Molecular Microbiology (2004) **51**(4), 925–936 doi:10.1046/j.1365-2958.2003.03893.x

A Gα subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*

Maita Latijnhouwers,[1†] Wilco Ligterink,[1‡] Vivianne G.A.A. Vleeshouwers,[2] Pieter van West[3] and Francine Govers[1*]

[1]*Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, NL-6709 PD Wageningen, and Graduate School Experimental Plant Sciences, The Netherlands.*
[2]*Laboratory of Plant Breeding, Wageningen University, Binnenhaven 5, NL-6709 PD Wageningen, The Netherlands.*
[3]*Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, UK.*

Summary

The heterotrimeric G-protein pathway is a ubiquitous eukaryotic signalling 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 in which signal transduction processes have not yet been studied at the molecular 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 six to eight times more frequently, causing them to swim only short distances compared with wild type. 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 20–45% in different *Pigpa1*-silenced mutants. Transformants expressing constitutively active forms of PiGPA1, containing amino acid substitutions (R177H and Q203L), showed no obvious phenotypic differences from the wild-type strain. Infection efficiencies on potato leaves ranged from 3% to 14% in the *Pigpa1*-silenced mutants, compared with 77% in wild type, showing that virulence is severely impaired. The results prove that PiGPA1 is crucial for zoospore motility and for pathogenicity in an important oomycete plant pathogen.

Accepted 15 October, 2003. *For correspondence. E-mail Francine.Govers@wur.nl; Tel. (+31) 317 483 138; Fax (+31) 317 483 412. Present addresses: †Sainsbury Laboratory, Colney Lane, Norwich, NR4 7UH, UK. ‡Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, NL-6703 PB, Wageningen, The Netherlands.

Introduction

Phytophthora infestans is the potato pathogen responsible for the notorious late blight disease that causes yearly losses of billions of dollars and is a threat to potato production all over the world. During the last decade, the aggressiveness of the pathogen population has increased significantly (Fry and Goodwin, 1997), and current control methods have become inadequate. We aim to elucidate signal transduction pathways that govern this pathogen's development and pathogenicity. Insight into 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. These wall-less spores possess two flagella and are thought to be attracted to the host, but the underlying mechanisms remain the subject of discussion (Zentmyer, 1961; Cameron and Carlile, 1978; Deacon and Donaldson, 1993; Gow *et al*., 1999; Morris *et al*., 1998; van West *et al*., 2002). 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. However, van West *et al*. (1999) described how the 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 transformants. This approach, based on homology-dependent gene silencing, was exploited to obtain *P. infestans* transformants deficient in the elicitin INF1. These mutants were used to demonstrate that INF1 acts as a 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 transmembrane (7-TM) 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 G$\beta\gamma$ subunits. Ion channels, adenylyl cyclase and phospholipase C are among the effectors of $G\alpha_{GTP}$, whereas G$\beta\gamma$ often feeds into the MAPK pathway and can also activate PI3kinase and the phospholipases C and A2 (reviewed by Hamm and Gilchrist, 1996). G-protein activation may eventually lead 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 7-TM receptors couple to hundreds of different possible G-protein trimer combinations, transducing a wide range of signals (reviewed by Dhanasekaran *et al*., 1998). In contrast, plants appear to have only a few genes encoding G-protein subunits (*Arabidopsis* Genome Initiative, 2000; Mason and Botella, 2000; 2001). They were shown recently to be involved in hormone and phytochrome signalling and organ development (Lease *et al*., 2001; Okamoto *et al*., 2001; Ullah *et al*., 2001; Wang *et al*., 2001; reviewed by Assmann, 2002). *Dictyostelium discoidium* and *Caenorhabditis elegans* both possess large numbers of Gα subunits (11 and 20 respectively) that were assigned prominent roles in perception and chemotaxis (reviewed by Parent and Devreotes, 1996; Jansen *et al*., 1999). Like plants, fungi possess only a few G-proteins (Lengeler *et al*., 2000). In *Saccharomyces cerevisiae*, they are implicated in mating, glucose sensing and dimorphic growth (Versele *et al*., 2001). In a number of fungal (plant) pathogens, G-proteins were also shown to affect developmental processes such as asexual sporulation, spore germination, pigmentation and appressorium formation and virulence (reviewed by Kronstad, 1997; Bölker, 1998; Borges-Walmsley and Walmsley, 2000; Lengeler *et al*., 2000).

Previously, 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. In a recent study, we found that mutants lacking the Gβ subunit formed aerial mycelium and failed to sporulate, demonstrating that PiGPB1 has a role in vegetative growth and sporulation. (Latijnhouwers and Govers, 2003). For the present study, which is focused on the Gα subunit gene *Pigpa1*, we developed PiGPA1-deficient mutants and transformants expressing constitutively activated forms of PiGPA1. Phenotypic characterization of the mutants revealed a clear role for PiGPA1 in zoospore swimming behaviour and taxis and modest roles in zoospore release and appressorium development. The virulence of the PiGPA1-deficient mutants was severely affected.

Results

Pigpa1-*silenced mutants*

Previous work in our laboratory has demonstrated that gene silencing can be achieved by transforming *P. infestans* with extra copies of the coding sequence of a target gene (van West *et al*., 1999). To obtain *P. infestans* transformants deficient in PiGPA1, strain 88069 was transformed with pCGR and pCGQ, two constructs containing the *Pigpa1* coding region, each with a single point mutation (see next section and *Experimental procedures*), under the control of the native promoter and terminator. pTH209, a plasmid carrying the geneticin resistance gene *nptII*, was used as a selection marker (Judelson *et al*., 1991). Transformants with one or more copies of pCGR or pCGQ integrated into the genome were screened for the absence of *Pigpa1* mRNA. Reverse transcription polymerase chain reaction (RT-PCR) on sporangial RNA of one transformant in which pCGR was introduced (gs1) and of two transformants in which pCGQ was introduced (gs2 and gs3) failed to give products using the normal number of 20 PCR cycles (Fig. 1B). 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 amplification was observed (results not shown). Southern blot analysis (Fig. 1A) revealed that, in these transformants, at least one to three extra copies of *Pigpa1* had integrated into the genome. Two transformants that were derived from the same transformation

