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Differential expression of G protein α and β subunit genes during development of *Phytophthora infestans*

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Abstract

A G protein α subunit gene (*pigpa1*) and a G protein β subunit gene (*pigpb1*) were isolated from the oomycete *Phytophthora infestans*, the causal agent of potato late blight. Heterotrimeric G proteins are evolutionary conserved GTP-binding proteins that are composed of α, β, and γ subunits and participate in diverse signal transduction pathways. The deduced amino acid sequence of both *pigpa1* and *pigpb1*, showed the typical conserved motifs present in Gα or Gβ proteins from other eukaryotes. Southern blot analysis revealed no additional copies of Gα or Gβ subunit genes in *P. infestans*, suggesting that *pigpa1* and *pigpb1* are single copy genes. By cross-hybridization homologues of *gpa1* and *gpb1* were detected in other *Phythophthora* species. Expression analyses revealed that both genes are differentially expressed during asexual development, with the highest mRNA levels in sporangia. In mycelium, no *pigpa1* mRNA was detected. Western blot analysis using a polyclonal GPA1 antibody confirmed the differential expression of *pigpa1*. These expression patterns suggest a role for G-protein-mediated signaling during formation and germination of asexual spores of *P. infestans*, developmental stages representing the initial steps of the infection process.

Keywords: Heterotrimeric G proteins; Gα subunit; Gβ subunit; Signal transduction; *Phytophthora infestans*; Oomycetes; Plant pathogen; Late blight

1. Introduction

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

In higher eukaryotes, G protein signaling has been implicated in mediating complex biological processes, such as the perception of taste, smell, and light, hormone action, chemotaxis, neurotransmission, and cell proliferation and differentiation (Dohlman et al., 1991). In lower eukaryotes also many processes involve G protein signaling. Studies of several ascomycetous and basidiomycetous fungi revealed a role for G proteins in

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

The subject of our studies is *Phytophthora infestans*, the causal agent of late blight and one of the most devastating pathogens of potato. *P. infestans* belongs to the oomycetes, a unique group of eukaryotes that includes significant pathogens of plants, insects, and animals. The genus *Phytophthora* comprises more than 60 species, which are all serious plant pathogens (Erwin and Ribeiro, 1996). *P. infestans* has a limited host range with, potato and tomato as the most important hosts. In contrast, other *Phytophthora* species can have a very broad host range; *P. nicotianae* and *P. palmivora*, for example, infect many plant species belonging to different families. Oomycetes have a fungus-like growth morphology but share little taxonomic affinity to filamentous fungi. This implies that oomycetous plant pathogens have evolved the ability to infect plants completely independently from true fungi. Yet, the way fungi and oomycetes infect and colonize plants is very similar. The evidence that G protein signaling is involved in fungal pathogenicity suggests that in oomycetes also G proteins may be crucial. As an extension of our studies on the pathogenicity of *P. infestans* we now report the cloning and characterization of the first oomycetous Gα and Gβ subunit genes and show that these genes are differentially expressed during the asexual life cycle, with the highest expression in sporangia.

2. Materials and methods

2.1. Strains and culture conditions

P. infestans strains 88069 (A1 mating type, race 1.3.4.7) and 90128 (A2 mating type, race 1.3.4.6.7. 8.10.11) from the Netherlands were used throughout this study. The isolates were routinely grown in the dark on rye agar medium supplemented with 2% (w/v) sucrose (RS medium) (Caten and Jinks, 1968) or modified Plich medium (van der Lee et al., 1997) at 18 °C. Mycelium for isolation of genomic DNA was obtained by growing the strains in liquid RS medium for 5–10 days. Mycelium, sporangia, zoospores, cysts, and germinating cysts for isolation of RNA and protein were obtained as described by van West et al. (1998). Other *Phytophthora* and *Pythium* species used in this study are listed in the legend of Fig. 5.

2.2. PCR amplification, cloning, and sequencing

Genomic DNA from *P. nicotianae* strain H1111 was used as template in a PCR reaction. The sequences of the degenerate primers that correspond to conserved regions of G protein α subunit genes were 5′-CGGATCCAA (A/G)TGGAT(T/C/A)CA(T/C)TG(T/C)TT-3′ and 5′-G GAATTC(A/G)TC(T/A/G)AT(T/C)TT(G/A)TT(T/A/C) AG(A/G)AA-3′. The underlined sequences correspond to *Bam*HI and *Eco*RI restriction sites, respectively. The primers, which were used previously to clone Gα subunit genes from a filamentous fungus (Choi et al., 1995), correspond to amino acid residues KWIHCF and FLNKID, respectively (see Fig. 1). The PCR was performed under the following conditions: 90 sec at 94 °C; 90 sec at 37 °C; 90 sec at 72 °C for 35 cycles. The PCR product was cloned in pBluescript(KS^+), resulting in plasmid pPngpa. The insert of pPngpa was used as a probe for screening a λEMBL3 genomic library of *P. infestans* strain 88069 (Pieterse et al., 1991). Filters containing plaques that covered four times the *P. infestans* genome were screened. Initially 14 λ clones were selected, of which 11 remained positive in the second screening. Positive plaques were purified and after mapping of restriction sites on the phage DNA, fragments from phage λP8 containing a putative Gα subunit gene were subcloned in pBluescript (SK^{II}) according to standard procedures (Sambrook et al., 1989) and sequenced.

