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Characterization of 1,3-β-glucanase and 1,3;1,4-β-glucanase genes from *Phytophthora infestans*

Adele McLeod, Christine D. Smart, and William E. Fry[*]

Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA

Abstract

Three putative exo-1,3-β-glucanase genes (*Piexo1*, *Piexo2*, *Piexo3*), one endo-1,3-β-glucanase (*Piendo1*) and one endo-1,3;1,4-β-glucanase (*Piendo2*) gene were cloned and characterized from the oomycete *Phytophthora infestans*. Southern hybridization revealed that *Piexo1*, *Piexo2*, *Piexo3*, and *Piendo2* are single copy, and that *Piendo1* is encoded by two copies. Furthermore, the analyses showed that for each gene, one or two closely related gene family members were present. The genes contain no introns. Nucleotide sequence analysis of the promoter regions (200 nt upstream of ATG start codon) showed that the regions have 56–81% similarity to a 16-nt core sequence hypothesized to be the initiation of transcription point in oomycetes. The predicted molecular weights (32–83 kDa), iso-electric points (4.2–6.7) and amino acid sequences of the five proteins are diverse. All the genes are expressed in in vitro grown mycelia and sporangia, as well as during infection of potatoes. Further, *Piendo1* and *Piendo2* are also expressed in germinating cysts, and *Piendo2* in zoospores.
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1. Introduction

Glycoside hydrolases that act on 1,3-β-glucans and 1,3;1,4-β-glucans (1,3-β-glucanases and 1,3;1,4-β-glucanases) are involved in development and signaling and have been studied extensively in plants and fungi (Cappellaro et al., 1998; Cid et al., 1995; Hrmova and Fincher, 2001; Pitson et al., 1993; Simmons, 1994). In yeast, 1,3-β-glucanases have been studied for their role in germination, sporulation, mating and cell growth since they are regulated in a cell cycle dependent manner, and are differentially expressed during vegetative growth, mating and the late stages of sporulating diploids (Cappellaro et al., 1998; Cid et al., 1995; Fontaine et al., 1997; Smits et al., 2001). In plants, 1,3-β-glucanases have been characterized for their major role in plant defense, as well as for their involvement in germination, microsporogenesis and embryogenesis (Cheong et al., 2000; Helleboid et al., 1998; McCormick, 1993; Simmons, 1994; Tucker et al., 2001). The plant 1,3;1,4-β-glucanases have been studied extensively for their role in coleoptile elongation and the mobilization of storage polysaccharides (Harvey et al., 2001; Inhouhe et al., 2000; Kotake et al., 2000; Thomas et al., 2000). The study of glucanases in fungi and plants over the past decades has led to the hypothesis that their original function might have been to promote cell growth and division of unicellular organisms by turning over cell wall β-glucan (Simmons, 1994).

The large and diverse number of glycoside hydrolases has created a need for a good classification system. The system that is most useful is based on amino acid sequence similarity, first proposed by Henrissat (Henrissat, 1991). The predictive power of this system lies within the conservation of catalytic residues and 3D structure within each family (Bourne and Henrissat, 2001; Henrissat and Davies, 1997). In this system, currently containing 87 families, plant, fungal and bacterial enzymes that hydrolyze 1,3- or 1,4-β-linkages in 1,3-β-glucan and 1,3;1,4-β-glucan are classified into nine different families (Henrissat and Davies, 1997).

Although a comprehensive set of molecular data is available for plant, bacterial and fungal glucanases, no glucanases have been characterized on a molecular level

*[*] Corresponding author. Fax: +607-255-4471.
E-mail address: wef1@cornell.edu (W.E. Fry).

in oomycetes. Oomycetes are a unique group of diploid fungal-like organisms, related to chromophyte algae and other heterokont protists (Baldauf et al., 2000; Cooke et al., 2000). The Oomycete taxon is important since it contains numerous devastating plant pathogens including species of *Phytophthora*, *Pythium*, and *Peronospora* (Alexopoulos et al., 1996). In oomycetes, glucanases have been studied on a biochemical level for their possible role in hyphal tip growth and branching where there is thought to be a delicate balance between cell wall synthesis and hydrolyses (Fevre, 1997; Meyer et al., 1976; Money and Hill, 1997; Thomas and Mullins, 1969). They have also been studied for their role in Ca^{2+} induced sporulation (Du and Mullins, 1998; Du and Mullins, 1999; Holten and Bartnicki-Garcia, 1972) and their role in the host pathogen interaction (Bodenmann et al., 1985; Stossel and Hohl, 1981).

Oomycetes contain large amounts of 1,3-β-glucan that is dynamic and specific to growth stages (Bartnicki-Garcia and Wang, 1983; Faro, 1972; Shapiro and Mullins, 1997). Glucans occur in the cell wall, where 1,3-β-glucan comprises between 48–68% of the wall dry weight depending on the developmental stage and growth conditions (Bartnicki-Garcia and Wang, 1983; Wang and Bartnicki-Garcia, 1980). Although the 1,3-β-glucan is the most abundant glucan in the cell wall, some 1,4-β-glucan is also present (Bartnicki-Garcia and Wang, 1983). Soluble 1,3-β-glucans in the cytoplasm account for 16–59% of the cell dry weight, depending on the developmental stage and growth condition. This soluble glucan seems to serve as principle storage polysaccharide for sporulation and germination, and has been designated mycolaminaran since it is similar, but not identical to algal laminaran (Bartnicki-Garcia and Wang, 1983; Faro, 1972; Lee and Mullins, 1994; Wang and Bartnicki-Garcia, 1974; Wang and Bartnicki-Garcia, 1980).

Investigation of the enzymes that hydrolyze and modify 1,3-β-glucan in the cell walls and cytoplasm of oomycetes will aid our understanding of the developmental biology of these organisms. In this study we describe the cloning and characterization of five putative glucanase genes from the oomycete, *Phytophthora infestans*. Glucanases were cloned based on expressed sequence tags (EST) of putative 1,3-β-glucanases and 1,3;1,4-β-glucanases available in the Phytophthora Genome Initiative database (Kamoun et al., 1999).

2. Materials and methods

2.1. Growth and culturing of Phytophthora and Pythium

Several *Phytophthora* and *Pythium* species were used in the study. *P. infestans* strains US940480 (ATCC 208834) and SA960008 were used throughout this study. *Phytophthora palmivora* (P. Van West, University of Aberdeen), *Phytophthora sojae* (J.K.C. Rose, Cornell University), *Pythium ultimum* and *Pythium graminearum* (E.B. Nelson, Cornell University) were used in Southern blot analyses. *Phytophthora* and *Pythium* strains were routinely cultured on Rye-A (Caten and Jinks, 1968) and 20% V8 media (Miller, 1955), respectively. For DNA extractions, *Phytophthora* and *Pythium* strains were grown in Pea-broth for 14 or 5 days respectively (Goodwin et al., 1992b). Developmental stages (mycelia, sporangia, zoospores, germinating cyst, and germinating sporangia) of *P. infestans* were obtained as described by Van West et al. (1998), except that germinating sporangia and cysts were obtained by incubation in water for 24 and 2–3 h, respectively.

