

A *Phytophthora infestans* G-Protein β Subunit Is Involved in Sporangium Formation

Maita Latijnhouwers† and Francine Govers*

Laboratory of Phytopathology and Graduate School of Experimental Plant Sciences,
Wageningen University, NL-6709 PD Wageningen, The Netherlands

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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 previous studies on the role of the G-protein α -subunit PiGPA1 in this organism. The expression of the *P. infestans* G-protein β -subunit gene *Pigpb1* was induced in nutrient-starved mycelium before the onset of sporangium formation. The gene was hardly expressed in mycelium incubated in rich growth medium. The 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 in rye sucrose medium and produced a denser mat of aerial mycelium than the wild type. Partially *Pigpb1*-silenced mutants showed intermediate phenotypes with regard to sporulation, and a relatively large number of their sporangia were malformed. The results show that PiGPB1 is important for vegetative growth and sporulation and, therefore, for the pathogenicity of this organism.

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 (22). The class comprises saprophytes and pathogens of plants, animals, and insects. The over 60 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 (5, 8, 15). In the vegetative stage, *Phytophthora* grows as coenocytic mycelium. Asexual spores (sporangia) are formed on branched sporangiophores that emerge from the mycelium. Some species sporulate spontaneously when growing on solid media. Moreover, sporangium production by many *Phytophthora* species can be induced by washing and transferring portions of the culture to water or salt solutions or to vegetable decoction medium with a low carbohydrate content (8).

Heterotrimeric G proteins are widespread among the animal, plant, and fungal kingdoms (1, 3, 25) and are also present in lower eukaryotes, such as *Dictyostelium discoideum* (26) and the ciliate *Stentor coeruleus* (23). 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 GTP by GDP on the α subunit of the protein causes activation of the trimer, leading to dissociation of the α subunit from the $\beta\gamma$ dimer. In the activated state, both the G-protein α subunit ($G\alpha$ subunit) and the G-protein $\beta\gamma$ dimer ($G\beta\gamma$ 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 $G\alpha$ and $G\beta\gamma$ subunits (25).

The heterotrimeric G-protein pathway has been identified as an important regulator of development and physiology in plant-pathogenic fungi (3, 17, 20). In particular, the function of $G\alpha$ subunits in fungal species has been studied extensively. For example, $G\alpha$ 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 $G\alpha$ subunits was also required for virulence (reviewed in references 3, 4, 17, and 20). The function of the $G\beta$ -subunit gene has been described for only one plant pathogen so far. In *C. parasitica*, disruption of the $G\beta$ -subunit gene *cpgb1* resulted in decreased pigmentation, conidiation, and virulence and in increased mycelial growth (14). Disruption of $G\beta$ -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 was induced in submerged cultures (28, 35).

The *P. infestans* $G\alpha$ -subunit (*Pigpa1*) and $G\beta$ -subunit (*Pigpb1*) genes are differentially expressed in the various stages of the life cycle of *P. infestans* (18). PiGPA1-deficient mutants displayed a pleiotropic phenotype. In particular, zoospore motility and taxis were altered and virulence was severely reduced (M. Latijnhouwers et al., submitted for publication). Because of the lack of a method for gene disruption, gene silencing based on cosuppression is the only way to inactivate genes in a targeted fashion in *Phytophthora*. So far, three such examples of targeted mutagenesis by gene silencing have been reported, two in *P. infestans* (33; Latijnhouwers et al., submitted) and one in *Phytophthora nicotianae* (9). The introduction of additional copies of the target genes (sense or antisense) into the genomes of the respective *Phytophthora* species induced gene

* Corresponding author. Mailing address: Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, NL-6709 PD Wageningen, The Netherlands. Phone: 31 317 483138. Fax: 31 317 483412. E-mail: Francine.Govers@wur.nl.

† Present address: Sainsbury Laboratory, Norwich NR4 7UH, England.

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.