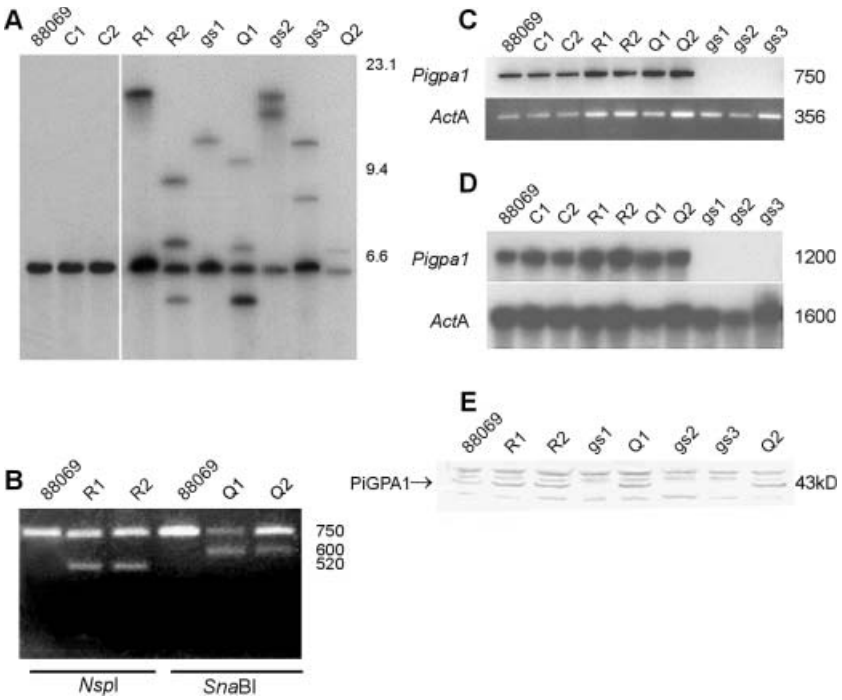

Fig. 1. Analysis of *P. infestans* transformants. Analysis of plasmid integrations of pCGR or pCGQ and of *Pigpa1* expression in the wild-type recipient strain (88069), the control transformants that were only transformed with the selection plasmid pTH209 (C1, C2), the PiGPA1 gain-of-function mutants 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*RI. The coding region of *Pigpa1* was used as a probe. Markers in kb are indicated on the right.
B. RT-PCR analysis and (C) Northern blot analysis of *Pigpa1* expression. The sizes of the RT-PCR products generated using primers corresponding to *Pigpa1* and *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.
D. 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 that was shown to be specific for PiGPA1 in a competition experiment (Laxalt *et al*., 2002). The arrow indicates the position of PiGPA1.
E. 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*BI. The sizes of the restriction fragments (in bp) are indicated on the right.

experiment but had integrations of the selection plasmid pTH209 only were used as control transformants (C1 and C2). The absence of *Pigpa1* mRNA in gs1, gs2 and gs3 was confirmed by Northern analysis (Fig. 1C). On a Western blot, antiserum recognizing PiGPA1 failed to detect a protein of the size of PiGPA1 in the lanes in which protein extract of gs1, gs2 and gs3 was loaded (Fig. 1D). These results show that the *Pigpa1* gene is completely silenced 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 6 months (≈ 12 subcultures) with similar outcome, indicating that the silencing is stable.

Gain-of-function mutations in the Gα subunit PiGPA1

Mutations of a conserved arginine (R201 in human $G\alpha_s$) into a histidine or 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). These amino acid substitutions have 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), *Ustilago maydis* (Regenfelder *et al*., 1997), *Cryphonectria parasitica* (Segers and Nuss, 2003) and *Neurospora crassa* (Yang and Borkovich, 1999). In these species, the mutations were dominant and conferred phenotypes opposite to, or otherwise related to, the phenotype of a knock-out of the same gene. The corresponding mutations were introduced into *Pigpa1* using plasmid pCG, resulting in the plasmids pCGR (R177H) and pCGQ (Q203L).

Transformants with integrations of pCGR or pCGQ in the genome were selected and, in those in which *Pigpa1* was not silenced, the expression of the mutant alleles of *Pigpa1* was analysed by RT-PCR on RNA isolated from sporangia. The R177H and Q203L mutations create unique restriction sites in *Pigpa1* for the restriction

enzymes *Nsp*1 and *Sna*B1, respectively, which allow the distinction of wild-type and mutant *Pigpa1* transcripts after digestion of the RT-PCR products with these enzymes. Figure 1E shows that only undigested product was obtained with the wild-type recipient strain 88069, whereas both undigested and digested RT-PCR products were obtained with the four selected transformants, R1 and R2 (R177H) and Q1 and Q2 (Q203L). 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, or slightly higher than, wild-type *Pigpa1* expression in these transformants (Fig. 1C and D). R1, R2, Q1 and Q2 were tentatively named 'PiGPA1 gain-of-function mutants'.

Pigpa1-*silenced mutants show normal colony morphology, but zoospore release and appressorium formation are less efficient*

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 RSA, and their colonies examined microscopically. No differences were observed between the wild-type 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 that of the wild type (≈ 80% of wild type), but they both grew at nearly the wild-type rate after subculturing several times. As only one out of the three *Pigpa1*-silenced transformants retained the reduced growth phenotype, it seems unlikely that *Pigpa1* silencing by itself affects the growth rate. 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.