2.2. Cloning and characterization of glucanase genes

The polymerase chain reaction (PCR) was used to amplify the 5′ region of putative glucanase genes (*Piexo1*, *Piexo3*, *Piendo1*, and *Piendo2*). Primers were designed based on expressed sequence tags (EST) present in the Phytophthora Genome Initiative database (Table 1) (Kamoun et al., 1999). PCR was performed with 1× amplification buffer (Invitrogen, Carlsbad, CA); 100 ng DNA template; 0.2 mM dATP, dCTP, dGTP, and dTTP (each); 0.2 μM each primer; and 0.5U Taq DNA polymerase (Invitrogen) in a final reaction volume of 30 μl. Amplification consisted of: one cycle of 5 min at 94 °C, and 36 cycles of denaturing for 1 min at 94 °C; annealing for 1 min at 55–65 °C (depending on primer pair used Table 1); and extension of 30 s for each 500 bp at 72 °C. A final extension step of 5 min at 72 °C was done for one cycle. PCR primers were synthesized by the Cornell Biotech Resource Center. PCR products were cloned using the TA cloning kit (Invitrogen), sequenced, and aligned with EST sequences to insure that the correct product was cloned.

Phytophthora infestans (isolate US940480) DNA partially digested with *Sau3A* was used to construct a genomic library in λ-Fix (Stratagene, La Jolla, CA) according to manufacturers instructions. The genomic library was screened with each of the five cloned glucanase PCR products (from above) to identify positive λ phage clones using standard protocols (Sambrook et al., 1989). Individual λ phage clones were selected and DNA was extracted using the Qiagen Lambda Midi kit according to manufacture's instruction (Qiagen, Valencia, CA). λ Phage restriction digest fragments that hybridized with the probes (Table 2) were gel purified and cloned. The full length cloned fragments were sequenced in both strands by primer walking. All sequencing was done at the Cornell Biotechnology Center.

Sequence analysis was performed using the software package Lasergene ver. 5.0 (DNASTAR, Madison, WI). Signal peptides and transmembrane helixes were predicted using the Signal P and Transmembrane WWW

Table 1
Primers and annealing temperatures used for polymerase chain reaction amplification of *P. infestans* glucanase genes

Gene	Primer	Primer sequence	Temperature[a]	EST clone name[b]	Size[c]
PCR amplification of 5′ ends					
Piexo1	Pexo1F	GCGCACTAGACTCTGTAT	55	piMY016aB09r	724
	Pexo1R	GTACGGGTTGAACATCCA		piMY007aH10r	
				piMY016aB08r	
				piMY023aA05r	
				piMY016aD05r	
				piMY018aF11r	
Piexo3	Pexo3F	CCACGATGAAGTTTCTAGGT	55	pMY-10-F-03	592
	Pexo3R	GCATGAAATCCTCCATAAAC			
Piendo1	Pendo1F	GTCCGCCTCGATAAGAGTA	55	piMY022aA09r	
	Pendo1R	ACATCCACCGTAATGTCTTT			
Piendo2	Pendo2F	TTGCTTACCCGGATATCTAC	60	piMY034aE05r	300
	Pendo2R	GAGCATATTCTCCACAATCC			
PCR amplification of full length genes					
Piexo1	Gexo1F	ATGTTGGTCGGGTCCAGCCTG	65		2238
	Gexo1R	TTACGCGCGGCCGCCAATGGG			
Piexo2	Gexo2F	ATGTACACCTTCTGCACGGCA	60		1701
	Gexo2R	TCATTAGGGATCAAACGTTCA			
Piexo3	Gexo3F	ATGTCCTGCGTAGCGTCCGAC	60		1116
	Gexo3R	TTAAAGTAGTCTCATGAC			
Piendo1	Gendo1F	ATGGTGACCGTGCTGCGTACG	60		1149
	Gendo1R	TTACATGGCACAGTCGTTGTG			
Piendo2	Gendo2F	ATGAGTCAAAGGAGCAAATT	60		891
	Gendo2R	TTACACGGGGCAGTTGGT			

[a] Annealing temperatures (°C) used for PCR reactions.
[b] Expressed sequence tags (EST) were obtained from the Phytophthora Genome Initiative database (Kamoun et al., 1999).
[c] Product size (base pairs) of polymerase chain reaction.

Table 2
Characterization of cloned genomic restriction fragments containing putative glucanase genes from *P. infestans*

Gene	Restriction fragment type	Fragment size (kb)	Open reading frames[a]
Piexo1	*Hind*III	6	PiEXO2
Piexo3	*Apa*I	10	Putative kinase and amino acid permease/transporter
Piendo1	Overlapping *Eco*RI and *Xho*I	7	
Piendo2	*Xho*I	6	L-galactono-1,4-lactone dehydrogenase

[a] Open reading frames with significant BLAST search similarities present on the cloned fragment.

server of the Center for Biological Sequence (http://www.cbs.dtu.dk/), as well as PSORT (http://psort.nibb.ac.jp) that also predicts vacuolar, mitochondrial, endoplasmic reticulum, nuclear and peroxisome localizations (Nakai and Kanehisa, 1992).

2.3. Phylogenetic analyses

Two phylogenetic trees were constructed, one containing proteins from glycoside hydrolase (GH) family 5 and one containing proteins from GH family 17 (Bourne and Henrissat, 2001; Henrissat and Davies, 1997). Accession numbers corresponding to the sequences used for construction of the phylogenies are presented in Table 3.

The Clustal X program (Thomas et al., 1997) was used to align sequences using the GONNET weight matrices. Phylogenetic trees were constructed using the built-in distance based NJ (Neighbour-Joining) algorithm. Phylogenetic trees were calculated using the full length amino acid sequence of the proteins, with the "exclude positions with gaps" function activated (alignment positions where any of the sequences had a gap were ignored) (Thomas et al., 1997). Branch support was determined by bootstrap analyses calculated using 1000 replicates. Trees were drawn using TREEVIEW (Page, 1996).