In order to isolate a cDNA clone of the Gα subunit gene, cDNA was synthesized with reverse transcriptase on mRNA isolated from sporangia (Sambrook et al., 1989). This cDNA was used as template in a PCR reaction with primers corresponding to the 5′ and 3′ ends of the open reading frame of the Gα subunit gene (gpa1; 5′-CCCTCGAGATGGGACTCTGTGCT-3′, and gpa2; 5′-GGGGTACCTTGCGCTTGCCTACAT-3′).

Fig. 1. Nucleotide sequences of *Phytophthora* Gα subunit genes and their deduced amino acid sequences. (a) Nucleotide sequence of *P. infestans gpa1*; position +1 is the first nucleotide of the ATG start codon. (b) Nucleotide sequence of *P. palmivora gpa1*; nucleotides identical to those in *pigpa1* are indicated by dots; dashes (–) are introduced for optimal alignment. (c) Deduced amino acid sequence PiGPA1. (d) Deduced amino acid sequence PpGPA1; amino acid residues identical to those in PiGPA1 are indicated by asterisks. (e) Deduced amino acid sequence of the 154-bp PCR fragment amplified on genomic *P. nicotianae* DNA. Lowercase letters in (a) and (b) indicate the 87-bp intron in *pigpa1* and the 61-bp intron in *ppgpa1*, respectively, with the intron boundary nucleotides and the branch point consensus in bold italics. The amino acids indicating the conserved regions that were chosen to design degenerated primers for PCR amplification are shaded in gray in (e). The gap in the gray shading indicates a different amino acid in GPA1 (K) than expected (I) based on the codon usage in the degenerate reverse primer. The primer sequences used to isolate *pigpa1* cDNA from sporangia and the *P. palmivora* homologue *ppgpa1* are underlined in (a). The first 15 and last 4 nucleotides of *ppgpa1* and the first five amino acids and the last amino acid of PpGPA1 (indicated with dotted lines in (b) and (d), respectively) are derived from the primer sequence and have not been verified by sequencing. The sequence of the peptide used to raise GPA1 antiserum is in bold in (c). The nucleotide sequence data are deposited under Accession Nos. AY050536 (*pigpa1*) and AY050537 (*ppgpa1*).

The underlined regions correspond to *Xho*I and *Kpn*I restriction sites, respectively. PCR conditions were 30 sec at 95 °C; 30 sec at 68 °C; 1 sec at 72 °C for 30 cycles. The PCR products were cloned in pGEM-T and sequenced. The same primers were used to amplify a fragment on genomic DNA isolated from *Phytophthora palmivora* strain P6390. The fragment was cloned into pCR4-Topo (Invitrogen) and sequenced.

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

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

2.3. DNA probes

As DNA templates for probe synthesis the following fragments were used: the 154-bp insert from pPngpa containing part of the coding region of a *P. nicotianae* Gα subunit gene, the 484-bp *Sal*I–*Bam*HI fragment from *pigpa*1, the 734-bp *Not*I–*Sal*I insert from EST MY-09-H-10, and the 796-bp *Hin*dIII fragment from pSTA31 containing the coding region of the *P. infestans* actin gene, *actA* (Unkles et al., 1991). All DNA templates were purified with the Qiaex II Agarose Gel Extraction kit (Qiagen GmbH, Hilden, Germany). The probes were radiolabeled with [α-^{32}P]dATP using the Random Primers DNA Labeling System (Gibco BRL, Gaithersburg, MD). To remove unincorporated nucleotides, the Qiaquick Nucleotide Removal kit (Qiagen GmbH) was used.

2.4. Southern and Northern blot analyses

Genomic DNA of *P. infestans* and related species was isolated from mycelia as described by Pieterse

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

2.5. Western blot analyses

Protein extracts were prepared by suspending sporangia, zoospores, cysts, or germinated cysts collected from one 14.5-cm rye agar plate in 300 μl of extraction buffer composed of 20 mM Tris, pH 7.5, 10 mM dithiothreitol, 5% glycerol, 1% SDS, 10 μg/ml of leupeptin and 1 mM phenylmethylsulfonyl fluoride and modified from Choi et al. (1995). For extracting proteins from mycelium, mycelium grown in liquid medium was frozen in liquid nitrogen and ground to powder. Powder (200 μl) was mixed with 300 μl of extraction buffer. Spores and cysts were disrupted by grinding with sand and disruption was checked under the microscope. Samples were centrifuged for 5 min at 14,000 rpm to remove cell debris. The protein content was determined using an assay developed by Bradford (1976) and according to the protocol provided by Biorad. Protein from each sample (50 μg) was separated by SDS/PAGE and blotted onto a nitrocellulose membrane (Schleicher and Schuell). GPA1 antiserum was raised against a peptide corresponding to 14 amino acids at the C-terminal end of GPA1 (in bold in Fig. 1) and supplemented with a cysteine at the N-terminus (Eurogentec, Belgium). The specificity of the GPA1 antiserum was tested in a competition experiment in which 40 μg/ml of the peptide was added during incubation of a Western blot containing protein extracts from sporangia with the antiserum. Western blots were incubated with GPA1 antiserum at a 1:500 dilution and with a horseradish peroxidase-conjugated antirabbit IgG antibody (Amersham) at a 1:2500 dilution. Proteins recognized by the antiserum were visualized using a chemiluminescence detection kit (Amersham).

