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RESEARCH ARTICLE

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The pipg 1 gene of the oomycete *Phytophthora infestans* encodes a fungal-like endopolygalacturonase

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Abstract Endopolygalacturonases (endoPGs) are plant cell wall-degrading enzymes that have been implicated in the invasion of plant tissue by pathogenic microbes. EndoPGs have been described from bacteria, plants, insects and numerous species of phytopathogenic fungi. In this study, we describe the first endoPG sequence from oomycetes, a unique group of eukaryotic plant pathogens that exhibit fungal-like filamentous growth but share little taxonomic affinity to fungi. The characterized gene, *pipg1*, was identified from the potato late-blight pathogen, *Phytophthora infestans*, and was predicted to encode a secreted glycoprotein with all the signature sequences of endoPGs. *Pipg1* was expressed during preinfection and infection stages. Phylogenetic analysis of endoPGs indicated that *pipg1* forms a unique class that is significantly more similar to fungal endoPGs than to plant or bacterial ones. This unexpected affinity between PIPG1 and fungal endoPGs contrasts with phylogenies obtained using ribosomal sequences or compiled protein sequences from mitochondrial and chromosomal genes, raising interesting questions about the evolution of these enzymes in oomycetes.

Keywords Endopolygalacturonase · Secreted glycoprotein · Plant–microbe interaction · Oomycete

Introduction

Most microbial plant pathogens produce an array of extracellular enzymes that degrade plant cell walls and aid the penetration and colonization of plant tissue. One of the first enzymes secreted by invading pathogens is endopolygalacturonase (endoPG, E.C. 3.2.1.15), which breaks up the α-1,4-polygalacturonic acid of pectins, a complex polysaccharide found in the middle lamella and primary cell wall of higher plants (Esquerre-Tugaye et al. 2000; ten Have 2001). EndoPGs have been described from plants, bacteria and from a large number of phytopathogenic fungi (Lang and Dornenburg 2000). The role of endoPGs in plant–fungal interactions centers around a complex cross-talk of signals and responses. Whereas it is generally accepted that endoPG degradation of plant cell walls is an important step in the penetration and invasion of host tissue, some of the pectic fragments released through the enzymatic activity of endoPGs can activate defense responses in plants (Esquerre-Tugaye et al. 2000; Lang and Dornenburg 2000; ten Have 2001). In addition, plants produce a class of proteins, known as polygalacturonase-inhibiting proteins, that can specifically bind and inactivate endoPGs (Esquerre-Tugaye et al. 2000; Lang and Dornenburg 2000; ten Have 2001). Therefore, the exact contribution of endoPGs to virulence remains unclear and is likely to vary depending on the pathosystem examined.

Oomycetes represent a diverse group of organisms that includes pathogens of plants, insects, crustaceans, fish and vertebrate animals, as well as saprophytic species. Traditionally and due to their filamentous growth habit, oomycetes have been classified in the kingdom Fungi. However, modern molecular and biochemical analyses suggest that oomycetes share little taxonomic affinity to filamentous fungi, but are more closely related to brown algae (heterokonts) in the kingdom Stramenopiles (Baldauf et al. 2000; Kumar and Rzhetsky 1996; Lang et al. 1999; Paquin et al. 1997; Van de Peer and De Wachter 1997). Among the oomycetes, *Phytophthora* spp cause some of the most destructive plant diseases in the world and are arguably the most devastating pathogens of dicotyledonous plants (Erwin and Ribeiro 1996). For example, *P. infestans*, also known as the cause of the Irish potato famine, remains a destructive pathogen resulting in multibillion-dollar

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losses in potato and tomato production (Fry and Goodwin 1997a, b; Kamoun 2000, 2001). Despite its economic importance and singular phylogenetic position, little is known about the molecular mechanisms that control pathogenicity in *P. infestans*. However, in recent years, structural genomics, such as large-scale sequencing of random cDNAs, emerged as a promising strategy for the identification of novel virulence genes (Kamoun et al. 1999; Qutob et al. 2000; Skinner et al. 2001). We and others in the oomycete research community generated thousands of expressed sequence tags (ESTs) from *Phytophthora* that were compiled into searchable databases (Kamoun et al. 1999; Qutob et al. 2000; Waugh et al. 2000). In this study, we describe and characterize *pipg1*, an endopolygalacturonase gene from *P. infestans* and one of the first putative virulence genes of *Phytophthora* identified using EST databases. Characterization of endoPGs from oomycetes should bring a new perspective to understanding the general role of these enzymes in interactions between plants and microbes.