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 48 h (% of wild type)[c]
88069	+	100
C1	+	98 ± 3
C2	+	93 ± 10
gs1	–	83 ± 5
gs2	–	82 ± 10
gs3	–	68 ± 8
R1	+[d]	100 ± 5
R2	+[d]	103 ± 8
Q1	+[e]	97 ± 3
Q2	+[e]	94 ± 10

a. All transformants were generated using 88069 as the recipient strain.
b. (+) present; (–) absent, see Fig. 1C and D.
c. The means of six independent measurements are presented.
d. Mixed pool of wild-type *Pigpa1* mRNA and mRNA of the R177H allele of *Pigpa1* (Fig. 1B).
e. Mixed pool of wild-type *Pigpa1* mRNA and mRNA of the Q203L allele of *Pigpa1* (Fig. 1B).

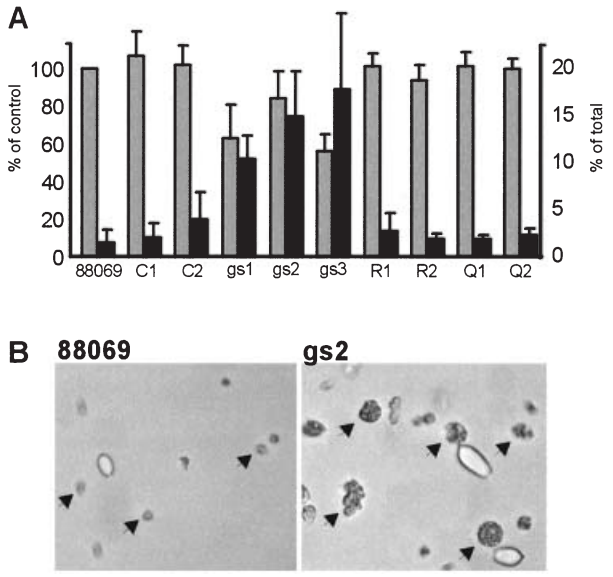

Fig. 2. Zoospore release and cytoplasmic cleavage in PiGPA1 gain-of-function mutants and *Pigpa1*-silenced mutants.
A. Grey bars show zoospore release as a percentage of the wild-type recipient strain 88069 (left *y*-axis). Black bars show the number of 'large', aberrant zoospores, as a percentage of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

Sporangia of wild-type 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 wild type (Fig. 2A). In contrast, zoospore release in the *Pigpa1*-silenced mutants was less efficient. In gs1, gs2 and gs3, the efficiency was reduced by 37%, 16% and 44%, respectively, compared with wild type. Incubation for longer than 2 h did not improve the efficiency of zoospore release in the mutants. Eleven percent (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 (Fig. 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%).

Germ tubes of cysts of *P. infestans* produce appressoria if they are in contact with a hydrophobic surface such as propylene foil or microtitre plate wells. In most cases, these appressoria are formed directly after emergence of the germ tube. 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 differences in the morphology of their appressoria or in the efficiency of appressorium formation were observed. The *Pigpa1*-silenced mutants produced fewer normal appressoria. Instead, the germ tubes became longer, and the tips only showed small swellings (results not shown). This phenotype was most severe in mutant gs3. Mutants gs2 and gs1 formed some normal among many abnormal appressoria, but their numbers were clearly reduced.

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 wild-type 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 minute, and Fig. 3B depicts the swimming patterns of wild-type and mutant zoospores as observed through the microscope. Wild-type 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 with wild-type zoospores. During the observations, the mutant zoospores rarely left the microscopic field, whereas the wild-type zoospores frequently did so.

Another difference between zoospores of the wild type and of the *Pigpa1*-silenced mutants that was detected by microscopic 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 microtitre plate or in a droplet on a microscope slide. The zoospores of the wild-type 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 defective in negative geotaxis. The aberrant, 'large' zoospores of the *Pigpa1*-silenced mutants were mainly detected at the bottom of the well or droplet, possibly because of their low speed or relatively high weight.

Zoospores of Pigpa1*-silenced mutants fail to autoaggregate*

When a suspension with a high concentration ($\geq 5 \times 10^5$ zoospores ml^{-1}) of wild-type *P. infestans* zoospores in water was poured into a Petri dish, aggregates of zoospores were visible as white spots within less than 1 min. Gentle jiggling of the Petri dish made the aggregates disappear, and new aggregates were formed

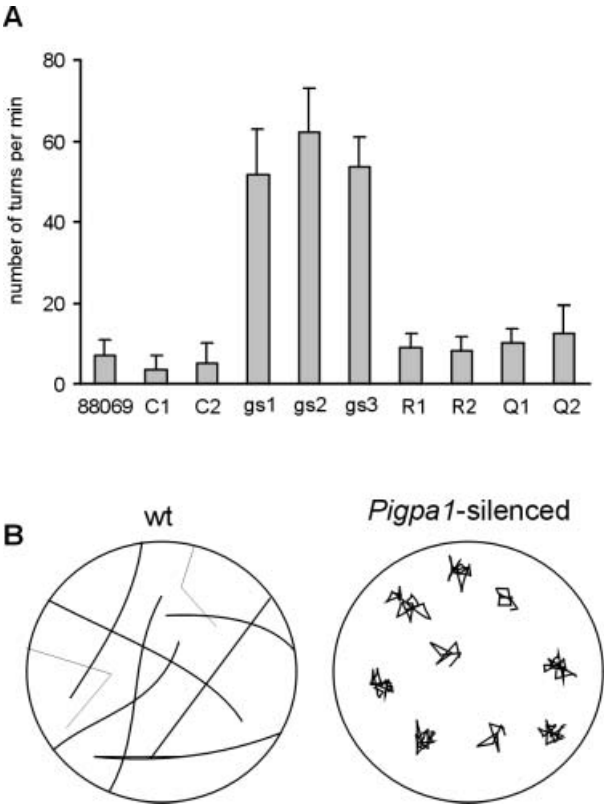

Fig. 3. A. Zoospores of *Pigpa1*-silenced mutants have higher turning frequencies. Individual zoospores were monitored for 1 min and the number of turns counted. The means of multiple zoospores from four independent zoospore isolations are presented. 88069, wild-type recipient strain; C1 and C2, control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

instantly. A real-time movie was made to illustrate the unusually high speed with which these aggregates are formed (*Supplementary material*, Fig. S1). In a volume of 1 ml, a minimal zoospore concentration of 2×10^5 ml^{-1} was required for the formation of visually detectable aggregates to take place. The wild type, 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 (Fig. 4). Even when incubation was prolonged up to 10-fold the time required for wild-type zoospores to aggregate, the zoospores of the *Pigpa1*-silenced mutants still showed a completely even distribution.