3. Results

3.1. Isolation and characterization of *P. infestans* Gα and Gβ subunit genes

Degenerate primers corresponding to conserved regions in Gα subunit genes were used for PCR amplification on *P. infestans* and *P. nicotianae* genomic DNA. Amplification was achieved with *P. nicotianae* DNA but not with *P. infestans* DNA. DNA sequencing revealed that the amplified *P. nicotianae* fragment was 154 bp long and that the deduced amino acid sequence was up to 68% identical to the comparable region in G protein α subunits of other organisms. This fragment was used to screen a *P. infestans* EMBL3 genomic library. Subclones from one of the most intensively hybridizing phages (λP8) containing the putative gene were sequenced. The nucleotide sequence of the region containing the gene of interest is shown in Fig. 1. The deduced amino acid sequence of the putative gene revealed open reading frames with significant regions of homology to deduced amino acid sequences of several other Gα subunit genes. The gene was designated *pigpa1* (*Phytophthora infestans* G protein α-subunit gene; Accession No. AY050536). Sequence comparison showed that the degenerate reverse primer used for the initial PCR on genomic DNA has three mismatches with the *pigpa1* sequence and this may explain why we were not successful in amplifying the corresponding fragment on *P. infestans* DNA. Another explanation may lie in the fact that the genome size of *P. infestans* is three- to fourfold larger than that of *P. nicotianae*.

A cDNA clone corresponding to *pigpa1* was obtained by PCR using cDNA synthesized on sporangia mRNA as template in combination with specific primers that were designed based on the *pigpa1* sequence (Fig. 1). Comparison of the cDNA and the genomic sequences revealed that *pigpa1* contains an intron between position 171 and 258. In *P. infestans* gene introns are rare. The first six and the last two nucleotides of the intron match consensus sequences for 5′ and 3′ intron splice sites in filamentous fungi (GTPuNGPy and PyAG, respectively; Unkles, 1992) and at 27 bp from the 3′ splice junction the intron contains a 7-bp sequence, CTCTAAC, which has five out of seven nucleotides identical to the yeast branch point consensus sequence TACTAAC that is necessary for intron splicing (Unkles, 1992). The intron position in *pigpa1* is not identical with intron positions in other Gα protein subunit genes. Finally, *pigpa1* lacks the consensus sequence that surrounds the transcription start site of several *Phytophthora* genes identified to date (Govers et al., 1997).

By PCR on genomic DNA of *P. palmivora* with 5′- and 3′-specific *pigpa1* primers a *P. palmivora* homologue of *pigpa1* was obtained and designated *ppgpa1* (Accession No. AY050537). At the amino acid acid level

PiGPA1 and PpGPA1 are 95% identical (Fig. 1). The intron in *ppgpa1* is at the same position but it is shorter, 61 instead of 87 bp, and has a completely different sequence than the intron in *pigpa1*. The 5′ and 3′ intron splice sites again match the consensus but an obvious yeast branch point consensus sequence is lacking.

One EST with high homology to known Gβ subunit genes was found among 1000 partial cDNA sequences derived from genes expressed in mycelium (Kamoun et al., 1999). The full-length gene corresponding to EST MY-09-H-10 was isolated from a BAC library and sequenced (Accession No. AY050538) and was designated *pigpb1*. The open reading frame is 1032 bp without introns. In the cDNA clone the ORF is followed by a 78-bp 3′ untranslated region preceding the poly(A) tail. As with *pigpa1*, *pigpb1* lacks the consensus sequence at the transcription start site.

Various organisms contain more than one Gα and Gβ subunit gene (Simon et al., 1991). To determine if *P. infestans* also has multiple Gα and Gβ subunit genes Southern blot hybridizations were performed using a *Bam*HI–*Sal*I fragment containing the 3′ part of the coding region of *pigpa1* and the insert of the *pigpb1* cDNA (EST MY-09-H-10) as probes. Under low stringency conditions, single hybridizing DNA fragments were detected on Southern blots containing genomic *P. infestans* DNA digested with *Hin*dIII (Fig. 5) and various other restriction enzymes (data not shown). The sizes of the fragments correspond to fragments present in phage λP8 (for *pigpa1*) or in the BACs (for *pigpb1*). This suggests that *P. infestans* does not contain additional Gα and Gβ subunit genes that are highly homologous to *pigpa1* and *pigpb1*, respectively.