2.4. Southern and Northern blot analyses

Nucleic acids for Southern and Northern blot analyses were extracted from *Phytophthora*, *Pythium* and potato tissue. DNA was isolated from *Phytophthora* and *Pythium* as previously described (Goodwin et al., 1992a). RNA from the different *P. infestans* developmental stages was extracted with the RNeasy Plant

Table 3
Organism, gene abbreviations and accession numbers of sequences used for phylogenetic analyses of glycoside hydrolase family 5 and 17

Organism	Protein abbreviation	Accession number	Function/putative function
Glycoside hydrolase family 5			
Erwinia chrysanthemi	CELZ	Y00540	endo-1,4-β-glucanase
Bacillus sp.	CELA	M14781	endo-1,4-β-glucanase
Acidothermus cellulolyticus	EI	U33212	endo-1,4-β-glucanase
Cryptococcus flavus CMC1	CMC1	D13967	endo-1,4-β-glucanase
Humicola insolens	CMC3	X76046	endo-1,4-β-glucanase
Trichoderma reesei	EGL2	M19373	endo-1,4-β-glucanase
Saccharomyces cerevisiae	SPR1	S52935	exo-1,3-β-glucanase
Yarrowia lipolytica	EXG1	Z46872	exo-1,3-β-glucanase
Paracoccidioides brasiliensis	GP43	U26160	exo-1,3-β-glucanase
Candida albicans	XOG1	X56556	exo-1,3-β-glucanase
S. cerevisiae	EXG1	M34341	exo-1,3-β-glucanase
S. cerevisiae	EXG2	Z46870	exo-1,3-β-glucanase
Cochliobolus carbodum	EXG2	AF229446	exo-1,3-β-glucanase
Agaricus bisporus		S72325	exo-1,3-β-glucanase
Debaryomyces occidentalis	EXG1	Z46871	exo-1,3-β-glucanase
Glycoside hydrolase family 17			
S. cerevisiae	BGL2	M31072	endo-1,3-β-glucanase/glucanosyltransferase
Candida albicans	BGL2	U12975	endo-1,3-β-glucanase/glucanosyltransferase
Aspergillus fumigatus	BGT1	AF038596	endo-1,3-β-glucanase/glucanosyltransferase
Pichia jadinii	BGL2	AF091241	glucanosyltransferase
S. cerevisiae	SCW10	Z49212	cell wall protein
S. cerevisiae	SCW11	Z72550	cell wall protein
S. cerevisiae	SCW4	Z73064	cell wall protein
Triticum aestivum	YPR2	Z22873	1,3;1,4-β-glucanase
Oryza sativa	GNS1	AF323610	1,3;1,4-β-glucanase
H. vulgare	EII	M13237	1,3;1,4-β-glucanase
Solanum tuberosum	GLUB2	U01901	endo-1,3-glucanase
Glyxine max	SGN1	U41323	endo-1,3-glucanase
Nicotiana tabacum	SP41B	X54431	endo-1,3-glucanase
Hordeum vulgare	GI	M96938	endo-1,3-glucanase

Mini-kit (Qiagen) after lyophilization and grinding of the tissue in liquid nitrogen. The purity of the different developmental stages was examined microscopically to ensure that at least 95% of the preparation contained the desired developmental stage. The germinating sporangia developmental stage consisted of approximately 60–70% germinated sporangia. The expression pattern of the genes was determined by extracting RNA from each of two independent experiments using isolate SA960008.

RNA for analyses of *P. infestans* glucanase gene expression *in planta*, were obtained from six-week-old potato plants (cultivar Kathadin) inoculated with *P. infestans* strain US940480 and from plants inoculated with strain SA960008. The plants were spray inoculated until run-off with *P. infestans* inoculum that was obtained as previously described (Mayton et al., 2001). The plants were placed in a humid chamber with 16 h light at 99% relative humidity (9 h/day). At each time point (1–6 days), one plant for each isolate-plant combination was removed and the leaf tissue harvested. Leaf tissue was also collected from healthy plants before inoculation to determine if the *P. infestans* glucanases would cross hybridize to potato transcripts during Northern blot analyses. RNA from infected and healthy potato plants was isolated using a hot phenol method (Perry and Francki, 1992).

Northern and Southern blot analyses were performed according to standard protocols (Sambrook et al., 1989). Gels used for Southern blot analyses were loaded with 2.5 μg DNA per lane and Northern blot analysis gels were loaded with 10–20 μg RNA per lane. Probes used for hybridization consisted of PCR products of the full length clones of each of the respective genes (Table 1), unless otherwise specified. Membranes were washed at high stringency (20 min in 2× SSC/0.1% SDS, followed by 10 min in 1× SSC/0.1% SDS and 10 min in 0.1× SSC/0.1% SDS at 65 °C). Southern blot analyses were done at high stringency to determine the number of highly homologous gene copies present for each gene.

2.5. RT-PCR

Transcript sizes of the full-length genes were determined by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from *P. infestans* (isolate US940480) mycelia using a hot phenol method (Perry and Francki, 1992). Poly(A)+ RNA was isolated from total RNA using the Oligotex m-RNA

Mini kit (Qiagen). The reverse transcriptase reaction that was incubated for 1 h at 42 °C consisted of m-RNA (200 ng), Superscript II RNase H-Reverse Transcriptase (1 U), random primers (150 ng), dNTPs (10 mM each), RNAseOut (1 U) and 1× first strand buffer (Invitrogen) in a total volume of 20 µl. PCR was done as described above, using the reverse transcriptase reaction at a 10% v/v concentration, and primers and annealing temperatures presented in Table 1. A control reaction without Superscript was included to confirm that no amplification was obtained due to DNA contamination in samples.

3. Results

3.1. Cloning and characterization of glucanase genes

Five putative glucanase genes were cloned from the *P. infestans* genomic library. The PCR product sizes amplified from the 5′ region of the genes are presented in Table 1. For each of the five putative glucanase genes one positive λ phage clone was identified and purified. The restriction fragment type and the size of the fragments that were sub-cloned and sequenced from each relevant λ phage for each glucanase gene, are presented in Table 2. Some of the cloned fragments also contained other open reading frames with significant BLAST similarity scores to other genes (Table 2). *Piexo1* and *Piexo2* were present in tandem on a 6 kb *Hind*III fragment, with the stop codon of *Piexo2* being 970 bp upstream of the start codon of *Piexo1* (Table 2). The full length sequences of *Piexo1*, *Piexo2*, *Piexo3*, *Piendo1*, and *Piendo2* were determined, as well as 350–1000 bp upstream and downstream sequence for each gene. These sequences have been submitted to Genbank under accession numbers AF494013 (*Piendo1*), AF494014

(*Piexo1*), AF494015 (*Piexo3*), AF494016 (*Piexo2*) and AF494017 (*Piendo2*).