MATERIALS AND METHODS

***P. infestans* strains and culture conditions.** *P. infestans* strains NL-88069 (mating type A1) and METV-618 (mating type A2) (hereafter referred to as 88069 and 618, respectively) and all transgenic isolates were routinely grown at 18°C in the dark on rye agar medium supplemented with 2% sucrose (RSA) (6). RSA was supplemented with 2.5 µg of G418 (Gibco-BRL, Bethesda, Md.)/liter for the transformants. For the isolation of sporangiospores, sporulating mycelium was flooded with modified Petri's solution (0.25 mM CaCl₂, 1 mM MgSO₄, 1 mM KH₂PO₄, 0.8 mM KCl) and rubbed with a sterile glass rod. Subsequently, the suspension was filtered through 50-µm nylon mesh. The number of sporangia in at least five different isolations from different plates of each transformant was determined by using a hemocytometer. To starve mycelium for nutrients, cultures were grown in liquid rye medium with sucrose (RS) in 9-cm petri dishes for 2 weeks. Mycelium was washed twice in fresh RS or minimal medium [10 mM Ca(NO₃)₂, 5 mM KNO₃, 4 mM MgCl₂, 20 µM FeSO₄, 20 µM Na₂EDTA, 10 mg of β-sitosterol/liter; modified from the medium of Marshall et al. (24)] and transferred to new petri dishes containing the respective media. Mycelium was harvested for RNA extraction after 6 h.

Plasmids used for the transformation of *P. infestans*. Plasmid pMH2 consists of a 2.3-kb *HindIII*-*BamHI* restriction fragment of *P. infestans* genomic DNA cloned into pBluescript SKII (GenBank accession no. AY204515). This fragment contains the full-length *Pigpb1* gene, a 911-bp promoter region, and 387 bp of the terminator (18). Plasmid pTH209 (13), consisting of the *hsp70* promoter of *Bremia lactuceae* fused to the *npII* coding sequence and the *ham34* terminator of *B. lactuceae*, was used as a selectable plasmid. A plasmid maxi kit (Qiagen GmbH, Hilden, Germany) was used for the isolation of plasmid DNA for the transformation of *P. infestans*. For transformation, 5 µg of pTH209 was used in combination with 30 µg (polyethylene glycol [PEG] transformation) or 15 µg (zoospore electroporation) of pMH2. Plasmid DNA used for transformation was digested with *EcoRI* (pTH209) and with *BamHI* or *HindIII* (pMH2).

Transformation of *P. infestans*. *P. infestans* strains 88069 and 618 were stably transformed by using the PEG protoplast method as described by van West et al. (32) with a slight modification. We found that the presence of Lipofectin (Gibco-BRL) negatively influenced the efficiency of transformation; Lipofectin therefore was omitted from the incubation mixture. Strain 88069 was also transformed by using zoospore electroporation (B. M. Tyler et al., unpublished data; M. Latijnhouwers et al., unpublished data).

Southern and Northern blot analyses. Mycelium for the isolation of genomic DNA was grown in modified liquid Plich medium (31). Genomic DNA was isolated as described by Raeder and Broda (27) with minor modifications. Following electrophoresis, DNA was transferred to Hybond N⁺ membranes (Amersham) by alkaline transfer. Total RNA was isolated by 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% dimethyl sulfoxide–10 mM sodium phosphate, electrophoresed, and transferred to Hybond N⁺ membranes in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (2, 29). Hybridizations of DNA and RNA blots were conducted at 65°C, and membranes were washed at 65°C in 0.5× SSC–0.5% sodium dodecyl sulfate. Purified PCR products (Qiaquick PCR purification kit; Qiagen) consisting of the complete coding region of *Pigpb1* (forward primer, 5'-TCCCCGGGCAATGGCGACGCTGCGGAG-3'; reverse primer, 5'-TCCCCGGGTAGGCCA GATCTTGAGTG-3') or a 796-bp *HindIII* restriction fragment of the *actA* gene from pSTA31 (30) were used as probes and radiolabeled with [³²P]dATP by using a random primer labeling kit (Gibco-BRL). To remove the unincorporated nucleotides, a Qiaquick nucleotide removal kit was used.