3.2. Sequence comparison of GPA1 with other Gα subunits

In order to compare the predicted amino acid sequence of the *P. infestans* Gα subunit GPA1 with amino acid sequences of other Gα subunits, sequences were aligned using ClustalW (European Bioinformatics Institute—www2.ebi.ac.uk) (Fig. 2). The size of GPA1, 355 amino acids, does not differ from that of other Gα subunits. The highest identity and similarity (43 and 73%, respectively) were found with the human $G\alpha_{olf}$ subunit (GB01), whereas the identities with Gα subunits of *Caenorhabditis elegans* (GB01) and the slime mold *Dictyostelium discoideum* (GBA1) were somewhat lower (40 and 42%, respectively). The identity with CPG1 from the ascomycetous fungus *C. parasitica* was even lower, 38%. GPA1 has key structural motifs typical of members of the Gα subunit family (Gilman, 1987, Simon et al., 1991). These include five GTPase domains (G1–G5), a glycine residue at position 2 within a N-terminal consensus myristoylation site (MGXXXS) which increases the affinity of the α subunit for the βγ subunit, and an arginine residue at position 177 within the G2 domain, which is an established ADP ribosylation site for cholera toxin protein (Fig. 2). GPA1 does not have a pertussis toxin-labeling site (CXXX) at its C terminus, suggesting that GPA1 is not a member of the Gα inhibitory subfamily (West et al., 1985).

3.3. Sequence comparison of GPB1 with other Gβ subunits

ClustalW was also used to align the predicted amino acid sequence of the *P. infestans* Gβ subunit GPB1 with other Gβ subunits (Fig. 3). GPB1 is 344 amino acids in size, similar to other Gβ subunits. The highest identity and similarity (57 and 81%, respectively) were found

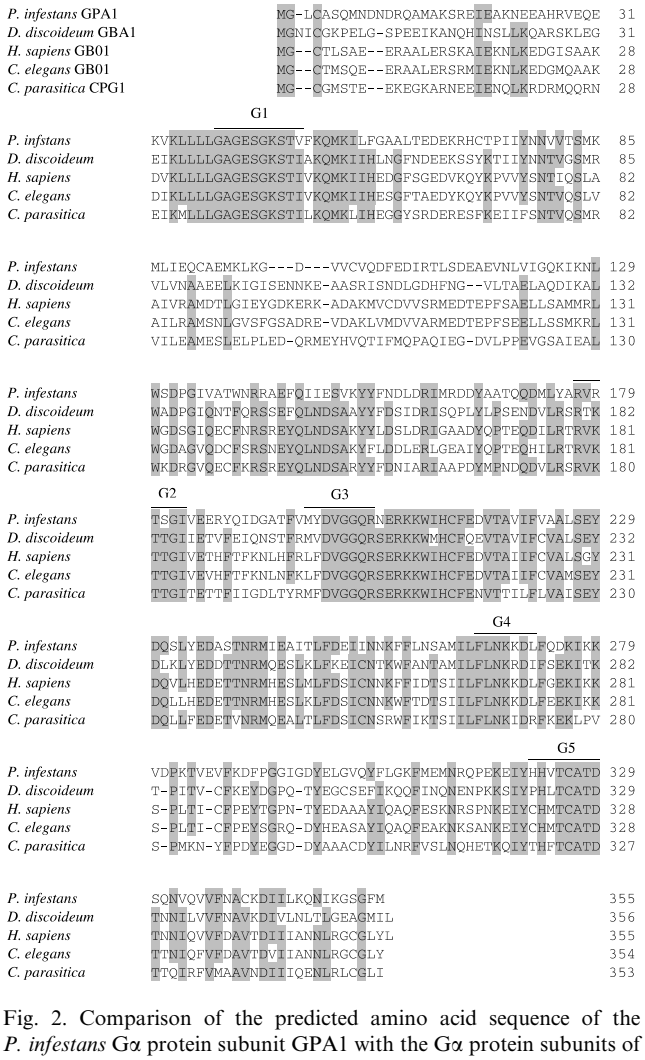

Fig. 2. Comparison of the predicted amino acid sequence of the *P. infestans* Gα protein subunit GPA1 with the Gα protein subunits of *D. discoideum* (GBA1, Accession No. P16894); *H. sapiens* (GB01, Accession No. P09471), *C. elegans* (GBO1, Accession No. AAA28059), and *C. parasitica* (CPG1, Accession No. L32176). Sequences of the Gα proteins were obtained from GenBank. Amino acids that are identical in four or five of the five homologues are shaded in gray. Spaces introduced for optimal alignment are indicated by a dash (−). The deduced locations of the G1–G5 regions of the GTPase domain (Gilman, 1987) are indicated by single lines above the alignment.

with the *D. discoideum* Gβ subunit GBB1, whereas the identities with Gβ subunits from *Homo sapiens* (GBB1), *C. elegans* (GBB1), and *C. parasitica* (CPGB1) were in the same range (55–56%). Gβ subunit proteins belong to an ancient regulatory protein family characterized by the occurrence of so-called WD repeats, highly conserved repeating units that usually end with Trp-Asp (WD) (Neer et al., 1994). As do all other Gβ subunit proteins, GPB1 has seven such repeats that, apart from 44 amino acids at the N-terminus, span the whole protein. The WD repeats determine its three-dimensional structure: a seven-bladed β-propeller (Sondek et al., 1996). Amino acid residues identified by crystallographic analysis as interacting with Gα subunits were found to be highly conserved among five Gβ homologue, while those residues identified as interacting with the Gγ subunit varied considerably (Fig. 3).

