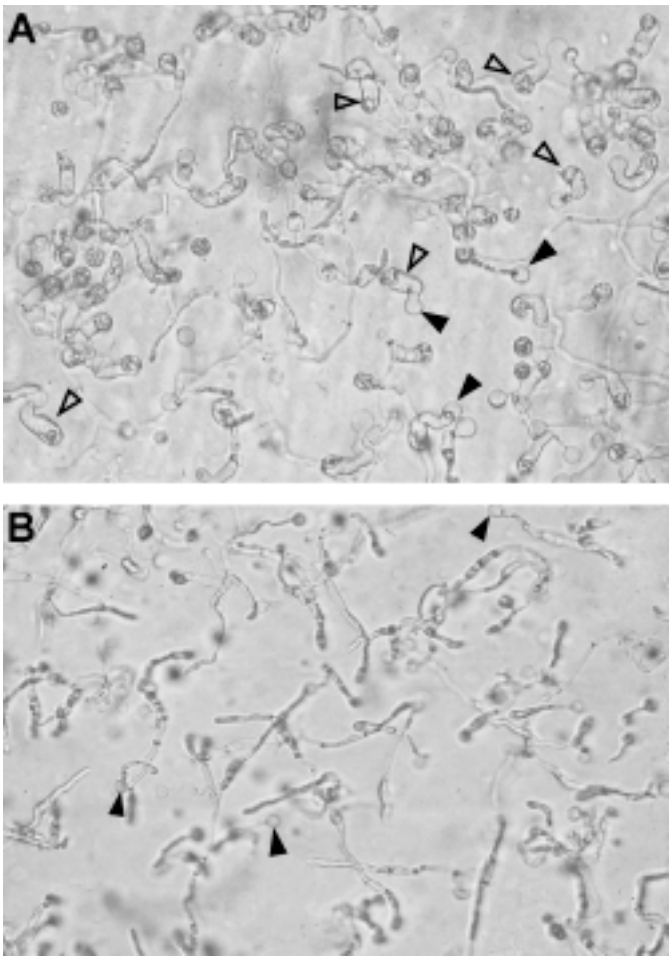

Figure 6. *Pigpa1*-silenced mutants show a defect in appressorium development.
Zoospores of 88069 (**A**) and of the *Pigpa1*-silenced mutant gs3 (**B**) were induced to encyst and the cysts were incubated for 14 h at 10 ºC, as described in the methods. In (**A**), appressoria are clearly visible; some are indicated by an open arrowhead. In (**A**) and (**B**), cysts are indicated by black arrowheads. Magnification, 200x

DISCUSSION

In this paper, we show that *P. infestans* possesses a heterotrimeric G-protein-dependent signaling pathway that controls multiple physiological and developmental processes and that is indispensable for virulence. Silencing of the Gα subunit gene *Pigpa1* resulted in a reduction in zoospore release and appressorium development, and zoospore motility and virulence were both severely affected. Disruption of a single Gα subunit gene also had severe effects in several species of plant-pathogenic fungi. The phenotypes of some fungal Gα-mutants partially overlap with the phenotypes of the *Pigpa1*-silenced mutants. For example, disruption of the *Magnaporthe grisea* Gα subunit gene *magB* resulted in reduced vegetative growth, conidiation and appressorium formation (Liu and Dean, 1997). This means that in spite of the fact that fungi are taxonomically unrelated to oomycetes, similarities can be found in signaling patterns involved in virulence and development.

Gene-silencing is often not absolute (Depicker and Montagu, 1997; van West et al., 1999). A small amount of RT-PCR product was generated from RNA of one of the three *Pigpa1*-silenced transformants (gs1) in an experiment that included 30 PCR cycles, whereas no product was observed in the other two mutants using this number of cycles. This means that gs1 likely expresses both the endogenous, wildtype gene and the mutant gene at a very low level. However, because gs1 behaved similarly to gs2 and gs3, the fact that gs1 is not 100% silenced and may express the mutant PiGPA1 protein in trace amounts is not expected to have influenced the phenotype of gs1. Transformant gs3 had a reduced growth rate on rye sucrose agar. As the two other *Pigpa1*-silenced mutants grew almost at wildtype rate, it is unclear whether the silencing of *Pigpa1* truly affected the growth rate or whether the growth retardation in the ase of gs3 had a different explanation.

Expression of *Pigpa1* is highest in sporangia and zoospores and no *Pigpa1* mRNA was detected in mycelium (Laxalt et al., 2002). This is consistent with aspects of zoospore development and physiology being largely affected by silencing of the gene. In the

Table 2. Virulence of *Pigpa1*-silenced mutants and PiGPA1-gain-of-function mutants.

strain	*Pigpa1*	infection efficiency (%)	lesion growth rate (mm/day)
88069	wildtype	84	2.3
gs1	silenced	11***	1.6
gs2	silenced	17***	1.3*
gs3	silenced	2***	1.8
R1	R177H	86	2.9*
R2	R177H	83	3.4**
Q1	Q203L	78	3.1*
Q2	Q203L	80	2.4

* P < 0.05
** P < 0.01
*** P < 0.001

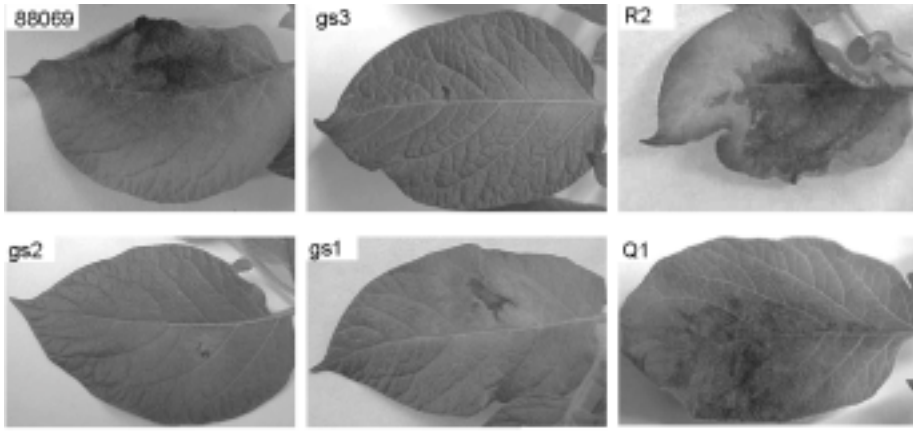

Figure 7. Virulence of PiGPA1 gain-of-function mutants and *Pigpa1*-silenced mutants.
Leaves of potato cultivar Nicola were spot-inoculated with equal numbers of zoospores (see materials and methods). Photographs were taken 5 days post inoculation. 88069, wildtype recipient strain; gs1, gs2, gs3, *Pigpa1*-silenced mutants; R2, Q1, PiGPA1-gain-of-function mutants.

Pigpa1-silenced mutants, zoospore release was reduced to an average of 66% of wildtype level and a considerable increase in the relative number of 'large', aberrant, zoospores was observed, suggesting that *Pigpa1* plays a role in the cytoplasmic cleavage in zoosporangia preceding zoospore formation. Alternatively, the function of *Pigpa1* may be related to stress tolerance because both reduced zoospore release and the formation of 'large' zoospores is sometimes observed in wildtype sporangia under suboptimal conditions (temperature, osmotic value or age, unpublished observations).

Zoospore motility and chemotaxis in the *Pigpa1*-silenced mutants

Comparison of zoospore behavior of wildtype and *Pigpa1*-silenced mutants in a number of different assays, clearly showed the involvement of *Pigpa1* in (directional) swimming and taxis. The zoospores of the *Pigpa1*-silenced mutants very frequently changed direction, as if a functional G-protein pathway is required to suppress turning. Moreover, negative geotaxis, the preference of *Phytophthora* zoospores to swim towards the liquid surface, was shown to be impaired in the *Pigpa1*-silenced mutants. The function of this phenomenon is thought to be associated with upward migration in flooded soil in root-pathogenic *Phytophthora* species (Cameron and Carlile, 1977) but the function for leaf pathogens is unknown. The *Pigpa1*-silenced mutants also appeared to be defective in chemotaxis towards at least two amino acids, glutamic and aspartic acid. Chemotaxis of zoospores to general compounds such as amino acids, sugars and alcohols has been observed in various *Phytophthora* species (Khew and Zentmeyer, 1973) and is thought to be important for root pathogens to localize plant roots. Autoaggregation (or autotaxis) of zoospores, although observed previously in *Phytophthora palmivora* (Ko and Chase, 1973; Reid et al., 1995), was not described before in *P. infestans*. This type of autoaggregation does not involve encystment. In our observations the autoaggregation in wildtype *P. infestans* was very rapid and massive, suggesting that this may be an

important process in the life cycle of *P. infestans* to intensify the infection pressure.

The defects in geotaxis, chemotaxis and autoaggregation in the *Pigpa1*-silenced mutants can be explained either by a defect in sensing an attractant or by the inability to master the direction of movements towards the aggregate or to the liquid surface. Interestingly, a mechanim for chemotaxis that is found in many organisms, called klinokinesis, simply involves an alteration of the frequency of change of direction without biasing the turns with respect to the stimulus (Erwin and Ribeiro, 1996; Dusenbery, 1989). The existence of this mechanism in *P. infestans* would explain the link between the fast-turning phenotype and the chemotaxis defect in our *Pigpa1*-silenced mutants. Mutations in calcium channels or in calmodulin were found to result in aberrant swimming patterns in case of the ciliate *Tetrahymena* (Watanabe et al., 1990; Schultz et al., 1990), This shows that Ca^{2+} is an important modulator of swimming direction in ciliates. Previous studies on zoospore behavior clearly showed that Ca^{2+} modulating drugs severly modified swimming patterns and induced encystment in several *Phytophthora* species (Byrt et al., 1982; Irving and Grant, 1984; Reid et al., 1995). It is conceivable that PiGPA1 influences Ca2+ channels or other Ca2+-binding proteins thus controlling flagellar movement in *P. infestans*.

Downstream targets of *Pigpa1*

Among the downstream targets of heterotrimeric G-proteins in other organisms are adenylyl cyclase, phospholipases and ion channels. In *Magnaporthe grisea* and *Cochliobolus heterostrophus*, the appressorium defect was restored by the addition of cAMP or IBMX. This was not the case with the *Pigpa1*-silenced mutants, meaning that at present there are no indications for the involvement of adenylyl cyclase or cAMP-dependent protein kinase in the pathway downstream of *Pigpa1.* G-protein signaling eventually alters gene expression in most systems. Therefore, transcript profiling of sporangial RNA of the *Pigpa1*-silenced mutants and the PiGPA1-gain-of-function mutants was carried out using cDNA-AFLP analysis. The characterization of genes that were differentially expressed between wildtype and the *Pigpa1*-silenced mutants is currently in progress (Dong et al., in preparation).

Pigpa1 is required for virulence

Each of the *Pigpa1*-silenced strains was severely affected in virulence. In most cases, growth was halted by a rapid necrotic response, as concluded from the presence of small black spots at the site of inoculation. It cannot be determined with certainty how each of the developmental and physiological defects of the *Pigpa1*-silenced mutants affected virulence. We corrected for the reduced zoospore release and the presence of large, deformed zoospores in the *Pigpa1*-silenced mutants by making inocula for each isolate with identical numbers of normal-sized zoospores. This implies that the *Pigpa1*-silenced transformants would be even more reduced in virulence in the natural field situation, where these corrections would be absent. The lack of aggregation and chemotaxis in the *Pigpa1*-silenced mutants is not expected to have a large effect on infection efficiency because the inoculation method we used, with a high number of zoospores in one droplet, ensures relatively high concentrations of cysts reaching the leaf surface in a limited area. This assumption was confirmed in the experiment where

cysts instead of zoospores were used for inoculation because this resulted in only a small increase in infection efficiency. The inability to form appressoria is expected to have a great impact on virulence since appressoria are required for epidermal cell wall penetration but this could not be tested (Erwin and Ribeiro, 1996).

The PiGPA1 gain-of-function mutants showed no phenotypes with respect to growth, zoospore behavior and appressorium formation, the processes that were clearly altered in the *Pigpa1*-silenced mutants. However, the two mutants with the R177H allele and one of the two with the Q203L allele grew at unusually high growth rate *in planta*, suggesting that their virulence is enhanced compared to the wildtype strain. The underlying mechanism of the increase in virulence is unknown but it is conceivable that PiGPA1 affects aspects of *in planta* growth that remained undetected. One of the PiGPA1 gain-of-function mutants, Q2, was shown to have only a slightly higher lesion growth rate than 88069. In this mutant, the proportion of the mRNA of the mutant allele of *Pigpa1* was smaller than in three other mutants (Figure 1B). This may explain why there is no clear effect on the lesion growth rate in Q2 compared to R1, R2 and Q1.

Many aspects of *Phytophthora* pathogenicity remain obscure and studying the role of specific genes in the infection process has always been very difficult. This study shows that gene-silencing is a tool that can help to overcome some of the difficulties. Elucidating the role of important signaling networks in *P. infestans*, such as the G-protein signaling pathway, will help forward the understanding of the biology and pathogenicity of *Phytophthora* and other oomycetes.

MATERIALS AND METHODS

Phytophthora infestans strains and culture conditions

P. infestans isolate NL-88069 (A1 mating type) and all transgenic isolates were routinely grown at 18 ºC in the dark on rye agar medium supplemented with 2% sucrose (RSA) (Caten and Jinks 1968). Radial growth was measured using a marking gauge linked to the IBREXDLL software (IBR Prozessautomation, Germany). For the isolation of sporangia, sporulating mycelium was flooded with modified Petri's solution (0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4 and 0.8 mM KCl) (Petri, 1917) and rubbed with a sterile glass rod. Subsequently, the suspension was filtered through a 50 µm nylon mesh. To obtain zoospores, sporulating mycelium was flooded with modified Petri's solution without $CaCl_2$ and incubated at 4 ºC for 2 h, after which the suspension was filtered. To obtain zoospores free of sporangia, the suspension was left on ice for 30 min to let the sporangia settle to the bottom, after which the zoospore suspension was transferred to a new tube. Cysts were obtained by vortexing a zoospore suspension for 2 min. Mycelium for isolation of DNA was grown in liquid modified Plich medium (van der Lee et al., 1997) or liquid rye sucrose medium.

Analysis of zoospore behavior, chemotaxis and appressorium development

To analyze zoospore release, swimming behavior and geotaxis, sporangia were incubated in 96-wells microtiter plates (Greiner) at 4 ºC. To observe zoospore aggregation, zoospore suspensions were poured or pipetted into a Petri dish or microtiter plate well (24 wells) and incubated at room temperature. To induce and observe appressorium development, cysts were incubated in microtiter plates or on polypropylene foil (Plastibrand GmbH, Wertheim) for 14 h at 10 ºC. Results of experiments with zoospores and appressoria were analyzed and photographed using an inverted microscope (Zeiss, Axiovert10). Zoospore autoaggregation was recorded in real time using a digital camera and the image was converted to MPEG format. Chemotaxis was examined according to Khew and Zentmyer (1973), by incubating equal numbers of zoospores in microscopic chambers into which glass capillaries

were inserted containing the specified concentrations of glutamic acid or aspartic acid in 1 % water agar on one side and water agar alone as control on the other side.

Construction of plasmids for transformation of *P. infestans*

All DNA manipulations were performed using standard protocols (Sambrook and Russel, 2001). Polymerase chain reaction (PCR) was performed with *Pfu* polymerase (Stratagene, La Jolla, CA) or AmpliTaq polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Restriction enzymes, Klenow polymerase and T4 ligase were from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Primers were synthesized by Amersham-Pharmacia (Buckinghamshire). The Plasmid Maxi kit (Qiagen GmbH, Hilden) was used for the isolation of plasmid DNA that was used for transformation of *P. infestans.* The authenticity of all cloned PCR fragments was confirmed by sequencing.

Plasmid pCG, which contains a 2.6 kb genomic fragment of *P. infestans* comprising the 1172 bp coding region of *Pigpa1* (1094 bp ORF + 78 bp intron), 700 bp of the promoter and 788 bp of the terminator, was used as template for PCR overlap-extension using primers that anneal to the vector and overlap primers: 5'-gtgcgtacatccggtatcgtagaag-3' and 5'-cttctacgataccggatgtacgc**acatgt**gcg-3' for the R177H amino acid substitution and 5'-cgtaacgagcgtaagaagtggatcca-3' and 5'-tggatccacttcttacgctcgt**tacgta**gtcca-3' for the Q203L amino acid substitution. The unique *Apa*I and *Bgl*II restriction sites in pCG were used to exchange a 1750 bp fragment of pCG for the products of the overlap extension PCR, after digesting them with the same enzymes. This resulted in the respective plasmids pCGR (R177H) and pCGQ (Q203L). In the primers, the mutated bases are underlined and the restriction sites for *Nsp*I and *Sna*BI are indicated in bold.

Transformation of *P. infestans*

Stable transformation of *P. infestans* strain 88069 was conducted according to the zoospore electroporation protocol of Tyler et al.,(in preparation). 0.5 – 1 x 10^7 zoospores in 500 µl were electroporated in the presence of 5 µg of the selectable plasmid pTH209 (Judelson et al.,1991) and 15 µg of a non-selectable plasmid, pCGR or pCGQ (this study), both linearized by digestion with *Eco*RI. The zoospores were plated on RSA containing 2 µg/ml geneticin (Gibco-BRL, Bethesda, MD). Colonies appeared after 6 – 8 days and were propagated on RSA containing 5 µg/ml geneticin. Approximately 105 transformants were screened for silencing of *Pigpa1*.

Southern and northern blot analysis

Genomic DNA of *P. infestans* was isolated from mycelium grown in liquid culture as described by Raeder and Broda (1985) with minor modifications. Following electrophoresis, DNA was transferred to Hybond N^+ membranes (Amersham) by alkaline transfer. Total RNA was isolated from sporangia using Trizol (Gibco-BRL, Bethesda, MD) according to the manufacturer's instructions. For northern blot analysis, 10 µg total RNA was denatured at 50 ºC in 1 M glyoxal, 50% DMSO, and 10 mM sodium phosphate, electrophoresed and transferred to Hybond N^+ membranes in 10x SSC (Ausubel et al., 1987; Sambrook and Russel, 2001). Hybridizations of DNA and RNA blots were conducted at 65 ºC and filters were washed at 65 ºC in 0.5x SSC + 0.5% SDS. Gel-purified DNA fragments (GFX kit; Amersham-Pharmacia, Buckinghamshire) consisting of a 1200 bp *Hinc*II fragment from pCG, the *Act*A gene from pSTA31 (Unkles et al., 1991) or a 1300 bp *Bam*H1/*Pvu*II fragment from pTH209 (Judelson et al.,1991) were used as probes and radiolabeled with α ^{32}P-dATP using a random primer labeling kit (Gibco-BRL, Bethesda, MD). To remove the unincorporated nucleotides, the Qiaquick Nucleotide Removal kit (Qiagen GmbH, Hilden) was used.

Reverse transcriptase (RT) -PCR analysis

To remove contaminating genomic DNA in RNA preparations, 10 µg total RNA was treated with 4 units RQ1 RNase-free DNase (Promega, Madison, WI) at 37 ºC for 1h. The removal of all DNA was verified in a PCR reaction under the same conditions as those used for the RT-PCR reaction, except that the 30 min cDNA synthesis step at 50 ºC was omitted. RT-PCR was performed using the One Step RT-PCR system (Gibco-BRL, Bethesda, MD) with 100 ng total RNA and 50 ng of each primer, following the manufacturer's instructions. To detect *Pigpa1* mRNA, the forward primer 5'-ccctcgagatgggactctgtgct-3' and reverse primer 5'-gtgatggccgcgatcatac-3', both annealing to the coding

region, were used. Similarly, to detect *Act*A, the forward primer 5'-cggctccggtatgtgcaaggc-3' and the reverse primer 5'-gcgggcacgttgaacgtctc-3', both annealing to the coding region, were used.

Western blot analysis

Protein extracts were prepared by vortexing 5 x 10^6 sporangia in 250 µl TEN buffer (50 mM Tris pH8, 5 mM EDTA and 150 mM NaCl), mixed with 0.5 ml 1 mm glass beads (Biospec products, Bartlesville, Oklahoma) for 30 s. The suspension was centrifuged for 5 min at 13,000 rpm, the pellet dissolved in extraction buffer composed of 20 mM Tris pH 7.5, 10 mM dithiothreitol, 5% glycerol, 1% SDS, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (modified from Choi et al. 1995) and the suspension centrifuged again for 5 min at 13,000 rpm. Western blot analysis of the protein extracts was performed as described by Laxalt et al. (2002).

Virulence assays

Detached potato leaves of cvs. Nicola, Bintje or G254 were placed in florist foam and inoculated on the abaxial side with one droplet (10 µl) containing 10^3 zoospores per leaflet. The leaves were incubated in a climate room at 18 ºC under 80 % humidity with 16 h light per 24 h. The length and width of the lesions were measured at three, four, five and/or six dpi using a marking gauge linked to the IBREXDLL software (IBR Prozessautomation, Germany). The average infection efficiencies (IE) and lesion growth rates (LGR) were estimated with ANOVA and REML respectively, using Genstat version 5.0 as described by Vleeshouwers et al. (1999).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Numbers

The GenBank accession number for the *P. infestans Pigpa1* gene is AY050536 (Laxalt et al., 2002).

ACKNOWLEDGEMENTS

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CHAPTER 4

A *PHYTOPHTHORA INFESTANS* G-PROTEIN β SUBUNIT IS INVOLVED IN SPORANGIA FORMATION

Maita Latijnhouwers and Francine Govers

Submitted for publication

ABSTRACT

The heterotrimeric G-protein pathway regulates cellular responses to a wide range of extracellular signals in virtually all eukaryotes. It also controls various developmental processes in the oomycete plant pathogen *Phytophthora infestans*, as was concluded from studies on the role of the Gα subunit PiGPA1 in this organism (Latijnhouwers et al., in preparation). Expression of the *P. infestans* G-protein β subunit gene *Pigpb1* was induced in nutrient-starved mycelium before the onset of sporangium formation. It was hardly expressed in mycelium incubated in rich growth medium. Introduction of additional copies of *Pigpb1* into the genome led to silencing of the gene and resulted in transformants deficient in PiGPB1. These *Pigpb1*-silenced mutants formed very few asexual spores (sporangia) when cultured on rye sucrose medium and produced a denser mat of aerial mycelium than wildtype. Partially *Pigpb1*-silenced mutants showed intermediate phenotypes with regard to sporulation and a relatively large number of their sporangia was malformed. The results show that PiGPB1 is important for vegetative growth and sporulation and, therefore, for pathogenicity in this organism.

INTRODUCTION

Phytophthora infestans causes potato late blight, a devastating disease posing a worldwide threat to potato production. This pathogen belongs to the oomycetes, a class of lower eukaryotes taxonomically related to golden-brown algae (Margulis and Schwartz, 2000). This class comprises saprophytes and pathogens of plants, animals and insects. The over sixty species in the genus *Phytophthora* are all plant pathogens and many of them infect economically important crops such as soybean, palm and cocoa. In addition, valuable forest trees in North America, Europe and Australia are threatened by *Phytophthora* species (Knight, 2002; Brasier et al., 1999; Erwin and Ribeiro, 1996). In the vegetative stage, *Phytophthora* grows as coenocytic mycelium. Asexual spores named sporangia, are formed on branched sporangiophores that emerge from the mycelium. Some species sporulate spontaneously when growing on solid media. Moreover, sporangia production can be induced in many *Phytophthora* species by washing and transferring portions of the culture to water or salt solutions or to vegetable decoction medium with low carbohydrate content (Erwin and Ribeiro, 1996).

Heterotrimeric G-proteins are widespread among the animal, plant and fungal kingdoms (Neer, 1995; Assman, 2002; Bölker, 1998) and are also present in lower eukaryotes such as *Dictyostelium discoidium* (Parent and Devreotes, 1996) and the ciliate *Stentor coeruleus* (Marino et al., 2001). They function as molecular switches that are activated by G-protein-coupled receptors upon binding of an extracellular ligand. According to the model, replacement of GDP for GTP on the protein's α subunit causes activation of the trimer leading to dissociation of the α subunit from the βγ dimer. In the activated state, both the Gα subunit and the Gβγ dimer can act on downstream targets. Upon hydrolysis of GTP to GDP by the intrinsic GTPase activity of the α subunit, the trimer reassociates, leading to inactivation of both Gα and Gβγ subunits (Neer, 1995).

The heterotrimeric G-protein pathway has been identified as an important regulator

of development and physiology in plant pathogenic fungi (Kronstad, 1997; Bölker, 1998; Lengeler et al., 2000). The function of the Gα subunits in particular has been studied extensively in fungal species. For example, Gα proteins were reported to control mating and filamentation in the corn smut fungus *Ustilago maydis*, conidiation, pigmentation and female fertility in the chestnut blight fungus *Cryphonectria parasitica,* and conidiation, appressorium formation and sexual development in the rice blast fungus *Magnaporthe grisea*. Each of these Gα subunits was also required for virulence (reviewed in Kronstad, 1997; Bölker, 1998; Borges-Walmsley and Walmsley, 2000; Lengeler et al.,2000). The function of the Gβ subunit gene has been described in only one plant pathogen so far. In *C. parasitica*, disruption of the β subunit gene *cpgb1* resulted in reduced pigmentation, conidiation and virulence and in increased mycelial growth (Kasahara and Nuss, 1997). Disruption of Gβ subunit genes in the saprophytes *Aspergillus nidulans* and *Neurospora crassa* had the opposite effect on vegetative growth and asexual sporulation: mycelial biomass was reduced and conidiation in submerged culture was induced (Yang et al., 2002; Rosén et al., 1999).

The *P. infestans* Gα (*Pigpa1*) and Gβ subunit (*Pigpb1*) genes are differentially expressed in the various stages of the life cycle of *P. infestans* (Laxalt *et al.,* 2002). PiGPA1-deficient mutants displayed a pleiotropic phenotype. In particular, zoospore motility and taxis were altered and virulence was severely reduced (Latijnhouwers *et al.,* in preparation). For lack of a method for gene disruption, gene silencing based on co-suppression is the only way to inactivate genes in a targeted fashion in *Phytophthora.* So far, three such cases of targeted mutagenesis by gene silencing have been reported, two in *P. infestans* (van West et al. 1999; Latijnhouwers et al. in preparation) and one in *Phytophthora nicotianae* (Gaulin et al., 2002). Introduction of additional copies of the target genes (sense or antisense) into the genome of the respective *Phytophthora* species induced gene-silencing in a subset of the transformants. In this report we describe the generation of *Pigpb1*-silenced mutants. The phenotypes of these mutants reveal that PiGPB1 plays a role in sporulation and is involved in the regulation of vegetative growth.

RESULTS

Pigpb1 expression is downregulated upon nutrient supply

The expression patterns of the *P. infestans* Gα and Gβ subunit genes *Pigpa1* and *Pigpb1* were previously shown to differ in one particular stage of development: *Pigpb1* was expressed in mycelium at a sufficiently high level for detection using northern blot analysis, whereas *Pigpa1* was not detectable (Laxalt et al., 2002 and Figure 1A). The mycelium that was used for the experiment was cultured for two weeks in a liquid, synthetic medium (modified Plich medium, van der Lee et al., 1997) and was about to sporulate (unpublished observations). To further explore the influence of growth conditions on the regulation of expression of *Pigpb1*, *P. infestans* was grown in a complex, rich medium (liquid Rye Sucrose (RS) medium) for two weeks. At this stage, few or no sporangia were observed in the cultures. The mycelium was subsequently rinsed and transferred to either fresh RS medium or a minimal medium (see methods). The mycelia

were harvested after 6 hours and the expression of *Pigpa1* and *Pigpb1* analyzed using RT-PCR. As shown in Figure 1B, *Pigpb1* mRNA was easily detected in the two-week-old mycelium before transfer and in the starved mycelium, whereas it was nearly untraceable in mycelium incubated in fresh, rich medium. After incubation in minimal medium for two days, the first sporangia appeared, whereas mycelium incubated in fresh RS did not sporulate within the same time interval. *Pigpa1* mRNA was not detected in starved mycelium nor in mycelium incubated in fresh RS, again showing the distinct expression patterns of *Pigpb1* and *Pigpa1*. *Pigpb1* expression precedes sporulation whereas *Pigpa1* is only expressed in sporangia and subsequent stages of the lifecycle (zoospores, cysts).

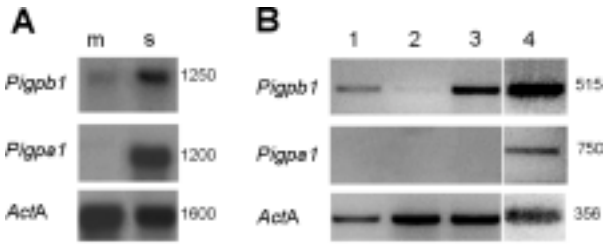


Figure 1.
Pigpb1 is strongly upregulated in nutrient-starved mycelium.
A. Expression of *Pigpb1* and *Pigpa1* analyzed by northern blot analysis. A northern blot containing RNA isolated from mycelium (m) grown in Plich medium for two weeks and from sporangia (s) was hybridized with probes derived from *Pigpb1*, *Pigpa1* and *Act*A. Sizes of the transcripts (in bases) of are indicated on the right.
B. RT-PCR analysis of *Pigpb1* and *Pigpa1* expression in two-weeks-old mycelium grown in rye sucrose (RS) medium before (lane 1) and after transfer to fresh RS medium (lane 2) or minimal medium (lane 3) and six hours incubation in the same medium. Expression in sporangia of wildtype strain 88069 was also analyzed (lane 4). Sizes (in bp) of RT-PCR products are indicated on the right.