RT-PCR analysis. To remove contaminating genomic DNA in RNA preparations, 10 µg of total RNA was treated with 4 U of RQ1 RNase-free DNase (Promega, Madison, Wis.) at 37°C for 1 h. The removal of DNA was verified in a PCR under the same conditions as those used for reverse transcriptase (RT)-PCR, except that the 30-min cDNA synthesis step at 50°C was omitted. RT-PCR was performed by using a 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. Incubation at 50°C for 30 min and at 94°C for 2 min and then 27 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and 72°C for 5 min were used for the detection of *Pigpb1* mRNA. To analyze the level of *actA* mRNA, the same

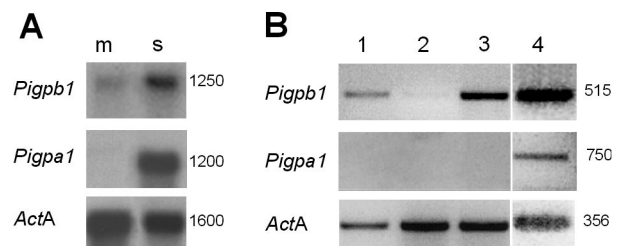


FIG. 1. *Pigpb1* is strongly upregulated in nutrient-starved mycelium. (A) Expression of *Pigpb1* and *Pigpa1* analyzed by Northern blot analysis. A Northern blot containing RNAs isolated from mycelium (m) grown in Plich medium for 2 weeks and from sporangia (s) was hybridized with probes derived from *Pigpb1*, *Pigpa1*, and *actA*. The sizes of the transcripts (in bases) are indicated on the right. (B) RT-PCR analysis of *Pigpb1* and *Pigpa1* expression in 2-week-old mycelium grown in RS just before transfer to fresh RS (lane 1) and 6 h after transfer to fresh RS (lane 2) or minimal medium (lane 3) and in sporangia (lane 4). RT-PCR products were visualized on ethidium bromide-stained gels; their sizes (in base pairs) are indicated on the right.

program was applied but with 24 cycles instead of 27. The forward primer 5'-TCCCCGGGCAATGGGCGACGCTGCGGAG-3' and the reverse primer 5'-GATGCAGTTGGAGTCCCCCG-3', both annealing to the coding region, were used for the amplification of *Pigpb1* from mRNA; the forward primer 5'-CGGTCGCGGTATGTGCAAGGC-3' and the reverse primer 5'-GCGGGC ACGTTGAACGTCTC-3' were used for *actA*; and the forward primer 5'-CCC TCGGATGGGACTCTGTGCT-3' and the reverse primer 5'-GTGATGGCC GCGATCATAAC-3' were used for *Pigpa1*. The level of *cdc14* mRNA was determined by RT-PCR with the forward primer 5'-TCCGAGGTTGAGTGG GCC-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, unpublished data).

Virulence assays. Detached potato leaves of cultivar Nicola were placed in florist foam and inoculated on the abaxial side with 1 drop (10 µl) containing 10³ zoospores per leaflet. The leaves were incubated in a climate-controlled room at 18°C and 80% humidity with 16 h of light in a 24-h period. The length and width of the lesions were measured at 3, 4, and 5 days postinoculation by using a marking gauge linked to IBREXDLL software (IBR Prozessautomation, Hanel, Germany). To test the growth of *P. infestans* strains on potato slices, agar plugs of identical sizes were placed on potato slices in a petri dish. The slices were incubated at 18°C in the dark for up to 10 days.

RESULTS

Pigpb1 expression is downregulated upon nutrient supply.

The patterns of expression of the *P. infestans* G α - and G β -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 by Northern blot analysis, whereas *Pigpa1* was not detectable (18) (Fig. 1A). The mycelium that was used for the experiment was cultured for 2 weeks in a synthetic liquid medium (modified Plich medium) (31) 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]) for 2 weeks. At this stage, the mycelium was starved, but few or no sporangia were observed in the cultures. The mycelium was subsequently rinsed and transferred to either fresh RS or minimal medium (see Materials and Methods). The mycelium was harvested after 6 h, and the expression of *Pigpa1* and *Pigpb1* was analyzed by RT-PCR. As shown in Fig. 1B, *Pigpb1* mRNA was easily detected in the 2-week-old mycelium before transfer