3.4. Expression of pigpa1 and pigpb1 during the life cycle of P. infestans

In the asexual lifecycle of *P. infestans* various distinct developmental stages can be recognized. When mycelium starts to sporulate upright branched sporangiophores are formed that produce sporangia as they grow. Sporangia are the asexual propagules and under natural conditions, sporangia are easily dispersed by wind and rain. When sporangia find the appropriate conditions, for example the moist surface of a leaf, they can either act as spores, now called sporangiospores, and germinate directly or develop into zoosporangia forming motile biflagellate zoospores that germinate after encystment. The tip of the germ tube develops into a specialized infection structure, the appressorium, from which a penetration hypha emerges that directly

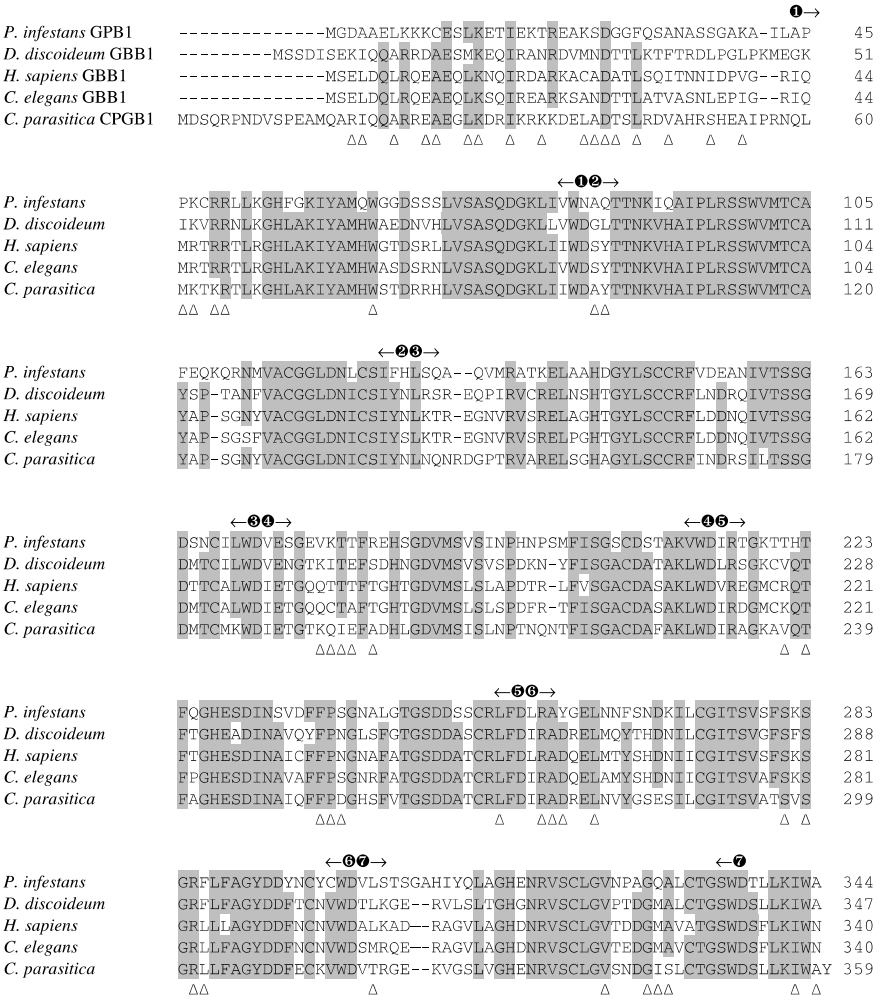

Fig. 3. Comparison of the predicted amino acid sequence of the *P. infestans* Gβ protein subunit GPB1 with the Gβ protein subunits of *D. discoideum* (GBB1, Accession No. P36408), *H. sapiens* (GBB1, Accession No. P04901), *C. elegans* (GBB1, Accession No. P17343), and *C. parasitica* (CPGB1, Accession No. O14435). Sequences of the Gβ proteins were obtained from GenBank. Amino acids that are identical in four or five of the five homologues are shaded in gray. Spaces introduced for optimal alignment are indicated by a dash (–). Numbers and arrows above the sequences delineate each of the seven WD repeats. Residues that contact the Gα protein subunit are indicated by black arrowheads (▲) while residues that contact the Gγ subunit are indicated by open arrowheads (△) (Sondek et al., 1996).

penetrates the leaf surface and grows into the epidermis. Here an infection vesicle is formed. Subsequently, hyphae emerging from the vesicle colonize the mesophyll cells thereby causing rapidly expanding water-soaked lesions. After a few days the center of the lesion becomes necrotic and this is soon followed by a complete collapse of the infected leaf. During lesion development numerous sporangiophores emerge from stomata, creating new infectious spores.