Silencing of the Gβ subunit gene *Pigpb1*

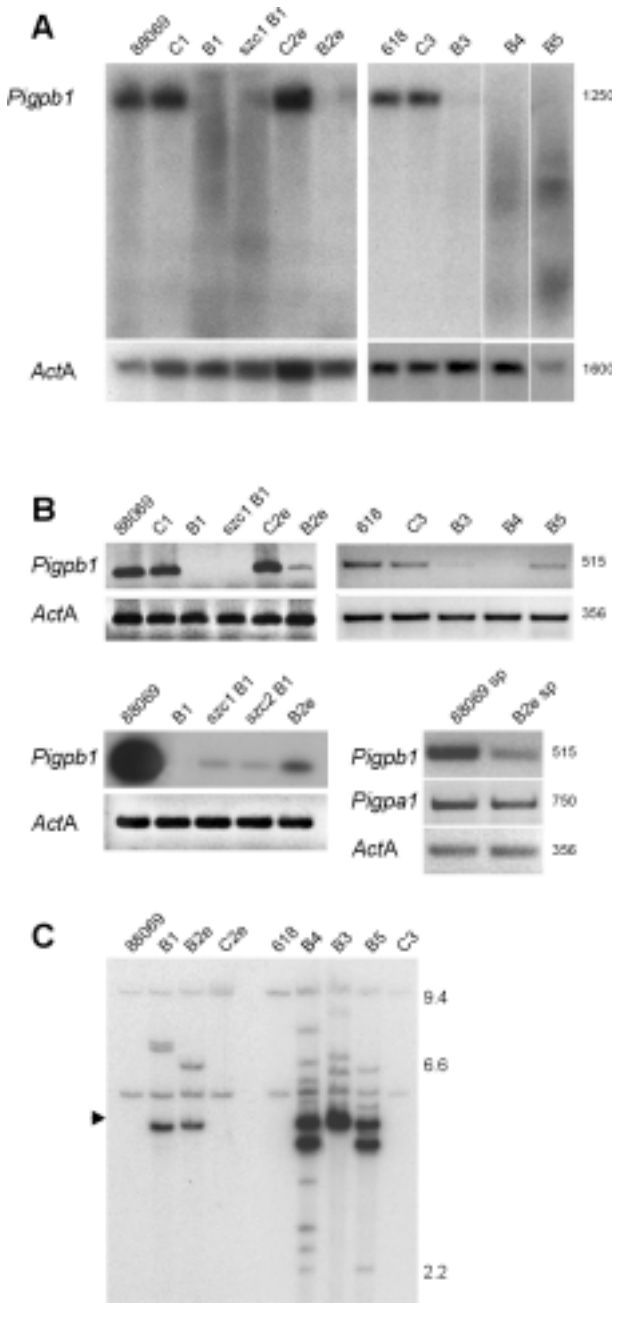
To obtain PiGPB1-deficient mutants, two different *P. infestans* strains, 88069 (mating type A1) and 618 (A2) were transformed with plasmid pMH2, containing a 2.5 kb genomic *Hin*dIII-*Bam*H1 fragment with the *Pigpb1* coding region flanked by its native promoter (911 bp) and terminator (387 bp). A total number of 17 transformants was generated using the PEG-protoplast transformation method (PEG transformants) (Judelson et al., 1991). Fifty-seven transformants were generated using electroporation-mediated transformation of zoospores of strain 88069 (electroporation transformants) (B.M. Tyler et al., in preparation). The levels of *Pigpb1* mRNA in (starved) mycelium of the transformants were determined using northern blot analysis. *Pigpb1* mRNA was absent in four of the PEG transformants (B1, B3, B4 and B5) and in one of the electroporation transformants (B2e) (Figure 2A). One of the four PEG transformants in which no *Pigpb1* mRNA was detected (i.e. B1) was derived from strain 88069 and the other three from strain 618. Curiously, *Pigpb1* mRNA was replaced by a smear in

transformants B1, B4 and B5, as if a mechanism involving mRNA breakdown was responsible for the silencing of *Pigpb1*. Since RT-PCR is more sensitive for detection of mRNA than northern blot analysis, this method was used to study the levels of *Pigpb1* mRNA in more detail (Figure 2B). Intermediate levels of *Pigpb1* were detected in two of the five previously mentioned transformants (B2e, B5), a low level was detected in B3 whereas no product could be amplified in transformants B1 and B4. We conclude that silencing of *Pigpb1* is complete in transformants B1 and B4, nearly complete in B3 and partial in B2e and B5. From the transformant B1, single zoospore cultures were isolated and in 4 of these cultures the expression of *Pigpb1* was analyzed (Figure 2A and B). No product was amplified from these single zoospore cultures when the normal number of cycles (i.e. 27) was used. However, a low level of expression was detected in three of the four after 30 cycles whereas no product was amplified from the parental strain B1 mRNA after 30 cycles (Figure 2B). This shows that silencing can be (partially) lost in a transformant when going through the zoospore stage. All RT-PCR experiments were repeated at least twice on independent RNA isolations.

Plasmid integration of pMH2 into the genome of the *Pigpb1*-silenced mutants was analyzed on Southern blot containing *Bam*H1 digested genomic DNA (Figure 2C). pMH2 contains a single recognition site for *Bam*H1. The blot was hybridized with a probe corresponding to the *Pigpb1* coding region. From the hybridization pattern it was concluded that pMH2 was integrated at different sites in the genome. The appearance of a strongly hybridizing band of the size of the plasmid (4.5kb) strongly indicates that tandem integrations have occurred in all *Pigpb1*-silenced transformants.

To verify that silencing of *Pigpb1* was not an effect of the transformation procedure alone, *Pigpb1* expression was analyzed in 15 independent PEG transformants containing plasmid integrations of a different plasmid not containing the *Pigpb1* coding region. Northern blot analysis revealed that all of these transformants expressed *Pigpb1* at levels comparable to the wildtype (results not shown). This strongly indicates that the absence of *Pigpb1* mRNA in the five selected transformants (B1-B5) is a result of the introduction of additional copies of the *Pigpb1* gene.

Figure 2. (next page)
Expression analysis of *Pigpb1*-silenced mutants. *Pigpb1* mRNA levels were determined in wildtype strains 88069 and 618, in control transformants in which only the selection plasmid was introduced (C1, C2e, C3), in the *Pigpb1*-silenced mutants (B1, B2e, B3, B4, B5) and in single zoospore cultures of B1 (szc1B1, szc2B1). C1, C3, B1, B3, B4, B5, PEG transformants; C2e, B2e, electroporation transformants.
A. Expression of *Pigpb1* in mycelium of the *Pigpb1*-silenced mutants, analyzed by northern blot analysis. The sizes (in bases) of the bands detected with probes derived from *Pigpb1* and *Act*A are indicated on the right. Left panel, 88069 wildtype and transformants derived from this strain. Right panel, 618 wildtype and transformants derived from this strain. Note that a smear has replaced the *Pigpb1* signal in the case of B1, B4 and B5.
B. RT-PCR analysis of gene expression in *Pigpb1*-silenced mutants. *Pigpb1* mRNA was amplified from total RNA extracted from starved mycelium of wildtype and the *Pigpb1*-silenced mutants using 27 cycles (upper two panels) or 30 cycles (bottom left panel). *Act*A mRNA was amplified using 24 cycles. Expression of *Pigpb1, Pigpa1* and *ActA* in sporangia of wildtype 88069 and partially *Pigpb1*-silenced mutant B2e was also analyzed (bottom right panel).
C. Southern blot analysis of *Pigpb1*-silenced mutants. Genomic DNA was digested with *Bam*H1 and the blot was hybridized with a probe derived from the coding region of *Pigpb1*. Arrow indicates the bands, which have the size of plasmid pMH2. Sizes are on the right, in kb.


A
86069
C1
B1
scc1 B1
C2e
B2e
618
C3
B3
B4
B5
Pigpb1
ActA
1250
1600
B
86069
C1
B1
scc1 B1
C2e
B2e
618
C3
B3
B4
B5
Pigpb1
ActA
515
356
86069
B1
scc1 B1
scc2 B1
B2e
Pigpb1
ActA
86069 sp
B2e sp
Pigpb1
Pigpa1
ActA
515
750
356
C
86069
B1
B2e
C2e
618
B4
B3
B5
C3
9.4
6.6
2.2

Pigpb1 is required for sporangia formation

Colonies of the (partially) *Pigpb1*-silenced mutants could easily be distinguished from wildtype colonies. When cultured on Rye Sucrose Agar (RSA), wildtype strains 88069 and 618 generally produce little mycelium and sporulate abundantly. Colonies therefore appear thin and translucent. The *Pigpb1*-silenced mutants and partially silenced mutants produced abundant aerial mycelium resulting in thicker colonies with a more intense white color (Figure 3A). However, their radial growth rates were not markedly different from wildtype. Mycelium of the mutants invaded poorly into the agar, as mycelium could easily be detached from this substrate. This growth habit will subsequently be referred to as 'fluffy'.

The *Pigpb1*-silenced mutants barely sporulated when cultured on RSA (Table1 and Figure 3B). The average numbers of sporangia of the two wildtype strains 88069 and 618 vary. However, there is a clear correlation between the levels of *Pigpb1* mRNA in the mutants and the number of sporangia when compared to the respective wildtype strain. E.g., mutants B1, B3 and B4 did not, or barely, express *Pigpb1* and produced less than 1% of the number of sporangia produced by a wildtype strain. Partially *Pigpb1*-silenced mutants formed reduced numbers of sporangia.

Table 1: *Pigpb1* mRNA levels and sporangia production on RSA of wildtype strains and transformants

recipient strain	transformants	*Pigpb1* mRNA level[a]	nr. of sporangia (x 10^4)[b]
88069	-	++++	370 ± 40
	B1	-	1 ± 2
	szc B1	+	23 ± 18
	B2e	++	140 ± 15
	C1, C2, C4[c]	++++	310 ± 93
618	-	++++	90 ± 10
	B3	+	1 ± 1
	B4	-	1 ± 2
	B5	++	58 ± 29
	C3	++++	230 ± 44

[a] based on results presented in Figure 2A and 2B. ++++ wildtype level, ++ partial silencing, + nearly complete silencing, - complete silencing.
[b] presented are the average numbers of sporangia from one RSA plate (∅ 9 cm)
[c] this number is an average of three independent control transformants

Microscopic observations revealed that a large proportion of the sporangia of the *Pigpb1*-silenced transformants were empty or extraordinarily small. In addition, sporangia with multiple hyphal outgrowths were observed (Figure 3C). The results show that fluffy growth, reduction in sporulation and the formation of malformed sporangia are associated with severely reduced levels of *Pigpb1* mRNA.

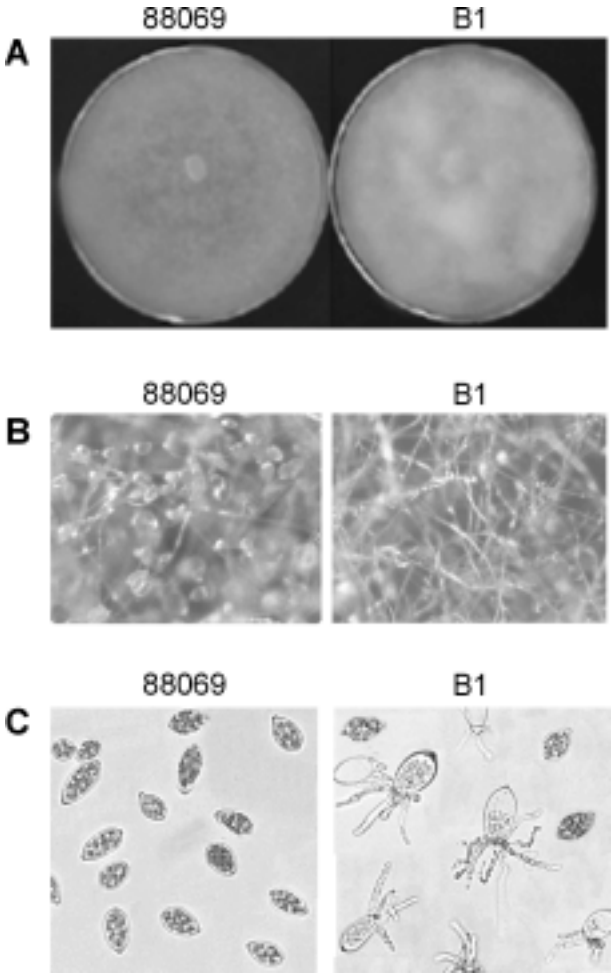


Figure 3.
Phenotypic characterization of *Pigpb1*-silenced mutants
A. Growth on rye sucrose agar (RSA) of wildtype recipient strain 88069 and *Pigpb1*-silenced mutant B1. B1 produces a denser mat of aerial mycelium than 88069. As a consequence, the colony of B1 is more opaque than the colony of 88069.
B. The colonies shown in (**A**) were photographed at 50x magnification. In the wildtype 88069, sporangia are visible as droplet-like structures. Note the absence of sporangia in the *Pigpb1*-silenced mutant B1.
C. Sporangia harvested from colonies of 88069 and B1 by flooding RSA plates with water, were concentrated and photographed (200x magnification). A large proportion of the sporangia of the *Pigpb1*-silenced mutants has multiple hyphal outgrowths and some are empty.

In *Saccharomyces cerevisiae* and several other fungi, the Gβγ dimer is required for mating and/or sexual development (Lengeler et al., 2000). To investigate a role for PiGPB1 in mating, *Pigpb1*-silenced mutants were co-cultivated with the opposite mating type (wildtype) and with *Pigpb1*-silenced mutants of the opposite mating type. However, no obvious differences were detected in the numbers of oospores and oospore-like structures that were formed at the interface of an A1 and an A2 colony irrespective of the expression levels of *Pigpb1* in the mating cultures. Even in complete absence of PiGPB1 (B1 as A1 strain and B4 as A2 strain) normal oospore formation was observed. Apparently, PiGPB1 is not required for the formation of gametangia nor for the actual mating resulting in the formation of sexual spores.

The expression of cdc14 phosphatase is downregulated in the *Pigpb1*-silenced mutants

CDC14 is a protein phosphatase playing a role in mitosis in *Saccharomyces cerevisiae* (Visintin et al., 1998). The gene encoding a *P. infestans* homologue of CDC14 is expressed at a very low level in vegetative hyphae. Its expression is strongly upregulated in sporulating hyphae and remains high in sporangia, zoospores and cysts (Audrey M. V. Ah Fong and H. S. Judelson, personal communication). Expression of *cdc14* in mycelium of wildtype incubated in rich and minimal medium for six hours (treated as described in the first paragraph) was analyzed by RT-PCR. This revealed that the expression of this gene is induced under starvation conditions (Figure 4). Very little *cdc14* mRNA was detected in both *Pigpb1*-silenced mutants, suggesting that PiGPB1 affects the expression of this *cdc14* homologue.

In planta growth

Virulence of *P. infestans* strains is routinely tested by inoculation of potato leaves with a droplet of zoospores. The partially silenced mutant B2e was the only *Pigpb1*-silenced mutant producing sufficient zoospores for inoculation of potato leaves. The level of *Pigpb1* mRNA in sporangia of B2e was determined by RT-PCR. The level was shown to be consistently lower than in wildtype sporangia (Figure 2B), albeit the difference between the two was smaller than in mycelium of the same strains. On leaves of potato cultivar Nicola, mutant B2e developed lesions of the same size as wildtype strain 88069 (data not shown). The infection efficiencies of wildtype and B2e was over 90%, showing that the reduction in *Pigpb1* mRNA in sporangia and mycelium did not affect the aggressiveness of mutant B2e. The lesions of B2e were covered with more mycelium than those of the wildtype strain, indicating that the 'fluffy' phenotype is maintained also during growth *in planta*.

In planta growth of the mutants not producing sporangia was analyzed by inoculating freshly cut potato slices with mycelium plugs. All (partially) *Pigpb1*-silenced mutants, including B2e, were able to grow on potato slices but the *Pigpb1*-silenced mutants grew consistently more slowly than the corresponding wildtype strain (data not shown). Altogether, it can be concluded that PiGPB1 does not seem to be required for *in planta* growth.

DISCUSSION

The G-protein β subunit gene *Pigpb1* is expressed in nutrient-starved mycelium and is downregulated in rich medium. Silencing of *Pigpb1* was shown to be associated with a defect in sporulation and with formation of abundant aerial mycelium that is only loosely attached to the agar surface. Partially silenced mutants displayed the same 'fluffy' growth habit and sporulation was reduced in these mutants as well. In the *Pigpb1*-silenced mutants, a large proportion of the sporangia was empty, small or possessed multiple hyphal extensions The *Pigpb1* expression pattern and the sporulation defect of the *Pigpb1*-silenced mutants indicate that PiGPB1 is involved in signaling events in mycelium leading to formation of sporangia. This means that

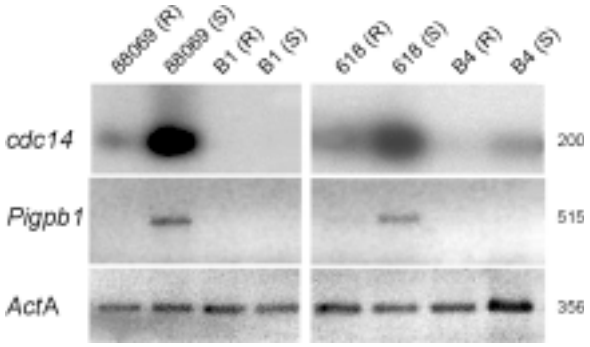


Figure 4.
Expression of *cdc14* and *Pigpb1* in two-weeks-old mycelium grown in rye sucrose (RS) medium after transfer to fresh rye sucrose medium (R) or minimal medium (S) and six hours incubation in the same medium. RT-PCR was performed using primers corresponding to *cdc14*, *Pigpb1* and *Act*A. 88069 and 618, wildtype strains. B1, B4, *Pigpb1*-silenced mutants

PiGPB1 is also indispensable for pathogenicity in *P. infestans*. We speculate that the increase in aerial mycelium in the *Pigpb1*-silenced mutants results from hyphae that continue to grow in the absence of a signal triggering formation of sporangia.

C. parasitica mutants in which the Gβ subunit gene *cpgb1* was disrupted showed reduced conidiation and an increase in hyphal density on rich media. The dense mycelial mat of this mutant could be easily peeled from the agar surface (Kasahara and Nuss, 1997). These phenotypes are strikingly similar to those of the *Pigpb1*-silenced mutants, showing that Gβ subunits control similar processes in these two evolutionary unrelated plant pathogens. In contrast, loss of the Gβ subunit gene *gnb-1* in *Neurospora crassa* and *sfaD* in *Aspergillus nidulans* had the opposite effect. In these species, the strains in which the Gβ genes were disrupted conidiated more profusely and showed inappropriate conidiation in submerged culture (Yang et al., 2002; Rosén et al., 1999). In *Dictyostelium discoidium*, starvation induces cells to secrete cAMP and to autoaggregate. cAMP sensing and chemotaxis are clearly regulated by G-protein signaling in this organism. Mutants in which the single copy Gβ gene, *gpb1,* is disrupted are completely impaired in chemoattractant-induced responses (Lilly et al., 1993; Jin et al., 1998). Such a relation between starvation and GPB1 function may also exist in *P. infestans* where we found that starvation induced *Pigpb1* expression, which, in turn, precedes sporulation.

We considered the possibility that the sporulation defect of the PiGPB1-deficient mutants is the cause and not the consequence of the absence of *Pigpb1* mRNA, since transformation itself occasionally results in a reduction in sporulation. However, a sustained reduction of sporulation to less than 1% as a side effect of transformation is extremely rare. Secondly, the frequency of silencing of *Pigpb1* in the PEG transformants was ca. 20% (5 / 17) and comparable to the frequency we observed for the silencing of the elicitin gene *inf1* (van West et al., 1999). In our experience, sporulation was never affected in 20% of the transformants as a result of transformation alone. Moreover, both sporulation and expression of *Pigpb1* was found to be normal in a series of 15 PEG transformants that were transformed with a different plasmid not containing *Pigpb1*. Finally, *Pigpb1* was expressed at wildtype level in a field isolate that sporulates poorly (80029) (results not shown), showing that reduced sporulation is not always associated with reduced *Pigpb1* expression. Based on these observations, there is little doubt that the sporulation defect in the mutants is caused by downregulation of *Pigpb1* expression. *Pigpb1* was silenced in only one of the 57 electroporation transformants. The finding that silencing was so rare among these transformants is not surprising because other genes were also silenced at low frequencies in electroporation transformants (Govers et al., unpublished results). The Gα subunit gene *Pigpa1*, for example was silenced in only 3% of the electroporation transformants (Latijnhouwers et al., in preparation). A possible explanation for this is that plasmid DNA integrates differently into the genome when using electroporation compared to PEG transformation: PEG-protoplast transformation generally results in many plasmid integrations (see Figure 2C and van West et al., 1999), whereas zoospore-electroporation usually results in only few plasmid integrations (unpublished results). However, we have so far no evidence that the number of plasmid integrations plays a role in triggering silencing (van West et al., 1999).

It was surprising to see that in four of the six *Pigpb1*-silenced mutants, a smear of RNA had replaced the distinct band representing *Pigpb1* mRNA on northern blots. This was not due to general RNA breakdown as the control probe hybridizes to a single band without a smear. RNA breakdown was not observed in the *Pigpa1*-silenced mutants (Latijnhouwers et al., in preparation), nor in the *inf1* and *cbel*-silenced mutants (Gaulin et al., 2002; van West et al., 1999). This raises the question of whether different mechanisms of gene-silencing may be operating in *Phytophthora*.

The Gα subunit gene *Pigpa1* is not expressed in mycelium, suggesting that PiGPB1 complexes with another *P. infestans* Gα subunit in mycelium. However, additional Gα subunit genes have not been detected in *P. infestans* despite considerable efforts (Laxalt et al., 2002). In *Saccharomyces cerevisiae* the Gα subunit GPA2 does not interact with βγ dimers but instead complexes with proteins containing seven kelch repeats (Harashima and Heitman, 2002). This example shows that G-protein signaling in lower eukaryotes does not always comply with the paradigm accepted for animals. It can therefore, not be excluded that PiGPB1 functions independently from Gα subunits.

The Gβγ dimer (STE4 / STE18) in *S. cerevisiae* is part of the pheromone response pathway. This pathway also involves a MAP kinase cascade (STE11 / STE7 / FUS3) (reviewed in Leberer et al., 1997). MAP kinase activation upon wounding and cold treatment was analyzed in the *P. infestans Pigpb1*-silenced mutants by means of in gel kinase assays but no differences were detected between wildtype and mutants (data

not shown). The cAMP pathway is another pathway known to be downstream of G-proteins in fungi (reviewed in Kronstad et al., 1998). Whether this pathway is also involved in processes controlled by G-proteins in *P. infestans* should be the focus of future research. G-protein signaling eventually alters gene expression (Gutkind, 1998). In wildtype, the expression of the gene encoding the *P. infestans* homologue of the *S. cerevisiae* CDC14 phosphatase was induced upon starvation and downregulated in mycelium that was incubated in rich medium for 6 hours. As the starvation conditions eventually led to sporulation, this expression pattern is in agreement with the finding that the expression of this gene correlates with sporulation (Audrey M. V. Ah Fong and Howard S. Judelson, personal communication). The level of *cdc14* mRNA in mycelium of the *Pigpb1*-silenced mutants was barely detectable, indicating that the expression of this gene is dependent on the presence of PiGPB1. Efforts to identify additional target genes of PiGPB1 are in progress. In view of the role of PiGPB1 in sporulation, genes under control of PiGPB1 may be of great help to increase our understanding of sporangia formation in *P. infestans*.

MATERIALS AND METHODS

Phytophthora infestans strains and culture conditions

P. infestans isolates NL-88069 (A1 mating type), METV-618 (A2 mating type) and all transgenic isolates were routinely grown at 18 ºC in the dark on rye agar medium supplemented with 2% sucrose (RSA) (Caten and Jinks 1968). RSA was supplemented with 2.5 mg/l of G418 (GIBCO-BRL, Bethesda, MD) for the transformants. For the isolation of sporangiospores, sporulating mycelium was flooded with modified Petri's solution (0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4 and 0.8 mM KCl) (Petri, 1917) and rubbed with a sterile glass rod. Subsequently, the suspension was filtered through a 50 µm nylon mesh. The number of sporangia was determined using a haemocytometer in at least 5 different isolations from different plates of each transformant. To starve mycelium for nutrients, cultures were grown in liquid RS medium in petri dishes (Æ 9 cm) for two weeks. Mycelium was washed twice in fresh RS or minimal medium (10 mM $Ca(NO_3)_2$; 5 mM KNO_3; 4 mM $MgCl_2$; 20 µM $FeSO_4$; 20 µM Na_2EDTA; 10 mg/l β-sitosterol; modified from Marshall et al., 2001) and transferred to a new petri dish containing the respective medium. The mycelia were harvested for RNA extraction after 6 hours.

Plasmids used for transformation of *P. infestans*

Plasmid pMH2 consists of a 2.3 kb *Hin*dIII-*Bam*H1 restriction fragment from *P. infestans* genomic DNA cloned into pBluescript (SK^{II}) (GenBank accession AY204515). This fragment contains the full length *Pigpb1* gene, 911 bp promoter region and 387 bp of the terminator (Laxalt et al., 2002). Plasmid pTH209 (Judelson et al., 1991), consisting of the *hsp70* promoter of *Bremia lactucea* fused to the *npt*II coding sequence and the *ham34* terminator of *B. lactucea*, was used as selectable plasmid. The Plasmid Maxi kit (Qiagen GmbH, Hilden) was used for the isolation of plasmid DNA for transformation of *P. infestans*. Per transformation, 5 µg of pTH209 was used in combination with 30 µg (PEG transformation) or 15 µg (zoospore electroporation) of pMH2. Plasmid DNA used for transformation was digested with *Eco*R1 in the case of pTH209 and with *Bam*H1 or *Hin*dIII in case of pMH2.

Transformation of *P. infestans*

P. infestans strains 88069 and 618 were stably transformed using the PEG protoplast method as described by van West et al. (1998) with a slight modification. We experienced that the presence of lipofectin (GIBCO-BRL, Bethesda, MD) negatively influenced the efficiency of transformation and lipofectin was therefore omitted from the incubation mixture. Strain 88069 was also transformed using zoospore electroporation (B.M. Tyler, et al., unpublished; Latijnhouwers et al., in preparation).

Southern and northern blot analysis

Mycelium for isolation of genomic DNA was grown in liquid modified Plich medium (van der Lee et al., 1997) as described by Raeder and Broda (1985) with minor modifications. Following electrophoresis, DNA was transferred to Hybond N^+ membranes (Amersham) by alkaline transfer. Total RNA was isolated using Trizol (Gibco-BRL, Bethesda, MD) according to the manufacturer's instructions. For northern blot analysis, 10 µg total RNA was denatured at 50 ºC in 1 M glyoxal, 50% DMSO, and 10 mM sodium phosphate, electrophoresed and transferred to Hybond N^+ membranes in 10x SSC (Ausubel et al., 1987; Sambrook and Russel, 2001). Hybridizations of DNA and RNA blots were conducted at 65 ºC and filters were washed at 65 ºC in 0.5x SSC + 0.5% SDS. Purified PCR products (Nucleotide removal kit, Qiagen, GmbH, Hilden) consisting of the complete coding region of *Pigpb1* (forward primer: 5'-tcccccgggcaatgggcgacgctgcggag-3'; reverse primer: 5'-tcccccgggttaggcccagatcttgagtg-3') or a 796 bp *Hind*III restriction fragment of the *Act*A gene from pSTA31 (Unkles et al., 1991) were used as probes and radiolabeled with α ^{32}P-dATP using a random primer labeling kit (Gibco-BRL, Bethesda, MD). To remove the unincorporated nucleotides, the Qiaquick Nucleotide Removal kit (Qiagen GmbH, Hilden) was used.

Reverse Transcriptase PCR analysis

To remove contaminating genomic DNA in RNA preparations, 10 µg total RNA was treated with 4 units RQ1 RNase-free DNase (Promega, Madison, WI) at 37 ºC for 1h. The removal of DNA was verified in a PCR reaction under the same conditions as those used for the RT-PCR reaction, except that the 30 min cDNA synthesis step at 50 ºC was omitted. RT-PCR was performed using the One Step RT-PCR system (Gibco-BRL, Bethesda, MD) with 100 ng total RNA and 50 ng of each primer, following the manufacturer's instructions. Incubation at 50 ºC for 30 min; 94 ºC for 2 min; 27 cycles of 94 ºC for 30 s, 58 ºC for 30 s, 72 ºC for 45 s and 72 ºC for 5 min was used for detection of *Pigpb1* mRNA. To analyze the level of *Act*A mRNA, the same program was applied using 24 cycles instead of 27. The forward primer 5'-tcccccgggcaatgggcgacgctgcggag-3' and reverse primer 5'-gatgcagttggagtcccccg-3', both annealing to the coding region, were used for amplification of *Pigpb1* from mRNA, the forward primer 5'-cggctccggtatgtgcaaggc-3' and the reverse primer 5'-gcgggcacgttgaacgtctc-3', for *Act*A and the forward primer 5'-ccctcgagatgggactctgtgct-3' and reverse primer 5'-gtgatggccgcgatcatac-3' for *Pigpa1*. The level of *cdc14* mRNA was determined in RT-PCR using the forward primer 5' tccgaggttgagtgggcc 3' and the reverse primer 5' tccgaggttgagtgggcc 3'. The GenBank accession number for *P. infestans* CDC14 is AY204881 (A.M.V. Ah Fong and H.S. Judelson, submitted). Primers were synthesized by Amersham-Pharmacia (Buckinghamshire).

Virulence assays

Detached potato leaves of cultivar Nicola were placed in florist foam and inoculated on the abaxial side with one droplet (10 µl) containing 10^3 zoospores per leaflet. The leaves were incubated in a climate room at 18 ºC and 80 % humidity with 16 h light per 24 h. The length and width of the lesions were measured at three, four and five days post inoculation using a marking gauge linked to the IBREXDLL software (IBR Prozessautomation, Germany). To test the growth of *P. infestans* strains on potato slices, agar plugs of identical size were placed on potato slices in a petri dish. The slices were incubated at 18 ºC in the dark for up to 10 days.

ACKNOWLEDGEMENTS

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CHAPTER 5

THE IDENTIFICATION OF POTENTIAL DOWNSTREAM TARGETS OF THE *PHYTOPHTHORA INFESTANS* Gα SUBUNIT PIGPA1 USING cDNA-AFLP

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Submitted for publication

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ABSTRACT

Phytophthora infestans is an oomycete plant pathogen causing late blight of potato. Previous work showed that the Gα subunit gene *Pigpa1* of this pathogen controls zoospore motility and is required for virulence (Latijnhouwers et al., in preparation). In addition, zoospore release and appressorium development were affected in the PiGPA1-deficient strains. So far, downstream target genes of G-proteins are unknown in oomycetes. To identify genes under control of PiGPA1, gene expression in sporangia of *Pigpa1*-silenced mutants was studied by cDNA amplified fragment length polymorphism (cDNA-AFLP) analysis and compared with gene expression in wildtype sporangia and in sporangia of a strain expressing a constitutively active form of PiGPA1. Gene expression in wildtype mycelium was also analyzed. A total of 2860 transcript derived fragments (TDFs) was amplified using 96 primer combinations. Seventy-seven TDFs were down- and 11 upregulated in the *Pigpa1*-silenced mutants. 34 TDFs were cloned and sequenced and 13 showed similarity to sequences present in the *P. infestans* and *P. sojae* EST databases. A subset of those showed homology to sequences in the NCBI database. The expression profiles of the genes from which these TDFs were derived, were verified by northern blot analysis or by RT-PCR. The power of the cDNA-AFLP approach for the identification of target genes in knock-down or gain-of-function mutants is discussed.