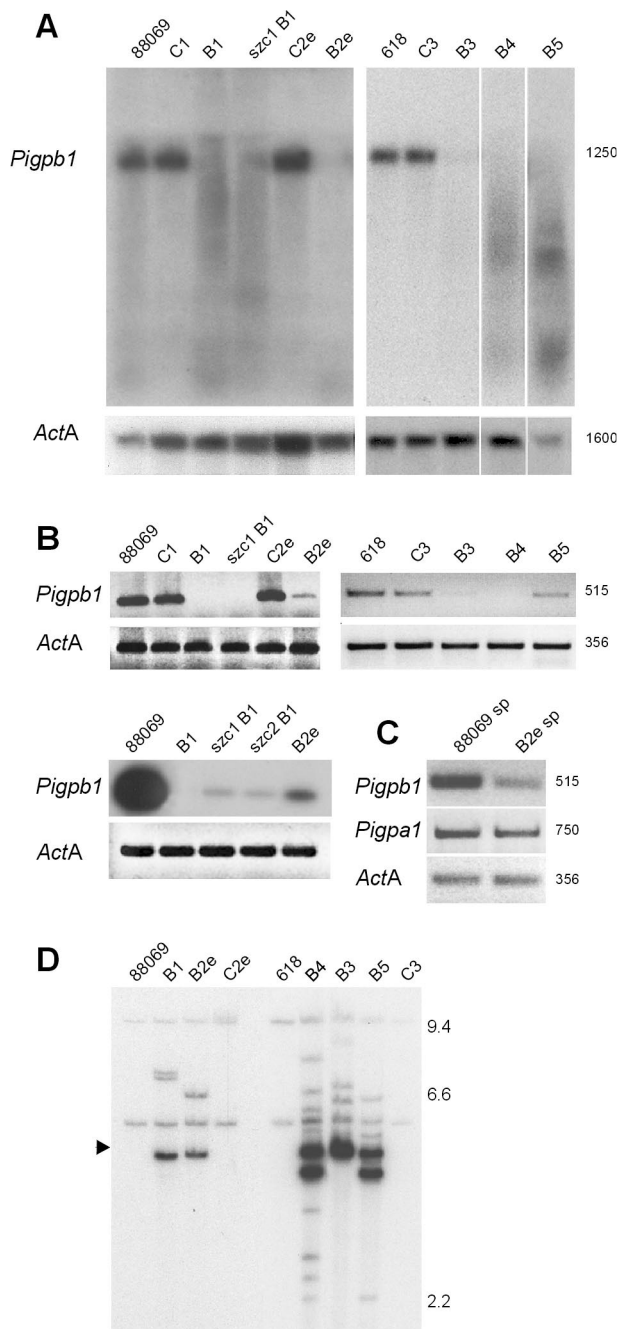


FIG. 2. Analysis of expression of *Pigpb1*-silenced mutants. *Pigpb1* mRNA levels in wild-type strains 88069 and 618, in control transformants in which only the selection plasmid was introduced (C1, C2e, and C3), in *Pigpb1*-silenced mutants (B1, B2e, B3, B4, and B5), and in single zoospore cultures of B1 (*szc1B1* and *szc2B1*) were determined. C1, C3, B1, B3, B4, and B5 are PEG transformants; C2e and B2e are electroporation transformants. (A) Northern blot analysis of expression of *Pigpb1* in mycelium of *Pigpb1*-silenced mutants. The sizes (in bases) of the bands detected with probes derived from *Pigpb1* and *actA* are indicated on the right. (Left panel) Wild-type strain 88069 and transformants derived from this strain. (Right panel) Wild-type strain 618 and transformants derived from this strain. Note that a smear has replaced the *Pigpb1* signal in B1, B4, and B5. (B) RT-PCR analysis of gene expression in wild-type strains, control strains, and *Pigpb1*-silenced mutants. Total RNA was extracted from starved mycelium. *Pigpb1* mRNA was amplified in 27 cycles (upper panels) or 30 cycles (bottom panels). *actA* mRNA was amplified in 24 cycles. RT-PCR

and in the starved mycelium, whereas it was nearly untraceable in the mycelium incubated in fresh RS. After incubation in minimal medium for 2 days, the first sporangia appeared, whereas the mycelium incubated in fresh RS did not sporulate within the same time interval. *Pigpa1* mRNA was not detected in the starved mycelium or in the mycelium incubated in fresh RS, again showing the distinct patterns of expression of *Pigpb1* and *Pigpa1*. *Pigpb1* expression precedes sporulation, whereas *Pigpa1* is expressed only in sporangia and subsequent stages of the life cycle (zoospores and cysts).