To determine the expression patterns of the *P. infestans* Gα and Gβ subunit genes in these distinct development stages, levels of *pigpa1* and *pigpb1* mRNA were analyzed by Northern blot hybridization. Total RNA isolated from different developmental stages was denatured, separated by electrophoresis, transferred to nylon membrane, and subsequently hybridized to a *pigpa1* and a *pigpb1* probe. As shown in Fig. 4A a major *pigpa1* transcript of approximately 1200 nt was detected. The highest level of *pigpa1* mRNA was found in sporangia. Also, zoospores, cysts, and germinating cysts contain *pigpa1* mRNA but, in contrast, no *pigpa1* mRNA could be detected in mycelium. A *pigpb1* transcript of approximately 1250 nt in length was found in all developmental stages but one: it was not detectable in zoospores. Similar to *pigpa1* the highest amount of *pigpb1* mRNA was found in sporangia. To exclude the possibility that the differential expression of *pigpa1* and *pigpb1* is strain specific, we analyzed the mRNA levels in another *P. infestans* strain. In strain 90128 the highest levels were also found in sporangia (data not shown). During in planta growth *pigpa1* mRNA was first detectable 5 days after inoculation, 3 days after the appearance of actin mRNA (data not shown). Since 5 days after inoculation, sporulation is abundant the observed accumulation of *pigpa1* mRNA is probably due to an increase in the relative amount of sporangium mRNA in the total pool of mRNA isolated from infected plant tissue.

To determine if the different *pigpa1* mRNA levels detected in the various developmental stages correlate with GPA1 protein levels we analyzed the presence of GPA1 by Western blot analyses. Polyclonal antibodies recognizing GPA1 were raised against a synthetic peptide identical to 14 amino acids at the C-terminus of GPA1 (Fig. 1). The antiserum specifically recognized a FLAG-GPA1 fusion protein produced in *Escherichia coli* upon expression of *pigpa1* cDNA cloned in a pFLAG-ATS expression vector (data not shown), demonstrating that the antiserum recognizes GPA1. On Western blots containing proteins extracted from zoospores, cysts, germinating cysts, and sporangia a protein was detected with the GPA1 antiserum and the size of 43 kDa corresponded to the size expected for GPA1 (Fig. 4B). With preimmune serum the band was not detected and also in the lane containing protein extracts from mycelium the band was absent. Since the protein has the expected molecular weight and is present in those cell types that also contain *pigpa1* mRNA it is very likely that this protein is GPA1. To confirm this we incubated a Western blot containing protein extracts from sporangia with the GPA1 antiserum in the presence of the peptide that was used as an antigen for generating the GPA1 antiserum. As shown in Fig. 4C the 43-kDa band is absent when the peptide is present,

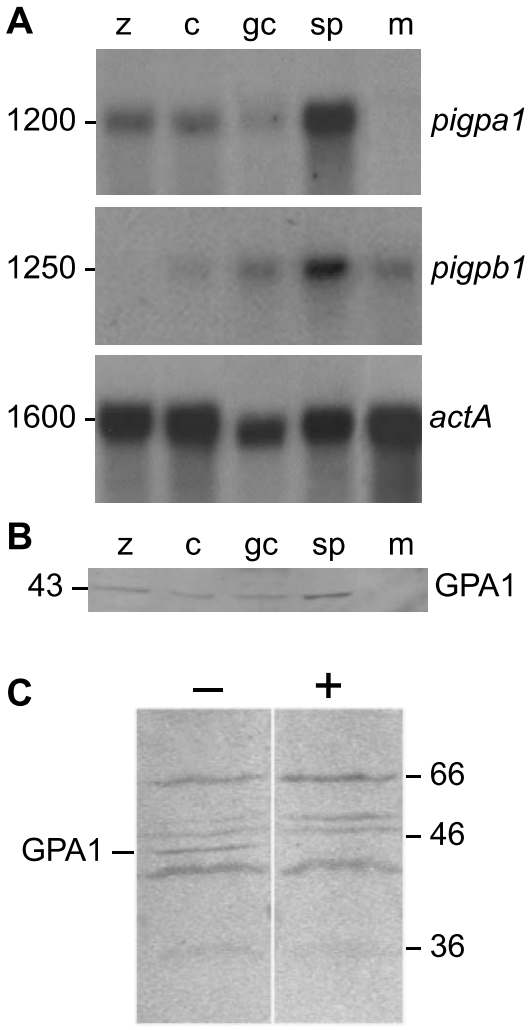

Fig. 4. Expression of *P. infestans pigpa1* and *pigpb1* in different development stages. Autoradiographs of a (A) Northern blot and (B and C) Western blots containing total RNA or protein extracts from *P. infestans* strain 88069 zoospores (z), cysts (c), cysts germinating for 2.5 h in water (gc), sporangia (sp), and mycelium (m). The Northern blot was sequentially hybridized with *gpa1*, *gpb1*, and *actA* probes. The *actA* probe, derived from a constitutively expressed *P. infestans* actin gene (Unkles et al., 1991), was used to check the amounts of RNA loaded on the gel. The approximate sizes of the transcripts in nucleotides are shown on the left. The Western blot was incubated with GPA1 antiserum. Only the region with the 43-kDa GPA1 protein is shown. In (C) two identical Western blots containing protein extracts from sporangia were incubated with GPA1 antiserum in the absence (−) or the presence (+) of 40 μg/ml of the peptide used as an antigen for the generation of GPA1 antiserum. Molecular weight markers are indicated on the right.

thereby demonstrating that the peptide competes away the primary antibodies and that the 43-kDa protein is indeed GPA1.