INTRODUCTION

Phytophthora infestans causes late blight on potato and tomato. This pathogen belongs to the oomycetes, a class of eukaryotic organisms evolutionary related to golden-brown algae (Margulis and Schwartz, 2000). The genus *Phytophthora* contains over 60 plant pathogens, among which those causing devastating diseases on economically important crops like potato, soybean, cocoa and pepper. *P. infestans* grows as coenocytic mycelium in the vegetative stage. It forms sporangia at the hyphal tips of so-called sporangiophores. Sporangia are asexual spores with two methods to propagate infection. Direct germination occurs at temperatures above ± 15 °C. At lower temperatures, sporangia undergo cytoplasmic cleavage and release swimming, uninucleate spores, called zoospores. Once these zoospores contact the host, they encyst, germinate and form appressoria from which a penetration hypha emerges that pierces the plant cuticle and the cell wall (Erwin and Ribeiro, 1996).

One of our approaches to throw light on *Phytophthora* genes involved in pathogenicity is by studying the role of conserved signaling pathways in development and virulence. One such pathway is the heterotrimeric G-protein pathway. Heterotrimeric G-proteins are key players in cellular signaling in virtually all eukaryotic taxa. The name G-protein comes from the ability of the Gα subunit to bind and hydrolyze GTP. G-protein linked membrane receptors can activate the G-protein trimer by replacing GDP that is bound to Gα, by GTP. This leads to dissociation of the trimer in a Gα and a Gβγ subunit, each acting on a range of downstream targets (Neer, 1995). In animals, G-protein targets include adenylyl cyclase, MAP kinases, phospholipases, ion channels and PI3kinase (reviewed in Albert and Robillard, 2002; Dhanasekaran et al., 1998).

Fungal G-proteins activate the cAMP and the MAP kinase pathways (Bölker, 1998), whereas their plant counterparts seem to activate phospholipases and ion channels (Bischoff et al., 1999; Assmann, 2002). These signaling cascades link external signals perceived by G-protein-coupled receptors to the nucleus by changing the affinity of transcription factors for their target sites thus altering expression of wide range of genes, enabling the cells to respond to changes in the environment (Gutkind, 1998).

P. infestans possesses at least one gene encoding a G-protein α subunit (*Pigpa1*) and one encoding a G-protein β subunit (*Pigpb1*) (Laxalt et al., 2002). PiGPA1-deficient mutants were generated by homology-dependent gene-silencing (Latijnhouwers, in preparation). Zoosporogenesis, zoospore aggregation and appressorium development were affected in the knock-down mutants and the virulence of these mutants was strongly reduced. In contrast, virulence of mutants expressing constitutively active forms of PiGPA1 seemed to be slightly enhanced (Latijnhouwers, in preparation). These so-called PiGPA1 gain-of-function mutants were created by introducing copies of *Pigpa1* with single basepair changes into a wildtype *P. infestans* strain, resultingin PiGPA1 proteins,where the conserved amino acids R177 or Q203 are substituted by a histidine or leucine, respectively (R177H and Q203L). These mutations are well characterized in other systems and are known to disrupt the GTPase activity of the protein, thereby preventing it from returning to the inactive form as would occur in the wildtype protein (Kurjan et al., 1991; Landis et al., 1989; Mendel et al., 1995; Regenfelder et al., 1997; Wong et al., 1991).

Because oomycetes are diploid organisms, it is impossible to isolate G-protein targets by screening a randomly mutagenized population for suppressor mutants, a method commonly used for fungi. Instead, we chose to use cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP) analysis to identify transcripts that are differentially expressed in the mutants compared to the wildtype strain. Those up- or downregulated transcripts may be derived from genes that are positively or negatively regulated by the G-protein signaling pathway. Compared to other methods employed to identify differentially expressed genes such as differential display and microarray analysis, cDNA-AFLP is a very sensitive and versatile method (Bachem et al., 1996; Donson et al., 2002). It has been successfully used in plants to identify organ-specific genes or genes the expression of which is altered during the defense response or during stress (Simoes-Araujo et al., 2002; van der Biezen et al., 2000; Durrant et al., 2000; Bachem et al., 1996). The technique was also used to identify a large *hrp*G-regulon in the plant-pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* (Noel et al., 2001) as well as genes that were differentially expressed under different growth conditions in the bacterium *Erwinia carotovora* (Dellagi et al., 2000).

With the purpose to isolate transcripts with differential expression in the *Pigpa1*-silenced mutants and the wildtype strain, gene expression was profiled by cDNA-AFLP in sporangia of those strains. Transcript derived fragments (TDFs) were cloned that were present in wildtype and in a mutant expressing a constitutively active PiGPA1, but that were absent in the *Pigpa1*-silenced mutants. The sequences of almost 50% of the cloned TDFs were derived from genes that are represented in the *P. infestans*and/or *P. sojae* Expressed Sequence Tag (EST) databases. Some of them share homology to known genes from other organisms.

RESULTS

cDNA-AFLP analysis in *Phytophthora infestans*

cDNA-AFLP analysis was used to display gene expression in sporangia of the wildtype *P. infestans* strain 88069 and in three mutants deficient in the Gα subunit PiGPA1, gs1, gs2 and gs3 (Figure 1). Sporangia of one PiGPA1 gain-of-function mutant (R2) and mycelium of wildtype strain 88069 were also included. The latter was added to evaluate the performance of cDNA-AFLP in our system. It is expected that a large number of genes is differentially expressed in mycelium and sporangia. Mycelium of the mutants was not included as *Pigpa1* is not expressed in mycelium (Laxalt, et al., 2002), making it less likely that genes expressed in mycelium are controlled by PiGPA1. RNA samples from two independent RNA isolations of each strain were pooled.

The cDNA pools were digested with the restriction enzymes *Apo*I and *Taq*I. These restriction enzymes were selected based on the predicted number of cDNA fragments to be generated and the expected size of the fragments (GenEST software (Qin et al., 2001; Dong et al., in preparation). Amplification primers containing a two selective nucleotide extension were used to obtain the desired specificity and a manageable number of TDFs per primer combination. Using 96 primer combinations, a total of 2860 TDFs was generated, ranging in length from 40 to 400 bp.

Table 1. Comparison of the profiles of sporangia and mycelium of wildtype. Numbers of sporangia-specific (absent in mycelium), mycelium-specific (absent in sporangia) and common bands (present in sporangia and mycelium of wildtype) are presented.

	sporangia and mycelium	sporangia only	mycelium only	total
number of TDFs	1876	557	392	2825

Differences in expression profiles

Of the total of 2860 TDFs, 1802 were amplified from all six samples tested and 1058 TDFs (37%) were absent in at least one of them. First, the mycelium profile was compared to the profile of wildtype sporangia (Table 1). They shared 1876 bands, 392 TDFs were mycelium-specific and 557 were sporangia-specific. Because the expression in stages other than mycelium and sporangia was not analyzed, the terms mycelium-specific and sporangia-specific, in this case, only refer to expression in sporangia relative to mycelium and *vice versa.*

The TDFs are ordered by types in table 2. Each type has a different expression pattern. TDFs of type 1 to 3 are either present or absent in all sporangia samples. They seem to be derived from genes the expression of which is unaltered in the mutants. Type 1 TDFs are common to all samples. Type 2 TDFs are sporangia-specific and type 3 mycelium-specific. TDFs of type 4 to 7 are altered in the *Pigpa1*-silenced mutants in

comparison to wildtype sporangia and the PiGPA1 gain-of-function mutant. Seventy-seven TDFs are absent in the *Pigpa1*-silenced mutants while present in the others (type 4 and 5) and 73 of those are sporangia-specific (type 4). These TDFs may be derived from genes the expression of which is normally induced by PiGPA1. Conversely, 11 TDFs are present in sporangia of the *Pigpa1*-silenced mutants and absent in the wildtype sporangia (type 6 and 7). Possibly, the expression of the genes represented by these TDFs is normally repressed by PiGPA1. A much smaller number of TDFs was dfferentially expressed in the gain-of-function mutant R2 (type 8-11), indicating that PiGPA1 activates rather than represses downstream genes. Only two sporangia-specific TDFs were absent in R2 but present in wildtype and the *Pigpa1*-silenced mutants (type 8 and 9) and five were unique for R2 (type 10 and 11). The TDFs of the latter types may be derived from genes the expression of which is specifically induced due to the presence of a constitutively active PiGPA1. Fifteen sporangia-specific TDFs were absent in both types of mutants (type 12 and 13) and eight TDFs were absent in wildtype sporangia but present in both types of mutants (type 14 and 15). An example of a gene from which a TDF of type 15 may be derived is the geneticin resistance gene *npt*II. This gene was used as selection marker and is expressed in both types of mutants but is evidently not present in wildtype.

TDFs present in only one or two of the three *Pigpa1*-silenced mutants were not included in this table because the expression of the genes from which they are derived is less likely to be controlled by G-proteins. This was the case with 6.3% of the total

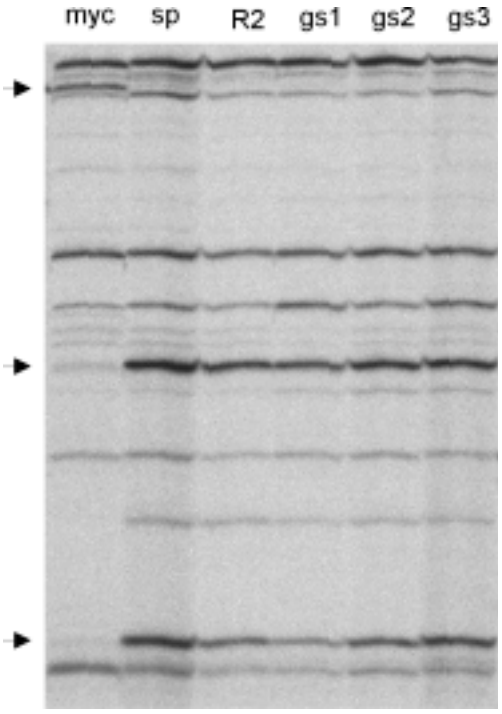

Figure 1.
cDNA-AFLP analysis of gene expression in wildtype mycelium and in sporangia of wildtype, of *Pigpa1*-silenced mutants and of a PiGPA1 gain-of-function mutant. Amplified fragments, separated on a denaturing polyacrylamide gel, are visualized by autoradiography. A section of an autoradiograph is shown in which fragments derived from genes differentially expressed in mycelium and sporangia are visible (arrows). myc, mycelium of wildtype strain 88069; sp, sporangia of wildtype strain 88069; R2, sporangia of PiGPA1 gain-of-function mutant; gs1, gs2, gs3, sporangia of *Pigpa1*-silenced mutants.

number of TDFs (the difference between the total number of 2860 TDFs and the number of TDFs listed in table 2). They may result from either statistical variation in the cDNA-AFLP procedure or from actual differences in gene expression between the mutants, possibly caused by coincidental differences e.g. in growth conditions.

Table 2. TDFs ordered based on differential expression in mycelium vs. sporangia (type 1-3), in the *Pigpa1*-silenced mutants vs. wildtype (type 4-7), in the PiGPA1 gain-of-function mutant vs. wildtype (type 8-11) or in both the *Pigpa1*-silenced mutants and the PiGPA1 gain-of-function mutant vs. wildtype (type 13-15).

type	mycelium	sporangia wild type	sporangia PiGPA1 gain-of-function	sporangia *Pigpa1*-silenced	number of TDFs	category
1	+	+	+	+	1802	general, mycelium-specific or sporangia-specific
2	-	+	+	+	419	
3	+	-	-	-	340	
4	+	+	+	-	4	altered in *Pigpa1*-silenced mutants
5	-	+	+	-	73	
6	+	-	-	+	1	
7	-	-	-	+	10	
8	+	+	-	+	0	altered in PiGPA1 gain-of-function mutants
9	-	+	-	+	2	
10	+	-	+	-	0	
11	-	-	+	-	5	
12	+	+	-	-	0	altered in both types of mutants
13	-	+	-	-	15	
14	+	-	+	+	1	
15	-	-	+	+	7	

+ band present; - band absent.

Cloning of differentially expressed cDNA fragments and verification of expression patterns

For further analysis and verification of the expression patterns, 30 of the cDNA-AFLP fragments that were up- or downregulated in the mutants were excised from gel, reamplified and cloned. Two mycelium-specific and two sporangia-specific TDFs were included as controls.The fragment types, designations (consisting ofprimer extensions and size) and the GenBank accession numbers are listed in Table 3. Based on the size of the fragment and the presence or absence of the expected two-base primer extension in the sequence it was concluded that in 32 of the 34 cases the correct fragment had been cloned.

To verify the cDNA-AFLP expression pattern, a northern blot containing sporangial RNA from wildtype and the four mutants was hybridized with a representative probe synthesized from TDF20. In the cDNA-AFLP analysis, this fragment was identified as a type 5 TDF, i.e. it was exclusively detected in sporangia of wildtype and in the PiGPA1 gain-of-function mutant R2 (Figure 2, upper panel). With the TDF20 probe, two bands of different sizes were recognized in RNA from both wildtype and R2 sporangia, whereas

Table 3. cloned transcript derived fragments

type*	clone	TDF
2	TDF2	A+AA/T+GTs363
2	TDF7	A+AA/T+CCs163
3	TDF16	A+AA/T+ATs143
3	TDF19	A+AA/T+GGs474
5	TDF20	A+AA/T+GGs233
7	TDF22	A+AG/T+TTs117
7	TDF23	A+AG/T+TTs109
7	TDF24	A+AG/T+TTs98
7	TDF26	A+AG/T+TTs60
4	TDF27	A+AG/T+TCs73
5	TDF28	A+AG/T+TCs54
7**	TDF29	A+AG/T+CTs48
5	TDF32	A+AT/T+GTs122
5	TDF33	A+AT/T+GTs93
11	TDF34	A+AT/T+CGs139
7**	TDF36	A+AT/T+TAs156
5	TDF37	A+AT/T+AAs287
5	TDF38	A+AT/T+ACs250
5	TDF40	A+TC/T+GTs40
5	TDF41	A+TC/T+TAs78
5	TDF42	A+TC/T+TAs76
5	TDF43	A+TC/T+GCs219
5	TDF44	A+TC/T+GCs115
5	TDF45	A+TC/T+GCs48
5	TDF49	A+TC/T+TTs61
5	TDF54	A+TC/T+CAs78
7**	TDF57	A+CA/T+AAs97
5	TDF60	A+CA/T+AGs46
5	TDF62	A+CA/T+CTs64
5	TDF64	A+TC/T+TTs54
5	TDF66	A+AT/T+GTs170
5	TDF67	A+AT/T+GTs139

* as defined in table 2
** only present in two out of three *Pigpa1*-silenced mutants

no bands were visible in the lanes containing RNA isolated from gs1, gs2 and gs3 sporangia (Figure 2, middle panel). This confirms that expression of the TDF20 gene is downregulated in the *Pigpa1*-silenced mutants. No explanation was found for the presence of two transcripts of different sizes. Primers specific for TDF20 were used to verify the expression pattern of the TDF20 gene by RT-PCR analysis. No product was generated from RNA isolated from sporangia of the *Pigpa1*-silenced mutants, again confirming the absence of mRNA of the respective gene in the three *Pigpa1*-silenced mutants (Figure 2, bottom panel).

Sequence-specific primer pairs were designed for 16 additional, cloned TDFs. These primers were used in RT-PCR analysis to verify the cDNA-AFLP patterns (Table 5, Figure 3). The RNA preparations used in the RT-PCR experiments were isolated

independently from RNA samples used for the cDNA-AFLP analysis. Fourteen primer pairs generated RT-PCR products of the expected size (See Figure 3 for a subset of them). The primer pairs for TDF45 and 66 failed to generate products. RT-PCR-mediated display of gene expression of six samples confirmed the results obtained with the cDNA-AFLP analysis for 13 of the 14 fragments. In the case of TDF29, the expression pattern generated by RT-PCR did not correspond to the expression of the fragment observed in the cDNA-AFLP analysis.

Sequence similarity of TDFs with known sequences

The TDF nucleotide sequences were compared to sequences in the National Center for Genome Resources (NCGR) Phytophthora Genome Consortium (PGC) and Syngenta Phytophthora Consortium (SPC) EST sequence databases using the BLAST algorithm (Altschul et al., 1997). 13 of the 29 (45%) cloned (and verified) TDFs showed similarities to *P. infestans* ESTs (*E*-value cutoff = 1e-05). For each TDF, Table 4 shows the EST to

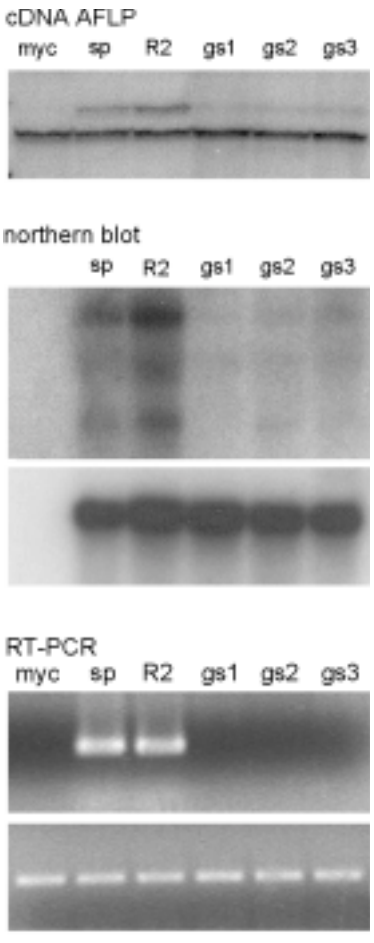

Figure 2.
Verification of cDNA-AFLP expression patterns. TDF20 was selected based on its differential expression pattern in cDNA-AFLP (upper panel) and was used as template to synthesize a probe for northern blot analysis (middle panel). The northern blot contained total RNA from sporangia (sp) of wildtype strain 88069, of PiGPA1 gain-of-function mutant (R2) and of three *Pigpa1*-silenced mutants (gs1, gs2 and gs3).
Sequence-specific primers corresponding to TDF20 were used to verify the expression of the TDF20 gene by RT-PCR (bottom panel). Total RNA of mycelium of wildtype strain 88069 (myc) and of sporangia of the previously mentioned strains (88069 (sp), R2, gs1, gs2, gs3) was used as template.

Table 4. EST Database hits of TDFs

	BLASTN (SPC database)		BLASTX (NCBI database)	
TDF	homology	score	closest similarity	score***
TDF2*	no hits		no hits	
TDF7*	no hits		no hits	
TDF16*	rpcy_10728.y1.abd	3e-71	gi|13474650 enoyl CoA hydratase [Mesorhizobium loti]	2e-92
TDF19*	rpcm_1853.y1.abd	0.0	gb|AAL73393.1 molecular chaperone MRJ [Bos taurus]	2e-19
TDF20*/**	rpvb_11347.y1.abd	4e-56	Blocks+: IPB001868 Amyloidogenic glycoprotein (Amyloid)	0.36
TDF22	rpcd_1921.y1.abd	5e-60	gi|6322544 Hypothetical ORF; Yjr085cp [Saccharomyces cerevisiae]	8e-13
TDF23*	no hits		no hits	
TDF24	PF002F4.XT7	6e-50	gb|AAB08700.1UbcB ubiquitin conjugating enzyme [Dictyostelium discoideum]	2e-38
TDF26	no hits		no hits	
TDF27	rpvb_10780.y1.abd	1e-34	Blocks+: IPB001479 Bact quinoprot dehydrogenase	0.11
TDF28	PM030G5.XT7	1e-21	>gb|AAL25565.1AT5g19750/T29J13_170 [Arabidopsis thaliana]	9e-10
TDF32	no hits		no hits	
TDF33	no hits		no hits	
TDF34	no hits		no hits	
TDF36	no hits		no hits	
TDF37*	rpwa_0101.y1.abd	e-158	pdb|1UTE|A Chain A, Pig Purple Acid Phosphatase Complexed	7e-07
TDF38*	no hits		no hits	
TDF40	rpch_6748.y1.abd	8e-16	no hits	
TDF41*	no hits		no hits	
TDF42	no hits		no hits	

TDF41*	no hits		no hits	
TDF42	no hits		no hits	
TDF43*	rpvb_9759.y1.abd	2e-73	Blocks+: IPR00636 AT2 angiotensin II receptor	0.36
TDF44*	PH010H6.XT7	2e-10	Blocks+ IPR01105 GPCR Bonzo	0.63
TDF49	rpch_1239.y1.abd	1e-25	gb|AAB88887.1|carnitine acetyl transferase [Magnaporthe grisea]	3e-16
TDF54*	no hits		no hits	
TDF57	no hits		no hits	
TDF60	PF037H7.XT7	3e-19	Blocks+: IPB001086 Prephenate dehydratase	0.24
TDF62	no hits		no hits	
TDF64	no hits		no hits	
TDF67*	no hits		no hits	

SPC: Syngenta Phytophthora Consortium database; NCBI: National Center for Biotechnology Information.
* used for expression analysis using RT-PCR
** used for expression analysis using northern blot analysis
*** denotes BLASTX match for cognate sequences in SPC database

which it has the highest similarity. Six of these ESTs are derived from genes with homologues stored in the National Center for Biotechnology Information (NCBI) sequence database and five additional ones contain motifs with homology to entries in the Blocks+ protein domain database (Henikoff and Henikoff, 1994) (Table 4). As an example, TDF24, which was only present in the three *Pigpa1*-silenced mutants, is similar to ESTs with homology to ubiquitin-conjugating enzymes. TDF37 and TDF 49, which were absent in the three *Pigpa1*-silenced mutants, show homology to purple acid phosphatases. and carnitine acetyl tranferase, respectively. In general, the cDNA-AFLP expression pattern correlated with the occurrence of the corresponding ESTs in the EST libraries from different stages. For example, most of the TDFs of type 5 only occurred in the EST libraries derived from cleaving sporangia or purified zoospores.

DISCUSSION

Gene expression analysis by cDNA-AFLP in sporangia of *Pigpa1*-silenced mutants and one PiGPA1 gain-of-function mutant resulted in the identification of TDFs that were differentially expressed in either type of mutant when compared to wildtype. A large proportion of this group of TDFs (77) was downregulated in the *Pigpa1*-silenced mutants. Twenty-three TDFs were up- or downregulated in both types of mutants. Only a small number of TDFs (7) were specifically up- or downregulated in the PiGPA1 gain-of-function mutant R2 and their sequences or expression patterns were not further investigated. Gene-expression patterns of wildtype mycelium and sporangia were also compared. Wildtype mycelium and sporangia shared 66% of the total number (2860) of bands, 14% was mycelium-specific and 19% was sporangia-specific. The remaining 1.2% of the total number of bands was not expressed in wildtype. The comparison of mycelium and sporangia demonstrates that the performance of the cDNA-AFLP analysis is accurate and efficient in this system. The technique has also been used successfully to identify stage-specific genes in *P. infestans* (Avrora et al., 2002).

45% of the TDF sequences were detected in the *Phytophthora* EST databases. A higher percentage of TDFs may be derived from genes represented in the EST databases since both the cDNA-AFLP fragments and the ESTs are only partial sequences. On the other hand, cDNA-AFLP is a very sensitive method (Donson et al., 2002) and is able to detect very low abundant mRNAs that may not be present in the EST database.

Only 6 of the 13 TDF sequences with similarity to ESTs are derived from genes with homologues in public sequence databases (NCBI). Entries of sequences of oomycetes and closely related organisms are underrepresented in public databases and therefore the chance to find homologues is low. For example, of the 760 unique sequences derived from the first 1000 *P. infestans* ESTs, only 55% had homologues in NCBI (Kamoun et al., 1999). Some of the TDFs with no homologues in the databases had similarities to protein domains in the Blocks+ database (Henikoff and Henikoff, 1994). Taking into account the evolutionary distance between *Phytophthora* and most of the organisms of which protein domains are represented in the Blocks+ database, these similarities to domains may be informative, even if the similarity scores are low.

In the cDNA-AFLP analysis only a subset of all genes was displayed. 96 (37.5%) of

the total number of 256 possible primer combinations were used. Moreover, the GenEST software (Qin, et al., 2001) detected *Apo*I-*Taq*I restriction fragments in only 30% of the *Phytophthora* ESTs (results not shown). Because EST sequences cover only parts of genes, the proportion of genes from which *Apo*I-*Taq*I fragments are generated is probably higher. Nevertheless, this particular enzyme combination is certainly not present in all *P. infestans* genes and therefore a more complete coverage would require additional primer and enzyme combinations.

The mutants in which the Gα subunit gene *Pigpa1* was silenced were affected in zoospore behavior and in virulence. The zoospores failed to aggregate and displayed altered swimming patterns. Zoospores encysted normally, but the cyst germ tubes formed appressoria less efficiently than wildtype (Latijnhouwers et al., in preparation). The TDFs that were up- or downregulated in the *Pigpa1*-silenced mutants will be used

Table 5. Primer pairs used in RT-PCR analysis

TDF Name	primer sequences	amplicon size (bp)	expression pattern confirmed (yes/no)
TDF2	5' accgtcgctgcgcttcaacaact 3' 5' agaggcccggatcatcaagacatt 3'	277	yes
TDF7	5' tggcgcaactggagaggt 3' 5' ccaatcgctgaggtgacg 3'	140	yes
TDF16	5' gaggtcacttccacacgctacag 3' 5' ttcgccaccaaggaccaga 3'	107	yes
TDF19	5' atggggtcagcggcagtcgtagc 3' 5' ggggcggcaacagcaccaatagt3'	284	yes
TDF20	5' gggatggcggcttggatac 3' 5' gatggcgctcgttgactgc 3'	184	yes
TDF23	5' agcgcccgaaggttttt 3' 5' cggattttgtcgggtctctg 3'	106	yes
TDF29	5' gccacggagccgtcaccg 3' 5' tggtttcggtggctatgc 3'	138	no
TDF37	5' tgacgaaaacgccaagtg 3' 5' gtcagcatcgcccgtgtc 3'	196	yes
TDF38	5' acccttgtgttgtgttgctttttc 3' 5' cttcttgttccccacgatgactaa 3'	143	yes
TDF41	5' gggctgcaaatgaacaaatgg 3' 5' tagggccgatgcactcactg 3'	63	yes
TDF43	5' ctgctgagcgtagaggactta 3' 5' gggctgtggcgcggtgaaat 3'	106	yes
TDF44	5' tggtccagcgtcaagat 3' 5' tggtccaccccgccgaatac 3'	79	yes
TDF45	5' gctaagcatacgattactc 3' 5' tcaagttctggccgtg 3'	-	no
TDF54	5' ctgcaaatgaacaaatggaaatg 3' 5' agggccgatgcactcactgt 3'	59	yes
TDF66	5' gtaccgcgacgagaacaacaag 3' 5' ttacgcggagcacctgaagc 3'	-	no
TDF67	5' ggtaattgcccctctgtcat 3' 5' aatcaccccgcgtttctatct 3'	62	yes
Pigpa1	5' ccctcgagatgggactctgtgct 3' 5' gtgatggccgcgatcatac 3'	745	yes
*Act*A	5' cggctccggtatgtgcaaggc 3' 5' gcgggcacgttgaacgtctc 3'	350	yes

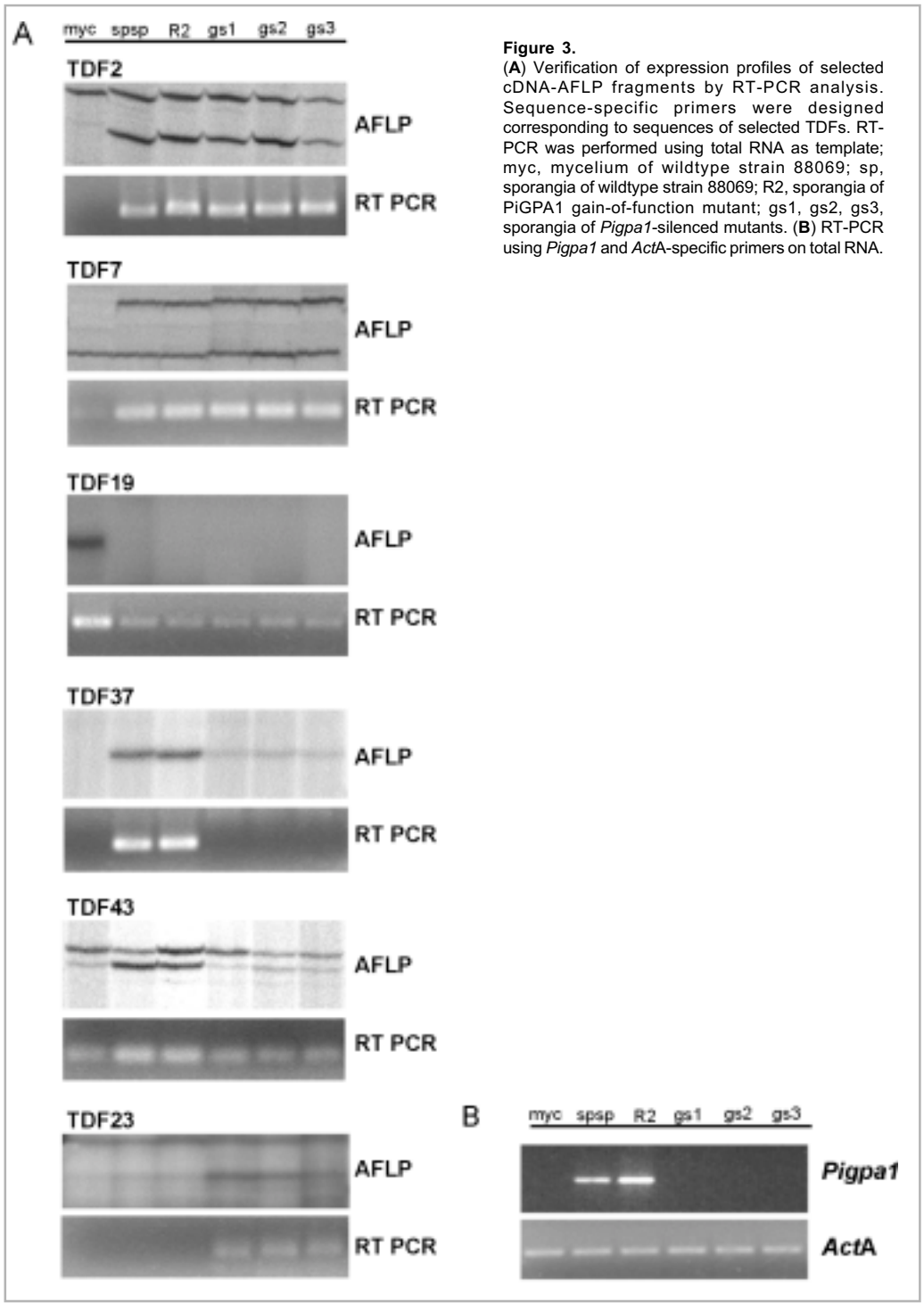

Figure 3.
(**A**) Verification of expression profiles of selected cDNA-AFLP fragments by RT-PCR analysis. Sequence-specific primers were designed corresponding to sequences of selected TDFs. RT-PCR was performed using total RNA as template; myc, mycelium of wildtype strain 88069; sp, sporangia of wildtype strain 88069; R2, sporangia of PiGPA1 gain-of-function mutant; gs1, gs2, gs3, sporangia of *Pigpa1*-silenced mutants. (**B**) RT-PCR using *Pigpa1* and *Act*A-specific primers on total RNA.

to isolate their corresponding genes. Future work should reveal whether the functions of these genes are related to the processes that are affected in the *Pigpa1*-silenced mutants. One of the genes that was upregulated in the *Pigpa1*-silenced mutants encodes a homologue of the *Dictyostelium discoidium* UbcB protein. This protein is a ubiquitin-conjugating enzyme and is required for development in the slime mold (Clark et al., 1997). It is conceivable that this protein is a G-protein target and that proper regulation of its expression is required for normal development in *P. infestans*. Few TDFs with altered expression in the PiGPA1 gain-of-function mutant R2 were found. Previous work showed that the PiGPA1 gain-of-function mutants showed normal development but their virulence was slightly enhanced. Therefore, the fact that we find fewer differentials in the PiGPA1 gain-of-function mutant than in the *Pigpa1*-silenced mutants is in agreement with its milder phenotype.