Silencing of the G β -subunit gene *Pigpb1*. To obtain PiGPB1-deficient mutants, two different *P. infestans* strains, 88069 (mating type A1) and 618 (mating type A2), were transformed with plasmid pMH2, containing a 2.5-kb genomic *HindIII-BamHI* fragment with the *Pigpb1* coding region flanked by its native promoter (911 bp) and terminator (387 bp). A total of 17 transformants were generated by the PEG protoplast transformation method (PEG transformants) (13). A total of 57 transformants were generated by electroporation-mediated transformation of zoospores of strain 88069 (electroporation transformants) (Tyler et al., unpublished). The levels of *Pigpb1* mRNA in starved mycelium of the transformants were determined by 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) (Fig. 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 were derived 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 the detection of mRNA than Northern blot analysis, this method was used to study the levels of *Pigpb1* mRNA in more detail (Fig. 2B). Intermediate levels of *Pigpb1* were detected in two of the five previously mentioned transformants (B2e and B5), a low level was detected in transformant B3, but no product could be amplified in transformants B1 and B4. We conclude that the silencing of *Pigpb1* is complete in transformants B1 and B4, nearly complete in B3, and partially complete in B2e and B5. From transformant B1, single zoospore cultures were isolated, and the expression of *Pigpb1* in four of these cultures was analyzed (Fig. 2A and B). No RT-PCR product was obtained 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 cultures after 30 cycles, whereas no product was obtained from parental strain B1 after 30 cycles (Fig. 2B). These results suggest that the level of silencing may change with passage through the zoospore stage. All RT-PCR

products were visualized on ethidium bromide-stained gels; their sizes (in base pairs) are indicated to the right of the upper panels. (C) RT-PCR analysis of the expression of *Pigpb1*, *Pigpa1*, and *actA* in sporangia (sp) of wild-type strain 88069 and partially *Pigpb1*-silenced mutant B2e. RT-PCR products were visualized on ethidium bromide-stained gels; their sizes (in base pairs) are indicated on the right. (D) Southern blot analysis of *Pigpb1*-silenced mutants. Genomic DNA was digested with *BamHI*, and the blot was hybridized with a probe derived from the coding region of *Pigpb1*. The arrowhead indicates bands of the size of plasmid pMH2. Sizes (in kilobases) are indicated on the right.

experiments were repeated at least twice with independent RNA isolations.

Integration of plasmid pMH2 into the genome of the *Pigpb1*-silenced mutants was analyzed on a Southern blot containing *Bam*HI-digested genomic DNA (Fig. 2D). pMH2 contains a single recognition site for *Bam*HI. 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 (4.5 kb) indicated that tandem integrations occurred in all *Pigpb1*-silenced transformants.

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

***Pigpb1* is required for sporangium formation.** Colonies of the *Pigpb1*-silenced mutants could be distinguished easily from wild-type colonies. When cultured on RSA, wild-type 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 (Fig. 3A). However, their radial growth rates were not markedly different from that of the wild type. The mycelium of the mutants invaded the agar poorly, as the mycelium could be detached from this substrate easily. This growth habit is subsequently referred to as “fluffy growth.”

The *Pigpb1*-silenced mutants barely sporulated when cultured on RSA (Table 1 and Fig. 3B). The average numbers of sporangia of wild-type strains 88069 and 618 varied. However, there was a clear correlation between the levels of *Pigpb1* mRNA in the mutants and the numbers of sporangia; e.g., mutants B1, B3, and B4 did not express or barely expressed *Pigpb1* and produced less than 1% the number of sporangia produced by a wild-type strain. Partially *Pigpb1*-silenced mutants formed reduced numbers of sporangia.