In summary, these results show that different developmental stages of *P. infestans* contain different levels of *pigpa1* and *pigpb1* mRNA. Moreover, in those cell types that contain *pigpa1* mRNA the transcript is translated into protein. We conclude that *pigpa1* and *pigpb1* have differential but different expression profiles in the various developmental stages of *P. infestans*. Yet, the two genes both have the highest expression levels in sporangia.

3.5. Sequences homologous to gpa1 and gpb1 in other Phytophthora species

To determine whether other oomycetes have sequences homologous to those of *pigpa1* and *pigpb1* we performed Southern blot analyses and hybridized *Hin*dIII digested genomic DNA isolated from various *Phytophthora* species and two *Pythium* species with probes from *pigpa1* and *pigpb1* under low-stringency conditions. In almost all lanes at least one fragment was found that hybridized specifically to the *pigpa1* and *pigpb1* probes, respectively (Figs. 5A and B). One exception was *Pythium sylvaticum,* in which we could not detect a fragment that is homologous to *pigpa1*. In contrast, a *pigpb1* homologue was present in this species. These results demonstrate that *pigpa1* and *pigpb1* are highly conserved within the genus *Phytophthora* and to some extent within the Pythiaceae family. In addition, in most species the homologues seem to be single copy genes.

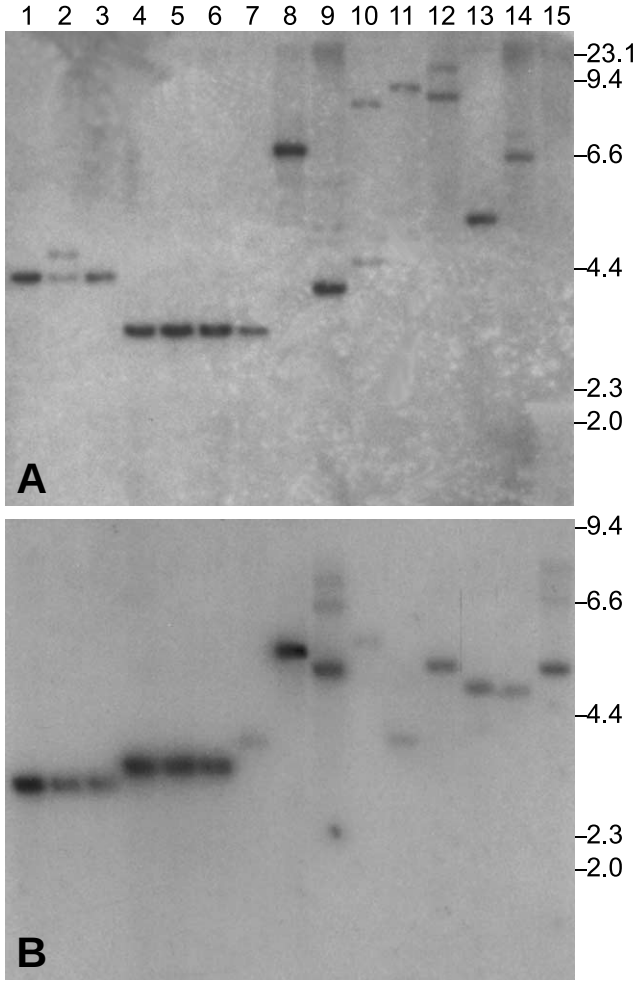

Fig. 5. Southern blot analysis of genomic DNA from various *Phytophthora* and *Pythium* species and hybridized at low stringency with (A) the 0.5-kb *Bam*H1–*Sal*I fragment from the coding region of the *pigpa1* gene and (B) the insert of the *pigpb1* cDNA clone MY-09-H-10. The DNA was digested with *Hin*dIII and isolated from *P. infestans* strains 88069 (lane 1), 90128 (2), and IPO-0 (3), *P. mirabilis* strains CBS678.85 (4), CBS136.86 (5), and CBS150.88 (6), *P. phaseoli* strain CBS556.68 (7), *P. nicotianae* strain USA1751 (8), *P. cactorum* strain 0436 (9), *P. palmivora* strain PD 93/56 (10), *P. porri* strain HH (11), *P. cinnamomi* strain 2 (12), *P. vignae* strain 20853 (13), *Pythium aphanidermatum* strain 933 (14), and *Pythium sylvaticum* strain 28 (15). Molecular size markers (in kb) are indicated on the right.