Materials and methods

Phytophthora strains and culture conditions

Two *P. infestans* isolates from the Netherlands, 88069 (A1 mating type, race 1.3.4.7) and 90128 (A2 mating type, race 1.3.4.7.8.9.10.11), were used throughout the study and in the infection assays. The two isolates infect tomato plants at different rates. Isolate 88069 produces slowly expanding necrotic lesions, whereas 90128 produces rapidly expanding and greenish (biotrophic) lesions. *P. infestans* strains were routinely grown on rye agar medium supplemented with 2% sucrose (Caten and Jinks 1968). For RNA extraction, plugs of mycelium were transferred to modified Plich medium (Kamoun et al. 1993) and grown for 2–3 weeks before harvesting. Germinating cysts and the time courses of *P. infestans* infection of tomato tissue were obtained exactly as described by Kamoun et al. (1997).

DNA manipulations

DNA manipulations were conducted essentially as described by Sambrook et al. (1989). The *pipg1* cDNA (GenBank accession number AAK98644) was sequenced by primer-walking, using an ABI Prism 377 automated sequencer (PE Applied Biosystems). In addition to vector primers, the following primers were used: 11B10F (5′-ACTGTGACTGGACCTGGAAC-3′), 11B10F2 (5′-GCCTCGCTATGCAGTCCAGCTC-3′) and 11B10R (5′-CTCCC TGACCATCAAGCGTTCC-3′).

RT-PCR analysis

Total RNA from *P. infestans* and from infected tomato was isolated using the Trizol reagent (Gibco-BRL, Bethesda, Md.) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 4.5 μg of total RNA with a universal oligo(dT) primer and the ThermoScript reverse transcriptase from the ThermoScript RT-PCR system (Gibco-BRL, Bethesda, Md.). Reactions were carried out according to the manufacturer's instructions and were incubated at 50 °C for 1 h. PCR amplifications were carried out with 0.004% of the cDNA product using the primer pair PIPG-RTF1 (5′-TCCGCACCTTCAGCATTCTC-3′) and PIPG-RTR1 (5′-CGCCCTTGGTCTTGTTGTAATCTG-3′), targeted towards a 450-bp region at the 3′ end of the *pipg1* open reading frame (ORF). Integrity of the mRNA and cDNA was controlled with primers EF2-F1 (5′-TGACGCTATCGCCAAG-GAATC -3′) and EF2-R1 (5′-TAACGCTGAGCCGTAATGGG-GG-3′), that are specific for the constitutive elongation factor 2 (*ef2*) gene of *P. infestans* (W.R. Morgan and S. Kamoun, unpublished data).

Phylogenetic analysis

Multiple alignment of endoPG amino acid sequences was conducted using the program CLUSTAL-X (Thompson et al. 1997). PAUP v4.0b8 (Sinauer Associates, Sunderland, Mass.) was used to reconstruct phylogenetic trees of the endoPG family, using the neighbor-joining method and maximum parsimony with 1,000 bootstrap replications.