Further research will be required to assess how many of the differentially expressed genes have functions that are truly related to PiGPA1. However, the results so far demonstrate that the cDNA-AFLP-based method for displaying differentially expressed genes is suitable for the analysis of gene expression in mutants and for the identification of genes under control of a signal transduction pathway.

MATERIALS AND METHODS

Phytophthora infestans strains

P. infestans strain NL-88069 (A1 mating type) wild type and the transgenic isolates gs1, gs2, gs3 and R2 (Latijnhouwers, in preparation) were routinely grown at 18°C in the dark on rye agar medium supplemented with 2% sucrose (RSA) (Caten and Jinks, 1968). In the case of the transformants, 5 µg/ml geneticin (Gibco-BRL, Bethesda, MD) was added to the medium. For the isolation of sporangiospores, sporulating mycelium was flooded with modified Petri's solution (0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4 and 0.8 mM KCl) (Petri, 1917) and rubbed with a sterile glass rod. Subsequently, the suspension was filtered through a 50 µm nylon mesh. To obtain mycelium free of sporangia, the strains were grown in liquid modified Plich medium (van der Lee et al., 1997). The mycelium was rinsed with water before harvesting.

cDNA-AFLP analysis

Total RNA was isolated using Trizol (Gibco-BRL, Bethesda, MD) according to the manufacturer's instructions and subsequently purified using phenol-chloroform extraction. RNA from two independent isolations was pooled 1:1. mRNA was isolated from 100 µg total RNA by using QIAGEN Oligotex mRNA kit. cDNA was synthesized using oligo(dT_{12-18}) and Superscript II reverse transcriptase (Gibco-BRL Superscript Choice System for cDNA Synthesis kit). The template for cDNA-AFLP was prepared according to Bachem et al. (1996) using *Apo*I and *Taq*I. Sequences of primers and adapters used for AFLP reactions were as follows: *Apo*I adapter top strand, 5'-CTCGTAGACTGCGTACC-3'; *Apo*I adapter bottom strand, 5'-AATTGGTACGCAGTCTAC-3'; *Taq*I adapter top strand, 5'-GACGATGAGTCCTGAC -3'; *Taq*I adapter bottom strand, 5'-CGGTCAGGACTCAT -3'; *Apo*I preamplification primer, 5'-CTCGTAGACTGCGTACCAATT-3'; *Apo*I selective amplification primers, 5'-GACTGCGTACCAATT(C/T)NN-3' (NN = AA, AC, AG, AT, CA, and TC; *Taq*I preamplification primer, 5'- GACGATGAGTCCTGAC CGA-3'; and *Taq*I selective amplification primers, 5'- GACGATGAGTCCTGACCGANN-3' (NN = GT, CG, TA, CC, TG, GC, AA, GG, AC, AG, TT, TC, CT, GA, AT, and CA). cDNA-AFLP analysis was performed as described (Bachem et al., 1996; Durrant et al., 2000).

Isolation, cloning and sequencing of TDFs

The bands of interest were cut out from the gel using a razor blade. The gel slices were rehydrated in 100 µl of water and incubated at 70°C for 15 min. The eluted fragment was reamplified with the primers with the same two basepair extension as used in the cDNA-AFLP analysis. PCR products were purified using QIAquick Nucleotide Removal kit (Qiagen, Hilden) and cloned into pGEM-T Easy (Promega, Madison, WI). Sequences of TDFs were determined using the dideoxy termination method (Sanger et al., 1977) (BaseClear, Leiden, The Netherlands).

Northern blot analysis

For northern blot analysis, 10 µg total RNA was denatured at 50 ºC in 1 M glyoxal, 50% DMSO, and 10 mM sodium phosphate, electrophoresed and transferred to Hybond N^+ membranes (Amersham-Pharmacia, Buckinghamshire) in 10x SSC (Ausubel et al., 1987; Sambrook et al., 1989). Probes were generated by PCR amplification of TDFs using the respective AFLP primers, incorporating ^{32}P-dCTP directly in the PCR reaction (http://www.bio.anl.gov/MR/resources/). Approximately 2 pmol of each appropriate primer was mixed with 33 pmol α^{32}P-dCTP (specific activity 1 mCi/mmol), 3.0 µl of 10 x PCR buffer, 3.0 µl of 25 mM $MgCl_2$, 1 unit of AmpliTaq (Perkin Elmer Applied Biosystems, Foster City, CA); 2.0 µl of dNTP mixture (25 µM dATP, dGTP, and dTTP each) and up to 5 ng of template in a total volume of 30 µl. A control probe was amplified from the *Act*A gene in pSTA31 (Unkles et al., 1991) in the same way using the forward primer 5'-cggctccggtatgtgcaaggc-3' and the reverse primer 5'-gcgggcacgttgaacgtctc-3', both annealing to the coding region. Hybridizations of DNA and RNA blots were conducted at 65 ºC in Nasmyth's buffer (Stergiopoulos et al., 2002) and filters were washed at 65 ºC in 0.5x SSC + 0.5% SDS.

Reverse Transcriptase (RT) PCR analysis

To remove genomic DNA from RNA preparations, 10 µg total RNA was treated with 4 units RQ1 RNase-free DNase (Promega, Madison, WI) at 37 ºC for 1h. The removal of all DNA was verified in a PCR reaction under the same conditions as those used for the RT-PCR reaction, except that the cDNA synthesis step was omitted. The first strand cDNA was synthesized using oligo(dT_{16}) and Superscript II reverse transcriptase for 30 min at 40ºC (Gibco-BRL, Bethesda, MD). Sequence-specific primers were used in the subsequent PCR with cDNA as template (see table 5) with 25 cycles (30 s at 94 ºC, 30 s at 58 ºC and 60 s at 72 ºC).

ACKNOWLEDGEMENTS

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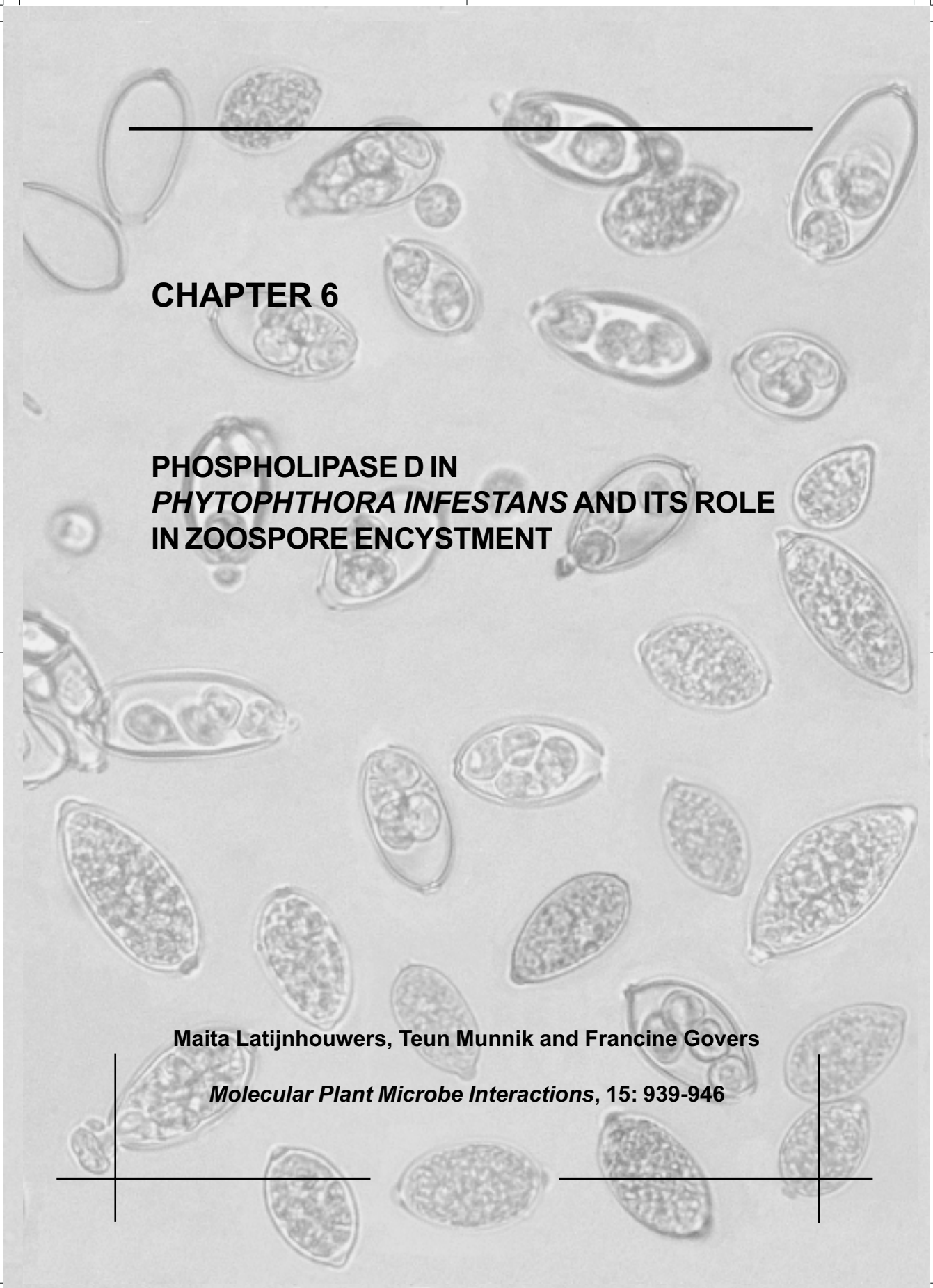

CHAPTER 6

PHOSPHOLIPASE D IN *PHYTOPHTHORA INFESTANS* AND ITS ROLE IN ZOOSPORE ENCYSTMENT

Maita Latijnhouwers, Teun Munnik and Francine Govers

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ABSTRACT

We show that differentiation of zoospores of the late blight pathogen *Phytophthora infestans* into cysts, a process called encystment, was triggered by both phosphatidic acid (PA) and the G-protein activator mastoparan. Mastoparan induced the accumulation of PA, indicating that encystment by mastoparan most likely acts through PA. Likewise, mechanical agitation of zoospores, which is often used to induce synchronized encystment, resulted in increased levels of PA. The levels of diacylglycerolpyrophosphate (DGPP), the phosphorylation product of PA, increased simultaneously. Also in cysts, sporangiospores and mycelium, mastoparan induced increases in the levels of PA and DGPP. Using an *in vivo* assay for phospholipase D (PLD) activity, it was shown that the mastoparan-induced increase in PA, was due to a stimulation of the activity of this enzyme. PLC in combination with diacylglycerol (DAG) kinase activity can also generate PA but activation of these enzymes by mastoparan was not detected under conditions selected to highlight ^{32}P-PA production via DAG kinase. Primary and secondary butanol, which, like mastoparan, have been reported to activate G-proteins, also stimulated PLD activity, whereas the inactive tertiary isomer did not. Similarly, encystment was induced by *n*- and *sec*-butanol but not by *tert*-butanol. Together, these results show that *Phytophthora infestans* contains a mastoparan- and butanol-inducible PLD pathway and strongly indicate that PLD is involved in zoospore encystment. The role of G-proteins in this process is discussed.

INTRODUCTION

The class oomycetes comprises many economically important plant pathogens and is grouped together with the brown algae, slime molds and other classes in the diverse kingdom Protoctista (Margulis and Schwarz 1998). The oomycete *Phytophthora infestans* causes the deleterious potato late blight disease, which was responsible for the notorious Irish potato famine in the 19th century. The vegetative stage of *P. infestans* is a diploid coenocytic mycelium. Sporangia, the vegetative propagules, are formed on branched sporangiophores that emerge from the mycelium. These can develop into sporangiospores that are able to germinate directly by formation of a germ tube. At temperatures below 12 degrees, however, sporangia develop into zoosporangia, releasing seven to eight zoospores, each possessing two flagella, enabling them to swim (Crosier 1934).

Little is known about the signal transduction pathways that regulate developmental processes in oomycetes. Our aim is to unravel cellular signaling in *P. infestans,* which may help to design new strategies to control oomycete plant pathogens. One of the crucial steps in the infection cycle of *P. infestans* is the differentiation of zoospores into cysts. This encystment process is characterized by retraction of the flagella and the formation of a solid cell wall (Cho and Fuller 1989; Hardham 1995). Cysts adhere to the leaf surface, where they germinate. Germ tubes develop appressoria and penetration hyphae emerging from the appressoria enter leaf tissue. In several *Phytophthora* species, including *P. infestans*, encystment is triggered by physical agitation and polyuronates

(Byrt et al. 1982b; Grant et al. 1985). In studies using calmodulin- and calcium channel modulating drugs, calcium was identified to play an important role in this process (Byrt et al. 1982a; Irving and Grant 1984).

Previous work suggested a role for phosphatidic acid (PA) in the differentiation of zoospores of *Phytophthora palmivora* (Zhang et al. 1992). PA is emerging as an important second messenger in a wide range of organisms (reviewed in English 1996; Munnik et al. 1998a; McPhail et al. 1999; Munnik 2001). It is generated when phospholipase D (PLD) hydrolyzes a structural phospholipid such as phosphatidylcholine (PC). Alternatively, PA is generated indirectly through phospholipase C (PLC) activity, since the diacylglycerol (DAG) which is formed from phosphatidyl 4,5-bisphosphate hydrolysis is readily phosphorylated by diacylglycerol kinase (DGK), to produce PA (Munnik et al. 1998b). In plants and fungi, PA can be phosphorylated further by PA kinase (PAK) producing diacylglycerolpyrophosphate (DGPP) (Munnik et al. 1996). The reaction is seen as attenuating PA levels and has been observed in plants as a response to pathogens (van der Luit et al. 2000), osmotic stress (Pical et al. 1999; Munnik et al. 2000), drought (Frank et al. 2000, Munnik et al. 2000) and during nodulation (Den Hartog et al. 2001).

The involvement of these PA-generating and metabolizing pathways in zoospore differentiation in oomycetes is investigated in this report and the enzymes' activities are characterized in various developmental stages. The PA-generating enzymes PLC and PLD can be activated by the tetradecapeptide mastoparan in plant cells (Quarmby et al. 1992; Legendre et al. 1993; Cho et al. 1995; Munnik et al. 1995; Munnik et al. 1998a; De Vrije and Munnik 1997; van Himbergen et al. 1999; Frank et al. 2000). Mastoparan is widely used as activator of heterotrimeric G-proteins (Higashima et al. 1990; Law and Northrop 1994; Ross and Higashijima 1994). Primary and secondary alcohols also act as G-protein activators since they activate adenylyl cyclase and PLC in a GTP-dependent manner. It is hypothesized that the interaction of these alcohols with certain membrane components activates G-protein signaling but the exact mechanism is unknown (Luthin and Tabakoff 1984; Hoffman and Tabakoff 1990; Hoek et al. 1987; Kiss and Anderson 1989; Musgrave et al. 1992; Munnik et al. 1995; Munnik et al. 1998b). In this study we incubated zoospores of *P. infestans* with mastoparan and alcohols and analyzed the effects on zoospore differentiation and phospholipid-based signaling pathways.

RESULTS

PA and mastoparan enhance encystment of *P. infestans* zoospores

Encystment of *Phytophthora* zoospores can be triggered by vortexing or by treatment with various compounds (Grant et al. 1985). We tested the effect of di-octanoyl phosphatidic acid (DOPA) and of mastoparan on this process. Zoospores of *P. infestans* were incubated for 10 min in the presence of DOPA and subsequently the number of cysts was counted. As shown in Figure 1A, encystment was triggered by PA. In 9 µg/ml DOPA, 80%, and in 15 mg/ml DOPA, all of the zoospores had encysted after 10 min, compared to 30% in its absence. Mas7, a potent mastoparan analogue, had a similar

effect (Figure 1B). 250 nM of this peptide was sufficient to cause 85% encystment. The same concentration of mas17, an inactive mastoparan analogue, did not significantly enhance encystment. In the presence of 250 nM mas7, cyst germination-efficiency was the same as in the water control.

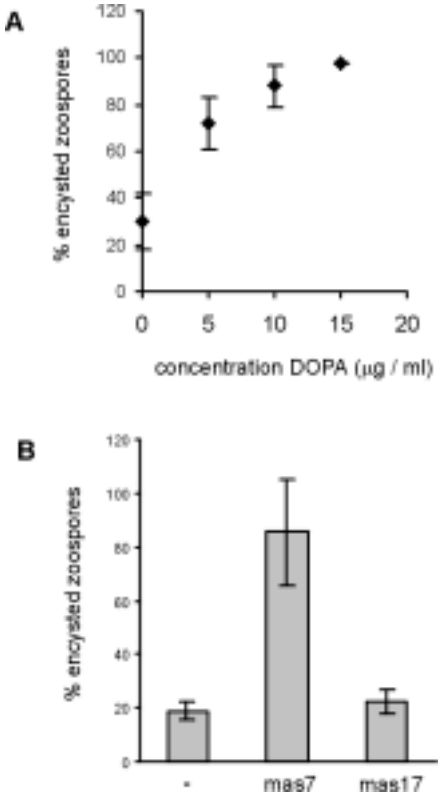

Figure 1.
Effect of PA and mastoparan on zoospore encystment. The experiments were repeated four times. The results of typical experiments consisting of ten measurements are shown. (**A**) 10 µl zoospore suspension was incubated with 1,2-dioctanoyl phosphatidic acid (DOPA) at different concentrations (5, 9 and 15 µg/ml) for 10 minutes in a total volume of 30 µl. (**B**) 10 µl suspension was incubated without (-) or with 250 nM mas7 or mas17 in a total volume of 30 µl for 10 minutes. The numbers of cysts were counted and expressed as percentages of the initial numbers of zoospores.

Mastoparan induces PA and DGPP formation in *P. infestans* zoospores, cysts, sporangiospores and mycelium

Since mastoparan mimicked PA in enhancing encystment, we tested whether it induced PA formation in zoospores and zoosporangia. Zoosporangia were incubated in the presence of $^{32}PO_4^{3-}$ to label the phospholipids and placed at 4°C to allow zoospore release. ^{32}P-Labeled zoospores were treated with mas7 for 5 min before extracting the lipids and separating the individual lipid species. As can be seen in the autoradiograph (Figure 2A), mas7 triggered the synthesis of two lipids, which were identified as PA and DGPP by co-chromatography with standards. The levels of these phospholipids were quantified using phosphoimaging (Figure 2B). To test the effect of mechanical agitation, ^{32}P-labeled zoospores were vortexed and the effects on the phospholipid composition analyzed. Although less pronounced, the effect on PA formation was

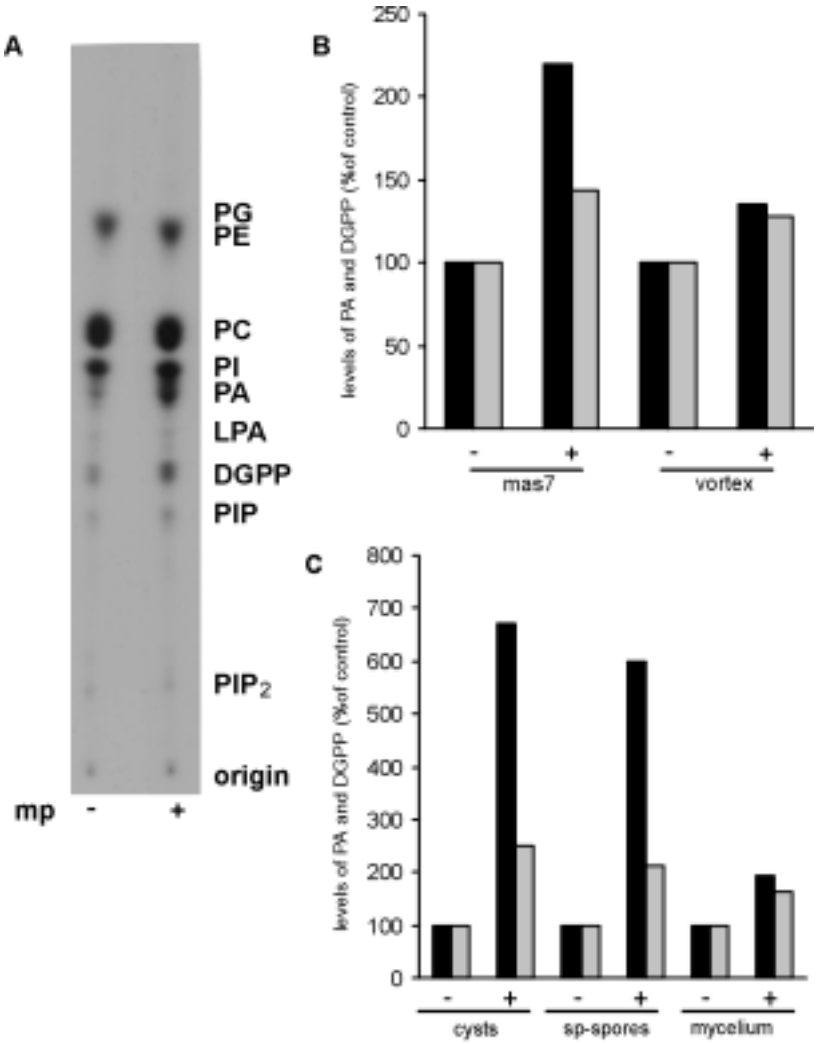

Figure 2.
Effect of mastoparan on the phospholipid composition of zoospores, cysts, sporangiospores and mycelium of *P. infestans*. Labeled cells were incubated with or without the mastoparan analogue, mas7, for 10 min. The concentration mas7 used for zoospores was 0.3 µM, while 4 µM was used for cysts, sporangiospores and mycelium. Phospholipids were extracted and separated by TLC using an alkaline solvent system. (**A**) TLC autoradiograph showing an increase in PA and DGPP in zoospores after incubation with (+) or without (-) mas7. phosphatidylglycerol, PG, phosphatidylethanolamine, PE, phosphatidylcholine, PC, lysophosphatidic, LPA, phosphatidyl inositolphosphate, PIP, phosphatidyl inositol bisphosphate PIP_2 (**B**) Levels of PA (black bars) and DGPP (grey bars) in zoospores incubated with (+) or without (-) mas7 and before (-) and after (+) vortexing for 1 min. (**C**) Levels of PA (black bars) and DGPP (grey bars) in the various tissues incubated with (+) or without (-) mas7, expressed as percentages of the water control (set at 100%). A typical result from two independent experiments is shown.

similar to that of mas7 treatment (Figure 2B), suggesting that increases in PA concentration are correlated with encystment. To investigate the effect of mastoparan on other developmental stages of *P. infestans*, cysts, sporangiospores and mycelium were also radio-labeled before treatment and the levels of ^{32}P-PA and ^{32}P-DGPP thereafter quantified (Figure 2C). Mastoparan stimulated PA and DGPP synthesis in all cells, although the effect was greater in cysts and sporangiospores than in zoospores and mycelium.

Mastoparan activates PLD

Increases in PA and DGPP are indicative of PLD and/or PLC activity. To investigate the origin of the mastoparan-induced PA formation, we exploited the ability of PLD to transfer the phosphatidyl moiety of its substrate to a primary alcohol rather than to water, producing a phosphatidylalcohol instead of PA, providing a quantitative measure of *in vivo* PLD activity (Munnik et al. 1995). For these experiments, sporangiospores were used because they are far easier to handle than zoospores. Labeled sporangiospores were incubated for 10 min with or without mastoparan in the presence or absence of 0.25% *n*-butanol. Lipids were then extracted and analyzed using a TLC system that separates phosphatidyl-butanol (PBut) and PA from the other phospholipids (Munnik et al. 1998b). As shown in Figure 3, mastoparan triggered the formation of PA and PBut. This clearly shows that mastoparan activates PLD.

In the absence of butanol, mastoparan enhanced PA production almost three-fold, but only two-fold in its presence (Figure 3B). However, the sum of PA and PBut, the total PLD product, was the same in both cases. The fact that PA production was reduced shows that butanol efficiently competed with H_2O for transphosphatidylation. Mastoparan also activated PBut formation in zoospores, mycelium and cysts (data not shown).

To monitor the synthesis of PA, PBut and DGPP in time, labeled sporangiospores were treated with mastoparan in the presence of *n*-butanol for different time periods. The radiolabel in all these lipids increased continuously over a 32 min period (Figure 4), with PA eventually accounting for 20% of the ^{32}P-labeled phospholipids (Figure 4B). In the absence of mastoparan, the levels of all three lipids increased slightly, indicating that 0.25% butanol may have stimulated PLD, as reported in the literature (Kiss and Anderson 1989; Musgrave et al. 1992; Munnik et al. 1995).

Other active mastoparan analogues (i.e. mas7 and mas8) also activated PLD, whereas the inactive analogue mas17 (Higashijima et al. 1990; van Himbergen et al. 1999) had no effect (data not shown).

PA is not generated via DAG kinase

To test whether mastoparan-activated PA synthesis took place via PLC and/or DAG kinase activation, a differential labeling strategy was applied in which preincubation in $^{32}PO_4^{3-}$ was reduced to 5 min (Munnik et al. 1998b). This is not enough to label the structural phospholipids, the substrates of PLD, but long enough for the synthesis of ^{32}P-ATP, the ^{32}P donor for DAG kinase. Therefore, any ^{32}P-PA formed under these conditions, can only arise from PLC/DAG kinase activity, because the PA formed by PLD will be non-radioactive.

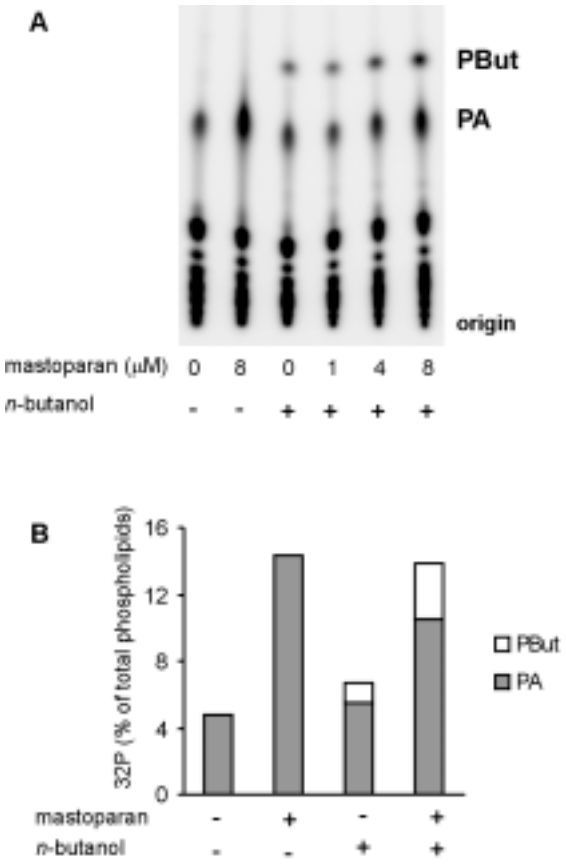

Figure 3.
Mastoparan activates PLD activity. Sporangiospores were labeled with $^{32}PO_4^{3-}$ for 16 h before adding mas7 in the presence (+) or absence (-) of 0.25% *n*-butanol for 10 min. (**A**) Lipids were extracted and separated by TLC using the ethyl acetate system. (**B**) Levels of PA (grey), and PBut (white) are shown as percentages of the total amounts of labeled phospholipids.

Accordingly, sporangiospores were radio-labeled for 5 min and then treated with different concentrations of mastoparan. Phospholipids were subsequently extracted and analyzed by TLC (Figure 5). Mastoparan did not increase the level of ^{32}P-PA. This shows that the PLC/DAG kinase route was not activated. However, since non-radioactive PA was generated by PLD, it was then converted to ^{32}P-DGPP by PA kinase using ^{32}P-ATP. The lack of ^{32}P-PA established that essentially all PA was derived from PLD activity. The DGPP pool migrated as two spots (Figure 5). We speculate that both spots are forms of DGPP that differ in their fatty acid composition.

PA is not only produced via PLC and PLD activity but also through acylation of lyso-PA (LPA) by acyltransferase (Weigert et al. 1999). Mastoparan treatment, however, did not have an effect on the level of LPA (Figure 2). Therefore, it is unlikely that the increase in PA is due to increased acyltransferase activity.

Primary and secondary alcohols activate PLD

In the time-course experiment described above (Figure 4), slight increases in the levels of PA, DGPP and PBut were observed when sporangiospores were incubated in 0.25%

n-butanol. Since primary and secondary alcohols can activate PLD in plant and mammalian cells (Kiss and Anderson 1989; Munnik et al. 1995), we investigated whether butanol was able to activate PLD in *P. infestans* sporangiospores. Labeled sporangiospores were treated with 2% primary, secondary or tertiary butyl alcohol in the presence of 0.5% ethanol as a reporter of PLD activity. Only *sec*-butanol caused clear increases in the levels of PA and phosphatidylethanol (PEt), the transphosphatidylation product of ethanol (Figure 6A and 6B). This shows that *sec*-butanol is a potent activator of PLD. PBut was only formed upon treatment with *n*-butanol (Figure 6C). This shows that *n*-butanol activated PLD and was the preferred substrate, because very little PA or PEt was formed. The sum of PA, PBut and PEt was similar for *n*- and *sec*-butanol, showing that these isomers activate PLD equally (Figure 6D). In comparison, *tert*-butanol hardly activated PLD.