Microscopic observations revealed that a large proportion of the sporangia of the completely silenced transformants were empty or extraordinarily small. In addition, sporangia with multiple hyphal outgrowths were observed (Fig. 3C). In the partially *Pigpb1*-silenced mutants, B2e and B5, only a small proportion of sporangia had multiple outgrowths, but the relative number of empty sporangia was much larger than that in the wild type. The results show that fluffy growth, a reduction in sporulation, and the formation of malformed or empty sporangia are associated with severely reduced levels of *Pigpb1* mRNA.

The number of sporangia in the completely *Pigpb1*-silenced mutants was too low to compare reliably *Pigpb1* expression in sporangia of the wild type and mutants. The partially *Pigpb1*-silenced transformant B2e, however, produced enough sporangia for RNA isolation. The level of *Pigpb1* mRNA in B2e

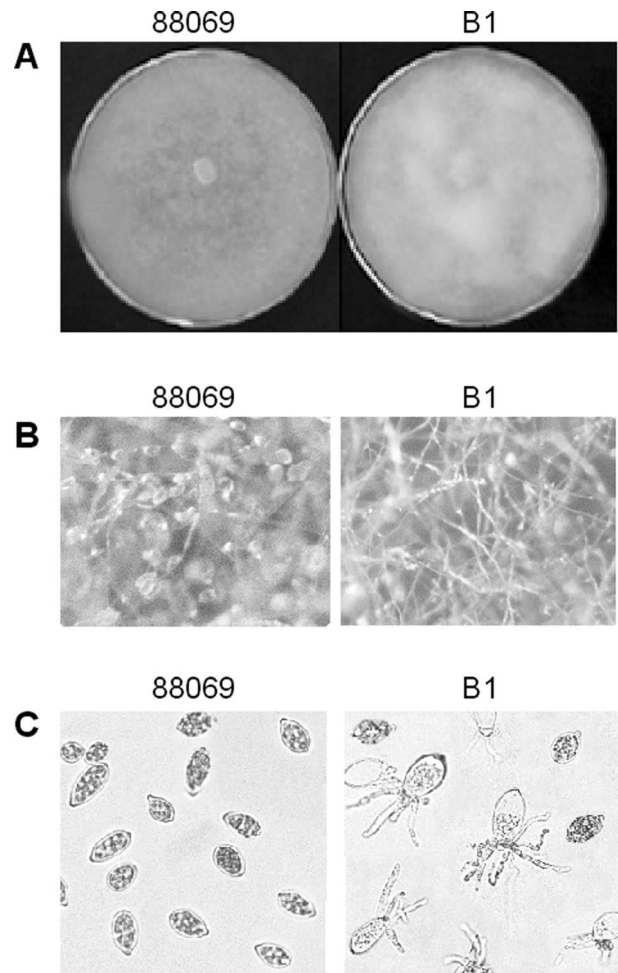


FIG. 3. Phenotypic characterization of *Pigpb1*-silenced mutants. (A) Growth on RSA of wild-type recipient strain 88069 and *Pigpb1*-silenced mutant B1. B1 produces a denser mat of aerial mycelium than 88069. As a consequence, the colony of B1 is more opaque than the colony of 88069. (B) The colonies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

sporangia, as determined by RT-PCR, was shown to be consistently lower than that in wild-type sporangia (Fig. 2C), albeit the difference between the two was smaller than that seen in the mycelia of the same strains. The expression of *Pigpa1*, a gene not expressed in mycelia but activated in sporangia, did not seem to be altered in B2e sporangia.

In *Saccharomyces cerevisiae* and several other fungi, the G β Y dimer is required for mating and/or sexual development (19, 20). To investigate a role for PiGPB1 in mating, *Pigpb1*-silenced mutants were cocultivated with the wild type 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 A1 and A2 colonies, irrespective of the levels of

TABLE 1. *Pigpb1* mRNA levels in and sporangium production by wild-type strains and transformants on RSA

Recipient strain	Transformant(s)	<i>Pigpb1</i> mRNA level ^a	Mean ± SD no. of sporangia (10 ⁴) ^b
88069	B1	—	370 ± 40
	szc1 B1	+	1 ± 2
	B2e	++	23 ± 18
	C1, C2, C4	++++	140 ± 15
618		++++	310 ± 93 ^c
	B3	+	90 ± 10
	B4	—	1 ± 1
	B5	++	1 ± 2
	C3	++++	58 ± 29
			230 ± 44

^a Based on results presented in Fig. 2A and B. +, wild-type level; ++, partial silencing; +, nearly complete silencing; —, complete silencing.