The predicted structural features of the proteins of the cloned genes are presented in Table 4. Similar signal peptide cleavage sites and transmembrane helixes were predicted for the genes using two different algorithms (Table 4). According to the BLASTX search program PiEXO1 and PiEXO2 have highest homology to an exo-1,3-β-glucanase of *Candida albicans* (CaEXG1) (E-value $= 10^{-41}$ and 10^{-36}, respectively) and PiEXO3 has highest homology to an exo-1,3-β-glucanase of the yeast *Yarrowia lipolytica* (E-value $= 10^{-33}$). PiENDO1 has highest homology to an endo-1,3-β-glucanase of *Saccharomyces cerevisiae* (BGL2) (E-value $= 10^{-23}$). PiENDO2 has low homology (E-value $= 10^{-7}$) to the amino acid sequence of a 1,3;1,4-β-glucanase purified from grass coleoptiles (Thomas et al., 2000).

Sequence analyses of *P. infestans* exo-1,3-β-glucanases (PiEXO1, PiEXO2, PiEXO3) showed that they are related to proteins of GH family 5 (Fig. 1). The *P. infestans* exo-glucanases all contain the eight conserved residues surrounding the catalytic active site of proteins from GH family 5, including the two catalytic active residues (Sakon et al., 1996). The conserved cellulase domain of GH family 5 was also identified in all three *P. infestans* exo-1,3-β-glucanases (E-values ranging between 10^{-5} and 10^{-14}) using reverse position specific (RPS)-BLAST 2.2.1 (Altschul et al., 1997).

Sequence analyses of the *P. infestans* endo-1,3-β-glucanase (PiENDO1) showed that this glucanase was closely related to glycosyl hydrolases classified into GH family 17 (Fig. 2). Alignment of PiENDO1 with several proteins from GH family 17 showed that the protein contained 11 of the 15 residues, including the two catalytic active glutamic acid residues, previously found to be conserved in plant endo-glucanases from GH family

Table 4
Predicted characteristics of 1,3-β-glucanases from *P. infestans*

Protein	aa[a]	Molecular weight (kDa)	pI[b]	Center for biological sequence prediction server[c]	PSORT prediction server[d]	N-glycosylation[e]
PiEXO1	745	82.99	6.13	Signal peptide (SP) cleavage site aa 27 and transmembrane helix (TMH) between aa 705 and 727	SP cleavage site aa 27 and TMH between aa 659 and 730	Three
PiEXO2	566	63.52	5.85	TMH between aa 524 and 546	TMH between aa 523 and 547 and peroxisome-target-signal (PTS) aa 499	Two
PiEXO3	371	43.20	6.73	None	PTS aa 11	None
PiENDO1	382	40.61	4.22	SP cleavage site aa 27	SP cleavage site aa 23	Three
PiENDO2	296	32.27	6.76	None	PTS aa 13	Three

[a] Number of amino acids (aa).
[b] Iso-electric point.
[c] N-terminal signal sequence and transmembrane helix predicted by the Signal P and transmembrane WWW server of the Center for Biological Sequence (http://www.cbs.dtu.dk/).
[d] Peroxisomal-target sequences (PTS), transmembrane helix (TMH) and N-terminal signal sequence (SP) as predicted by the PSORT algorithm (http://psort.nibb.ac.jp).
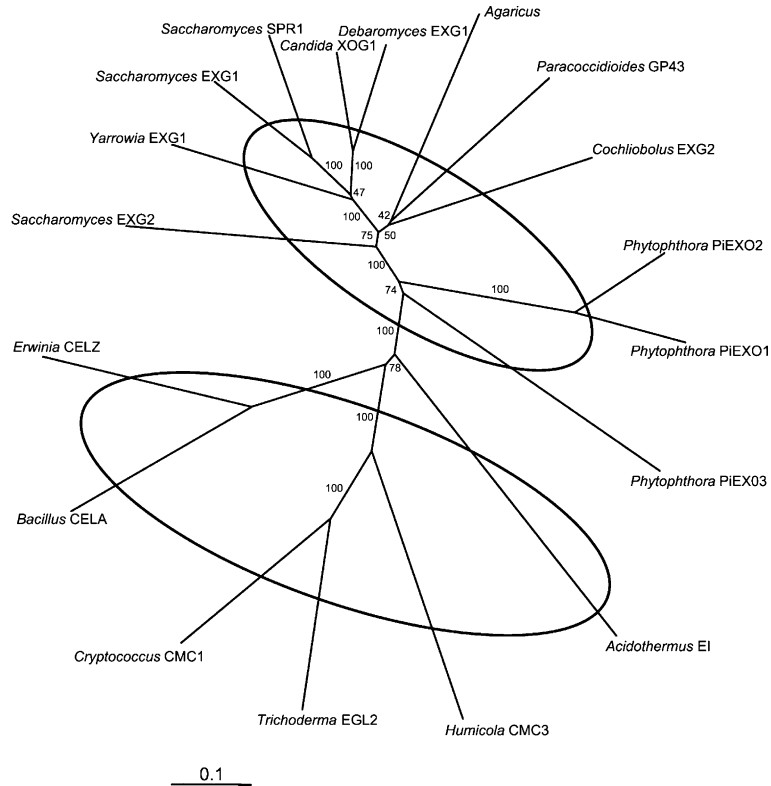[e] Number of predicted N-glycosylation sites.

Fig. 1. Phylogram of glycoside hydrolase family 5 endo-1,4-β-glucanases, exo-1,3-β-glucanases and three putative *P. infestans* exo-1,3-β-glucanases, obtained by the neighbour-joining method. The numbers on branches indicate the percentages of bootstrap values (based on 1000 bootstraps). The two main clades of the tree are encircled.
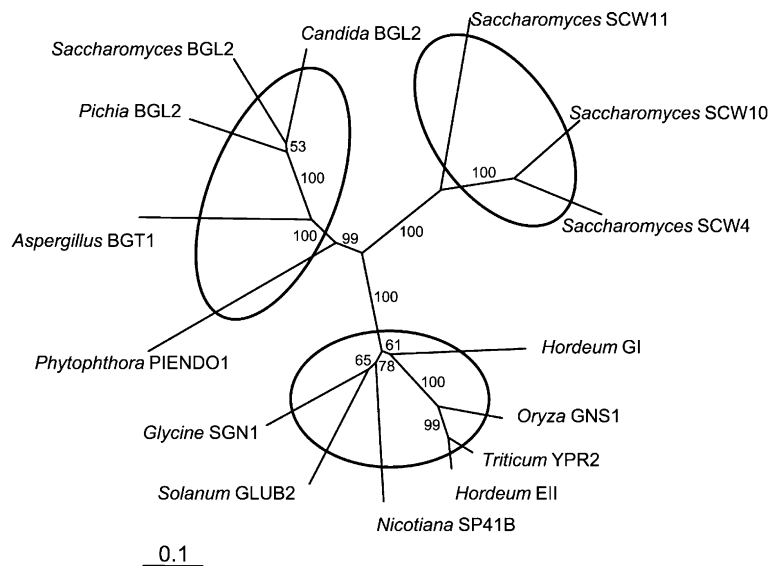

Fig. 2. Phylogram of glycoside hydrolase family 17 fungal endo-1,3-β-glucanases and plant endo-1,3-β- and 1,3;1,4-β-glucanases, as well as a putative endo-1,3-β-glucanase from *P. infestans*. The phylogram was constructed using the neighbour-joining method. The numbers at the branch points indicate the percentages of bootstrap values (based on 1000 bootstraps). The three main clades of the tree are encircled.