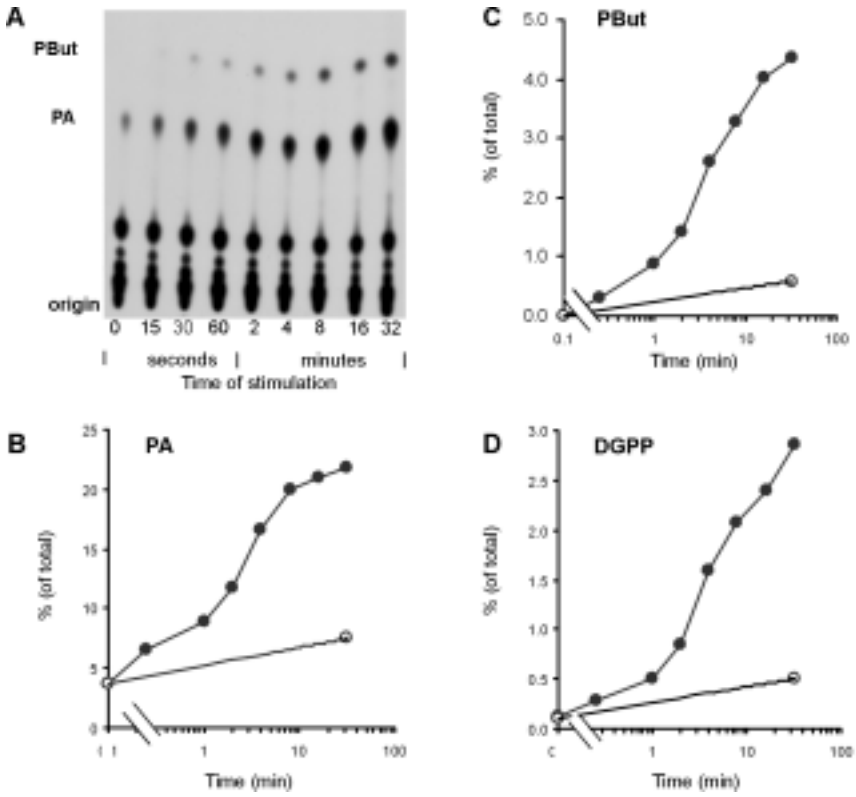

Figure 4.
Time course of mastoparan-induced changes in phospholipid composition. Sporangiospores were labeled with $^{32}PO_4^{3-}$ for 16 h and treated with 4 µM mas7 in the presence of 0.25% *n*-butanol. Samples were taken when indicated. A control sample was treated with 0.25% *n*-butanol for 32 min. Phospholipids were extracted and separated by TLC using the ethyl acetate system. (**A**) Autoradiograph showing increases in PBut and PA with time. (**B-D**) Levels of PA, PBut and DGPP were quantified and expressed for mas7 (closed circles) or control (open circles) treatments as percentages of the total labeled phospholipids.

Primary and secondary butanol trigger encystment

Because butanol isomers activated PLD, their effects on zoospore encystment were also investigated. After 10 min in 1% *n*-butanol, 85% of the zoospores had encysted compared to 21% in the absence of butanol (Figure 7). Secondary butanol was less active (37%). The activity of the tertiary isomer was not significantly different from the control (23 %). This demonstrates that the efficiency with which butanol activates PLD is related to its potency to trigger encystment.

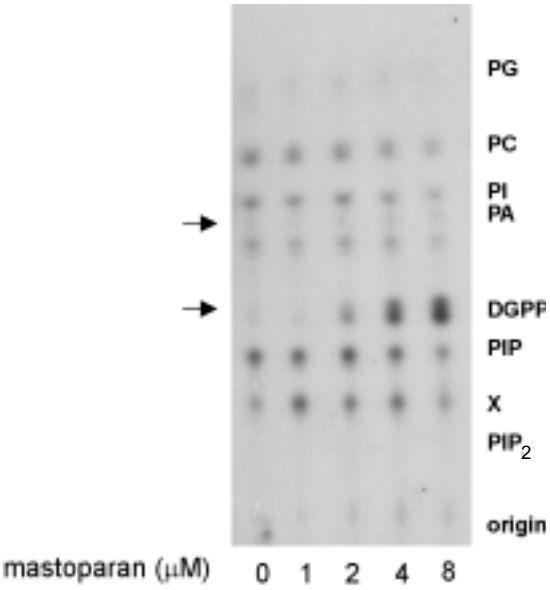

Figure 5.
DAG kinase is not activated in mastoparan-treated sporangiospores of *P. infestans* . Sporangiospores were labeled with $^{32}PO_4^{3-}$ for 5 min and treated with mas7 for 5 min. Phospholipids were extracted, separated by alkaline TLC and visualized by autoradiography. Mastoparan did not affect labeling of the PA spot. Arrows indicate location of the spots of PA and DGPP. X represents a phospholipid that could not be identified.

DISCUSSION

Late blight epidemics spread at the highest rate under cool weather conditions, when zoospores rather than sporangiospores are the infectious particles. Therefore, encystment is one of the key steps in the infection process, culminating in host penetration and lesion development. The better we understand the factors involved, the greater the prospect of developing strategies to control large scale pathogenesis by *P. infestans.*

In this study we have shown that PA is a potent inducer of encystment in *P. infestans*. For *P. palmivora,* Zhang et al. (1992) showed that of the four phospholipids tested, only PA was active. To investigate whether PA generated *in vivo* by natural signaling mechanisms was also biologically active, we tested the G-protein activators mastoparan and butanol for their ability to induce PA synthesis and zoospore encystment. They were chosen for their reported efficacy in generating PA in lower and higher plants (Munnik et al. 1995; Munnik et al. 1998b; Frank et al. 2000; Den Hartog et al. 2001).

When zoospores were labeled with $^{32}PO_4^{3-}$, ^{32}P-PA production increased roughly 2-fold when treated with mas7 and 1.5-fold when treated with *n*-butanol. The same treatments in zoospores induced 80 to 100% encystment. Mastoparan and butanol isomers that did not stimulate PA synthesis (mas17 and *tert*-butanol), were biologically inactive. Since we usually induce encystment by mechanical agitation, we also tested this treatment's effect on ^{32}P-PA production and found a 1.5-fold increase. This confirms the previous report (Zhang et al. 1992) that shaking stimulated an increase in PA in *P. palmivora*. Together these data establish a clear correlation between natural increases in PA and encystment in *Phytophthora* species. They highlight the exciting possibility that PA is acting as a morphogen, triggering this specific differentiation process.

In cysts and sporangia, the activation of PLD by mastoparan resulted in even larger increases in PA levels than in zoospores and mycelium. This could be due to the presence of higher concentrations of PLD enzyme or of different PLD isoforms in cysts and sporangia compared to zoospores and mycelium, but also to differences in their membrane composition. The presence of a mastoparan-inducible PLD pathway in each of the developmental stages tested suggests that there are multiple roles for PA in *P. infestans.*

PA production was dramatic and fast, being initiated within 1 min. This in itself indicates that PA is being generated as part of a signaling pathway. This is supported by the fact that both mastoparan and butanol are activators of G-proteins in animals and have extensively been used as such in plants (Luthin and Tabakoff 1984; Hoffman and Tabakoff 1990; Hoek et al. 1987; Kiss and Anderson 1989; Higashima et al. 1990; Law and Northrop 1994; Ross and Higashijima 1994). It suggests that natural agonists stimulate cyst formation by means of G-protein-coupled receptors. We are studying such signaling mechanisms in *P. infestans* and have recently charaterized genes encoding α and β subunits of heterotrimeric G-proteins (Laxalt et al. 2002). They are the first reported G-protein genes for oomycetes and add credence to the idea that PA formation was stimulated via a G-protein-regulated signaling mechanism.

It is known that high concentrations of mastoparan analogues can form molecular pores in the plasma membrane (Suh et al. 1996) and so permeabilize them to Ca^{2+}, thereby activating other signaling pathways**.** However, we emphasize that sub-micromolar concentrations (250 nM) were used for zoospores and micromolar concentrations (4 μM) for cysts, sporangia and mycelium, while permeabilization of animal and plant cells has been reported for concentrations that were 200 to 2000 higher (Suh et al. 1996; Munnik et al. 1998b; van Himbergen et al. 1999). Zoospores responded to much lower concentrations of mastoparan compared to cysts, sporangia and mycelium. This might be due to the absence of a cell wall in zoospores, as opposed to the three other stages.

If PA is generated via G-protein activation, the question is which effector enzyme is responsible. We considered the possibilities that PA could be generated through PLD, PLC/DAG kinase or via acylation of LPA by an acyltransferase. We thought the last possibility most unlikely, because we did not detect any changes in LPA concentration, but the first two possibilities were seriously considered. PLD activity was measured by its ability to transfer the phosphatidyl group of its substrate to a primary alcohol. As was shown for numerous animal and plant PLDs (Cockcroft 2001;

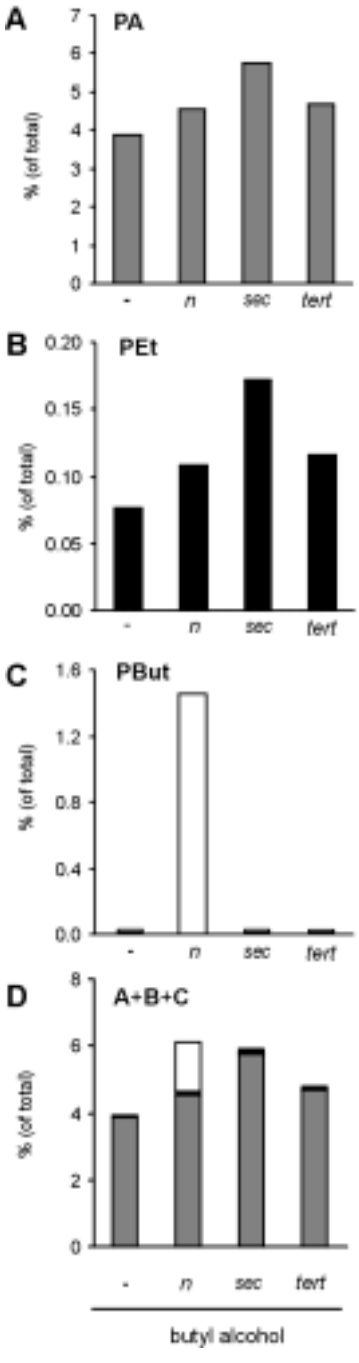

Figure 6.
Effect of butanol isomers on PLD activity and their ability to function as a transphosphatidylation substrate. Sporangiospores were labeled with $^{32}PO_4^{3-}$ for 16 h and treated with H_2O (-) or with 2% primary (*n*), secondary (*sec*) or tertiary (*tert*) butyl alcohol in the presence of 0.5% EtOH. This concentration of EtOH did not affect PLD activity (results not shown). Phospholipids were extracted and separated by TLC. (**A-C**). Levels of PA, PEt and PBut, as percentages of the total amount of phospholipids. (**D**). The sum of PA (grey), PEt (black) and PBut (white) is given as a percentage of the total amount of phospholipids. A typical result from two independent experiments is shown.

Munnik 2001), the production of phosphatidylalcohol provides a quantitative unequivocal measure of PLD activity. Based on this criterion, both mastoparan and butanol rapidly activated PLD. This is the first report of PLD signaling in an oomycete.

Since PLD was activated, we were obliged to monitor PLC/DAG kinase activity because these two signaling pathways are usually activated at the same time in both animals and plants, with PLD sometimes being downstream from PLC (Munnik et al. 1998a). However, we could not detect PLC's substrate PI(4,5)P_2 in *P. infestans*, and could thus not follow its metabolism. We therefore resorted to measuring DAG production via its subsequent conversion to PA. Since DAG kinase uses ATP for this reaction, short pre-labeling times were used to label ATP, which were not long enough to label PLD's substrate, the structural lipids. In this way PLC/DAG kinase activity could be selectively monitored because PA_{PLC} is radioactive under these conditions, whereas PA_{PLD} is not. Using these conditions, neither mastoparan nor butanol stimulated ^{32}P-PA formation, establishing that PLC/DAG kinase was not activated. We emphasize this unusual characteristic for we could only find two examples of PLC-independent PLD activation in the animal literature (Pettitt et al. 1997; Melendez et al. 1998) and only one very recent example in the plant literature (Meijer et al. 2002). The use of this assay was restricted to sporangiospores since zoospores did not take up sufficient PO_4^{3-} in these short labeling times. Given that this situation can be extended to zoospores, PLD seems to be solely responsible for PA production in *P. infestans* during encystment and may have the sole responsibility in *P. palmivora* as well. This would explain why Zhang et al. (1992) measured clear increases in PA, but failed to find an increase in $InsP_3$. One immediately wonders whether PLD-generated PA is involved in zoospore encystment in other, non-oomycete species.

In living organisms signals must be rapidly attenuated so that cells can respond to a second stimulus. Lower and higher plants have developed a PA-attenuation

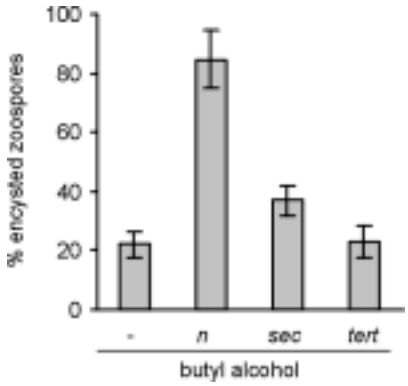

Figure 7.
Effect of butanol isomers on zoospore encystment. 10 µl zoospore suspension was incubated with 1% *n*-, *sec*- or *tert*-butanol or without butanol (-) in a total volume of 30 µl. The numbers of cysts were counted and expressed as a percentage of the initial number of zoospores.

mechanism not yet reported for animals, in which it is phosphorylated to DGPP by PA kinase (Munnik 2001). *P. infestans* also makes use of this mechanism, for increases in PA were always rapidly followed by increases in DGPP. Since DGPP can hardly be detected in resting cells and is only detected *in vivo* during cell stimulation, its presence together with PA in *P. infestans* confirms that we are dealing with cells in a state of signaling. Although DGPP is considered an inactive metabolite of PA, the fact that its synthesis is always coupled to signaling suggests that it could itself be a signal molecule (Munnik 2001). This possibility was given a serious boost when DGPP was shown to activate a MAP kinase pathway in macrophages (Balboa et al. 1999).

Ca^{2+} plays an important role in the encystment of zoospores of several *Phytophthora* species (Byrt et al. 1982a; Irving and Grant 1984). Millimolar concentrations of Ca^{2+} induced encystment and encystment is associated with transmembrane Ca^{2+} fluxes in *Phytophthora parasitica* (Warburton and Deacon 1998). It would be interesting to analyze the effect of Ca^{2+} on PLD activation during encystment, since Ca^{2+} activates many plant PLD isozymes (reviewed in Munnik et al. 1998a; Pappan and Wang 1999).

Vesicle trafficking and exocytosis are prominent events during encystment of zoospores. Peripheral vesicles fuse with the plasma membrane and release their contents to the outer surface to form a cell wall (Hardham 1995). In humans, PLD and vesicle trafficking are tightly linked (Liscovitch et al. 2000; Cockcroft 2001; Munnik and Musgrave 2001). In accordance with these findings, it is plausible that PLD has a role in these processes in *P. infestans.*

A role for PA as second messenger in cell signaling in a wide variety of organisms is now well established. In animals, it is not only involved in vesicle trafficking, but also in other cellular processes such as actin polymerization, secretion, oxidative burst and Ca^{2+} signaling (reviewed in English 1996; Cockcroft 2001). In plants, PA plays a role in the response to a number of stress conditions including, wounding, pathogen challenge and water stress and is important for ethylene- and ABA signaling (reviewed in Munnik 2001). During the last few years, several PA targets have been identified, including protein kinases, phosphatases, small G-proteins and NADPH oxidase (McPhail et al.1999; Cockcroft 2001; Manifava et al. 2001; Munnik 2001), illustrating the wide range of pathways in which it can participate.

Thus far, PLD signaling has been studied in only a few lower eukaryotes and in no other plant-pathogen. In the *Saccharomyces cerevisae* PLD, SPO14, is known to be required for sporulation and secretion (Rudge and Engebrecht 1999; Rudge et al. 2001). In *Candida albicans*, deletion of the *pld1* gene has been shown to affect the infections of mice, but had no detectable growth effect on growth (Hube et al. 2001). The identification of PA as a natural inducer of encystment, a crucial cell differentation event for the pathogenicity of *P. infestans*, is, therefore, an important step in unraveling phospholipid signaling in eukaryotic plant pathogens.

MATERIALS AND METHODS

Phytophthora infestans strains and culture conditions

P. infestans isolates NL-88069 (A1 mating type) and NL-98014 (A1 mating type) were routinely grown at 18ºC in the dark on rye agar medium supplemented with 2% sucrose (Caten and Jinks 1968). For the isolation of sporangiospores, sporulating mycelium was flooded with water, rubbed with a sterile glass rod and the suspension filtered through a 50 µm nylon mesh. To obtain zoospores, sporulating mycelium was flooded with water and incubated at 4ºC for 2-3 h, before filtering through a 10 µm nylon mesh to separate the zoospores from the sporangiospores. Cysts were obtained by centrifuging a zoospore suspension at 500 g for 5 min at room temperature. Mycelium free of sporangiospores was grown in Petri dishes containing liquid modified Plich medium (Kamoun et al. 1993).

Radio-labeling phospholipids

Suspensions of sporangiospores or cysts were centrifuged at 500 g for 5 min at room temperature. Pellets were washed twice with 50 ml labeling medium (10 mM MES pH5.5, 1 mM Mg_2SO_4, 6.7 mM L-asparagine, 139 mM glucose, 3 µM thiamine) and resuspended in the same buffer to a final concentration of 0.5 x 10^7 sporangiospores per ml or 2 x 10^7 cysts per ml. Samples of 100 µl were used.

For labeling the mycelium, 0.2 g plugs (including liquid) were harvested using forceps, washed twice in 50 ml labeling medium and then taken up in 100 µl of the same medium. Samples were incubated in 10 µCi carrier-free ^{32}P-PO_4^{3-} (Amersham International, The Netherlands) for the periods indicated. Prelabeled cells were incubated with mastoparan analogues (Peninsula laboratories) or alcohols (Sigma) in a final volume of 150 µl at the concentrations and for the times indicated.

To assay PLD activity, *n*-butanol (0.25%) or ethanol (0.5%) was added at the same time as the agonist (Munnik et al. 1995; De Vrije and Munnik 1997). Reactions were stopped by adding perchloric acid (PCA) to a final concentration of 5%, after which the samples were frozen in liquid nitrogen.

Zoospores were labeled by incubating sporangia in 1.5 ml solution containing 0.25 mM Ca^{2+}, 1 mM $MgSO_4$ and 0.8 mM KCl at 4ºC in the presence of 20 µCi carrier-free ^{32}P-PO_4^{3-}. Before adding mastoparan, zoospores were separated from the sporangia (at the bottom of the tube) by carefully transferring the zoospore suspension to a new tube without disturbing the sporangia at the bottom of the tube. The reaction was stopped by addition of 5% PCA. The cells were subsequently centrifuged for 2 min at 10,000 g. Phospholipids were extracted and analyzed as described below.

Phospholipid extraction and separation

Phospholipids were extracted as described in Munnik et al. (1996). In short, 3.75 volumes of $CHCl_3$/CH_3OH/1M HCl (50:100:1 by vol.) were added and the samples were again frozen in liquid nitrogen for 5 min. Subsequently, the extraction was carried out by vigorously shaking the samples for 30 min at room temperature. A two-phase system was induced by adding 1 vol. of 0.9% NaCl and 3.75 vol. of $CHCl_3$. Samples were vortexed for 15 s and centrifuged for 1 min. The lower organic phase was washed twice with 3.75 vol. of $CHCl_3$/CH_3OH/1M HCl (3:48:47 by vol.) and dried by vacuum centrifugation.

Lipids were dissolved in 20 µl $CHCl_3$ and chromatographed on heat-activated silica 60 TLC plates (0.25 x 200 x 200 mm; Merck, Darmstadt, Germany). Two different solvent systems were used. For the separation of PBut and PA from other phospholipids, an ethyl acetate system was used (the upper phase of ethyl acetate/*iso*-octane/formic acid/H_2O (13:2:3:10 by vol. Munnik et al. 1998b). For separation of the different phospholipid classes, potassium-oxalate impregnated TLC plates were used, combined with an alkaline solvent system (Munnik et al. 1996). Radiolabeled phospholipids were detected and quantified by phosphoimaging (Storm, Molecular Dynamics; Sunnyvale, CA).

Encystment assay

Zoospores were isolated in 0.25 mM $CaCl_2$; 1 mM $MgSO_4$; 1 mM KH_2PO_4, 0.8 mM KCl (modified from Petri's solution (Petri et al. 1917)). The zoospore suspension was transferred to a beaker and kept on ice. In microslide wells (well diameter 7 mm; Flow Laboratories, McLean, VA), 10 µl zoospore suspension containing approximately 5 x 10^5 zoospores, was mixed with 20 µl solution containing 5 mM MES pH 5.5 and 1,2-dioctanoyl phosphatidic acid (DOPA) (Sigma), mastoparan or alcohol in the concentrations

indicated. Photographs were made using a 1000x magnification with either the bottom of the microslide or the liquid surface in focus (Zeiss, Axiovert10). These photographs were used to determine the fraction of the zoospores that had encysted.

ACKNOWLEDGEMENTS

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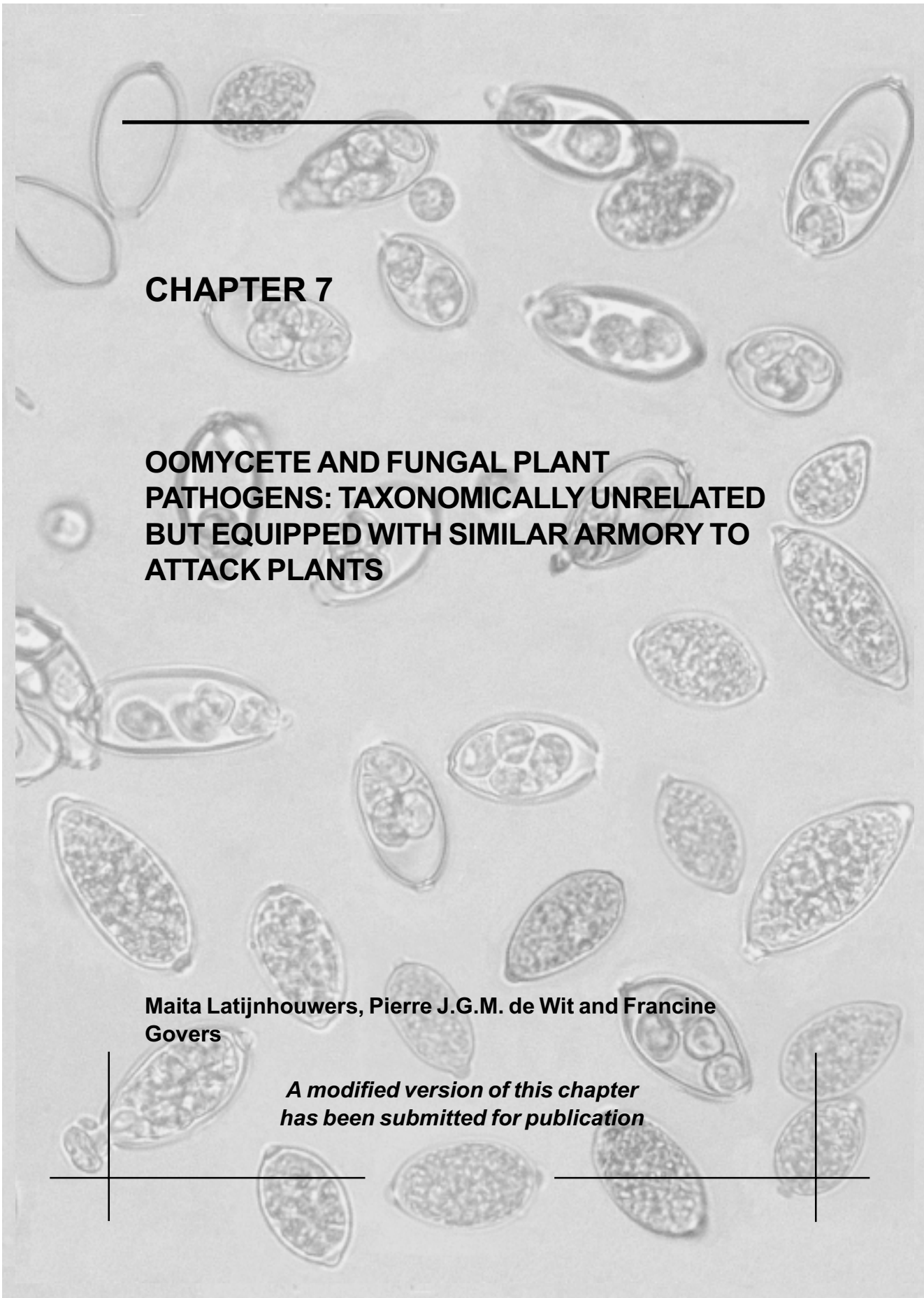


CHAPTER 7

OOMYCETE AND FUNGAL PLANT PATHOGENS: TAXONOMICALLY UNRELATED BUT EQUIPPED WITH SIMILAR ARMORY TO ATTACK PLANTS

Maita Latijnhouwers, Pierre J.G.M. de Wit and Francine Govers

A modified version of this chapter has been submitted for publication

ABSTRACT

The fungi and oomycetes are the two most important groups of eukaryotic plant pathogens. The fungi form a separate kingdom and are evolutionarily related to animals. The oomycetes are classified in the kingdom Protoctista and are related to heterokont (biflagellate) golden-brown algae. Both fungi and oomycetes show filamentous growth, produce mycelia and form sexual and asexual spores. Several fundamental differences in physiology, biochemistry and genetics between fungi and oomycetes have been described in the past. These differences are also reflected in large variations between the two groups in sensitivity or tolerance to conventional fungicides. Recently, more pronounced differences are revealed by genomics approaches. In this review we compare the mode of colonization employed by the two taxonomically distinct groups and show that their strategies have much in common. A better understanding of these strategies may be helpful to develop novel approaches to control the devastating diseases caused by oomycete plant pathogens.

INTRODUCTION

Together, the fungi and oomycetes cover the majority of all eukaryotic plant pathogens. At first glance the growth patterns of these two groups of pathogens are similar and oomycetes were long considered a class within the kingdom Fungi. Fungi and oomycetes both show filamentous growth in their vegetative stage, produce mycelia and form spores for asexual and sexual reproduction that are often air-borne. However, taxonomic analyses of phenotypic characteristics and sequence comparisons have unambiguously shown that the two groups appear on different eukaryotic branches of phylogenetic trees. Based on comparisons of genes encoding the small ribosomal subunit, actin and tubulin, it was concluded that fungi share a common ancestor with animals (van de Peer and de Wachter, 1997; Baldauf et al., 2000), whereas the oomycetes' closest relatives are the heterokont (biflagellate) golden-brown algae (Ben Ali et al., 2002; van de Peer and de Wachter, 1997; Baldauf et al., 2000). Although the reclassification of the oomycetes is still under construction, it is no longer disputed that oomycetes are more related to plants than to animals (Figure 1).

The ca. 10,000 plant pathogenic fungal species show enormous variation in host range and infection strategies. Some fungi, such as *Cladosporium fulvum,* causing tomato leaf mold, and the species causing smut diseases, grow and reproduce in living plant tissue. These so-called biotrophs obtain nutrients through intimate interactions with living plant cells. Necrotrophic species, in contrast, feed on dead plant cells. They kill host tissue before colonizing it. *Cochliobolus* and *Botrytis* species are examples of fungal necrotrophs. Hemibiotrophs such as *Colletotrichum* and *Venturia* species have intermediate lifestyles. They initially establish a biotrophic relationship with their host but subsequently the host cells die as the infection proceeds. Some biotrophs, e.g. the species causing rusts and powdery mildews, can not be cultured on artificial media. As they can grow exclusively on living tissue, they are called obligate parasites (Agrios, 1997).

Presently, twenty percent of all costs spent on control of plant diseases is used to

control oomycete diseases (Lyr et al., 1999). Oomycete plant pathogens belonging to the genera *Phytophthora*, *Pythium* and *Aphanomyces* cause foliage blights, damping-off, and root and stem rots. These pathogens have necrotrophic or hemibiotrophic lifestyles. Obligate biotrophic oomycetes are found within the families *Albuginaceae* and *Peronosporaceae,* and usually cause diseases called downy mildews.

With the exception of the rust and smut fungi, nuclei in vegetative fungal tissue are generally haploid. Oomycete nuclei, in contrast, are diploid throughout almost the entire life cycle (Zentmyer, 1983; Dick, 2001a). In contrast to fungi, oomycete hyphae are non-septate (Dick, 2001b). Many oomycetes are (partial) sterol auxotrophs (Nes, 1987) and their membranes contain lipids with unusual structures and long-chain fatty acids that presumably replace sterols in mycelial membranes (Nes, 1988; Erwin and Ribeiro, 1996). In addition, fungi and oomycetes synthesize lysine by different pathways (LéJohn, 1972). Most manifest, however, are the differences in cell wall composition of fungi and oomycetes. Cell walls of most oomycetes consist mainly of β-glucans and cellulose. Chitin, a major constituent of fungal cell walls, has been detected in small amounts in only a few oomycetes (Bartnicki-Garcia and Wang, 1983; Bulone et al., 1992; Werner et al., 2002). These differences between oomycetes and fungi are also reflected in their distinct sensitivities to conventional fungicides. Azole fungicides, for example, act by inhibition of the ergosterol biosynthesis pathway (Buchenauer, 1977). Since oomycetes do not synthesize ergosterol, they are insensitive to this important group of fungicides (Griffith et al., 1992). Because chitin is only a minor component of oomycete cell walls, chitin synthase inhibitors, such as Nikkomycin and Polyoxin D, have no effect on oomycetes. Conversely, phenylamides, such as metalaxyl, display highly selective activity against oomycetes. These compounds interfere with the synthesis of ribosomal RNA (Davidse, 1990).