4. Discussion

In this paper we describe the characterization of two novel *P. infestans* genes that encode two of the three subunits that constitute heterotrimeric G proteins. The *pigpa1* gene encodes an α subunit and *pigpb1* a β subunit. To our knowledge these are the first cloned heterotrimeric G protein genes of an organism that belongs to the class of oomycetes. For cloning the Gα subunit gene we exploited the highly conserved nature of Gα subunits found in many eukaryotes and used a degenerate primer PCR approach. The Gβ subunit gene was selected from a database containing a limited number of *P. infestans* ESTs obtained from a cDNA library from mycelium (Kamoun et al., 1999). We cloned the full-length genes, studied expression of both *pigpa1* and *pigpb1* during the life cycle of *P. infestans*, and analyzed various *Phytophthora* species for the presence of *pigpa1* and *pigpb1* homologues.

The predicted amino acid sequence of *pigpa1* shows a high degree of similarity to known Gα subunits and all conserved characteristic key structural motifs necessary for GTPase activity are present. Intensive attempts to clone additional Gα subunit genes from*P. infestans* have so far been unsuccessful. It is possible that a single gene produces all Gα subunit molecules required for signaling in *P. infestans*. Also, in the basidiomycetous fungus *Ustilago hordei* (Lichter and Mills, 1997) and the ascomycetes *C. heterostrophus* (Horwitz et al., 1999) and *C. trifolii* (Truesdell et al., 2000) only a single copy of a Gα subunit gene was detected, but in contrast, several other fungi contain more than one Gα subunit gene and each of these seems to function in a different signaling pathway. Examples are *Saccharomyces cerevisiae* (Nakafuku

et al., 1988), *S. pombe* (Isshiki et al., 1992), *N. crassa* (Ivey et al., 1996), *U. maydis* (Regenfelder et al., 1997), *M. grisea* (Mitchell and Dean, 1995), and *C. parasitica* (Gao and Nuss, 1996).

As with *pigpa1*, *pigpb1* seems to be a single copy gene in *P. infestans*. Gβ subunit proteins belong to the ancient regulatory protein family of WD repeat proteins and eukaryotic organisms contain a whole range of these proteins. In the current *P. infestans* EST database we found at least four different cDNA clones with homology to WD repeat proteins. However, the nonredundant EST we selected for isolating *pigpb1* is the only true candidate for a Gβ subunit gene among a total of 2400 ESTs. It is highly homologous to known Gβ subunits, with many of the conserved amino acids at sites that interact with Gα. In several organisms only a single copy of the Gβ subunit gene is found. Examples are *Arabidopsis* and *S. cerevisiae*. In *S. cerevisiae* the only Gβ subunit is the pheromone activated STE4 that is quite different in its primary structure than all other Gβ subunits known so far. *C. elegans* has two Gβ subunits genes, of which only GBB1 is essential (van der Linden et al., 2001).

RNA blot analysis showed that different cell types contain different levels of *pigpa1* and *pigpb1* mRNA, demonstrating differential expression in the various developmental stages of *P. infestans* with the highest mRNA accumulation in sporangia. In mycelium neither GPA1 nor *pigpa1* mRNA was detectable. This is consistent with the fact that the current EST database from the mycelial cDNA library, with over 2400 sequences, does not contain a *pigpa1* sequence. With only one Gα subunit gene and a universal role for heterotrimeric G proteins in many cellular signaling processes one would expect to find GPA1 in every cell type. It may well be that the amounts of GPA1 and *pigpa1* mRNA in mycelium are below the detection levels but the possibility that GPA1 is dispensable in mycelium cannot be excluded. Recent findings in yeast suggest that some G protein subunits participate in signaling independently of other subunits (Lengeler et al., 2000). Signaling pathways involving only one of the G protein subunits may also occur in *P. infestans* and this may explain why the expression patterns of the two single copy genes are different.

The finding that both genes are expressed in preinfection stages, albeit at different levels, suggests that in those stages Gα and Gβ, together with a yet unknown Gγ subunit, form a heterotrimeric G protein that is activated by extracellular signals binding to heptahelical transmembrane receptors. For pathogens it is important to sense the environment and to find the appropriate hosts for extracting nutrients. For several fungal plant pathogens, both plant and human, it has been demonstrated that G protein signaling is crucial in the preinfection stage and for the subsequent invasion of host tissue (Lengeler et al., 2000). Despite the fact that fungi and oomycetes are taxonomically very divergent they have many features in common and it is very likely that oomycete pathogens also rely on G protein signaling to invade and colonize host tissue. With the availability of *P. infestans* genes encoding subunits of heterotrimeric G proteins it is now possible to analyze the role of G protein mediating signaling in late blight pathogenesis in more detail. The diploid nature of oomycetes hampers "knockout" mutagenesis in these organisms but via targeted gene silencing (van West et al., 1999) it will be possible to obtain GPA1- and GPB1-deficient *P. infestans* strains. These mutants will help to elucidate the function of GPA1 and GPB1 and to unravel signal transduction pathways in *P. infestans* that may be required for pathogenicity. Interference with such pathways may form the basis for new strategies to control late blight.

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