17 (Chen et al., 1993; Hoj and Fincher, 1995; Varghese et al., 1994). The RPS-BLAST program also identified a domain characteristic of GH family 17 in PiENDO1 (*E*-value = 0.007).

PiENDO2 as well as the maize 1,3;1,4-β-glucanase, with which it has highest homology, belong to an unspecified family of glycosyl hydrolases (Thomas et al., 2000). Hence, the RPS-BLAST program was unable to

identify any of the known glycosyl hydrolase consensus sequences in the genes. However, a dienelactone domain characteristics of the dienelactone hydrolase family was identified in both the *P. infestans* (E-value $= 10^{-10}$) and maize (E-value $= 10^{-9}$) endo-glucanases.

The upstream regions of the glucanase genes (hereafter referred to as promoter regions) were searched to determine if a previously described conserved oomycete promoter sequence (GCTCATTYYNCAWTT) was present (Pieterse et al., 1994). Each gene promoter had partial homology to this sequence. The homology ranged from a high (81%) for *Piexo1* to a low (56%) for *Piexo2* (Table 5).

3.2. Phylogenetic analyses

The phylogram of proteins from GH family 5 obtained by the NJ method is shown in Fig. 1. The tree divided the proteins into two major clades, one containing all the exo-1,3-β-glucanases, including the putative *P. infestans* glucanases, and the other the endo-1,4-β-glucanases (bacteria and fungi) of GH family 5. However, there was some branch support for grouping *P. infestans* exo-glucanases separate from the fungal exo-glucanases. The phylogeny also showed that *Piexo3* was distinct from *Piexo1* and *Piexo2* and from the fungal exo-glucanases (Fig. 1).

The phylogram constructed of proteins of GH family 17 is shown in Fig. 2. The tree divided the proteins into three main clades (Fig. 2). One clade, clearly separate from all the fungal glucanases and *P. infestans* glucanase, included all the plant glucanases. The second clade included the *P. infestans* endo-glucanase and the fungal cell wall localized glucanase/glucanosyltransferase homologues (BGL2 and BGT1). The third clade included all the yeast cell wall proteins (SCW homologues) involved mainly in the mating interaction (Cappellaro et al., 1998).

3.3. Gene copy number and orthologues in P. palmivora, P. sojae, P. ultimum, and P. graminearum

Four of the five genes (*Piexo1*, *Piexo2*, *Piexo3*, and *Piendo2*) are present in single copy in the *P. infestans* (strains SA960008 and US940480) genome. There are two copies of *Piendo1*. When *P. infestans* DNA was digested with enzymes that do not cut within the genes, there was only one band in Southern blot analysis. However, when this DNA was digested with enzymes with a restriction site in the gene (*Sst*I for *Piexo1*, *Eco*RI for *Piexo2*, and *Kpn*I for *Piendo2*), there were two bands in Southern blot analysis (Figs. 3a, b, and e). Southern hybridization with the N-terminal fragments of *Piexo3* showed that the gene is present as a single copy, since *Piexo3* hybridized to only one band when the DNA was digested with *Hin*dIII (data not shown). However, when hybridization was done with the full length gene of *Piexo3* three bands were revealed (Fig. 3c), thus indicating the presence of a second gene highly homologous to *Piexo3*. The presence of one or two weak bands in Southern blot analyses of all five genes indicates the presence of related family members (Fig. 3).

Piendo1 is present as two copies. This can be deduced from the *Xho*I and *Hin*dIII restriction hybridization patterns with the full length gene (Fig. 3d) and N-terminus fragment probe (data not shown). *Piendo1* contains one *Xho*I restriction site and no *Hin*dIII restriction site. Southern hybridization using a third *P. infestans* isolate (1306) (kindly provided by H.S. Judelson, University of California, Riverside) confirmed the presence of two copies of *Piendo1* and a highly homologous copy of *Piexo3* (data not shown).

All five cloned *P. infestans* glucanase genes have orthologues in *P. palmivora* and *P. sojae* and some also have orthologues in *Pythium* spp. (Fig. 3). *Piexo1*,

Table 5
Alignment of the 5′ upstream region of *P. infestans* glucanase genes to a conserved sequence GCTCATTYYNCAWTT found in the promoter region of oomycete genes (Pieterse et al., 1994)

Gene[a]	% Sim[b]	nt Sequence with highest similarity to the conserved sequence[c]																
			G	C	T	C	A	T	T	Y	Y	N	C	A	W	T	T	
P. megasperma actin	88	−61	C	G	T	C	A	T	T	C	C	G	C	A	A	T	T	T	−45
Piexo1	81	−73	T	C	T	C	A	T	T	T	C	C	G	C	A	T	T	T	−58
Piexo2	56	−152	C	T	C	C	A	T	T	C	A	A	C	A	A	G	C	A	−137
Piexo3	75	−44	G	C	T	C	A	C	T	T	T	G	A	A	A	C	T	C	−29
Piendo1	81	−83	G	G	T	C	A	T	T	T	C	C	C	A	A	A	C	T	−68
Piendo2	62	−70	G	C	C	C	A	T	A	G	T	G	A	C	A	G	T	T	−55
P. cryptogea elicitin	81		G	C	T	C	A	C	T	C	C	A	C	A	A	C	T	A	
P. cinnamomi pyruvate	81	−36	G	C	T	C	A	T	T	C	C	T	C	A	A	C	T	A	−51
P. infestans ipiO1	81	−31	G	C	T	C	A	T	T	T	G	T	G	A	A	T	T	C	−16

[a] Phytophthora genes *Piexo1*, *Piexo2*, *Piexo3*, *Piendo1*, *Piendo2* (the manuscript), *P. cryptogea* elicitin (Panabieres et al., 1995), *P. infestans* ipiO1 (Pieterse et al., 1994), *P. cinnamomi* pyruvate phosphate dikinase (Marshall et al., 2001) and *P. megasperma* actin (Dudler, 1990).

[b] The promoter region of each glucanase gene was searched upstream of the ATG for highest similarity to the consensus sequence GCTCATTYYNCAWTT (where Y = C or T; N = any nucleotide; W = A or T).