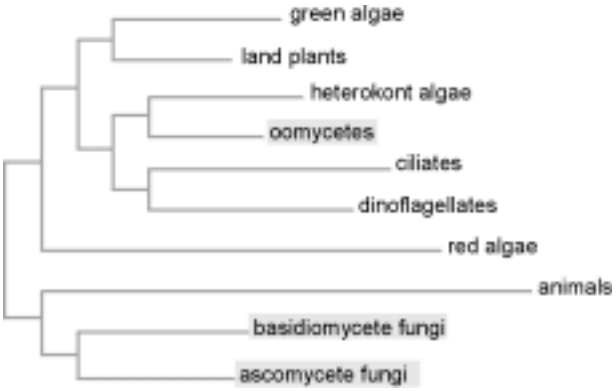


Figure 1.
Phylogenetic tree showing the evolutionary relationships between the major eukaryotic groups (adapted from van de Peer and de Wachter, 1997; Kamoun et al., 1999b). The oomycetes and the (ascomycetous and basidiomycetous) fungi are shaded in grey. Note the evolutionary distance between the oomycetes and fungi.

As their vegetative stage is diploid and homologous recombination has not been achieved, oomycetes are far less tractable to genetic manipulation than many fungi. Of all oomycetes, *Phytophthora* is the best-studied genus causing economically important diseases of staple crops such as potato, cocoa and soybean. Valuable resources including

genetic linkage map(s), bacterial artificial chromosome (BAC) and large EST libraries of numerous different developmental stages have been generated in the last few years (reviewed in Tyler, 2001) and several new methods have been developed for DNA transformation of *Phytophthora* species using zoospore electroporation (Tyler, B. and Govers, F. unpublished), microprojectile bombardment (Cvitanich and Judelson, 2001) and *Agrobacterium tumefaciens*- mediated transformation (Vijn and Govers, submitted). To circumvent the need for homologous recombination to obtain gene knock-out strains, gene-silencing has been successfully used as a method to accomplish targeted mutagenesis of some genes (Latijnhouwers et al., in preparation; Gaulin et al., 2002; van West et al., 1999) but it still requires further optimization.

Thanks to the development of these new methods and resources, significant progress has been made in elucidating infection strategies of oomycetes. In this paper, important aspects of fungal and oomycete virulence, including histopathology of infection structures, the production of virulence factors, and the role of conserved signaling pathways in development and virulence, are compared and similarities and differences are highlighted.

Spores and infection structures

Efficient spore production and dispersal is a prerequisite for successful infection. Asexual spores of oomycetes and fungi are both dispersed by wind. Sporangia, the oomycete asexual spores, can undergo cytoplasmic cleavage in most species, resulting in the formation of zoospores, wall-less, uninucleate cells possessing two flagella (Carlile, 1983). They are dispersed in water drops, water films or through water-rich soils. The mechanism by which zoospores are attracted to the host is not yet fully understood, but there are reports indicating that chemotaxis, electrotaxis and autoaggregation play a role (Morris et al., 1998; Reid et al., 1995; van West et al., 2002; reviewed in Tyler, 2002). Once zoospores have reached the host, they retract their flagella and become immobile cysts. Zoospores are not produced by fungi (Margulis and Schwartz, 2000). Sexual spores of both fungi and oomycetes can usually survive under extreme environmental conditions (cold, heat, drought) encountered during overwintering or oversummering on leaf debris or in soil (Agrios, 1997).

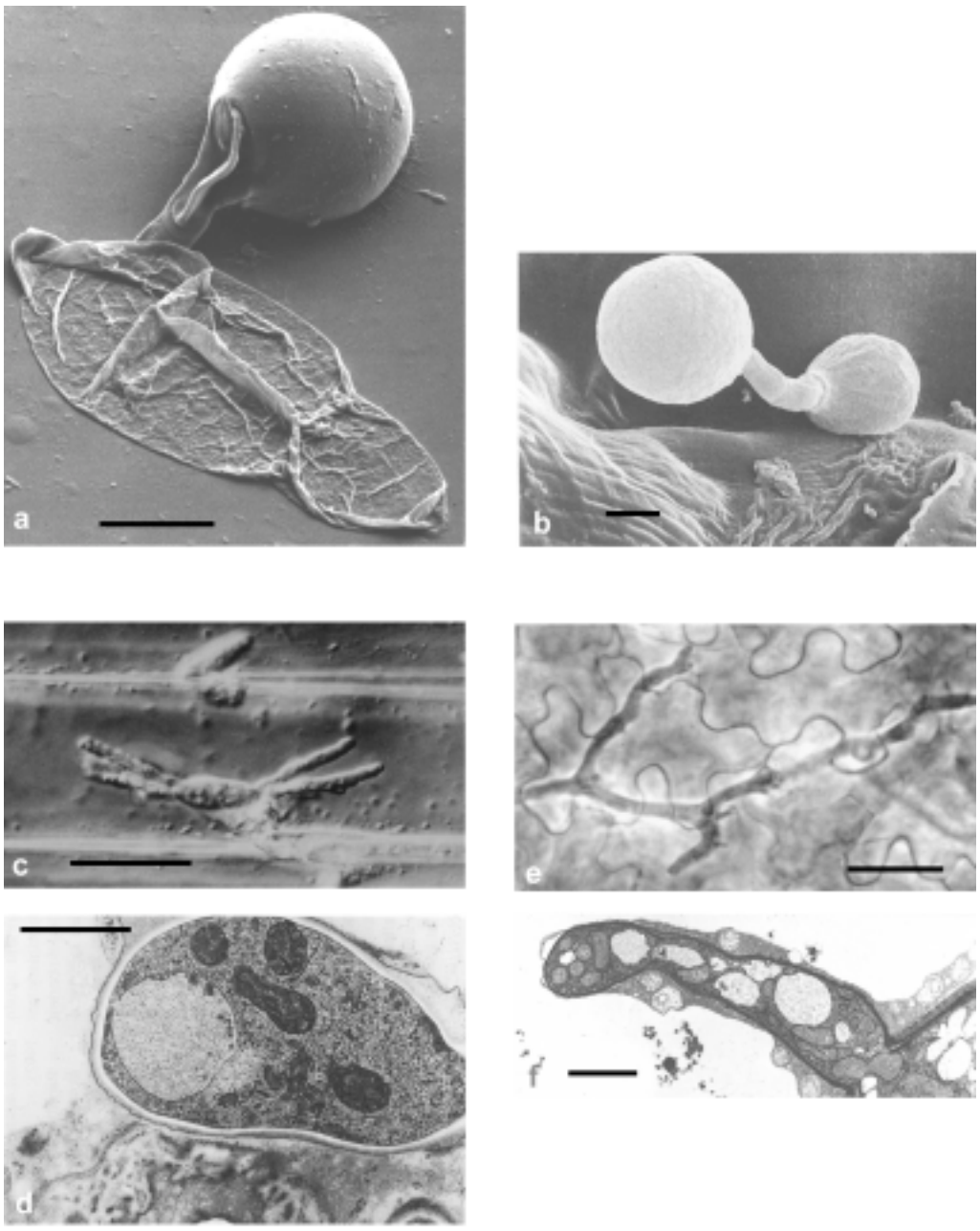
Fungal spores generally employ a combination of water-insoluble glycoproteins, lipids and polysaccharides for host surface attachment (reviewed in Tucker and Talbot, 2001; Hardham, 2001). *Magnaporthe grisea* secretes pre-formed adhesive material from the spore tip (Hamer et al., 1988), whereas in other fungi spore attachment requires *de novo* protein synthesis. Adhesive substances of the fungi *Erisyphe graminis* and *Uromyces viciae-fabae* contains enzymes that can change surface hydrophobicity, thus improving spore attachment (Deising et al., 1992; Nicholson et al., 1993). Germinating sporangia of the oomycete *Peronospora parasitica* were shown to secrete an adhesive layer containing glycoproteins and β-1,3-glucans that may contribute to germling attachment (Carzaniga et al., 2001). The oomycetes *Pythium* and *Phytophthora* store adhesive material in small vesicles inside zoospores, which is released upon encystment (Hardham and Gubler, 1990). Germinating cysts of *Phytophthora infestans* produce proteins with many repeats with homology to human mucins, thought to play a role in protecting germlings from dessication or physical damage (Görnhardt et al., 2000).

Some fungi avoid breaching the cell wall by entering the plant tissue exclusively through stomata and remain confined to the apoplastic space. Other fungi and many oomycete species form specialized infection structures named appressoria enabling them to rupture plant cell walls (Figure 2A and B). An appressorium is a swelling of the tip of a germ tube from which a penetration peg emerges to pierce the cell wall allowing the pathogen to enter the epidermis cell (reviewed in Hardham, 2001a; Tucker and Talbot, 2001). Many fungi require a hard, hydrophobic surface for induction of appressorium formation and the topology of the surface, e.g. the presence of ridges, can be an important trigger (thygmotropism). Fungal appressoria are separated from the spore by a septum (reviewed in Staples and Hoch, 1997). Appressoria of *M. grisea* accumulate high concentrations of glycerol causing a turgor pressure of up to 8 MPa (de Jong et al., 1997). The presence of melanin in appressorial walls is required for cell wall enforcement enabling build-up of spectacular turgor pressure, and mutants in melanin synthesis are unable to penetrate the plant cell wall due to the inability to build up turgor pressure (Chumley and Valent, 1990; Howard et al., 1991; Perpetua et al., 1996; de Jong et al., 1997). During the last five years, many genes have been identified that are required for appressorium formation, turgor generation or for appressorial penetration in *M. grisea* and an overall picture of appressorium functioning is beginning to emerge (Xue et al., 2002; Clergeot et al., 2001; Balhadere and Talbot, 2001; deZwaan et al., 1999; Kershaw et al., 1998; Talbot et al., 1996).

Many oomycetes produce appressoria, although those of *Phytophthora*, *Pythium* and *Peronospora* species are generally small compared to the fungal ones (Krämer et al., 1997; Endo and Colt, 1974). They do not seem to contain melanin and the cytoplasm of germ tubes and appressoria is uninterrupted, only separated by a so-called false septum (Carzaniga et al., 2001; Krämer et al., 1997; Enkerli et al., 1997). Like in fungi, topological features on the surface of host tissues such as ridges and irregularities can induce formation of appressoria in *P. parasitica, P. infestans* and *Phytophthora sojae* (Krämer et al., 1997; Bircher and Hohl, 1997; Morris et al., 1998; Koch and Slusarenko, 1990). The turgor pressure in oomycete appressoria has not been quantified nor has the chemical composition of the low molecular solutes in appressoria been analyzed.

Both in fungi and oomycetes, the infection hyphae, formed immediately after entering the plant tissue, are larger in diameter than the colonizing hyphae (Mendgen and Hahn, 2002; Krämer et al., 1997). Intercellular hyphae of many biotrophic fungi and oomycetes form spherical or lobed structures named haustoria that penetrate the

Figure 2.
Infection structures of plant-pathogenic fungi and oomycetes. **a.** Cryoscanning electron micrograph of mature appressorium of *Magnaporthe grisea*. bar 5 µm (micrograph courtesy Richard J. Howard, DuPont Crop Genetics). **b.** Cryoscanning electron micrograph of cyst (left) with germ tube forming appressorium (right). Site of penetration is adjacent to a stoma. bar 8 µm (Coffey and Gees, 1991). Because cyst and appressorium are not separated by a septum in *P. infestans*, the cyst does not collapse as in *M. grisea* (**a**) **c.** Haustorium of *Erisyphe graminis* f. sp. *hordei* with 'lobed structure' in epidermal host cell of *Hordeum vulgare*, stained with $DiOC_7$, as viewed by differential interference microscopy. bar 20 µm (Bushnell et al., 1987). **d.** Transmission electron micrograph of haustorium of *E. graminis* f. sp. *hordei* in *H. vulgare*. bar 1 µm (Hippe-Sanwald et al., 1992). **e.** Fluorescence micrograph of trypan blue-stained tissue showing branching hypae of *P. infestans* with haustoria in mesophyll cells of potato cultivar 'Bintje'. bar 35 µm (Vleeshouwers et al., 2000). **f.** Transmission electron micrograph of haustorium of *P. infestans* in potato cultivar 'Majestic'. bar 2 µm (Coffey and Wilson, 1983). Note digit-like shape of *P. infestans* haustoria in **e** and **f**.


a
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adjacent plant cells (Figure 2C-F). Haustoria are organelles that are specialized to retrieve nutrients from the host (reviewed in Mendgen and Hahn, 2002; Hahn and Mendgen, 2001; Hardham, 2001a; Hardham, 2001b). At the site of haustorium formation, the plant plasma membrane invaginates and surrounds the pathogen's cell wall. The electron dense material between the pathogen cell wall and the plant plasma membrane is known as the extrahaustorial matrix. A so-called neckband separates the extrahaustorial matrix from the plant's intercellular space. In the case of *Puccinia hemerocallidis* infection, the extrahaustorial membrane was recently reported to form tubular extensions into the plant cytoplasm, possibly to enlarge the interacting surface (Mims et al., 2002). Genes encoding hexose- and amino acid transporters and thiamine biosynthesis genes are highly expressed in haustoria of the rust fungus *U. viciae- fabae* (Voegele et al., 2001; Sohn et al., 2000), adding credence to the idea that haustoria play a central role in primary metabolism and import of nutrients (reviewed in Mendgen and Hahn, 2002). Uptake of nutrients was proposed to be driven by a proton gradient generated by H^+ ATPases (Szabo and Bushnell, 2001). Haustoria of rust fungi and of biotrophic oomycetes such as *P. parasitica* are similar in size but the organization of the electron-dense 'neckband' is different (Lucas et al., 1995). In contrast, hemibiotrophic oomycete species belonging to the genera *Phytophthora* and *Pythium* form small finger- or digit-like haustoria during the biotrophic phase (Enkerli et al., 1997; Coffey and Wilson, 1983) (Figure 2F).

Cytological examinations of the events following spore and cyst germination clearly suggest a high degree of similarity in infection mechanisms between fungi and oomycetes. However, more in-depth molecular and biochemical studies will be required, especially in oomycetes, to enable more detailed comparisons of the composition of appressoria and extrahaustorial matrices, as well as of the identity of transport proteins and transport mechanisms in haustoria.

Virulence factors

Compounds secreted by pathogens are designated virulence factors when they contribute to virulence, as determined by replacement or silencing of the encoding genes. Cell wall-degrading enzymes (CWDEs), proteins involved in protection against plant defense compounds (counter defense) and toxins are examples of virulence factors. Some pathogen-derived proteinaceous compounds, called elicitors or avirulence factors, were discovered by their ability to elicit defense responses in host plants carrying the matching resistance genes (Keen, 1990). Many of these so-called elicitors may intrinsically be virulence factors, but, as plants have evolved the ability to recognize them, they have become the telltales of the pathogens' presence. How these elicitors elicit plant defense responses has been the subject of several recent reviews (Knogge, 2002; van 't Slot and Knogge, 2002; Luderer and Joosten, 2001). Here, we will focus on putative virulence functions of the elicitors.

Cell wall-degrading enzymes

CWDEs are often expressed in germ tubes and infection hyphae suggesting that they are used to loosen the plant cell wall facilitating successful penetration. (Mendgen and Deising, 1993; Pryce-Jones et al., 1999). On the other hand, necrotrophic fungi are thought

to use CWDEs to break down the plant cell wall, thus killing their hosts' cells to feed on the released nutrients. A large number of fungal CWDE genes, encoding polygalacturonases, pectin-lyases, pectinases, cellulases, xylanases and glucanases have been cloned (ten Have et al., 2002). Targeted disruption of many of these genes clearly showed their role in fungal virulence (Oeser et al., 2002; Isshiki et al., 2001; Rogers et al., 2000; ten Have et al., 1998; Shieh et al., 1997). However, there are several examples where disruption or replacement of genes encoding CWDEs did not alter virulence, possibly because these genes are redundant and other isozymes encoded by paralogous genes were able to take over the function of the lacking enzyme (reviewed in Lebeda et al., 2001).

Oomycete species of the genera *Phytophthora* and *Pythium* are known to produce and secrete CWDEs in liquid cultures and on solid media (Jarvis et al., 1981; Endo and Colt, 1974). Recently, a gene encoding an extracellular endopolygalacturonase was identified in *P. infestans*. Polygalacturonases (PGs) are pectinases hydrolyzing the homogalacturonan backbone of pectin (ten Have et al., 2002). The *P. infestans* PG gene *Pipg1* is expressed in germinating cysts, suggesting that this PG plays a role in the initial stage of infection. However, the gene is also expressed during all stages of growth in tomato, and hence, the same enzyme could also be involved in inducing or maintaining necrotrophic growth (Torto et al., 2002). A remarkably large PG gene family consisting of 19 members was identified in *Phytophthora cinnamomi*. These genes are located in three clusters in the *P. cinnamomi* genome (Götesson et al., 2002). In addition, five genes encoding exo- and endo-1,3(1,4)-β-glucanases were cloned from *P. infestans* and low-stringency hybridization suggested the presence of additional glucanase genes in the *P. infestans* genome (McLeod et al., 2002). *Phytophthora* PGs and glucanases are more similar to the corresponding fungal and insect enzymes than to their plant counterparts, which seems to be in disagreement with the previously described evolutionary distance between oomycetes and fungi. However, the enzymes likely perform similar functions and encounter a similar selection pressure. It was therefore suggested that sequence convergence may explain the similarities between the *Phytophthora* and fungal CWDEs (Götesson et al., 2002; Torto et al., 2002; McLeod et al., 2002).

Plants produce CWDEs directed against fungal and oomycete cell walls. These CWDEs presumably function by affecting the invader's cell wall integrity (ten Have et al., 2002). In addition, CWDE-mediated release of oligosaccharides from the pathogen's cell wall is known to elicit defense responses in the plant (reviewed in Côté and Hahn, 1994; Ebel and Cosio, 1994). To escape from damage by plant glucanases, the oomycete *P. sojae* secretes proteins that can inhibit those CWDEs. (Ham et al., 1997). One of the Glucanase Inhibiting Proteins, GIP1, specifically inhibits EgaseA, the soybean endo-β-1,3-glucanase that is responsible for the release of glucan oligosaccharide elicitors from the pathogen's cell wall. By secreting this protein into the apoplast, *P. sojae* prevents the release of oligo-β-glucans and thus avoids or slows down early recognition by the host (Rose et al., 2002). Fungi have also evolved strategies to escape from the action of fungal cell wall degrading enzymes, in particular plant chitinases. AVR4 is an extracellular protein that contains a chitin-binding domain. It is produced *in planta* by the fungus *C. fulvum*. Fungi that are sensitive to plant chitinases such as *Trichoderma*

viride and *Fusarium solani* are protected against lytic effects of this enzyme after incubation *in vivo* with the AVR4 protein. Thus binding of AVR4 to the chitin exposed at the surface of the cell walls of these fungi protects the chitin from being hydrolyzed chitin. Chitin of *C. fulvum* is only exposed *in planta* and binding of AVR4 to chitin might protect the fungus against the deleterious lytic effects of plant chitinases (van de Burg et al. submitted). These results show that oomycetes and fungi have both found ways to defend themselves against some plant CWDEs, the first by binding to the enzyme itself and the latter by binding to chitin preventing the enzyme to bind to and act on its substrate. Similarly, plants protect their walls from being hydrolyzed by microbial polygalacturonases by producing polygalacturonase-inhibiting proteins (PGIPs) (reviewed in de Lorenzo and Ferrari, 2002).

Inactivation of plant defense compounds and toxins

Plants not only produce CWDEs to inhibit pathogen proliferation, but they also synthesize antimicrobial secondary metabolites such as phytoanticipins (pre-existing) and phytoalexins (induced by pathogens) for the same purpose. Fungi have developed ways to prevent damage by these compounds. Some fungi produce enzymes detoxifying pre-existing and induced antimicrobial plant secondary metabolites and some of these enzymes have been shown to contribute to pathogenicity (reviewed in Morrissey and Osbourn, 1999). Another mechanisms of resistance to antimicrobial plant compounds in plant pathogenic fungi seem to be based on the secretion of the compounds. ABC-transporters are membrane pumps that are involved in transporting a wide variety of compounds over the plasma membrane. ABC-transporter knock-out strains of *Botrytis cinerea* and *Gibberella pulicaris* were more sensitive to secondary metabolites of their respective hosts. These knock-out strains, as well as ABC-transporter disruptants of *Mycosphaerella graminicola* and *M. grisea,* were reduced in virulence (Schoonbeek et al., 2001; Fleissner et al., 2002; Stergiopoulos et al., submitted; Urban et al., 1999). In many cases, oomycete plant pathogens have to cope with the same antimicrobial compounds in their hosts, suggesting that they too must have evolved mechanisms to withstand them. For discovery of counter-defensive mechanisms in pathogenic oomycetes, the mechanisms described to occur in fungi should be fully explored.

Toxin biosynthesis remains a similarly unexplored area in oomycetes. Some necrotrophic fungi produce toxins to kill host cells and the genes involved in the biosynthesis of these toxins have been studied in great detail. Two important groups of fungal phytotoxins are nonribosomally synthesized linear but often cyclic peptides and polyketide derivatives (Panaccione et al., 2002). Peptide synthetases and polyketide synthetases involved in the synthesis of the respective groups of toxins possess domains that are conserved between different fungal species. A large number of oomycete pathogens are necrotrophic or have a necrotrophic growth phase. Therefore, the possibility that they too use toxic compounds to kill their host cells should certainly be considered.

Plants produce reactive oxygen species (ROS) as an early response to infection known as the hypersensitive response (HR) (Levine et al., 1994). ROS are expected to be involved in limiting pathogen growth. Enzymes produced by pathogens, such as superoxide dismutases, catalases and peroxidases, are thought to be involved in

breakdown or inactivation of ROS (Mayer et al., 2001). A number of genes encoding ROS-inactivating enzymes have been cloned from fungi (Mayer et al., 2001). However, their role in virulence remains uncertain (Schouten et al., 2002). Sequences derived from ROS-inactivating enzymes were identified in *Phytophthora* EST databases, indicating that oomycetes may also defend themselves against damage by ROS generated by the host (Kamoun et al., 1999).

Elicitors

Plants have evolved surveillance systems to specifically recognize pathogen-derived molecules to quickly mount efficient defense responses often including an HR. These molecules are called elicitors and are produced by fungal and oomycete pathogens (reviewed in Knogge, 2002; van 't Slot and Knogge, 2002; Luderer and Joosten, 2001). One elicitor homologue has been detected in both fungi and oomycetes. This so-called necrosis inducing protein causes necrosis and elicits defense responses including HR in a broad range of dicotylenous plants (Bailey, 1995; Bailey et al., 2000; Qutob et al., 2002). This protein was first detected in the fungus *Fusarium oxysporum* f.sp.*erythroxyli* (Bailey, 1995) and homologues were subsequently found in several oomycetes (Qutob, 2002; Fellbrich et al., 2002; Veit et al., 2001) and in bacteria (*Bacillus halodurans, Streptomyces coelicolor* and *Vibrio* species) (Bentley et al., 2002; Takami et al., 2000). A mechanism involving horizontal gene transfer was proposed to explain the occurrence of the gene in microbial species belonging to the different kingdoms (Qutob et al., 2002).

Elicitors are not produced by plant pathogens just for the sake of being recognized by the host, unless the pathogen could benefit from HR or necrosis often induced in plant tissue after recognition of elicitors. It is hypothesized that most elicitors are secreted proteins with virulence functions that have acquired the ability to elicit defense responses in plants at a later stage of (co-)evolution between plants and their pathogens (Dangl and Jones, 2001; Luderer and Joosten, 2001). This explains why the elicitors form such a heterogeneous group of molecules. Searching for oomycete elicitors based on homology with fungal elicitors, or *vice versa*, is therefore expected not to be successful. For that reason, we will focus on possible virulence functions of fungal and oomycete elicitors.

To date, there is evidence for intrinsic functions in virulence for only very few elicitors (Table 1). Examples of those include the necrosis inducing protein 1 (NIP1) from the fungus *Rhynchosporium secalis*, which functions as elicitor in barley cultivars carrying the corresponding resistance gene *Rrs1.* As a result, only *R. secalis* strains lacking NIP1 are able to grow on Rrs1 barley cultivars (Rohe et al., 1995). However, NIP1 causes necrosis in resistant and susceptible cultivars of barley and other cereals (Wevelsiep et al., 1991) and disruption of the *nip1* gene resulted in reduced virulence of *R. secalis* on susceptible plants. This suggests that NIP1 functions as a virulence factor (Hahn et al., 1993). Another example of elicitors with proposed functions in virulence are the oomycete elicitins. Elicitins are 10 kD proteins that are found in most *Phytophthora* and in a few *Pythium* species (reviewed in Tyler, 2002; Ponchet et al., 1999). They elicit a wide range of defense responses in *Nicotiana* species. Elicitins were shown to bind sterols, suggesting that they could function in recruiting sterols from the environment (Mikes et al., 1997, 1998; Vauthrin et al., 1999). Since *Phytophthora* species can not

Table 1: fungal and oomycete elicitors with proposed intrinsic function

elicitor	organism	name and putative function	virulence function confirmed by gene-disruption / silencing	reference(s)
Fungi				
AvrPita	*Magnaporthe grisea*	zinc metallo protease	No	Jia et al., 2000 Orbach et al., 2000
ECP1	*Cladosporium fulvum*	unknown	Yes	Lauge et al., 1997
ECP2	*Cladosporium fulvum*	unknown	Yes	Lauge et al., 1997
AVR4	*Cladosporium fulvum*	chitin binding / protection against chitinases	No	van den Burg et al., in preparation
NIP1	*Rynchosporium secalis*	unknown	Yes	Wevelsiep et al., 1991
*ace*1*	*Magnaporthe grisea*	polyketide synthese / nonribosomal peptide synthetase	No	Fudal et al., 2003
Oomycetes				
elicitins	*Phytophthora* spp	sterol binding	No	Mikes et al., 1997
GPE1	*Phytophthora sojae*	transglutaminase; crosslinking of cell wall proteins	No	Scheel et al., 2000
CBEL	*Phytophthora parasitica*	cellulose binding glycoprotein	Yes	Mateos et al., 1997 Gaulin et al., 2002

* it is hypothesized that ACE1 is required for synthesis of a sofar unknown secondary metabolite elicitor

synthesize their own sterols, this seems a plausible assumption.

Large-scale screening of pathogen proteins for elicitor or necrosis-inducing activity using *Agrobacterium* and virus-mediated expression *in planta* has rapidly identified new elicitors from both fungal and oomycete origin (Takken et al., 2000; Torto et al., 2002). Unraveling the intrinsic functions of additional elicitors in the future is very important as it will increase our understanding of the infection strategies employed by different pathogens.

Signal Transduction Involved in Pathogenesis

In the last decade, evolutionarily conserved signaling pathways that underlie fungal development and virulence, such as G-protein, cAMP and MAPK signaling have received much attention (D'Souza and Heitman, 2001; Borges-Walmsley and Walmsley, 2000; Xu, 2000). These pathways function to communicate extracellular signals over the plasma membrane to the interior of the cell, allowing the pathogen to respond adequately and timely to changes in its environment. In most fungi, the number of different G-protein α subunits ranges from 2 to 4. In each of the plant pathogenic fungi studied, at least one of the Gα subunit genes plays and important role in development and virulence (Lengeler et al., 2000; Kronstad, 1997; Bölker, 1998). For example, the Gα subunit GPA3 of the corn smut fungus *Ustilago maydis* controls mating, filamentation, melanization and virulence (Regenfelder et al., 1997). In *Cryphonectria parasitica* disruption of the Gα subunit CPG1 negatively affects female fertility, conidiation and pigmentation (Choi *et al.,* 1995; Gao and Nuss, 1996) and in *M. grisea*, disruption of the Gα subunit gene *magB* resulted in reduced conidiation and appressorium development (Liu and Dean, 1997). Moreover, a *B. cinerea* Gα subunit (BCG1) is responsible for secretion of proteases (Gronover et al., 2001). The function of a G-protein β subunit has been studied in only one plant pathogenic fungus so far. The *C. parasitica* G-protein β subunit, CPGB1, regulates conidiation, pigmentation and vegetative growth (Kasahara and Nuss, 1997). These results show that G-proteins are key players in development and virulence of plant pathogenic fungi.

P. infestans is the only oomycete in which the functions of G-protein subunits have been analyzed. Silencing of the Gβ subunit gene *Pigpb1* resulted in a defect in sporangia formation, indicating that a function can be assigned to this gene comparable to its fungal counterpart from *C. parasitica* (Latijnhouwers et al., in preparation). Mutants deficient in the Gα subunit PiGPA1 show a reduction in zoospore release and appressorium formation and their virulence is severely impaired. The zoospores of the PiGPA1-deficient mutants change direction with high frequency and they fail to aggregate or to be attracted to chemotactic compounds (Latijnhouwers et al., in preparation). The function of PiGPA1 in zoospore aggregation seems related to functions of Gα subunits in the slime mold *Dictyostelium discoidium.* Also in this organism, heterotrimeric G-proteins control chemotaxis and aggregation (Parent and Devreotes, 1996).

Heterotrimeric G-proteins often feed into the cAMP pathway. Fungal strains with mutations in the catalytic subunits of cAMP- dependent protein kinase (PKA) or adenylyl cyclase display phenotypes similar to those of G-protein deletion mutants. Moreover, levels of cAMP were altered in several G-protein mutants and there are

examples where addition of cAMP was able to restore normal development (Klimpel et al., 2002; Krüger et al., 1998; Xu et al., 1997; Gold et al., 1994; Dürrenberger et al., 1998; Mitchell and Dean, 1995; Adachi and Hamer, 1998; Xu and Hamer, 1996; Choi and Dean, 1997). In *U. maydis* and *M. grisea*, the cAMP and MAP kinase pathways are clearly linked although there is no evidence for direct activation of MAP kinases by G-proteins in plant pathogenic fungi. The *M. grisea* MAP kinase PMK1 and its homologues in other fungi have been shown to be crucial for appressorium formation and pathogenesis (Xu, 2000; Xu and Hamer, 1996). Another *M. grisea* MAP kinase, MPS1, is dispensable for formation of appressoria but is required for appressorial penetration (Xu et al., 1998). As the genes encoding the respective kinases are well represented in the *Phytophthora* EST databases, a role for the cAMP and the MAP kinase pathway downstream of G-proteins is conceivable but this has not yet been investigated.

Experiments using the G-protein-activating peptide mastoparan in *P. infestans* indicated that phospholipase D is located downstream of the G-protein pathway and it was suggested that it plays a role in encystment (Latijnhouwers et al., 2002). Other studies have shown that also Ca^{2+} signaling is involved in this process (Warburton and Deacon, 1998; Judelson and Roberts, 2002). Curiously, phospholipases and Ca^{2+} signaling have so far received little or no attention in fungal plant pathogens. To identify genes involved in the processes affected in the *Pigpa1*-silenced mutants, i.e. in zoospore development and behavior, appressorium development and virulence, cDNA Amplified Fragments Length Polymorphism (cDNA-AFLP) analysis of gene expression in the PiGPA1-deficient mutants was carried out (Dong et al., in preparation). This resulted in the isolation of transcript derived fragments (TDFs) that show differential expression profiles when compared between wildtype and the *Pigpa1*-silenced mutants and possibly represent G-protein target genes.