Results

Molecular analysis of a *P. infestans* cDNA encoding an endoPG

We scanned the *P. infestans* EST database described by Kamoun et al. (1999) for sequences encoding cell wall-degrading enzymes. One EST, MY-11-B-10, was identified by similarity searches to show significant similarity to endoPGs. DNA sequencing of the full cDNA revealed an ORF of 1,125 bp, corresponding to a predicted translated product of 374 amino acids. SignalP (Nielsen et al. 1997) analysis of the predicted protein identified a 20-amino acid signal peptide with a significant mean *S* value of 0.88. Ten potential N-glycosylation sites, corresponding to the consensus sequence NX(S/T), were also identified. Similarity searches of the predicted protein against the nonredundant database of GenBank, using the BLASTP program (Altschul et al. 1997), revealed highly significant matches to fungal endoPGs ($E = 10^{-44}$ for best hit), insect endoPGs ($E = 10^{-31}$), plant endoPGs ($E = 10^{-17}$) and bacterial endoPGs ($E = 10^{-10}$). Searches against the InterPro database (Apweiler et al. 2001) revealed similarity to InterPro domain IPR000743 for polygalacturonase. We then used CLUSTAL-X (Thompson et al. 1997) to generate a multiple alignment of the *P. infestans* endoPG with 38 endoPGs from fungi, insects, plants and bacteria (Fig. 1). The length of the *P. infestans* endoPG (374 amino acids) was within the range of known endoPGs (361–452 amino acids). Five conserved amino acid blocks defining the endoPG family signature and catalytic sites were conserved, including the amino acid blocks NTDG, DD, GHGXSIGS, RIK and Y (Stratilová et al. 1993; Stratilova et al. 1996). The only substitution in these motifs that was unique to the *P. infestans* sequence was a serine in the first residue of the GHGXSIGS motif. However, histidine, the key catalytic residue in this motif (Rao et al. 1996; Stratilová et al. 1993), remained conserved. Based on these results, we propose that the analyzed *P. infestans* cDNA is likely to encode a functional endoPG and we designated the cDNA *pipg1*.