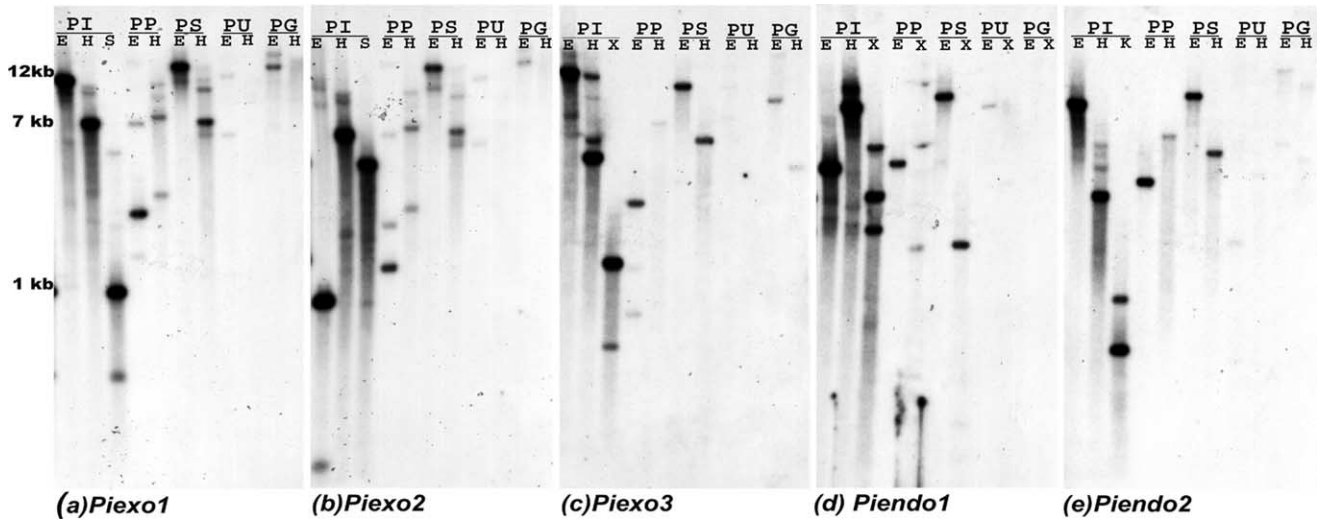[c] Nucleotides that are similar to the consensus sequence are shaded.

Fig. 3. Autoradiographs of Southern blots containing 2.5 μg genomic DNA of *P. infestans* isolate US940480 (PI), *P. sojae* (PS), *P. palmivora* (PP), *P. ultimum* (PU), and *P. graminearum* (PG). Southern blots were hybridized and washed at high stringency with the full length gene sequence of *Piexo1* (a), *Piexo2* (b), *Piexo3* (c), *Piendo1* (d), and *Piendo2* (e). Genomic DNA was digested with different restriction enzymes (*Eco*RI (E), *Hin*dIII (H), *Sst*I (S), *Xho*I (X) or *Kpn*I (K)).

Piexo2, and *Piendo2* also have orthologues in *P. ultimum* and *P. graminearum* (Figs. 3a, b, and e). *Piexo3* has an orthologue in *P. graminearium* and *Piendo1* an orthologue in *P. ultimum* (Figs. 3c and d).

3.4. RT-PCR

RT-PCR analysis using mycelial RNA, showed that amplification products from the transcripts of the five genes are the same size as the open reading frames identified in the cloned genomic DNA fragments. Primers complementary to the 5′ and 3′ regions of the genes were used (Fig. 4).

3.5. Expression analysis

Phytophthora infestans exo-1,3-β-glucanases, *Piexo1* and *Piexo3*, are expressed in in vitro grown mycelia, sporangia and germinated sporangia (Figs. 5a and c). The germinated sporangia consisted mainly of young hyphal tips. *Piexo2* is expressed at a low level in mycelia, sporangia and in zoospores (Fig. 5b). There were two different transcript sizes detected in blots hybridized with the *Piexo3* probe, a transcript size of approximately 1.30 kb in mycelia and 1.6 kb transcript in sporangia (Fig. 5c). The same expression pattern was seen for *Piexo3* whether the N-terminal fragment (data not shown) or full length sequence (Fig. 5c) of the gene was used as a probe.

Northern blot analysis of the *P. infestans* endo-1,3-(*Piendo1*) and endo-1,3;1,4-β-glucanase (*Piendo2*) genes in vitro showed that they are expressed in mycelia, sporangia, germinating cyst and germinating sporangia (Figs. 5d and e). *Piendo2* is also expressed in zoospores (Fig. 5e).

Northern blot analyses of expression of the glucanase genes during infection of potato plants showed that they are all expressed *in planta* (Fig. 6). Infection of potato plants with US940480 (Fig. 6) showed the same pattern of gene expression as did infection with SA960008 (data not shown). High levels of *in planta* expression can be seen starting at 4 days post inoculation for *Piexo3*, *Piendo1*, and *Piendo2* (Figs. 6c–e). High levels of expression of *Piexo1* can already be seen 3 days post inoculation (Fig. 6a). A very low level of expression of *Piexo2* can be seen starting 4 days post inoculation (Fig. 6b).

4. Discussion

We have cloned and characterized the full length sequence, as well as up-stream regions of five glucanase genes from the oomycete *P. infestans*. The structurally diverse glucanases are differentially expressed during the life cycle of the pathogen in vitro, and are also expressed during host infection. The five glucanases belong to three different glycoside hydrolase families and have highest homology to fungal genes, except for *Piendo2*.