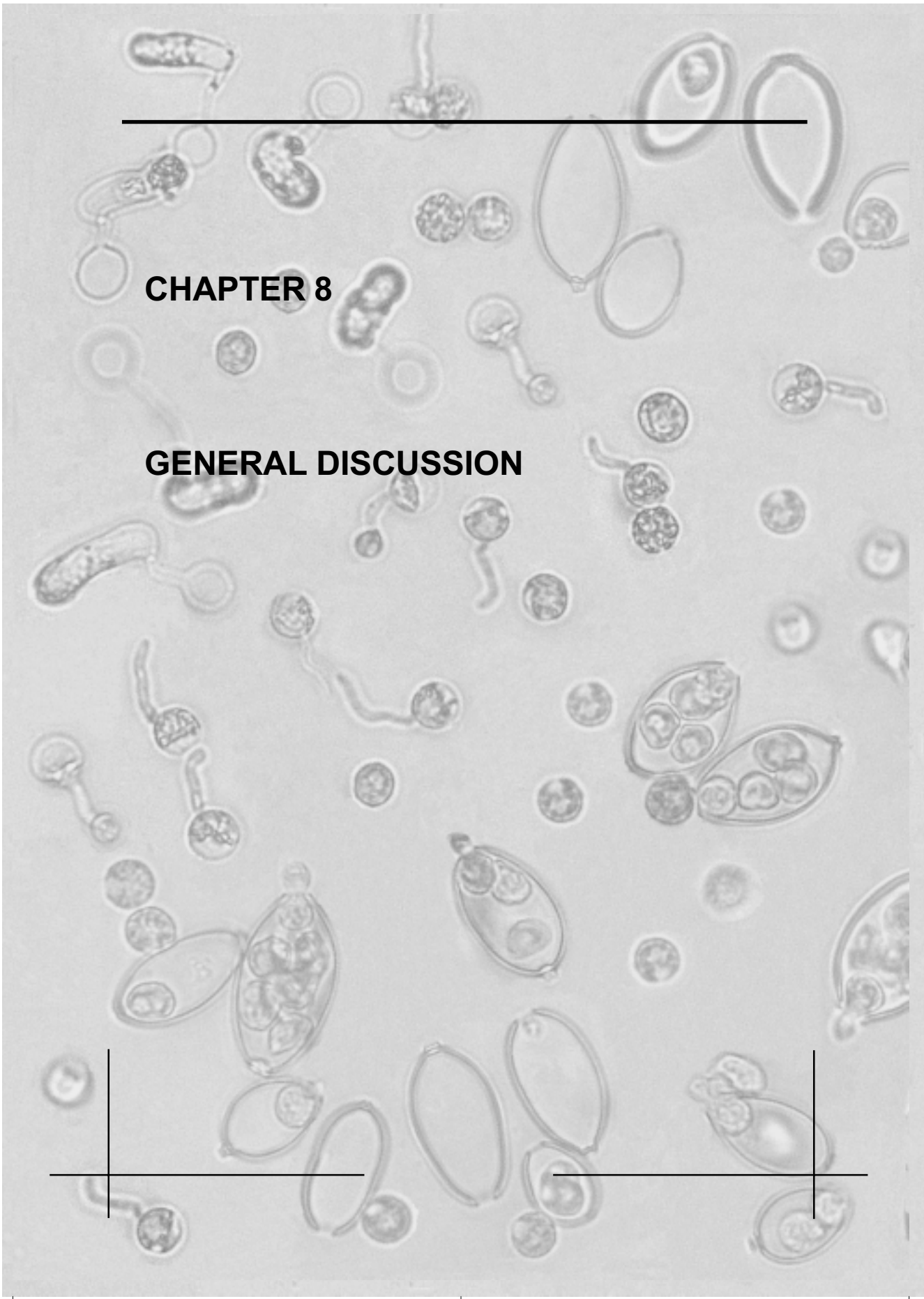
Unraveling the role of conserved signal transduction pathways by means of gene disruption, gene replacement or gene silencing has been proven to be a powerful method to dissect developmental processes in plant pathogens. It has become clear that both oomycetes and fungi rely on a functional G-protein pathway for normal development and virulence, whereas downstream pathways and targets of G-proteins have been studied only in fungi. The pheromone receptors in *Ustilago* species are the only receptors coupling to G-proteins described in plant pathogens so far (Kronstad and Staben, 1997). To better understand how environmental cues affect development of these pathogens, the identification of receptors and ligands deserves more attention.

Concluding remarks

In spite of the distinct evolutionary origin of the two groups, fungi and oomycete use infection strategies that have much in common. Their specialized infection structures (appressoria and haustoria) share many features and the same signaling pathway (the heterotrimeric G-protein pathway) is a key-regulator in important developmental processes and virulence in both groups of pathogens. Moreover, they both use an extensive toolbox of CWDEs to penetrate into and to degrade plant cell walls. This in itself may not be surprising, but the protein sequences of many CWDEs of fungi and oomycetes appear to be more conserved than would be expected from their evolutionary relation alone. This high degree of similarity suggests that the *in planta* conditions are

very similar for both plant pathogenic fungi and oomycetes where they encounter a wide range of preformed and induced antimicrobial compounds. This must have forced evolution into similar directions in fungi and oomycetes. Thus, convergent evolution seems to have forced the development of similar infection strategies in plant pathogenic oomycetes and fungi. Horizontal gene transfer may have occurred, either between fungi and oomycetes or between these organisms and other species. It is hypothesized that fungi have acquired gene clusters from other organisms (Rosewich and Corby, 2000) and, as discussed before, the occurrence of homologues of the *Fusarium oxysporum* f.sp.*erythroxyli* necrosis inducing protein in many oomycete species might have been the result of horizontal gene transfer. However, the frequency of occurrence of this phenomenon and its impact on evolution has not yet been investigated.

Important differences concerned classes of virulence factors that occur only in one of the two groups of plant pathogens. So far, antimicrobial plant secondary metabolite-detoxifying enzymes and fungal phytotoxins have not been detected in oomycetes. Complete genomic sequences of plant pathogenic fungi will soon become available (Dean, 2002; Kahmann, 2002). Moreover, funds have been awarded to produce genomic sequences of *P. sojae* and *Phytophthora ramorum* (Tyler, 2002). Complete sequence information of these oomycetes will be very useful in discovering novel virulence factors based on homology with fungi or in high-through-put functional genomics approaches employing gene silencing.



CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

G-proteins in *P. infestans*, why and how?

Mechanisms underlying *Phytophthora* pathogenesis are largely unknown. *Phytophthora* genes playing a role in the interaction with the host have been and are still isolated using several different approaches. For example, a differential hybridization procedure was applied to isolate *P. infestans* genes that are specifically expressed during colonization of leaf tissue. This resulted in the identification of several *in planta* induced (*ipi*) genes (Pieterse et al., 1993). Whether these genes are important for pathogenesis remains to be determined. Genes encoding *Phytophthora* avirulence factors, also called cultivar-specific elicitors, are being isolated using positional cloning strategies (van der Lee et al., 2001; MacGregor et al., 2002; Shan and Tyler, unpublished). Cultivar-specific elicitors trigger defense responses in host cultivars carrying the corresponding resistance gene (Keen, 1990 and chapter 7). In other studies, *Phytophthora* proteins eliciting defense responses in hosts or non-hosts were purified from culture filtrate or from cell wall preparations. Their (partial) sequences were determined and the corresponding genes cloned (reviewed in Knogge, 2002). The recent development of methods for high throughput virus- or *Agrobacterium tumefaciens*-mediated expression of *Phytophthora* cDNAs *in planta* has greatly facilitated the screening for necrosis-inducing proteins from *Phytophthora.* Several novel necrosis-inducing proteins and potential elicitors have been isolated by these methods (Torto et al., 2002). Another frequently used method to identify genes involved in virulence is often referred to as the 'candidate gene approach'. In this method the decision to study the functions of certain genes is based on the known function of homologues or orthologues. This method is gaining popularity because of the rapidly expanding databases with sequence information from many organisms, including *Phytophthora*. The choice to study G-protein-mediated signaling in *P. infestans* was the outcome of a candidate gene approach. In plant pathogenic fungi, heterotrimeric G-protein signaling plays a role in development of infection structures, and is essential for full virulence (chapter 1 and 7 and Lengeler et al., 2001; Kronstad, 1997; Bölker, 1998). This knowledge inspired us to investigate the role of G-protein-mediated signaling in *P. infestans*. This research question is not only of interest with regard to pathogenesis of *P. infestans* but also from an evolutionary perspective: to what extent is G-protein-signaling conserved in oomycetes?

Like most fungal genes encoding G-protein subunits (Bölker, 1998), the two *P. infestans* G-protein subunit genes described in this thesis were isolated based on homology (chapter 2). Unfortunately, the third subunit of the G-protein, the Gγ subunit, was not identified. Gγ subunits are far less conserved than the Gα and Gβ subunits (Neer, 1995). For this reason also the Gγ subunit genes from *Dictyostelium discoidium* and from *Arabidopsis thaliana* were identified only recently (Zhang et al., 2001; Mason and Botella, 2000). The latter Gγ proteins were purified biochemically, based on their interaction with Gβ. Hopefully, common structural characteristics of the *D. discoidium*, fungal and plant Gγ subunits will guide us towards identifying a *P. infestans* Gγ subunit gene in the future.

Pigpa1 and *Pigpb1* are both highly expressed in the infection stages (sporangia,

cysts, zoospores), indicating that they play a role in relation to these different stages of the *P. infestans* life cycle. The expression studies also revealed that *Pigpb1* is expressed at a detectable level in mycelium, whereas *Pigpa1* is not. This suggested that PiGPB1 interacts with a different Gα subunit in mycelium. However, attempts to isolate additional Gα subunit genes using degenerate PCR have failed so far, nor did extensive EST sequencing of cDNA clones from a large number of libraries from different developmental stages of *P. infestans* and *Phytophthora sojae* reveal the presence of any additional Gα subunits. An alternative explanation is that PiGPB1 functions independently from Gα subunits in mycelium (discussed in chapter 2 and 4). Six months from now, the complete genome sequences of *P. sojae* and *P. ramorum* will be available and this will bring us closer to an answer to the question of whether or not additional Gα subunits are present in *Phytophthora* that may interact with PiGPB1 in mycelium.

Gene-replacement to obtain gene knock-outs is more or less routine in many fungi. The mutants that are generated in this way usually have no change in their genome other than the replacement of the target gene by an antibiotic resistance cassette. However, homologous recombination has never been achieved in *P. infestans*. Even if homologous recombination were feasible, it would be very difficult to obtain knock-out mutants by gene replacement since *P. infestans* is diploid almost throughout its life cycle. For that reason, we obtained G-protein-deficient mutants by gene silencing. Gene silencing was initially discovered in plants as a side effect in an experiment dealing with flower pigmentation. Extra copies of a gene involved in flower pigment synthesis were introduced in Petunia in an attempt to overexpress this gene. It appeared, however, that introduction of additional copies of the gene did not always result in overexpression. Instead, it sometimes led to the disappearance of transcripts of both the endogenous gene and the introduced copies (van der Krol et al., 1990). In plants, gene silencing serves as a resistance mechanism to viral infection and is indispensable for normal development (reviewed in Voinnet, 2002; Matzke et al., 2002; Moss, 2001). Gene silencing has also been described to occur in fungi, nematodes, flies and vertebrate animals (reviewed in Cogoni and Macino, 2000), although it sometimes carries a different name. Gene silencing is now widely used as a method to obtain knock-down mutants of genes of interest (reviewed in Baulcombe, 1999).

Like in plants, gene silencing in *P. infestans* can be triggered by introduction of additional copies of an endogenous gene (van West et al., 1999). The work described in the chapters 3 and 4 of this thesis has confirmed that gene silencing can be successfully used for targeted mutagenesis in *P. infestans*. Unfortunately, silencing is still difficult to accomplish in this organism. Both in the case of *Pigpa1* and of *Pigpb1,* silencing occurred at very low frequencies in transformants generated using the electroporation of zoospores. In contrast, in the case of *Pigpb1*, it occurred at a frequency of ca. 20% in transformants obtained by PEG-protoplast transformation. Van West et al. (1999) also used the PEG-protoplast transformation protocol to obtain *inf1*-silenced mutants and also in their study the frequency of silencing reached 20%. However, transformation using the PEG-protoplast protocol has several disadvantages: it is time-consuming, results in low yields and a crucial enzyme preparation required for protoplasting is no longer manufactured. A third method, based on *Agrobacterium tumefaciens*-mediated transformation, has been developed in our laboratory (Vijn and Govers, submitted) but

so far attempts to obtain *inf1*-silenced mutants using this method were unsuccessful. For these reasons, the generation of G-protein-deficient mutants was a protracted, laborious process. A more efficient procedure for targeted mutagenesis is a prerequisite for further dissection of *Phytophthora* pathogenicity. It is therefore of utmost importance to study the mechanism of gene-silencing in *P. infestans* and to develop new methods yielding silenced strains with a higher efficiency. Introduction of an inverted repeat of a fragment of the target gene has been useful in increasing the frequency of silencing in a number of organisms (Matzke et al., 2002). This is one of the approaches to improve gene-silencing in *P. infestans* that is currently being tested in our laboratory.

The functions of PiGPA1 and PiGPB1

The characteristics of the *Pigpa1* and *Pigpb1*-silenced mutants have been extensively discussed elsewhere in this thesis. In this chapter, I will restrict myself to briefly touch upon the most important phenotypic effects of silencing the G-protein subunit genes and discuss possible directions for further research.

The most pronounced effect of silencing of *Pigpa1* involved the change in zoospore swimming behavior and the defect in chemotaxis and aggregation. Previous work on two biological phenomena in other organisms should be considered when trying to further understand the mechanism underlying aggregation in *P. infestans*. The first phenomenon is chemotaxis leading to cell polarity in eukaryotic cells like *Dictyostelium discoidium* cells or mammalian neutrophils and leukocytes. In these systems, cells aggregate by moving up a chemoattractant gradient. They move in a amoeboid fashion and the movement is caused by polymerization of filamentous actin at the front and depolymerization at the cell's posterior. The signaling pathway triggering the cytoskeletal rearrangements involves G-protein coupled receptors and heterotrimeric G-proteins. The G-protein pathway activates phosphoinositide 3-kinase (PI3K), resulting in (i) recruitment of PH-domain-containing proteins to the membrane of the leading edge and (ii) the activation of the small GTPases Rac and Cdc42. Both activities of PI3K are necessary for the actin rearrangements leading to cell polarity in these organisms (reviewed in Weiner, 2002; Chung et al., 2001). Knowing that the G-protein pathway is also involved in aggregation of zoospores in *P. infestans* it would be worthwhile to investigate whether the pathway regulating (chemo- or auto-) taxis in *P. infestans* contains similar components as those in *D. discoidium* and neutrophils.

However, aggregation in *P. infestans* is based on directional swimming rather than on amoeboid movements. The most conspicuous feature of the *Pigpa1*-silenced mutants was the excessive turning in the zoospores. Therefore, it is possible that the mechanism underlying zoospore motility is more equivalent to the mechanism controlling ciliary movements in for example *Paramecium* and *Tetrahymena*. Swimming direction in these organisms is regulated by ciliary reversal, the ciliary beat change from forward to backward swimming. The level of external calcium is an important modulator of this process. Ca^{2+} enters the flagellum or cilium through calcium channels present in the flagellar membrane and alters the swimming direction when it binds to calcium binding proteins present in the flagellar axoneme. Some nonreversal mutants of *Tetrahymena* have been identified with mutations in calcium channels, others with point mutations in calmodulin (Watanabe et al., 1990). Calcium-calmodulin has been shown to activate

guanylyl cyclase, accounting for a role for cGMP in ciliary movement. In addition, the second messenger cAMP is also implicated in this process (Pech-Louis, 1995). In *P. infestans*, Ca^{2+} is known to affect motility and swimming patterns in zoospores (Judelson and Roberts; 2002; Warburton and Deacon, 1998; Reid et al., 1995), showing that there may be similarities between ciliates and zoospores. I speculate that the data on ciliate movement and on chemotaxis in amoebae will both prove to be valuable in dissecting zoospore aggregation.

Another matter deserving attention regarding the role of G-proteins in zoospore aggregation is the identity of the receptors involved. As described in chapter 1, animal and fungal G-protein-coupled receptors (GPCRs) have seven transmembrane domains as a common feature. Also, the GPCRs required for autoaggregation in *D. discoidium* belong to the class of 7TM receptors. In contrast, plants lack this type of GPCRs and it remains unclear how external signals in plants are transferred to plant G-proteins. Apart from containing seven TM domains, GPCRs have little in common, making it difficult to isolate the genes based on homology. So far, one gene encoding a receptor-like protein possessing seven transmembrane domains has been cloned from *P. infestans* (W. Ligterink and F. Govers, in preparation). As yet, the function of this protein is a mystery as no obvious phenotypic changes were observed in strains in which the gene was silenced.

Chapter 4 reveals a link between PiGPB1 and sporangia formation as a response to starvation. We attempted to find components in the pathway leading to sporulation by analyzing mycelium proteins in wildtype and *Pigpb1*-silenced mutants. Protein extracts were separated by two dimensional SDS polyacrylamide gel electrophoresis (2D-SDS-PAGE) and digitally analyzed. We did not detect consistent differences between wildtype and the *Pigpb1*-silenced mutants using this approach. In another approach we analyzed MAPK activation, as the Gβγ dimer (STE4/STE18) activates the MAPK cascade in *Saccharomyces cerevisiae* (reviewed in Leberer et al., 2001). In our experiments, however, we found no indication of MAPK activation by PiGPB1: there were no differences in MAPK activation in response to cold or wounding between wildtype or the *Pigpb1*-silenced mutants. EST sequencing has now resulted in the identification of *Phytophthora* genes encoding MAPKs, regulatory and catalytic subunits of cAMP (or cGMP) dependent protein kinase (PKA) and adenylyl (or guanylyl) cyclases. The isolation of these genes will facilitate elucidating possible functions of the pathways they are involved in.

G-proteins are known to activate or inactivate some of their downstream targets post-transcriptionally. Other downstream targets are controlled at the transcriptional level (Gutkind, 1998). What would be the best way to isolate those downstream targets and close the gap between PiGPA1 and pathogenicity, or between PiGPA1 and zoospore motility? Random mutagenesis to uncover suppressor mutations would have been the method of choice in fungi but, unfortunately, this is barely possible in a diploid organism. Screening for direct interactors of PiGPA1 using a biochemical approach is laborious and prone to errors. Therefore, we chose to use transcript profiling and compared transcript levels in wildtype and *Pigpa1*-silenced mutants to identify transcriptionally regulated components. Roughly 15-20% of the total sporangial transcriptome was analyzed using cDNA-AFLP and this yielded 77 fragments derived from differentially

expressed genes (chapter 5). The limited size of the fragments hampered identification of the corresponding ESTs and homologous sequences in public databases. The next step, therefore, involves the isolation of the full length genes and the selection of interesting candidates for further study. To find additional genes that are up- or downregulated in the *Pigpa1*-silenced mutants, the expression of 9,000 *P. infestans* genes will be analyzed. This set of genes has been constructed from approximately 80,000 ESTs that were obtained during the last three years. Currently, a DNA chip is manufactured carrying all 9,000 genes. Hybridizations of chips with wildtype and *Pigpa1*-silenced sporangial RNA may lead to identification of additional genes the expression of which is controlled by PiGPA1. The chips will also be used to identify genes that are differentially expressed between wildtype mycelium and mycelium of the *Pigpb1*-silenced mutants.

The role of PLD in *Phytophthora infestans*

Chapter 6 describes the activation of phospholipase D (PLD) by G-protein activators in different developmental stages of *P. infestans*. G-proteins agonists were previously shown to activate PLD in a similar way in plants and algae (Munnik et al., 1995; Lein and Saalbach, 2001) and G-proteins are also implicated in PLD activation in animals (Xie et al., 2002). We tested whether PiGPA1 or PiGPB1 could play a role in activation of PLD. However, mastoparan was as effective in activating PLD in the *Pigpa1*- and *Pigpb1*-silenced mutants as in wildtype, indicating that G-proteins other than PiGPA1 and PiGPB1 are involved. Genes encoding PLDs have been cloned from animals, plants and yeast, and have been characterized in detail. We identified *P. infestans* EST clones with significant homology to PLD genes from animals and plants. These ESTs are expected to be derived from 3 to 5 different genes. Characterization of the *P. infestans* PLD gene family and analysis of the functions of the genes using gene-silencing are in progress (W. Ligterink et al., unpublished results).

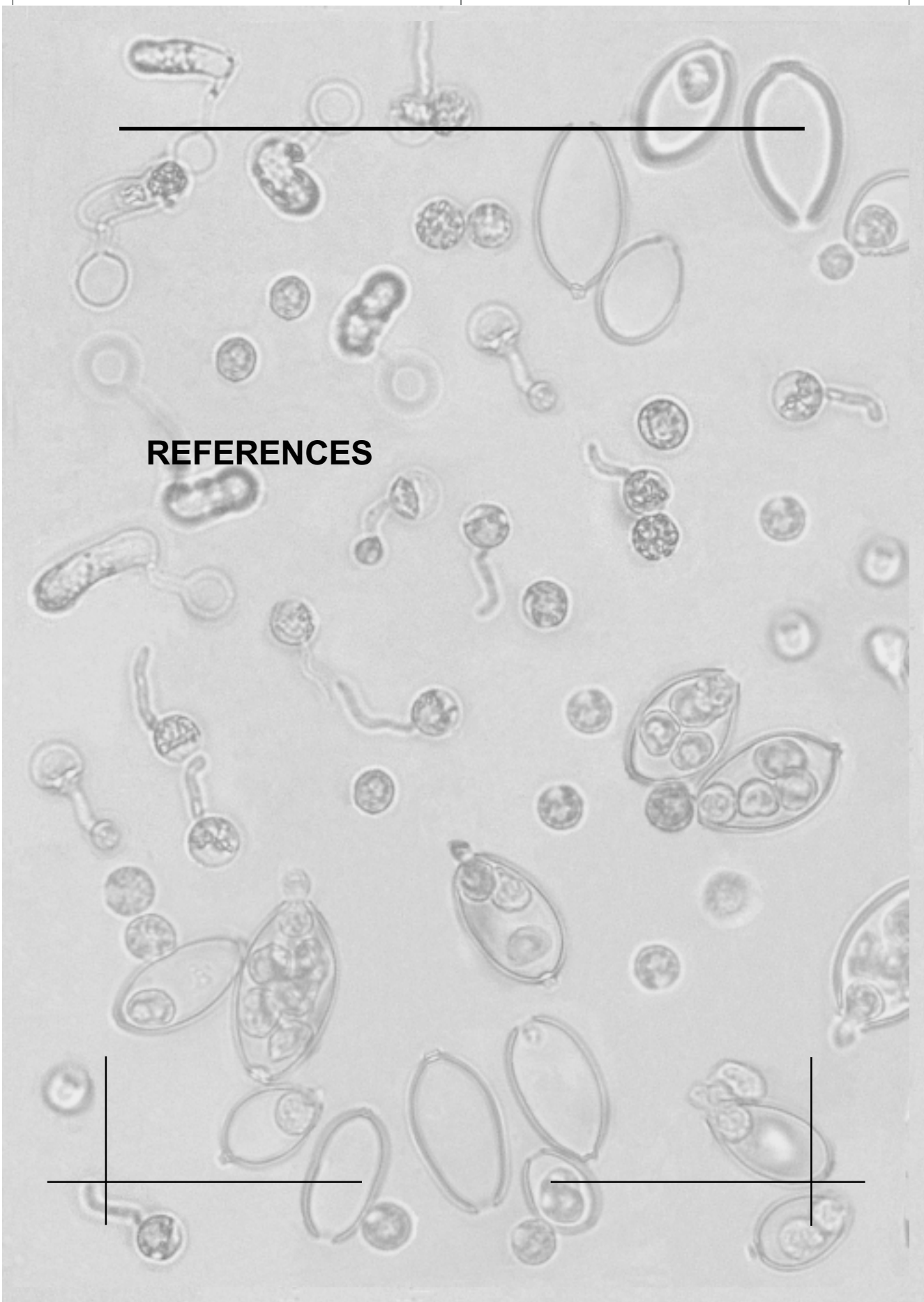
Does unraveling G-protein signaling in *Phytophthora infestans* help to fight late blight?

Late blight infections are threatening potato crops in an ever growing part of the world and disease incidences have increased over the past years. The economic damage to agriculture caused by outbreaks of late blight and the costs of control measures together reach values of several billions of dollars worldwide. Farmers lacking access to fungicides have traditionally used biological and cultural control measures such as soil sanitation and crop rotation to fight the disease. These methods have been proven useful but are inadequate when disease pressure is high. Only the farmers in Western Europe and North America can afford to apply sufficient amounts of fungicides required to control the disease. In this way, they can keep losses limited to about 15% (Garelik, 2002). The dependence of the potato cultivation on fungicides is a matter of concern as two issues will limit the effectiveness and use of present day fungicides in the near future. First of all, the heavy use of fungicides has created resistance in *P. infestans* populations. For example, resistance to the fungicide metalaxyl has been reported in *P. infestans* populations in most areas around the world (Lee et al., 1999). Moreover, people are becoming more and more aware of potential risks posed by fungicides to the environment and to human health. Therefore, governments plan to ban some of the

fungicides that are presently used in large quantities to control late blight in the course of a few years (Proost and Matteson, 1997).

Not the potato producers but industry and consumer decide which potato cultivars are grown in our fields. Clearly, industry and consumer prioritize size, shape and taste over high levels of resistance against late blight. The continuing challenge for potato breeders, therefore, is to combine resistance with these commercial values in one potato cultivar. This has proven utterly difficult. Priority has been given to quantitative resistance in *Solanum tuberosum*, based on multiple genes (Landeo, 2002). In addition, wild Solanum species are being explored for resistance. Resistance present in *Solanum bulbocastanum* was shown to endure the exceptionally high disease pressure in Toluca Valley in Mexico (Niederhausen and Mills, 1953). Using a map-based cloning approach, a *S. bulbocastanum* gene coding for a coiled coil-nucleotide binding site-leucine rich repeat (CC-NBS-LRR) type of resistance protein was recently cloned. When introduced into a susceptible *S. tuberosum* cultivar, this gene (*Rpi-blb*), was shown to confer complete resistance to late blight (van der Vossen et al., 2002). This (transgenic) approach may bring us closer to the resistant potato cultivar with the desired characteristics. However, *P. infestans'* flexibility to overcome barriers has surprised researchers over the years. The pathogen may develop strategies to avoid each resistance gene introduced into *S. tuberosum*. Moreover, many people have concerns about the use of genetically engineered crops on ethical grounds or because of the possible risks for human health and the natural environment.

Every strategy to develop a durable solution for the late blight problem, either fungicides or resistant cultivars, should consider both the role of the pathogen and the host. For this reason, unraveling the infection strategy of *P. infestans* deserves more attention and the work described in this thesis is in line with this idea. It formed the start of a new line of research, studying the role of conserved signal transduction pathways in development and virulence of *P. infestans*. The choice to start off with the G-protein signaling was evident as the ample knowledge of this signaling route in other organisms greatly facilitated our work. The results of over four years of work revealed functions of two components of the G-protein pathway (PiGPA1 and PiGPB1) in development and virulence, and a possible relation to PLD signaling. These results have significantly added to our understanding of development in *P. infestans* and provided a strong basis for further research. G-proteins are conserved in plants and animals and are therefore unsuitable as fungicide targets. However, the fragments that were isolated in the cDNA-AFLP study (chapter 5) based on their differential expression in wildtype and *Pigpa1*-silenced mutants, are likely derived from G-protein-regulated genes. Among those, there may be *Phytophthora*-specific genes involved in the developmental processes regulated by PiGPA1 or in virulence. The possibility that those genes encode useful targets for disease control should certainly be explored. In addition, G-protein coupled receptors are specific and often bind unique ligands. For that reason, potentially unique receptor-ligand combinations linked to PiGPA1 and/or PiGPB1 may also be useful as fungicide target. To investigate this, we need to expand the scope of the study to these downstream components and try to identify those receptors. By doing so, we will at last be able to draw a picture of how *P. infestans* perceives and responds to extracellular signals.



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SUMMARY

Ever since the 1840s, when the disease first appeared in Europe and the United States, the threat of new outbreaks of late blight has terrified potato growers. In years when cool and wet weather conditions are prevailing, the disease is most destructive and nearly impossible to control. Decades of breeding efforts failed to yield resistant cultivars able to satisfy potato producers and consumers. In addition, many of the present-day fungicides are either ineffective or hazardous to human health or the natural environment. *Phytophthora infestans*, the organism causing late blight, is an oomycete. The class oomycetes encompasses eukaryotic pathogens and saprophytes with morphological similarities to fungi. Since fungi and oomycetes are evolutionary unrelated organisms, the ability to infect plants also evolved independently in both classes of organisms.
Heterotrimeric G-proteins are ubiquitously occurring signaling components that function in the translation of extracellular cues into intracellular signals and are indispensable for cells to adapt to alterations in environmental conditions. Animals, plants, fungi and lower eukaryotes such as the slime molds, all possess heterotrimeric G-proteins. In fungal plant pathogens, heterotrimeric G-proteins have been shown to control developmental processes such as mating, sporulation and formation of infection structures, including appressoria. Moreover, the virulence of plant pathogenic fungal species in which genes encoding G-protein subunits were disrupted, was often reduced. To gain more insight in the regulation of development and virulence in *P. infestans*, we aimed at elucidating the role of conserved signaling pathways in this pathogen. We set off by studying the role of heterotrimeric G-proteins, the results of which are described in this thesis.

Heterotrimeric G-proteins consist of three subunits (α, β and γ) of which the α and β subunits are highly conserved. This high level of conservation was utilized to isolate *P. infestans* genes encoding these two subunits (**chapter 2**). Degenerate primers corresponding to conserved regions in Gα were used in a polymerase chain reaction (PCR) resulting in the amplification of a fragment of a *Phytophthora* Gα subunit gene. The gene was named *Pigpa1*. A Gβ gene, *Pigpb1*, was isolated based on sequence information from a *P. infestans* mycelium expressed sequence tag (EST) database with high homology to Gβ genes from other organisms. *Pigpa1* and *Pigpb1* expression was developmentally regulated. Both genes were transcribed at the highest level in sporangia, the asexual spores of *P. infestans*. Both genes displayed lower levels of expression in zoospores and cysts. In mycelium, *Pigpb1* was only lowly expressed while no expression of *Pigpa1* could be detected.