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

We tested whether chemotaxis was also affected in zoospores of the *Pigpa1*-silenced mutants. To analyse 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 >25 mM (unpublished observations). Wild-type 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 (Fig. 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 wild-type 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 the 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.

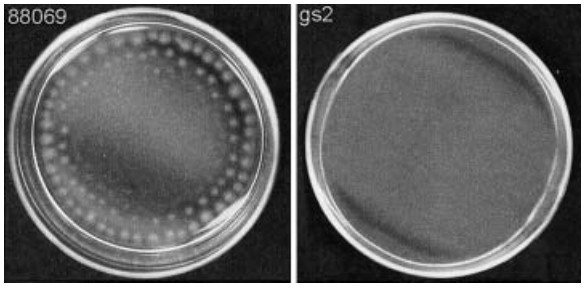

Fig. 4. *Pigpa1*-silenced mutants fail to autoaggregate. Zoospore suspensions of wild-type strain 88069 and of *Pigpa1*-silenced mutant gs2 were poured into a Petri dish (Ø 5 cm) and allowed to autoaggregate. Pictures were taken after 2 min. The control transformants (C1, C2) and the four PiGPA1 gain-of-function mutants (R1, R2, Q1, Q2) all aggregated similarly to 88069. A video showing autoaggregation of zoospores of wild-type strain 88069 is appended as *Supplementary material*.

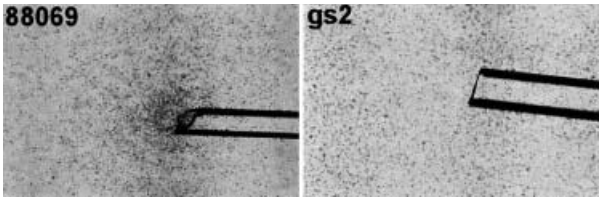

Fig. 5. Zoospores of the *Pigpa1*-silenced mutants do not show chemotaxis to glutamic acid. Microscopic chambers, in which capillaries with 50 mM glutamic acid were inserted, were filled with equal numbers of zoospores of wild-type 88069 or of *Pigpa1*-silenced mutant gs2. Photographs were taken after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

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

Strain	*Pigpa1*	Infection efficiency (%)	Lesion growth rate (mm^2 day^{-1})
88069	Wild type	77a[a]	3.1a
C1	Wild type	52b	3.3a
C2	Wild type	49b	2.8a
gs1	Silenced	11c	1.1c
gs2	Silenced	14c	1.3c
gs3	Silenced	3c	0.8c
R1	R177H	76a	3.2a
R2	R177H	75a	3.8b
Q1	Q203L	68a	3.3a
Q2	Q203L	75a	2.9a

a. Values with the same letter are not significantly different ($P < 0.05$).

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 with regular intervals between 3 and 6 days post inoculation (dpi). The infection efficiencies and the average lesion growth rates of the successful infections, as observed in six independent experiments, are presented in Table 2. The majority of the leaves inoculated with the wild-type strain 88069 showed large, sporulating lesions (Fig. 6) with lesion growth rates typically observed for wild-type strains in the experimental set-up (Vleeshouwers *et al.*, 1999). The

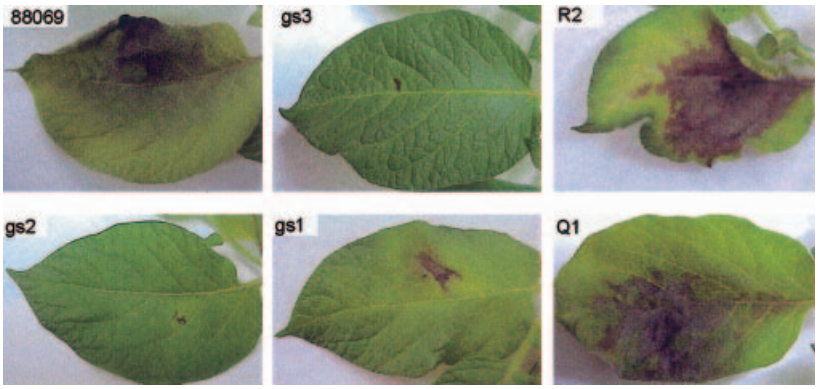

Fig. 6. Virulence of PiGPA1 gain-of-function mutants and *Pigpa1*-silenced mutants. Leaves of potato cultivar Nicola were spot-inoculated with equal numbers of zoospores (see *Experimental procedures*). Photographs were taken 5 days post inoculation. 88069, wild-type recipient strain; gs1, gs2 and gs3, *Pigpa1*-silenced mutants; R2 and Q1, PiGPA1 gain-of-function mutants.

infection efficiencies of the two control strains were slightly lower, but the lesion growth rates were similar to that of the wild-type strains. 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. Moreover, those few lesions showed a significantly lower growth rate than the lesions caused by 88069 and the control strains (35%, 42% and 26% of wild type respectively), and they generally showed limited sporulation. The virulence of the PiGPA1 gain-of-function mutants was unaltered compared with the wild-type strain, except for the lesion growth rate of one of the PiGPA1 gain-of-function mutants (R2), which is significantly higher than the lesion growth rate of the wild-type strain.

To confirm that the silencing was maintained during *in planta* growth, the pathogen was reisolated from 12 of the expanding lesions caused by inoculations with *Pigpa1*-silenced mutants. Phenotypic analysis showed that the growth rate and zoospore behaviour were still similar to those of the primary mutants, indicating that *Pigpa1* was still silenced in all 12 isolates.