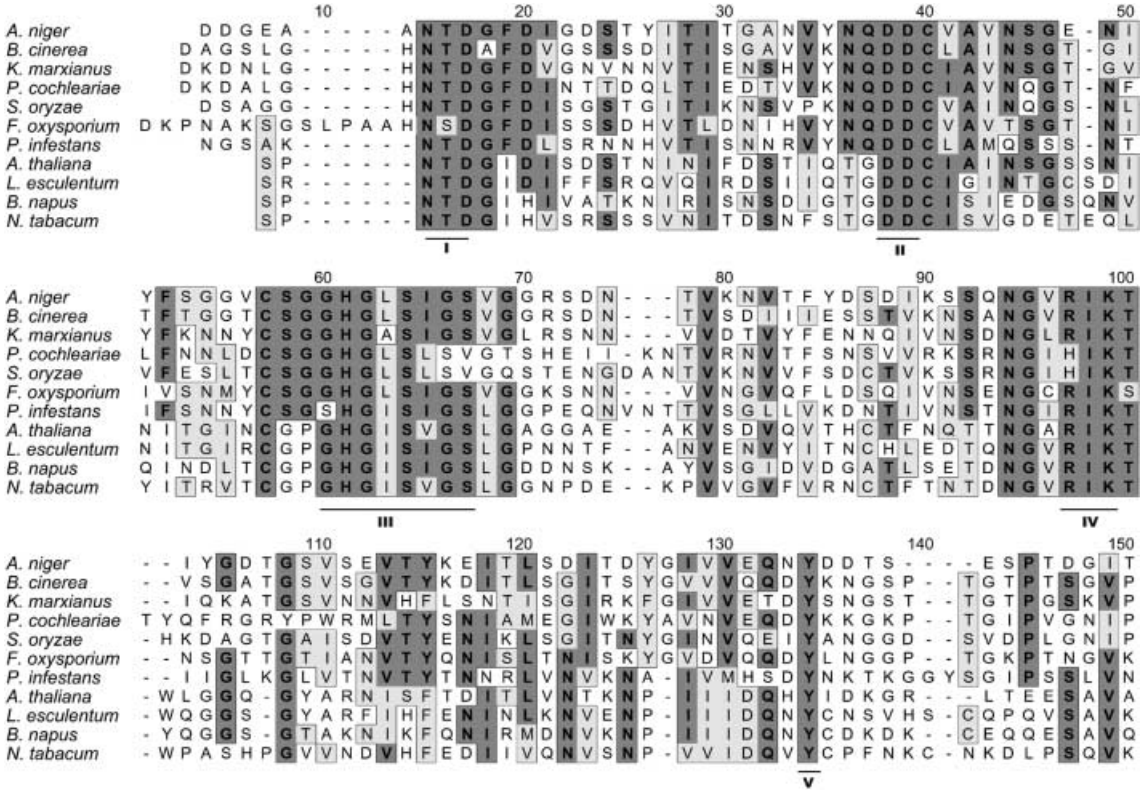

Fig. 1. Multiple alignment of a representative set of deduced endopolygalacturonase (endoPG) amino acid sequences from *Aspergillus niger* (GenBank CAA74744), *Botyritis cinerea* (AAC64374), *Kluyveromyces marxianus* (CAA03900), *Phaedon cochleariae* (CAA76930), *Sitophilus oryzae* (AAG35693), *Fusarium oxysporum* (BAA20555), *Phytophthora infestans* (AY052571), *Arabidopsis thaliana* (CAA20037), *Lycopersicon esculentum* (AAD17250), *Brassica napus* (CAA65702) and *Nicotiana tabacum* (S32008). Identical amino acids are *shaded in dark gray* and similar amino acids *shaded in light gray*. Residue numbers are *indicated above the sequences*. The conserved endoPG family signatures are *underlined* and are: *I* NTD, *II* DD, *III* GHGXSIGS, *IV* RIK and *V*

Phylogenetic analyses

To further investigate the relationship between PIPG1 and endoPGs, we reconstructed the phylogeny of the endoPG family, using both the neighbor-joining distance matrix method and maximum parsimony (Fig. 2). A total of 1,000 bootstrap replications were conducted with each method to determine the statistical significance of the obtained branches. The phylogenetic trees generated were overall similar. When considering bootstrap values higher than 85%, three main branches grouping the bacterial, plant and fungal/insect endoPGs were observed. PIPG1 clustered outside these main branches, suggesting that *Phytophthora* endoPGs may form a new class of these enzymes. Interestingly, PIPG1 was significantly more closely related to the fungal/insect clade than to the plant or bacterial ones.

Expression of *pipg1* during preinfection and infection stages of *P. infestans*

The *pipg1* cDNA was identified from a library constructed from mRNA isolated from mycelium grown in synthetic medium (Kamoun et al. 1999). To determine whether *pipg1* is expressed under conditions relevant to the interaction with plants, we used RT-PCR analysis to detect *pipg1* mRNA in a preinfection stage (germinating cysts) and in a time course of *P. infestans* infection of tomato (Fig. 3). PCR amplifications were carried out with equal amounts of cDNA from the various stages, using primers specific for *pipg1*. Transcripts for *pipg1* were detected in germinating cysts, in mycelium of both *P. infestans* isolates 88069 and 90128, in tomato leaves infected with 88069 2–5 days post-inoculation and in tomato leaves infected with 90128 1–5 days post-inoculation. No *pipg1* mRNA could be detected in leaves infected with isolate 88069 1 day post-inoculation, in uninfected tomato leaves, or in a water control. Control RT-PCR amplifications were conducted on the same cDNAs with primers specific for the constitutively expressed *ef2* gene.

Discussion

EndoPGs have been described from bacteria, plants, insects and numerous species of phytopathogenic fungi. In this study, we describe and characterize the first endoPG gene from oomycetes, a unique group of