^b From one 9-cm RSA plate.

^c Average for three independent control transformants.

expression of *Pigpb1* in the mating cultures. Even in the complete absence of PiGPB1 (B1 as an A1 strain and B4 as an A2 strain), normal oospore formation was observed. Apparently, PiGPB1 is not required for the formation of gametangia or for the actual mating resulting in the formation of sexual spores.

The expression of CDC14 phosphatase is downregulated in *Pigpb1*-silenced mutants. CDC14 is a protein phosphatase that plays a role in mitosis in *S. cerevisiae* (34). 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 (A. M. V. Ah Fong and H. S. Judelson, personal communication). RT-PCR analysis of *cdc14* gene expression in mycelium of the wild type grown in rich and starved media showed that the expression of this gene is induced under starvation conditions (Fig. 4), again indicating that sporulation is initiated under these conditions. Very little *cdc14* mRNA was detected in the *Pigpb1*-silenced mutants, suggesting that PiGPB1 affects the expression of the *cdc14* homologue.

In planta growth. The 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 that produced sufficient zoospores for the inoculation of potato leaves. On leaves of potato cultivar Nicola, mutant B2e produced lesions of the same size as those produced by wild-type strain 88069 (data not shown). The efficiencies of infection by the wild type and B2e were over 90%, showing that the reduction in *Pigpb1* mRNA levels in sporangia and mycelia did not affect the aggressiveness of mutant B2e. The lesions of B2e were covered with more mycelium than those of the wild type, indicating that the fluffy growth phenotype is maintained during growth in planta as well.

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

DISCUSSION

The Gβ-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 the formation of abundant aerial mycelium that was only loosely attached to the agar surface. Partially silenced mutants displayed the same fluffy growth, and sporulation was reduced in these mutants as well. In the *Pigpb1*-silenced mutants, a large proportion of the sporangia were empty, were 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 mycelia leading to the formation of sporangia. This means that PiGPB1 is also indispensable for pathogenicity in *P. infestans*. We speculate that the increase in the formation of aerial mycelium in the *Pigpb1*-silenced mutants results from hyphae that continue to grow in the absence of a signal triggering the formation of sporangia.

C. parasitica mutants in which the Gβ-subunit gene *cpgb1* was disrupted showed a decrease in conidiation and an increase in hyphal density on rich medium. The dense mycelial mat of this mutant could be peeled from the agar surface easily (14). These phenotypes are strikingly similar to those of the *Pigpb1*-silenced mutants, showing that Gβ subunits control similar processes in these two evolutionarily unrelated plant pathogens. In contrast, loss of the Gβ-subunit gene *gpb1* in *N. crassa* and *sfaD* in *A. nidulans* had the opposite effect. In these species, the strains in which the Gβ-subunit genes were disrupted showed more profuse conidiation and inappropriate conidiation in submerged cultures (28, 35). In *D. discoïdium*, starvation induces cells to secrete cyclic AMP (cAMP) and to autoaggregate. cAMP sensing and chemotaxis are clearly regulated by G-protein signaling in this organism (7). Mutants in which the single-copy Gβ-subunit gene, *gpb1*, is disrupted are completely impaired in chemoattractant-induced responses (12, 21). Such a relationship between starvation and GPB1 function may also exist in *P. infestans*, where we found that