The homology of the *P. infestans* glucanase genes to fungal genes is unexpected since ribosomal phylogenies and several biochemical studies have shown a closer evolutionary relationship between oomycetes and plants, than oomycetes and fungi (Baldauf et al., 2000; Cavalier-Smith, 2000; Cooke et al., 2000). However, we believe that the phylogenic relatedness of *P. infestans* glucanases to fungal glucanases (Figs. 1 and 2) is evidence of convergent evolution of enzymes with the same
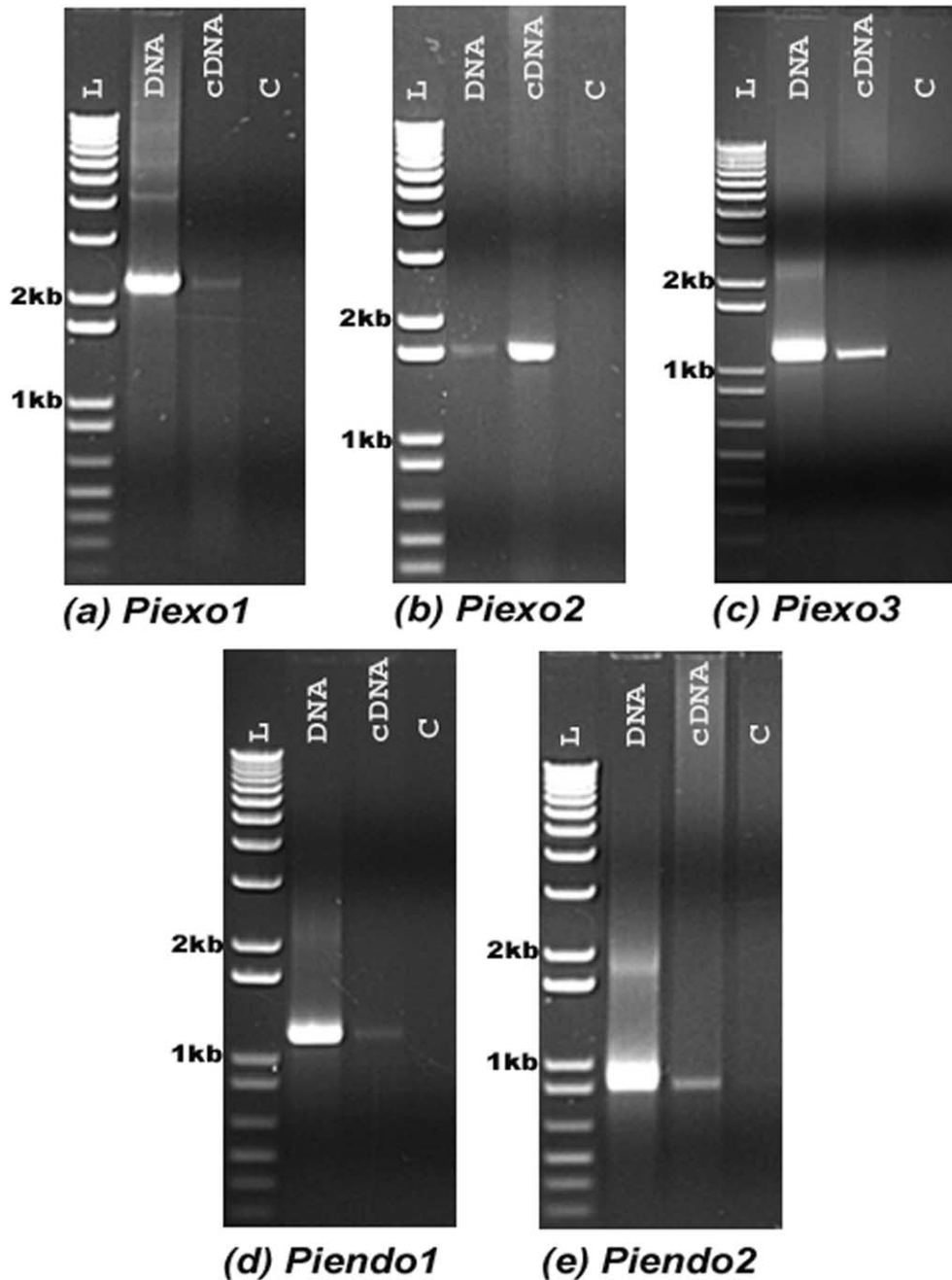
Fig. 4. RT-PCR analysis of *Piexo1*, *Piexo2*, *Piexo3*, *Piendo1*, and *Piendo2* from mycelia of *P. infestans* using primers complementary to the 5′ and 3′ termini of the genes. PCR reactions were done using as template: DNA of the cloned genomic fragments (DNA); in vitro transcribed m-RNA from *P. infestans* mycelia (cDNA); and m-RNA from *P. infestans* mycelia in an in vitro transcribed reaction without Superscript Reverse transcriptase (C). A molecular marker (L) was included in each analysis.

substrate specificity and/or function, rather than an indication of evolutionary relationships. This is further supported by the fact that a *P. infestans* β-glucosidase from GH family 30 also did not have highest homology to plant genes, but had homology to human and mouse glycoside hydrolases (Brunner et al., 2002). Furthermore, the current under representation of glycoside hydrolases cloned from oomycetes and related organisms, also contribute to phylogenies that do not represent evolutionary relationships.