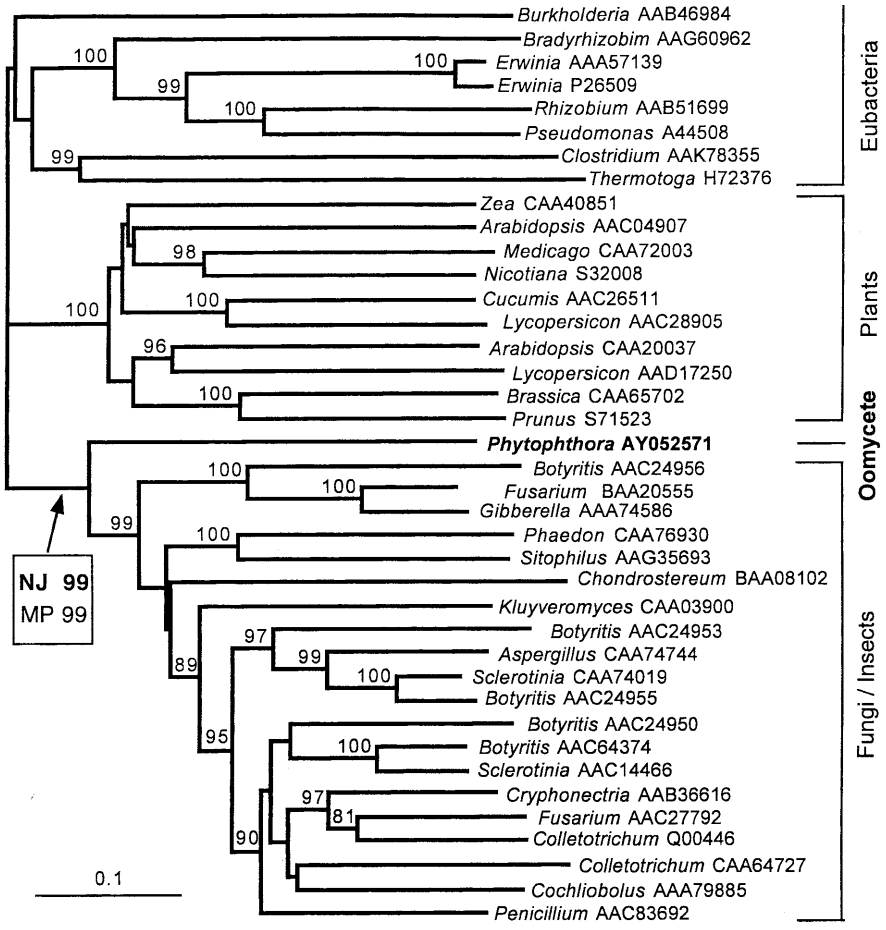

Fig. 2. Phylogenetic analysis of 39 endoPGs from fungi, plants, insects, bacteria and the oomycete *P. infestans*. The phylogenetic tree was constructed using the neighbor-joining method, based on the conserved overlapping portion of the endoPGs. Highly significant bootstrap values (>80%) from 1,000 replications are *indicated at the nodes*. The branch grouping PIPG1 with the fungal/insect sequences is *indicated by the arrow* and the bootstrap values for both neighbor-joining (*NJ*) and maximum parsimony (*MP*) analyses are *indicated*. The *length of the branches* reflect weighted amino acid substitutions; and the *scale bar* represents 10% weighted sequence divergence. The genus name and the GenBank accession number are given for all sequences

eukaryotic plant pathogens that exhibit fungal-like filamentous growth but share little taxonomic affinity to fungi. The characterized gene, *pipg1*, was identified from the potato late-blight pathogen *P. infestans* and was predicted to encode a secreted glycoprotein with all the signature sequences of endoPGs.

The recent taxonomic positioning of oomycetes as stramenopiles suggest a certain level of evolutionary relationship to brown algae and possibly to plants (Baldauf et al. 2000). Based on systematic analyses of cDNA sequences, Kamoun et al. (1999) concluded that *P. infestans* genes are not particularly related to fungal sequences and a bias towards plant sequences was apparent. This has been further confirmed using a series of phylogenetic analyses of *Phytophthora* genes encoding metabolic enzymes and other conserved proteins (A. LaLumia, W.R. Morgan and S. Kamoun, unpublished results). Therefore, the affinity observed here between PIPG1 and fungal endoPGs is unexpected and contrasts with phylogenies obtained using ribosomal sequences or compiled protein sequences from mitochondrial and chromosomal genes (Baldauf et al. 2000; Kumar and Rzhetsky 1996; Lang et al. 1999; Paquin et al. 1997; Van de Peer and De Wachter 1997).