To investigate whether the aberrant zoospore swimming behaviour of the *Pigpa1*-silenced mutants had an 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 zoospores was determined. For wild-type 88069, inoculation with cysts resulted in a 10% reduction in infection efficiency compared with 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 varied among experiments and ranged from 10% to 88% (data not shown). It was, however, obvious that this broad variation resulted from the low number of successful infections caused by the mutant strains and that the increase was not large enough to explain the difference in virulence between wild type and the *Pigpa1*-silenced mutants. This indicates that defects other than aberrant zoospore swimming behaviour also negatively affected virulence.

Apart from the six independent inoculation experiments performed on cultivar Nicola, the experiment was repeated on two very susceptible potato clones, i.e. cultivar Bintje and breeding clone G254. The outcome of this test confirmed the reduction in virulence of the *Pigpa1*-silenced mutants (results not shown).

Discussion

In this paper, we show that *P. infestans* possesses a heterotrimeric G-protein-dependent signalling pathway that controls multiple physiological and developmental processes and is indispensable for virulence. Silencing of the Gα subunit gene *Pigpa1* severely affected zoospore motility and virulence and resulted in reductions in zoospore release and appressorium development. Expression of *Pigpa1* is highest in sporangia and zoospores (Laxalt *et al*., 2002), and this is consistent with aspects of zoospore development and physiology being affected by silencing of the gene. Disruption of a single Gα subunit gene also had severe effects in several species of plant-pathogenic fungi. Disruption of the *Magnaporthe grisea* Gα subunit gene *magB*, for example, resulted in reduced vegetative growth, conidiation and appressorium formation and in loss of virulence (Liu and Dean, 1997). It has also been demonstrated that alterations in the accumulation of one G-protein subunit can have an effect on the accumulation of other G-protein subunits (Yang *et al*., 2002; Parsley *et al*., 2003; Segers and Nuss, 2003). In the *Pigpa1*-silenced mutants, the mRNA levels of the *P. infestans* Gβ subunit, PiGPB1, were unaltered (results not shown). However, we cannot rule out the possibility that the mutants have altered levels of PiGPB1 protein and/or Gα protein, partially accounting for the observed phenotypic effects.

Gene silencing is often not absolute (Depicker and Montagu, 1997; van West *et al*., 1999). In this study, 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, indicating that gs1 expresses *Pigpa1* at a very low level. This low level of *Pigpa1* mRNA may also involve mRNA from the introduced, mutant copy of *Pigpa1* and may have affected the phenotype of this mutant. However, gs1 behaved similarly to gs2 and gs3. Therefore, the fact that gs1 is not silenced for 100% is not expected to have influenced the phenotype of gs1.

Effects of PiGPA1 deficiency on development of Phytophthora infestans

In the *Pigpa1*-silenced mutants, zoospore release was reduced to an average of 66% of the wild-type 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 wild-type sporangia under suboptimal conditions (temperature, osmotic value or age; unpublished observations).

Comparison of zoospore behaviour of wild type and *Pigpa1*-silenced mutants clearly showed the involvement of *Pigpa1* in (directional) swimming and taxis. The zoospores of the *Pigpa1*-silenced mutants changed direction very frequently, as though 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 Zentmyer, 1973) and is thought to be important for root pathogens to localize plant roots. Autoaggregation of zoospores, although observed previously in *Phytophthora palmivora* (Ko and Chase, 1973; Reid *et al*., 1995), has not been described before in *P. infestans*. In our observations, the autoaggregation in wild-type *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 by either a defect in sensing an attractant or the inability to master the direction of movements. Interestingly, a mechanism for chemotaxis that is found in many organisms, called klinokinesis, involves an alteration in the frequency of change of direction without biasing the turns with respect to the stimulus (Dusenbery, 1989; Erwin and Ribeiro, 1996). 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.

Previous studies on zoospore behaviour clearly showed that Ca^{2+}-modulating drugs severely modified swimming patterns in *Phytophthora* species (Byrt *et al*., 1982; Irving and Grant, 1984; Reid *et al*., 1995). Mutations in calcium channels or in calmodulin result in aberrant swimming patterns in the case of ciliates, e.g. *Tetrahymena* (Schultz *et al*., 1990; Watanabe *et al*., 1990), showing that Ca^{2+} is an important modulator of swimming direction in ciliates. It is conceivable that PiGPA1 influences Ca^{2+} channels or other Ca^{2+}-binding proteins, thus controlling flagellar movement in *P. infestans*.

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. The very few successful infections expanded much more slowly than lesions caused by the wild-type and control strains. The two control strains had lower infection efficiencies than wild type, suggesting that the transformation procedure can have some effect on virulence. However, the infection efficiencies of the four PiGPA1 gain-of-function mutants, derived from the same transformation experiments, were not affected. Remarkably, one of the PiGPA1 gain-of-function mutants, R2, had a significantly higher lesion growth rate than wild type and, occasionally, the other three gain-of-function mutants also showed higher lesion growth rates. The PiGPA1 gain-of-function mutants were generated to investigate the effect of constitutive activation of the Gα protein. Several examples have been reported in the literature in which these mutations act as dominant mutations, and the observed phenotypes are usually attributed to functioning of the Gα subunit independent of Gβγ (Landis *et al*., 1989; Wong *et al*., 1991; Mendel *et al*., 1995). In the present study, we observed no obvious phenotypic effects of the presence of constitutively active PiGPA1 protein on development or zoospore behaviour. Further experiments are required to confirm the virulence phenotypes of the gain-of-function mutants preferably in gain-of-function strains completely lacking the wild-type allele. Unfortunately, gene replacement in *P. infestans* is not feasible.

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 from 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, with these corrections being 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 that we used, with a high number of zoospores in one droplet, ensures that high concentrations of cysts reach the leaf surface at one spot. This assumption was confirmed in the experiment in which cysts instead of zoospores were used for inoculation because this resulted in only a modest increase in infection efficiency. The *Pigpa1*-silenced mutants produced reduced numbers of appressoria, and many appressoria were deformed. It is likely that this negatively influences virulence as appressoria are required for epidermal cell wall penetration (Erwin and Ribeiro, 1996). Further experiments are required to establish the role of PiGPA1 in appressorium formation in more detail.