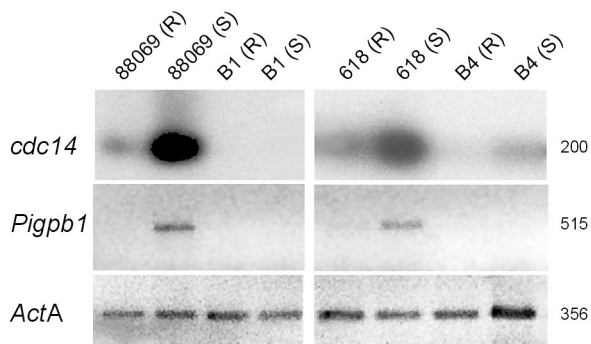


FIG. 4. Expression of *cdc14* and *Pigpb1* in 2-week-old mycelium grown in RS medium after transfer to fresh RS (R) or minimal medium (S) and 6 h of incubation in the same medium. RT-PCR was performed with primers corresponding to *cdc14*, *Pigpb1*, and *actA*. 88069 and 618 are wild-type strains, and B1 and B4 are *Pigpb1*-silenced mutants. RT-PCR products were visualized on ethidium bromide-stained gels; their sizes (in base pairs) are indicated on the right.

starvation induced *Pigpb1* expression which, in turn, preceded sporulation.

We considered the possibility that the sporulation defect in 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 in sporulation to less than 1% as a side effect of transformation is extremely rare. Second, the frequency of silencing of *Pigpb1* in the PEG transformants was ca. 20% (5 of 17) and comparable to the frequency that we observed for the silencing of the elicitor gene *inf1* (33). 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* were found to be normal in a series of 15 PEG transformants that were transformed with different plasmids not containing *Pigpb1*. Finally, *Pigpb1* was expressed at the wild-type level in a field isolate that sporulated 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 the downregulation of *Pigpb1* expression. *Pigpb1* was silenced in only 1 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 (unpublished results). The α -subunit gene *Pigpa1*, for example, was silenced in only 3% of the electroporation transformants (Latijnhouwers et al., submitted). A possible explanation for this finding is that plasmid DNA integrates differently into the genome when electroporation rather than PEG is used for transformation: PEG protoplast transformation generally results in many plasmid integrations (Fig. 2D) (33), whereas zoospore electroporation usually results in only a few plasmid integrations (unpublished results). However, so far we have no evidence that the number of plasmid integrations plays a role in triggering silencing (33).

It was surprising to find that in three of the five *Pigpb1*-silenced mutants, a smear of RNA replaced the distinct band representing *Pigpb1* mRNA on Northern blots. This result was not due to general RNA breakdown, as the control probe hybridized to a single band without a smear. RNA breakdown was not observed in the *Pigpa1*-silenced mutants (Latijnhouwers et al., submitted) or in *inf1*- and *cbel*-silenced mutants (9, 33). These data raise the question of whether different mechanisms of gene silencing may be operating in *Phytophthora*.

The α -subunit gene *Pigpa1* is not expressed in mycelium, suggesting that PiGPB1 complexes with another *P. infestans* α -subunit in mycelium. However, additional α -subunit genes have not been detected in *P. infestans*, despite considerable efforts (18). In *S. cerevisiae*, the α subunit GPA2 does not interact with $G\beta\gamma$ dimers but instead complexes with proteins containing seven kelch repeats (11). This example shows that G-protein signaling in lower eukaryotes does not always comply with the paradigm accepted for animals. Therefore, the possibility that PiGPB1 functions independently from α subunits cannot be excluded.

The $G\beta\gamma$ dimer (STE4/STE18) in *S. cerevisiae* is part of the pheromone response pathway. This pathway also involves a mitogen-activated protein kinase cascade (STE11/STE7/FUS3) (reviewed in reference 19). Mitogen-activated protein

kinase activation in the *P. infestans* *Pigpb1*-silenced mutants upon wounding and cold treatment was analyzed by means of in-gel kinase assays, but no differences between the wild type and mutants were detected (data not shown). The cAMP pathway is another pathway known to be downstream of G proteins in fungi (reviewed in reference 16). 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 (10). In the wild type, the expression of the gene encoding the *P. infestans* homologue of the *S. cerevisiae* CDC14 phosphatase was induced upon starvation and downregulated in mycelium incubated in rich medium for 6 h. 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 (Ah Fong and 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 depends 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, differentially expressed genes may be of great help for increasing the understanding of sporangium formation in *P. infestans*.

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