There are several possible explanations for the exceptional phylogeny of PIPG1. For example, the observed affinity to fungal endoPGs may reflect convergent

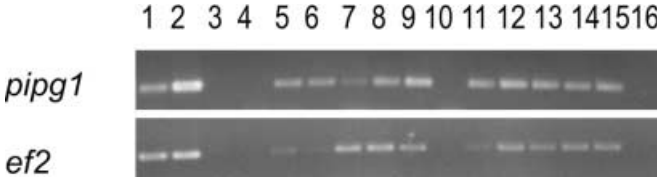

Fig. 3. Expression of *pipg1* during preinfection and infection stages of *P. infestans*. Lanes show: *1* total RNA from *P. infestans* isolate 88069 mycelium, *2* 88069 germinating cysts, *3* uninfected tomato, *4–8* 88069-infected leaves of tomato at 1 day (*4*), 2 days (*5*), 3 days (*6*), 4 days (*7*) and 5 days (*8*) after inoculation, *9* *P. infestans* isolate 90128 mycelium, *10* uninfected tomato, *11-15* 90128-infected leaves of tomato at 1 day (*11*), 2 days (*12*), 3 days (*13*), 4 days (*14*) and 5 days (*15*) after inoculation and *16* a water control. RT-PCR amplifications were as described in the text. Amplification of the *P. infestans* elongation factor 2 gene (*ef2*) was used as a control to determine the integrity of the RNA

evolution through which phylogenetically distinct enzymes have evolved to share significant similarity. This is supported by the fact that both plant pathogenic fungi and oomycetes occupy comparable ecological niches and their endoPGs may target similar substrates, host pectins, and face similar selective forces. Alternatively, the endoPG phylogenetic tree could reflect horizontal gene transfer events, in which plant pathogenic oomycetes acquired endoPG genes from fungi. An exhaustive

survey and phylogenetic analysis of endoPG sequences from plant-pathogenic, animal-pathogenic, saprophytic oomycetes and other stramenopile species should help test the horizontal gene transfer hypothesis and distinguish between these two explanations.

In a recent study, ten Have et al. (2001) demonstrated that the endoPG genes of *Botrytis cinerea* are differentially expressed in various hosts and stages of infection. The emerging view is that endoPGs are not always expressed in a particular host plant and are likely to also contribute to saprophytic survival (ten Have 2001; ten Have et al. 2001). To determine whether *pipg1* is expressed during host infection, expression studies were conducted using RT-PCR and two *P. infestans* isolates, 88069 and 90128, that infect tomato plants at different rates. These experiments showed that *pipg1* is expressed during both preinfection and infection stages and thus could play a role in the penetration and invasion of plant tissue by *P. infestans*. Even though RT-PCR is at best a semi-quantitative assay, the levels of *ef2* mRNA detected appeared to correlate with the expected differences in infection rate between the two *P. infestans* isolates tested. Messenger RNAs for *ef2* and *pipg1* were detected earlier in the interaction between 90128 and tomato than in the 88069 interaction, consistent with the differences in symptom development between the two interactions. Thus, the *pipg1*-expression pattern appeared to resemble that of the constitutive gene *ef2* and *pipg1* was expressed in both types of interaction.

Phytophthora spp and other oomycetes have been reported to secrete a variety of extracellular enzymes, including plant cell wall-degrading enzymes, such as endoPGs (Jarvis et al. 1981; McIntyre and Hankin 1978; Moreau and Seibles 1985). In this study, we initiated the molecular genetic characterization of plant cell wall-degrading enzymes in *Phytophthora* through the identification and characterization of *pipg1*, the first oomycete cDNA encoding an endoPG. Preliminary analyses using DNA hybridizations and PCR analyses suggest that multiple *pipg1*-like sequences occur in the *P. infestans* genome and that endoPGs form a gene family in *P. infestans* (T. Torto and S. Kamoun, unpublished results). Future studies on the endoPG gene family of *P. infestans* should provide additional insights into the mechanisms of pathogenicity of this important and distinct group of plant pathogens and should improve our general understanding of endoPGs in interactions between plants and microbes.

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