Many aspects of *Phytophthora* pathogenicity remain obscure, and studying the role of specific genes in the infection process has always been arduous. This study shows that gene silencing is an appropriate tool to knock down target genes and to obtain hypovirulent strains. Elucidating the role of important signalling networks in *P. infestans*, such as the G-protein signalling pathway, will help to forward the understanding of the biology and pathogenicity of *Phytophthora* and other oomycetes.

Experimental procedures

Phytophthora infestans *strains and culture conditions*

Phytophthora infestans isolate NL-88069 (A1 mating type) and all transgenic isolates were grown routinely at 18°C 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). For the isolation of sporangia, sporulating mycelium was flooded with modified Petri's solution (5 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°C 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 the isolation of DNA was grown in liquid modified Plich medium (van der Lee *et al*., 1997) or liquid rye sucrose medium.

Analysis of zoospore behaviour, chemotaxis and appressorium development

To analyse zoospore release, swimming behaviour and geotaxis, sporangia were incubated in 96-well microtitre plates (Greiner) at 4°C. To observe zoospore aggregation, zoospore suspensions were poured or pipetted into a Petri dish or microtitre plate well (24 wells) and incubated at room temperature. To induce and observe appressorium development, cysts were incubated in microtitre plates or on polypropylene foil (Plastibrand) for 14 h at 10°C. Results of experiments with zoospores and appressoria were analysed 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 (*Supplementary material*).

Chemotaxis was examined according to the method of 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 a 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). PCR was performed with *Pfu* polymerase (Stratagene) or AmpliTaq polymerase (Perkin-Elmer Applied Biosystems) according to the manufacturer's instructions. Restriction enzymes, Klenow polymerase and T4 ligase were from Promega or New England Biolabs. Primers were synthesized by Amersham-Pharmacia. The Plasmid Maxi kit (Qiagen) 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 transformants of *P. infestans* strain 88069 were obtained using zoospore electroporation (B. M. Tyler, personal communication). Briefly, zoospores were harvested in 80 ml of modified Petri's solution from five RSA plates (Ø 15 cm) and divided over two 50 ml tubes (Sarstedt). Two layers of Percoll (Amersham; 33% and 133%) were placed

underneath the zoospore suspension. The zoospores were immobilized by adding 0.1 M LiCl and subsequently concentrated by centrifugation (700 *g* for 5 min at 4°C) such that the zoospores accumulated between the two Percoll layers. Approximately 0.5–1 × 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^{-1} geneticin (Gibco BRL). Colonies appeared after 6–8 days and were propagated on RSA containing 5 μg ml^{-1} geneticin. Approximately 100 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. After electrophoresis, DNA was transferred to Hybond N^+ membranes (Amersham) by alkaline transfer. Total RNA was isolated from sporangia using Trizol (Gibco BRL) according to the manufacturer's instructions. For Northern blot analysis, 10 μg of total RNA was denatured at 50°C in 1 M glyoxal, 50% DMSO and 10 mM sodium phosphate, electrophoresed and transferred to Hybond N^+ membranes in 10× SSC (Ausubel *et al*., 1987; Sambrook and Russel, 2001). Hybridizations of DNA and RNA blots were conducted at 65°C, and filters were washed at 65°C in 0.5× SSC + 0.5% SDS. Gel-purified DNA fragments (GFX kit; Amersham Pharmacia) 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*HI–*Pvu*II fragment from pTH209 (Judelson *et al*., 1991) were used as probes and radiolabelled with [α-^{32}P]-dATP using a random primer labelling kit (Gibco BRL). To remove the unincorporated nucleotides, the Qiaquick nucleotide removal kit (Qiagen) was used.

RT-PCR analysis

To remove contaminating genomic DNA in RNA preparations, 10 μg of total RNA was treated with 4 units of RQ1 RNase-free DNase (Promega) at 37°C for 1 h. The removal of all DNA was verified in a PCR under the same conditions as those used for the RT-PCR, except that the 30 min cDNA synthesis step at 50°C was omitted. RT-PCR was performed using the One Step RT-PCR system (Gibco BRL) with 100 ng of total RNA and 50 ng of each primer, according to 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 × 10^6 sporangia in 250 μl of TEN buffer (50 mM Tris, pH 8, 5 mM EDTA and 150 mM NaCl), mixed with 0.5 ml of 1 mm glass beads (Biospec Products) for 30 s. The suspension was centrifuged for 5 min at 15 000 *g*, the pellet dissolved in extraction buffer composed of 20 mM Tris, pH 7.5, 10 mM dithiothreitol, 5% glycerol, 1% SDS, 10 μg ml^{-1} leupeptin and 1 mM phenylmethylsulphonyl fluoride (modified from Choi *et al*., 1995) and the suspension centrifuged again for 5 min at 15 000 *g*. 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°C under 80% humidity with 16 h light per 24 h. The length and width of the lesions were measured at three, four and five, or four, five and six dpi using a marking gauge linked to the IBREXDLL software (IBR Prozessautomation). 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).

Accession numbers

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

Acknowledgements

The assistance of Fred van Eeuwijk in the statistical analysis of the virulence data and of Dirk-Jan Huigen in keeping the plants in excellent condition has been of great value. We thank Grardy van den Berg-Velthuis for technical assistance, Brett M. Tyler for establishing the zoospore transformation procedure, Tijs Ketelaar, Alex Appiah and the Aberdeen Medical Illustration Service for their help in recording zoospore swimming, Neil Gow for his hospitality, and Pierre de Wit for critical reading of the manuscript. We acknowledge the NWO-Council Earth and Life Sciences (M.L.), the Syngenta *Phytophthora* Research Consortium co-ordinated by Syngenta (Research Triangle Park, NC, USA) (W.L.) and The Royal Society (P.v.W.) for financial support.

Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3893/mmi3893sm.htm

Fig. S1. This video shows the autoaggregation of wild-type zoospores of strain 88069 in a real-time fashion.

References

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408:** 796–815.

Assmann, S.M. (2002) Heterotrimeric and unconventional

GTP binding proteins in plant cell signaling. *Plant Cell Suppl* **2002:** S355–S373.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J., Smith, J.A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*. New York: John Wiley & Sons.

Bölker, M. (1998) Sex and crime: heterotrimeric G proteins in fungal mating and pathogenesis. *Fungal Genet Biol* **25:** 143–156.

Borges-Walmsley, M.I., and Walmsley, A.R. (2000) cAMP signalling in pathogenic fungi: control of dimorphic switching and pathogenicity. *Trends Microbiol* **8:** 133–141.

Byrt, P.N., Irving, H.R., and Grant, B.R. (1982) The effect of cations on zoospores of the fungus *Phytophthora cinnamoni*. *J Gen Microbiol* **128:** 1189–1198.

Cameron, J.N., and Carlile, M.J. (1977) Negative geotaxis of zoospores of the fungus *Phytophthora*. *J Gen Microbiol* **98:** 599–602.

Cameron, J.N., and Carlile, M.J. (1978) Fatty acids, aldehydes and alcohols as attractants for zoospores of *Phytophthora palmivora*. *Nature* **271:** 448–449.

Caten, C.E., and Jinks, J.L. (1968) Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Can J Bot* **46:** 329–348.

Choi, G.H., Chen, B., and Nuss, D.L. (1995) Virus-mediated or transgenic suppression of a G-protein alpha subunit and attenuation of fungal virulence. *Proc Natl Acad Sci USA* **92:** 305–309.

Deacon, J.W., and Donaldson, S.P. (1993) Molecular recognition in the homing response of zoosporic fungi, with special reference to *Pythium* and *Phytophthora*. *Mycol Res* **97:** 1153–1171.

Depicker, A., and Montagu, M. (1997) Post-transcriptional gene silencing in plants. *Curr Opin Cell Biol* **9:** 373–382.

DeSimone, S.M., and Kurjan, J. (1998) Switch-domain mutations in the *Saccharomyces cerevisiae* G protein α-subunit Gpa1p identify a receptor subtype-biased mating defect. *Mol Gen Genet* **257:** 662–671.

Dhanasekaran, N., Tsim, S.T., Dermott, J.M., and Onesime, D. (1998) Regulation of cell proliferation by G proteins. *Oncogene* **17:** 1383–1394.

Dusenbery, D. (1989) Efficiency and the role of adaptation in klinokinesis. *J Theor Biol* **136:** 281–293.

Erwin, D.C., and Ribeiro, O.K. (1996) *Phytophthora Diseases Worldwide.* St Paul, MN: The American Phytopathological Society, pp. 54–58.

Freissmuth, M., and Gilman, A.G. (1989) Mutations of GS α designed to alter the reactivity of the protein with bacterial toxins. Substitutions at ARG187 result in loss of GTPase activity. *J Biol Chem* **264:** 21907–21914.

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

Gow, N.A.R., Campbell, T.A., Morris, B.M., Osborne, M.C., Reid, B., Shepherd, S.J., and Van West, P. (1999) Signals and interactions between phytopathogenic zoospores and plant roots. In *Microbial Signaling and Communication.* England, R., Hobbs, G., Bainton, N.McL., and Roberts, D. (eds). Society for Microbiology Symposium 57. Edinburgh: Cambridge University Press, pp. 285–305.

Hamm, H.E., and Gilchrist, A. (1996) Heterotrimeric G proteins. *Curr Opin Cell Biol* **8:** 189–196.

Irving, H.R., and Grant, B.R. (1984) The effect of calcium on zoospore differentiation in *Phytophthora cinnamomi*. *J Gen Microbiol* **130:** 1569–1576.

Jansen, G., Thijssen, K., van Werner, P., derHorst, M., Hazendonk, E., and Plasterk, R. (1999) The complete family of genes encoding G proteins of *Caenorhabditis elegans*. *Nature Genet* **21:** 414–419.

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

Kamoun, S., van West, P., Vleeshouwers, V., deGroot, K.E., and Govers, F. (1998) Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. *Plant Cell* **10:** 1413–1425.

Khew, K.L., and Zentmyer, G.A. (1973) Chemotactic response of zoospores of five species of *Phytophthora*. *Phytopathology* **63:** 1511–1517.

Ko, W., and Chase, L. (1973) Aggregation of zoospores of *Phytophthora palmivora*. *J Gen Microbiol* **78:** 79–82.

Kronstad, J.W. (1997) Virulence and cAMP in smuts, blasts and blights. *Trends Plant Sci* **2:** 193–199.

Landis, C.A., Masters, S.B., Spada, A., Pace, A.M., Bourne, H.R., and Vallar, L. (1989) GTPase inhibiting mutations activate the α chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature* **340:** 692–696.

Latijnhouwers, M., and Govers, F. (2003) A *Phytophthora infestans* Gβ subunit is involved in sporangium formation. *Eukaryotic Cell* **2:** 971–977.

Laxalt, A.M., Latijnhouwers, M., van Hulten, M., and Govers, F. (2002) Differential expression of G protein α and β subunit genes during development of *Phytophthora infestans*. *Fungal Genet Biol* **36:** 137–146.

Lease, K.A., Wen, J., Li, J., Doke, J.T., Liscum, E., and Walker, J.C. (2001) A mutant *Arabidopsis* heterotrimeric G-protein β subunit affects leaf, flower, and fruit development. *Plant Cell* **13:** 2631–2641.

van der Lee, T., de Witte, I., Drenth, A., Alfonso, C., and Govers, F. (1997) AFLP linkage map of the oomycete *Phytophthora infestans*. *Fungal Genet Biol* **21:** 278–291.