Introduction of extra copies of *Pigpa1* in the *P. infestans* genome triggered gene-silencing in a subset of the transformants, resulting in mutants that no longer transcribed the *Pigpa1* gene and failed to synthesize the PiGPA1 protein (**chapter 3**). Sporangial cytoplasmic cleavage and zoospore release was less efficient in these *Pigpa1*-silenced

mutants compared with the wildtype strain. Wildtype zoospores generally swim in straight lines and form aggregates at the liquid surface. The zoospores of the *Pigpa1*-silenced mutants turned much more frequently than wildtype and showed no preference to move to the liquid surface. Neither did they aggregate and chemotaxis towards amino acids was absent in mutant zoospores. This indicates that PiGPA1 is either involved in the regulation of flagellar movements, perception of chemotactic compounds, or both. The *Pigpa1*-silenced mutants were severely reduced in virulence. In contrast, PiGPA1 gain-of-function mutants expressing a constitutively active form of PiGPA1, showed increased virulence in three out of four of these mutants. These observations point to a role for PiGPA1 in virulence of *P. infestans*.

Pigpb1-silenced mutants were generated using a similar procedure as used for obtaining *Pigpa1*-silenced mutants (**chapter 4**). Two transformation methods were used to introduce copies of the *Pigpb1* gene. The traditional polyethylene glycol (PEG)-mediated protoplast transformation resulted in a higher frequency of gene silencing than transformation by electroporation of zoospores. *Pigpb1*-silenced mutants formed only very few sporangia when cultured on rye sucrose agar. Sporulation in the completely *Pigpb1*-silenced mutants was even below 1% of wildtype sporulation. Instead, the mutants produced a denser mat of aerial mycelium than wildtype on the same growth medium. *Pigpb1* expression in mycelium was induced under starvation conditions. PiGPB1 seems to represent an important link between starvation and sporulation.

Activation of G-proteins triggers a signaling cascade, which eventually results in changes in expression of particular genes. In **chapter 5,** experiments are described that aimed at finding genes, the expression of which is under control of the Gα subunit PiGPA1. Gene expression in sporangia of the wildtype strain, one of the PiGPA1 gain-of-function mutants and three *Pigpa1*-silenced mutants was profiled using cDNA amplified fragment length polymorphism (cDNA-AFLP) analysis. Gene expression in wildtype mycelium was used as an internal control. Seventy-seven transcript-derived fragments (TDFs) were generated that were present in the wildtype profile and absent in the profile of the *Pigpa1*-silenced mutants. *Vice versa*, 11 TDFs were present in the *Pigpa1*-silenced mutants but absent in the wildtype strain. These two groups of TDFs are highly interesting as they are potentially derived from genes the expression of which is normally up- or downregulated by PiGPA1, respectively. The expression patterns of a subset of the genes from which those TDFs are derived were confirmed by reverse transcriptase (RT)-PCR and northern blot analysis. Twenty-seven of the differentially expressed TDFs were cloned and sequenced. A subset of these 27 clones showed homology with sequences present in *P. infestans* EST databases. Further analysis of these genes will reveal their possible functions in signaling and virulence.

Chapter 6 describes the first steps in the characterization of phospholipase D (PLD) signaling in *P. infestans*. PLD hydrolyzes phospholipids that are abundant in membranes, such as phosphatidylcholine or phosphatidylserine. The products are phosphatidic acid (PA) and the respective headgroup. PLD and PA are involved in vesicle trafficking in animals and possibly also in plants. PLD is activated by a variety

of stress conditions in plants and conversion of PA to diacylglycerolpyrophosphate (DGPP) is often observed, presumably as a means to quench the PA signal. Treatment of *P. infestans* sporangia, zoospores, cysts and mycelium with the G-protein-activating peptide mastoparan resulted in increases in the levels of both PA and DGPP. This can be due to activation by mastoparan of the phospholipases PLD, PLC, or both. If available, PLD favors alcohols over water as the 'acceptor' of the phosphatidyl moiety, producing phosphatidylalcohol instead of PA. This feature was exploited to monitor PLD activity in *P. infestans*. Mastoparan treatment in the presence of butanol resulted in an increase in the level of phosphatidylbutanol, evidencing activation of PLD by mastoparan. Similarly, treatment with primary and secondary alcohols also activated PLD. Furthermore, it was demonstrated that mastoparan did not activate PLC in sporangia of *P. infestans*. PLD was activated during zoospore encystment. All PLD activators tested, as well as the product of PLD, PA, induced encystment. Taken together, we consider it likely that PLD plays a role in encystment.

Chapter 7 is a treatise on differences and similarities between strategies of fungal and oomycete pathogens to colonize plants. These groups are taxonomically unrelated. The comparison of the infection strategies of these two groups based on published data led us to conclude that they are more similar than anticipated. There are clear resemblances in infection structures, virulence factors and signal transduction pathways that govern development and virulence. Of course, there are also differences and we mention in this chapter the presence of certain classes of virulence factors that are described in fungi but have not (yet) been detected in oomycetes. We concluded that convergent evolution has had a large impact on the development of infection of plants in the two groups.

Finally, we discuss the results described in this thesis in a broader context (**chapter 8**). These results have increased our understanding of the biology of *P. infestans* and are likely to be applicable to other oomycete species. The value of this work for developing novel methods to control late blight is also touched upon. Moreover, directions for future research are suggested.

SAMENVATTING

In Europa en de Verenigde Staten verscheen de Aardappelziekte, in het Engels "late blight" geheten, voor het eerst in het midden van de negentiende eeuw. Sindsdien is een mogelijke uitbraak van de ziekte elk jaar weer een grote zorg voor aardappeltelers. Bij koel en nat weer is het risico voor een uitbraak het grootst. Het is dan bijna onmogelijk infecties in de hand te houden. Veredelaars proberen al tientallen jaren resistente cultivars te ontwikkelen maar dat heeft tot nu toe nog niet de resistente aardappel opgeleverd die tevens een goede vorm, grootte en smaak heeft van de aardappel die we gewend zijn te eten zoals bijvoorbeeld het "Bintje". De meeste fungiciden die op dit moment gebruikt worden om de Aardappelziekte te bestrijden zijn òf niet effectief genoeg, bijvoorbeeld omdat de ziekteverwekker resistentie heeft opgebouwd tegen het fungicide, òf ze vormen een gevaar voor de gezondheid van de mens of voor het milieu. De ziekteverwekker die de Aardappelziekte veroorzaakt heet *Phytophthora infestans* en hoort taxonomisch bij de oömyceten. De oömyceten vormen een klasse van eukaryoten waaronder pathogenen van dieren, insecten en planten vallen, alsmede een groot aantal saprofyten. Oömyceten lijken uiterlijk op schimmels. Ze zijn echter taxonomisch niet verwant aan schimmels en hebben daarom het vermogen om planten te infecteren onafhankelijk van de echte schimmels verworven. Morfologisch vertonen ze echter veel gelijkenis met schimmels omdat ze ook hyfen en een netwerk aan mycelium kunnen vormen.
Heterotrimere G-eiwitten zijn zeer algemeen voorkomende signaaltransductie componenten die een rol spelen bij het vertalen van extracellulaire signalen naar intracellulaire. Cellen hebben G-eiwitten nodig om adequaat te kunnen reageren op veranderingen in omstandigheden buiten de cel. Dieren, planten, schimmels en andere lagere eukaryoten bezitten allemaal heterotrimere G-eiwitten. Sommige planten-pathogene schimmels hebben G-eiwitten waarvan is aangetoond dat ze betrokken zijn bij belangrijke processen in de normale ontwikkeling. Voorbeelden van zulke processen zijn de sexuele en asexuele voortplanting, en het vormen van infectiestructuren. Bovendien zijn G-eiwitten vaak onmisbaar voor de virulentie van de schimmel. Om beter te begrijpen welke processen ten grondslag liggen aan de ontwikkeling en virulentie van *P. infestans*, proberen we de functie van geconserveerde signaaltransductieroutes te ontrafelen. De signaaltransductieroute die gebruik maakt van heterotrimere G-eiwitten was de eerste die in dit kader onderzocht werd en de resultaten van die studie staan beschreven in dit proefschrift.

Heterotrimere G-eiwitten bestaan uit drie componenten, Gα, Gβ en Gγ. De aminozuurvolgorde van zowel Gα als Gβ is in hoge mate geconserveerd. Deze eigenschap hebben we gebruikt om de genen van *P. infestans* die coderen voor Gα en Gβ te isoleren (**hoofdstuk 2**). Met gedegenereerde oligonucleotiden die corresponderen met geconserveerde delen van Gα werd door middel van de polymerase chain reaction (PCR) een fragment van een Gα-gen geamplificeerd. Het gen kreeg de naam *Pigpa1*. Een tweede gen dat codeert voor Gβ, *Pigpb1*, werd geïsoleerd door gebruik te maken van een

sequentie uit een bank van expressed sequence tags (ESTs), korte sequenties van complementair-DNA (cDNA) die gegenereerd zijn van messenger RNA (mRNA) uit mycelium. Deze sequentie was duidelijk zeer homoloog aan genen uit andere organismen die coderen voor Gβ's. Het expressieniveau van *Pigpa1* en *Pigpb1* bleek afhankelijk te zijn van het ontwikkelingsstadium van *P. infestans*. Beide genen komen hoog tot expressie in sporangia, de asexuele sporen van *P. infestans.* In zoösporen en cysten zijn de expressieniveaus lager en *Pigpb1* komt slechts zeer laag tot expressie in mycelium. Van *Pigpa1* was geen mRNA detecteerbaar in mycelium.

Door extra copieën van *Pigpa1* te introduceren in het genoom van *P. infestans* bleek in enkele transformanten een mechanisme in werking te worden gesteld dat in het Engels "gene silencing" heet. Gene silencing bewerkstelligde dat geen of niet detecteerbare transcriptie van *Pigpa1* meer plaatsvindt en dientengevolge wordt in deze transformanten het PiGPA1-eiwit ook niet meer gemaakt (**hoofdstuk 3**). In de transformanten waarin *Pigpa1* gesilenced was, vond de vorming van zoösporen in de sporangia minder efficiënt plaats dan in het wildtype. De zoosporen van het wildtype zwemmen normaal rechtuit en ze vormen agregaten aan het vloeistofoppervlak. De zoösporen van de mutanten waarin *Pigpa1* gesilenced was veranderden veel frequenter van richting en ze zwommen niet naar het vloeistofoppervlak toe. Bovendien vormden ze geen agregaten en vertoonden ze ook geen chemotaxis in de richting van een gradient van aminozuren, hetgeen wel werd waargenomen bij zoösporen van het wildtype. Dit laat zien dat PiGPA1 betrokken is bij de regulatie van de bewegingen van de flagellen, bij de perceptie van externe signaalstoffen of bij beide. De mutanten vormden bovendien minder appressoria en de virulentie van de mutanten waarin *Pigpa1* gesilenced was, was ernstig aangetast. Daarentegen leken mutanten die een constitutief actieve vorm van PiGPA1 tot expressie brengen virulenter te zijn dan het wildtype. Die toename in virulentie namen we waar in drie van de vier gevallen waarin de virulentie gemeten werd. Deze resultaten laten zien dat PiGPA1 belangrijk is voor de virulentie van *P. infestans*.

In **Hoofdstuk 4** staat beschreven hoe transformanten werden gegenereerd waarin het *Pigpb1* gen niet langer tot expressie kwam, oftewel, waarin *Pigpb1* gesilenced was. Daarvoor werden twee verschillende methoden gebruikt. Bij de ene werden protoplasten geproduceerd en deze werden vervolgens getransformeerd. In de andere methode werden zoösporen getransformeerd met behulp van electroporatie. De eerste methode gaf een veel hogere silencingfrequentie dan de tweede methode. De mutanten waarin *Pigpb1* gesilenced was, waren duidelijk te onderscheiden van het wildtype wanneer ze op rogge-suiker agar groeiden. Ze vormden zeer weinig sporangia (minder dan 1% van het wildtype-niveau in transformanten waarin *Pigpb1* volledig gesilenced was) en ze produceerden meer en langere hyphen. De experimenten die in dit hoofdstuk beschreven staan lieten ook zien dat de expressie van *Pigpb1* in mycelium toenam als onvoldoende voedingsstoffen aanwezig waren. Daarmee lijkt het PiGPB1eiwit een belangrijke schakel te zijn tussen voedselgebrek/schaarste en sporulatie.

Wanneer G-eiwitten worden geactiveerd wordt een signaaltransductiecascade aangeschakeld. Het signaal bereikt de celkern en dit leidt uiteindelijk tot veranderingen

in de expressie van bepaalde genen. Naar verwachting kan PiGPA1 na activatie genen ook weer uitschakelen. M.a.w., de activatie is vaak tijdelijk. Met de experimenten die in **hoofdstuk 5** zijn beschreven wilden we die genen opsporen. Daartoe werd de genexpressie in sporangia van de mutanten waarin *Pigpa1* gesilenced was, en ook in het wildtype, in kaart gebracht door gebruik te maken van een analysemethode die in het Engels 'cDNA amplified fragment length polymorphism (cDNA-AFLP)' genoemd wordt. Met deze techniek worden op grote schaal cDNA fragmenten geamplificeerd. Als interne controle werd ook genexpressie in mycelium van het wildtype in kaart gebracht. In totaal werden 77 geamplificeerde cDNA fragmenten opgespoord die aanwezig zijn in het wildtype maar afwezig waren in de mutanten waarin *Pigpa1* gesilenced was. Anderzijds waren 11 van deze fragmenten wel aanwezig in de mutanten maar afwezig in het wildtype. Het zou kunnen dat deze fragmenten genen vertegenwoordigen die gereguleerd worden door PiGPA1. Een aantal van de fragmenten (27) werd daarom gekloneerd en hun sequentie werd bepaald. Het bleek dat een aantal van de sequenties ook aanwezig was in een of meer van de *Phytophthora* EST databanken en een enkeling tevens in de DNA sequentiedatabank van het National Center for Biotechnology Information (NCBI). De expressiepatronen van de genen die coderen voor deze fragmenten werden geverifieerd met behulp van reverse transcriptase (RT)-PCR en northern blot analyse. De fragmenten zullen in de toekomst worden gebruikt om genen te vinden die processen sturen die niet meer normaal functioneren in de mutanten waarin *Pigpa1* gesilenced is, zoals bijvoorbeeld het zwemgedrag van de zoösporen, het vormen van appressoria en virulentie.

Hoofdstuk 6 beschrijft hoe de activiteit van het enzym phospholipase D (PLD) werd gekarakteriseerd in *P. infestans*. PLD kataliseert de hydrolyse van phospholipiden, zoals phosphatidylcholine of phosphatidylserine. Dit zijn phospholipiden die veel voorkomen in celmembranen. Phosphatidylzuur (in het Engels "phosphatidic acid (PA)" genoemd) en de kopgroep van het betreffende phospholipide zijn de producten van de activiteit van PLD. In dierlijke systemen en mogelijk ook in planten zijn PLD en PA betrokken bij het transport van blaasjes (in het Engels "vesicles" genoemd). Daarnaast wordt PA in planten vaak omgezet tot diacylglycerolpyrophosphaat (DGPP). Waarschijnlijk is dat nodig om het PA signaal op tijd uit te doven. Door sporangia, zoösporen, cysten en mycelium van *P. infestans* met een peptide te behandelen dat G-eiwitten activeert, mastoparan geheten, namen de niveaus van PA and DGPP toe. Zulke toenames in PA en DGPP kunnen veroorzaakt worden door PLD activiteit, maar ook door phospholipase C (PLC) activiteit. Wanneer alcoholen aanwezig zijn gebruikt PLD deze tijdens dechemische reactie in plaats van water. Het product van deze alternatieve reactie is phosphatidylalcohol en de aanwezigheid van dit product kan eenvoudig zichtbaar gemaakt worden. In aanwezigheid van de alcohol butanol leverde behandeling van sporangia met mastoparan inderdaad een bepaalde hoeveelheid phosphatidylbutanol op. Daarmee werd bewezen dat mastoparan PLD activeert. Primaire en secundaire alcoholen kunnen PLD ook activeren. Daarnaast lieten we zien dat PLC niet geactiveerd wordt door mastoparan in sporangia van *P. infestans*. Tijdens de vorming van cysten uit zoösporen werd activatie van PLD waargenomen. Bovendien bleken alle stoffen die PLD activeren ook de vorming van cysten te activeren. Daarom

wordt verondersteld dat PLD betrokken is bij de cystevorming.
Hoofdstuk 7 geeft een verhandeling over verschillen en overeenkomsten tussen schimmels en oömyceten met betrekking tot hun strategieën om planten te infecteren. Aangezien deze twee groepen zich taxonomisch gezien onafhankelijk ontwikkeld hebben zullen hun infectiestrategieën ook los van elkaar ontstaan zijn. Na een vergelijkend literatuuronderzoek, concluderen we dat de infectie-strategieën van beide pathogenen meer overeenkomen dan aanvankelijk werd aangenomen. Er zijn duidelijke overeenkomsten tussen infectiestructuren, virulentiefactoren en signaaltransductieroutes van oömyceten en schimmels. Er zijn uiteraard ook verschillen. Zo zijn in schimmels bepaalde groepen van virulentiefactoren gerapporteerd die dusver nog niet zijn aangetroffen in oömyceten. Convergerende evolutie moet van groot belang zijn geweest bij de ontwikkeling van het vermogen om planten te infecteren in oömyceten en schimmels.

Tot slot bespreken we de resultaten die in dit proefschrift beschreven staan in een bredere context (**hoofdstuk 8**). Deze resultaten hebben bijgedragen aan een beter begrip van de ontwikkeling van de *P. infestans* en ze zijn hoogstwaarschijnlijk ook toepasbaar op de ontwikkeling van andere oomyceten. De betekenis van dit werk voor het ontwikkelen van nieuwe bestrijdingsmethoden voor de Aardappelziekte wordt ook behandeld. Bovendien worden suggesties voor vervolgonderzoek naar voren gebracht.

Boven: voorbereidingen voor een grootschalig experiment om de virulentie van transformanten te testen. >500 inoculaties

NAWOORD

Met veel plezier kijk ik terug op de afgelopen viereneenhalf jaar. Mijn OIOperiode bij Fyto begon in 1998 in het oude gebouw aan de Binnenhaven 9. Dat gebouw, met rommelige, overvolle labs met houten vloeren en tafels, en met ramen die tenminste nog open konden. We begonnen er de dag met een kop koffie uit de zuurkast in het souterrain. Willem Twijssel regeerde er over de keuken en het magazijn en er was nota bene een kantine! Tja,……laat ik mij echter niet verliezen in nostalgische mijmeringen maar dit slotwoord gebruiken om een aantal mensen hulde te brengen aan wie ik het te danken heb dat ik het in de achterliggende periode zo naar m'n zin had en zonder wie dit proefschrift er nooit was gekomen.

Allereerst mag genoemd worden: 88069, onze *P. infestans* labstam. Deze was de hoofdrolspeler in alle experimenten die ik heb uitgevoerd. Echter, dank uitspreken voor een labstam zullen veel mensen als een vorm van vakidiotie beschouwen. Bovendien, van medewerking was ook niet altijd sprake……..

Francine, ik ben je dankbaar voor al je steun. Je hebt me alle kansen geboden zodat ik me kon ontplooien tot een (hopelijk) volwaardig wetenschapper. Door de grote drukte was het niet altijd gemakkelijk voor je om het signaaltransductiewerk, en de lading literatuur die daarbij hoort, bij te houden. Toch is alles in goede harmonie verlopen en ik denk dat je begeleiding daarom nog een extra compliment verdient. Het werk aan *Phytophthora* is geen makkelijke klus. Echter, uit het feit dat je je niet laat ontmoedigen blijkt dat de stelling van W. Fry die ik in het proefschrift citeer op jou zeker van toepassing is!

Pierre, als promotor hield je alles waardig vanaf de zijlijn in de gaten. Vol bewondering merkte ik dat je ondanks je hectische bestaan het hele proefschrift doornam om het te corrigeren waar nodig, en dat je bereid was daar je weekeinden aan op te offeren. Ook ben ik je veel dank verschuldigd voor je bijdrage aan hoofdstuk zeven van dit proefschrift.

Wilco, we waren veroordeeld tot hetzelfde lot: het silencen van signaaltransductiegenen van *P. infestans*. We hebben allebei laten zien dat het kàn. Het was je nooit teveel moeite me te helpen bij mijn onnozele vragen over computerprogramma's en soms minder onnozele vragen over experimenten, die je met je nuchtere aanpak gewoonlijk snel opgelost had. Het is me in de loop van de tijd opgevallen dat je altijd goed gezind bent en dat maakte dat de sfeer in onze gezamenlijke werkkamer altijd erg gezellig was. Ik wens je heel veel succes in je verdere carrière.

Frank, elke dag zat je daar met een brede glimlach in de achterste coupe van de trein die om 7.50u uit Utrecht naar Nijmegen vertrok. Niet alleen werd het forensen verlicht door onze intensieve trein / fiets relatie maar ik kon ook binnen het lab met alle vragen bij je terecht. Gelukkig betekende jouw vertrek naar de UvA geen einde aan onze vriendschap. Ik herinner me veel culinaire uitspattingen waarop ik door jou en Christa onthaald werd en waarbij een goede fles wijn nooit ontbrak. Dit jaar zullen we onze wandelvakantie wellicht moeten overslaan maar hopelijk zullen we de traditie weer hervatten. Ik wens jullie veel geluk met jullie mooie nieuwe stek en met de grote dingen

die te gebeuren staan…..

Camiel, onze skate-fiets combi is onovertroffen. Wat hebben we genoten van mooie zomeravonden op hoge snelheid over de Rijndijk! Dank je voor alle hulp, de gezellige kletspraat in het lab en voor de gastvrijheid. "Chez Camille" bood altijd wel een plekje om de nacht in Wageningen door te brengen. Aanvankelijk in de logeerkamer, maar ja, die heeft nu moeten plaatsmaken voor een volwaardig orchideënkweeklaboratorium……..

Teun, het is alweer een tijd geleden dat je me onder je hoede nam om me in te wijden in de wereld van de lipids en de TLCs. In de discussies praatte je me meestal snel onder tafel maar ik heb veel van je geleerd en ben heel blij met de resultaten die het PLDwerk heeft opgeleverd. Mijn dank gaat ook uit naar Alan voor zijn hulp bij het schrijven van het PLDmanuscript en naar alle leden van de plantenfysiologie groep, omdat ze altijd klaarstonden om me vooruit te helpen.

Vivianne, je was mijn personal advisor op het gebied van virulentietests. Het spijt me dat ik je steeds weer opzadelde met onmogelijke (lees: ongebalanceerde) datasets. Wat kunnen Genstat, ANOVA en REML een mens het leven zuur maken! Gelukkig lukte het je uiteindelijk toch telkens weer om er mooie tabellen uit te laten rollen. John, bedankt voor je belangstelling voor mijn werk en privé-leven (of was het enkel nieuwsgierigheid?). Je aanwezigheid in het lab houdt de boel levendig! Renier, als een grote broer heb je me gesteund tijdens de afronding van het proefschrift. Veel dank daarvoor. We zien elkaar weer in Norwich! Marieke, jij bent de enige student die ik begeleid heb. Voor jou was het je eerste afstudeervak en daarom was het spannend voor allebei. Ik heb je met veel plezier de basis van het moleculaire werk bijgebracht. Bedankt voor het werk dat je hebt verricht. Dan nog de *Phytophthora* groep: Rays, Peter, Andre en Rob, en voormalig groepsleden, Wubei, Irma, Theo, Andrea, Wilko en Guo, veel dank voor jullie hulp en gezelligheid in het lab, voor de literatuurdiscussies, borrels en barbecues, en voor het verdragen van mijn muziekvoorkeur. Alle andere fytonezen wil ik heel hartelijk bedanken. Een leuke vakgroep, waar veel gebeurt en waar tijdens de koffie en in de kroeg veel gekletst wordt en onzin uitgekraamd en waar men bereid is elkaar vooruit te helpen is een onontbeerlijke steun bij promotieonderzoek. Ik had het geluk om in zo'n groep terecht te komen!

Over the years I met a whole bunch of Phytophthorologists from other parts of the world. Those meetings always garanteed a lot of fun and were truly inspiring. Sophien, Steve, Paul, Shuang, Alex, Neil, Howard, Brett, Dinah and all others, thanks for the fruitful discussions and for your support.

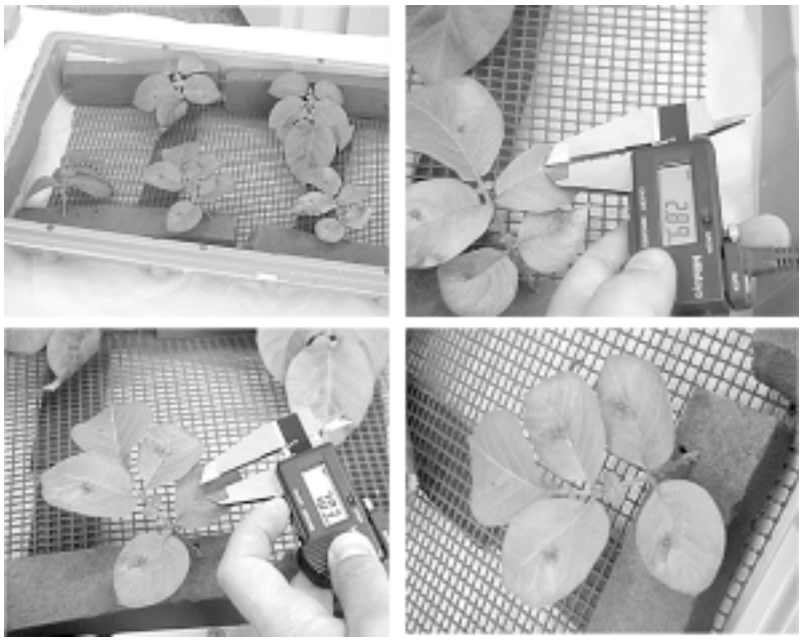
Gelukkig bestonden de afgelopen jaren niet alleen uit labwerk en *Phytophthora*. De achterhoede in Utrecht zorgde ervoor dat muziek, sport en gezelligheid nooit ontbraken. Brita, de afgelopen jaren hebben we samen de meest uiteenlopende dingen meegemaakt. Hoe gelukkig is me prijs met jouw vriendschap laat zich niet in woorden uitdrukken! Ineke, Ramon, Caroline, Janneke, Martien, en alle anderen, Norwich is niet ver, ik hoop jullie spoedig te mogen verwelkomen aan de andere kant van de 'Pond'. Ik zal het de komende tijd zonder VU-kamerorkest, Arezzo, NSG of Philharmonie moeten doen. Lieve mede-strijkers en andere muziekvrienden, met name ook de organisatoren van de evenementen, heel erg bedankt voor de gezelligheid en voor de fantastische muziek.

Maria en Jo, dat ik me binnenkort doctor mag noemen heb ik natuurlijk op de eerste plaats aan jullie te danken. Jullie hebben me altijd onvoorwaardelijk gestimuleerd door te gaan met studeren en er veel voor opzij gezet. Een grote familie is een grote zegen: ouders, zusjes en (schoon)broertjes, hoewel ik jullie het minst zag kregen jullie het altijd het eerst te horen als het werk tegenzat of als ik het gewoon even niet meer zag zitten. Gelukkig stonden jullie dan altijd klaar om me te troosten en me nieuwe moed in te spreken. Niet tevergeefs zoals jullie zien! Stasia, jouw leven is enorm veranderd in de laatste paar jaar. Het gezin vraagt veel van je. Gelukkig bleef je tijd houden voor lange telefoongesprekken, vaak laat op de avond. Mieke, samen vormden we een ideaal team en we brachten het voor elkaar om het boekje in korte tijd in de goede vorm te gieten. Bedankt voor je hulp daarbij.

Pieter mag ik ook zeker niet vergeten. Pieter, ontzettend bedankt voor je gastvrijheid, de levendige discussies en voor je hulp, aanvankelijk bij protoplasttransformatie en vervolgens bij de proteomicsexperimenten. Helaas konden de resultaten van de proteomics niet opgenomen worden in het proefschrift. Je bent iemand met gevoel voor *Phytophthora* en dat laat zich zien. Dank je voor de fijne tijd in Aberdeen.

This visit to Aberdeen changed my life. In fact, I should thank KLM for the three hours' delay of the flight Aberdeen – Amsterdam on the 22nd of March of last year. Rob, on that friday afternoon, a marvellous adventure took off and hopefully, the story will go on for a lóóóóóóóng time! You are of great support and a source of inspiration to me.

Dankjewel.



Boven: Deze foto laat zien hoe lesies op aardappelcultivar *Nicola* werden gemeten ten einde de virulentie van G-eiwit mutanten van *Phytophthora infestans* te bepalen.

CURRICULUM VITAE

Maita Latijnhouwers werd op 17 maart 1973 geboren te Berkel-Enschot in Noord-Brabant. Ze behaalde in 1992 haar diploma *cum laude* aan het Stedelijk Gymnasium in 's Hertogenbosch. Het jaar erna besteedde ze aan het leren van de Spaanse taal in Salamanca, in het noorden van Spanje, waarna ze zich in 1993 op de biologie ging richten. Utrecht bood haar naast de studie biologie ook een interessant studenten-muziekleven. Een eerste onderzoeksstage voerde zij uit aan *Pseudomonas* bacteriën bij de sectie moleculaire microbiologie aan de Universiteit Utrecht onder leiding van Wilbert Bitter. Vervolgens vertrok ze voor een verblijf van negen maanden naar Stanford University in Californië. Daar deed ze in het laboratorium van Virginia Walbot onderzoek naar het proces van splicing in planten. Tijdens het staartje van haar studietijd in Utrecht zette zij zich in voor een grootse operatoernee ter gelegenheid van het 35ste lustrum van het Utrechtsch Studenten Concert. In 1998 sloot zij de studie biologie af met het behalen van een doctoraalbul *cum laude* en slaagde zij voor haar propedeutisch examen filosofie. In september van dat jaar begon zij haar promotie-onderzoek bij het Laboratorium voor Fytopathologie aan Wageningen Universiteit. Dat onderzoek nam ruim vier jaar in beslag en de resultaten ervan staan beschreven in dit proefschrift. Korte tijd na haar promotie zal ze als post-doc aan het werk gaan in de groep van dr. Anne Osbourn in het Sainsbury Laboratory in Norwich (UK). Daar zal zij onderzoek gaan verrichten naar de rol die specifieke secundaire metabolieten van planten, saponines genaamd, spelen in de interactie tussen schimmelpathogenen en planten.

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