Lengeler, K.B., Davidson, R.C., D'Souza, C., Harashima, T., Shen, W.C., Wang, P., *et al.* (2000) Signal transduction cascades regulating fungal development and virulence. *Microbiol Mol Biol Rev* **64:** 746–785.

Liu, S.H., and Dean, R.A. (1997) G protein α subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol Plant–Microbe Interact* **10:** 1075–1086.

Margulis, L., and Schwartz, K.V. (2000) *Five Kingdoms: an Illustrated Guide to the Phyla of Life on Earth*, 3rd edn. New York: W.H. Freeman & Co.

Mason, M.G., and Botella, J.R. (2000) Completing the heterotrimer: isolation and characterization of an *Arabidopsis thaliana* G protein α-subunit cDNA. *Proc Natl Acad Sci USA* **97:** 14784–14788.

Mason, M.G., and Botella, J.R. (2001) Isolation of a novel G-protein α-subunit from *Arabidopsis thaliana* and its interaction with Gβ. *Biochim Biophys Acta* **1520:** 147–153.

Mendel, J.E., Korswagen, H.C., Liu, K.S., Hajdu-Cronin,

Y.M., Simon, M.I., Plasterk, R.H., and Sternberg, P.W. (1995) Participation of the protein Go in multiple aspects of behavior in *C. elegans*. *Science* **267:** 1652–1655.

Morris, P.F., Bone, E., and Tyler, B.M. (1998) Chemotropic and contact responses of *Phytophthora sojae* hyphae to soybean isoflavonoids and artificial substrates. *Plant Physiol* **117:** 1171–1178.

Neer, E.J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* **80:** 249–257.

Okamoto, H., Matsui, M., and Deng, X.W. (2001) Overexpression of the heterotrimeric G-protein α-subunit enhances phytochrome-mediated inhibition of hypocotyl elongation in *Arabidopsis*. *Plant Cell* **13:** 1639–1652.

Parent, C.A., and Devreotes, P.N. (1996) Molecular genetics of signal transduction in *Dictyostelium*. *Annu Rev Biochem* **65:** 411–440.

Parsley, T.D., Segers, G.C., Nuss, D.L., and Dawe, A.L. (2003) Analysis of altered G-protein subunit accumulation in *Cryphonectria parasitica* reveals a third Gα homologue. *Curr Genet* **43:** 24–33.

Petri, L. (1917) Ricerche sulla morfologia e biologia della *Blepharospora cambivora*, parasitica del Castagno. *Atti R Accad Lincei Rend Cl Sci Fis Mat Nat Series* **5:** 297–299.

Raeder, U., and Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Lett Appl Microbiol* **1:** 17.

Regenfelder, E., Spellig, T., Hartmann, A., Lauenstein, S., Bolker, M., and Kahmann, R. (1997) G proteins in *Ustilago maydis*: transmission of multiple signals? *EMBO J* **16:** 1934–1942.

Reid, B., Morris, B.M., and Gow, N.A.R. (1995) Calcium-dependent, genus-specific, autoaggregation of zoospores of phytopathogenic fungi. *Exp Mycol* **19:** 202–213.

Sambrook, J., and Russel, D.W. (2001) *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schultz, J., Klumpp, S., and Hinrichsen, R. (1990) Calcium and membrane excitation in *Paramecium*. In *Calcium as an Intracellular Messenger in Eucaryotic Microbes*, O'Day, D.H. (ed.). Washington, DC: American Society for Microbiology Press, pp. 124–140.

Segers, G.C., and Nuss, D.L. (2003) Constitutively activated Gα negatively regulates virulence, reproduction and hydrophobin gene expression in the chestnut blight fungus *Cryphonectria parasitica*. *Fungal Genet Biol* **38:** 198–208.

Ullah, H., Chen, J.G., Young, J.C., Im, K.H., Sussman, M.R., and Jones, A.M. (2001) Modulation of cell proliferation by heterotrimeric G protein in *Arabidopsis*. *Science* **292:** 2066–2069.

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

Versele, M., Lemaire, K., and Thevelein, J.M. (2001) Sex and sugar in yeast: two distinct GPCR systems. *EMBO Rep* **2:** 574–579.

Vleeshouwers, V., van Dooijeweert, W., Keizer, L., Sijpkes, L., Govers, F., and Colon, L. (1999) A laboratory assay for *Phytophthora infestans* resistance in various *Solanum* species reflects the field situation. *Eur J Plant Pathol* **105:** 241–250.

Wang, X.Q., Ullah, H., Jones, A.M., and Assmann, S.M. (2001) G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. *Science* **292:** 2070–2072.

Watanabe, Y., Hirano-Ohnishi, J., and Takesama, T. (1990) Calcium-binding proteins and ciliary movement regulation in *Tetrahymena*. In *Calcium as an Intracellular Messenger in Eucaryotic Microbes*. O'Day, D.H. (ed.). Washington, DC: American Society for Microbiology Press, pp. 343–361.

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

van West, P., Morris, B.M., Reid, B., Appiah, A.A., Osborne, M.C., Campbell, T.A., *et al.* (2002) Oomycete plant pathogens use electric fields to target roots. *Mol Plant–Microbe Interact* **15:** 790–798.

Wong, Y.H., Federman, A., Pace, A.M., Zachary, I., Evans, T., Pouyssegur, J., and Bourne, H.R. (1991) Mutant α subunits of Gi2 inhibit cyclic AMP accumulation. *Nature* **351:** 63–65.

Yang, Q., and Borkovich, K.A. (1999) Mutational activation of a Gαi causes uncontrolled proliferation of aerial hyphae and increased sensitivity to heat and oxidative stress in *Neurospora crassa*. *Genetics* **151:** 107–117.

Yang, Q., Poole, S.I., and Borkovich, K.A. (2002) A G-Protein β subunit required for sexual and vegetative development and maintenance of normal G protein levels in *Neurospora crassa*. *Eukaryotic Cell* **1:** 378–390.

Zentmyer, G.A. (1961) Chemotaxis of zoospores for root exudates. *Science* **133:** 1595–1596.