The *P. infestans* exo-1,3-β-glucanase genes all contain the conserved domain and residues of GH family 5, and are clearly distinct from the endo-1,4-β-glucanases of GH family 5 (Fig. 1). The *P. infestans* exo-1,3-β-glucanases are single copy as is true in fungi (Esteban and Aldana, 1999a,b; Morais et al., 2000), although there is
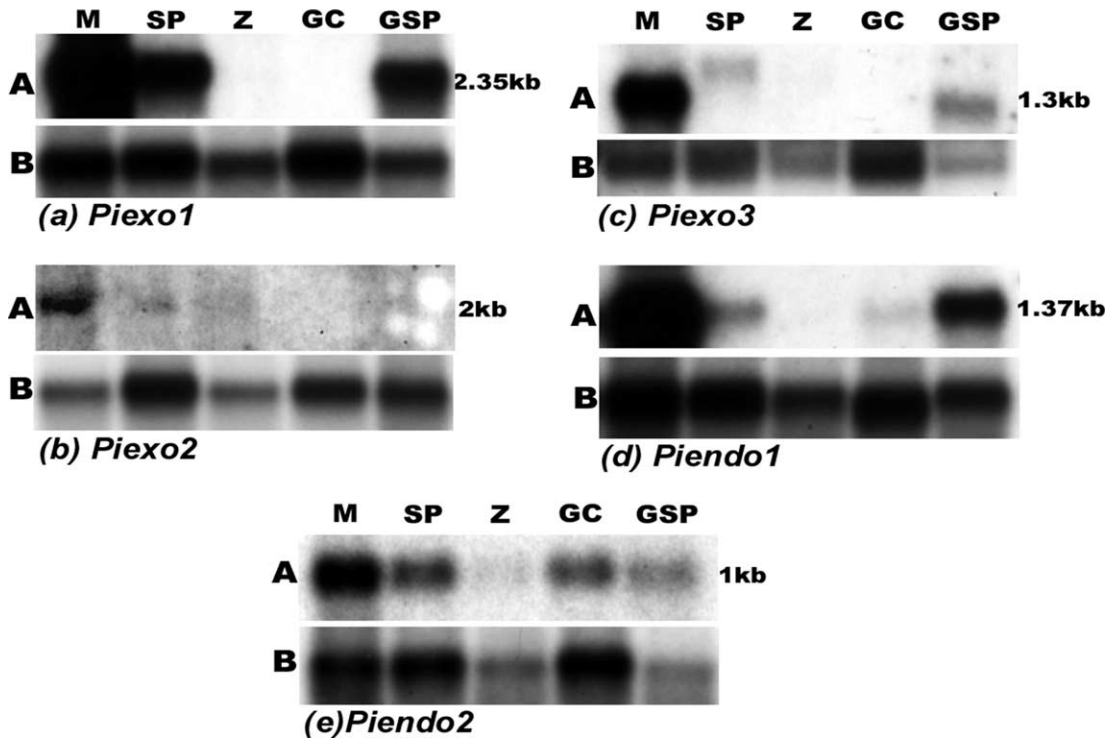
Fig. 5. Expression of *Piexo1*, *Piexo2*, *Piexo3*, *Piendo1*, and *Piendo2* in different in vitro developmental stage of *P. infestans*. (A) Autoradiographs of Northern blots containing 10 μg total RNA from *P. infestans* (SA960008) mycelia (M), sporangia (SP), zoospore/cysts (Z), germinating cysts (GC) and germinating sporangia (GSP). The membranes were hybridized with the full length gene sequences of *Piexo1* (a), *Piexo2* (b), *Piexo3* (c), *Piendo1* (d), and *Piendo2* (e). (B) Autoradiographs of A were stripped and probed with rDNA to show equal loading of samples.
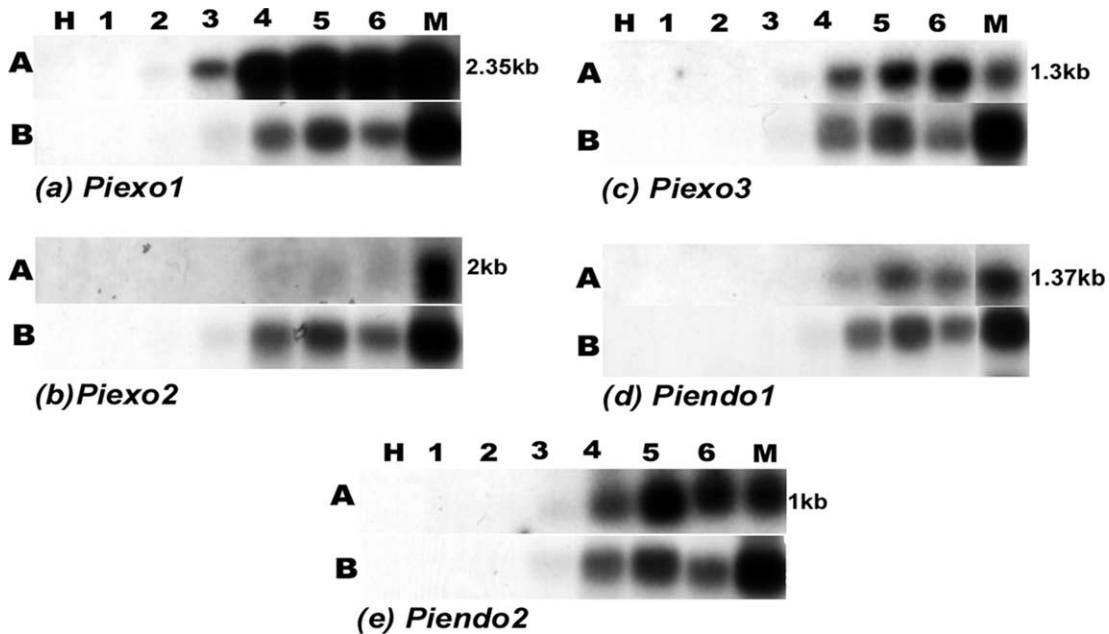

Fig. 6. Expression of *Piexo1*, *Piexo2*, *Piexo3*, *Piendo1*, and *Piendo2* during growth of *P. infestans* in potatoes. (A) Autoradiographs of Northern blots containing 20 μg total RNA isolated from uninoculated potato leaves (H) and potato leaves infected with *P. infestans* 1–6 days post inoculation. Total RNA (20 μg) of in vitro grown *P. infestans* mycelia (M) was also included in the Northern blots. The membranes were hybridized with the full length gene sequences of *Piexo1* (a), *Piexo2* (b), *Piexo3* (c), *Piendo1* (d), and *Piendo2* (e). (B) Autoradiographs of A were stripped and probed with *P. infestans* rDNA.

evidence of related family members in *P. infestans* (Fig. 3). Similar to yeast glucanases, the *P. infestans* glucanases lack introns, a trait characteristic of most oomycete

genes (Cid et al., 1995; Pieterse et al., 1994). The yeast and *P. infestans* exo-1,3-β-glucanases are characterized by diverse amino acid sequence homologies (Fig. 1), as

well as diverse molecular weights and structural features including signal peptides, N-glycosylation sites and membrane spanning/attachment regions (Table 4) (Esteban and Aldana, 1999a; Muthukumar et al., 1993).

The *P. infestans* endo-1,3-β-glucanase (PiENDO1) is closely related to glycoside hydrolases of family 17 and also contain the conserved domain of this family, although with low homology. Interestingly, PiENDO1 is more related to the major cell-wall-associated endo-1,3-β-glucanses/glucanosyltransferases from yeast and *Aspergillus* in GH family 17, than to the yeast cell wall proteins (SCW) involved in the mating interaction (Fig. 2). The *P. infestans* endo-glucanase contains no introns, whereas plant glucanases contain multiple introns (Cheong et al., 2000; Jin et al., 1999; Romero et al., 1998).

The *P. infestans* putative 1,3;1,4-β-glucanase (PiENDO2) is the only glucanase gene that is more closely related to plant genes than to fungal genes. PiENDO2 has very low homology to a 1,3;1,4-β-glucanase from maize which belongs to a new, as yet unspecified family of glycoside hydrolases (Thomas et al., 2000). The maize protein, may be involved in plant growth, because it is expressed in coleoptile cell walls and hydrolyzes β-1,4-linkages of several substrates including 1,3;1,4-β-glucan, xylo-β-glucan and carboxymethylcellulose (Hatfield and Nevins, 1987; Thomas et al., 2000). This protein, as well as PiENDO2, contains a region with homology to dienelactone hydrolase. In microbes, this enzyme hydrolyzes dienelactone to maleylacetate and also has esterase activity towards other substrates (Pathak et al., 1991). The function of dienelactone domains in higher organisms has not been described, but the domain has been detected in a protein from *Arabidopsis* (ATHCOR1). ATHCOR1 is thought to belong to a family of enzymes involved in the biosynthesis/hydrolysis of plant cell wall components (Benedetti et al., 1998). The dienelactone hydrolase domain in PiENDO2 suggests that it could function in cell wall metabolism.

The *P. infestans* glucanase genes are highly conserved in two other *Phytophthora* species and some are also conserved in *Pythium* species (Fig. 3). This suggests that, as expected, glucanases are highly conserved proteins in oomycetes. However, it is interesting to note that a recently cloned *P. infestans* β-glucosidase/xylosidase did not have a homologue in *P. ultimum* (Brunner et al., 2002).

Our current knowledge of sequences required for gene expression in oomycetes is limited (Judelson et al., 1992). It has been established that oomycetes do not contain the TATAAA and CAAT motifs found in higher eukaryotes, or the motifs found in filamentous fungi involved in initiation of transcription (Pieterse et al., 1994). However, a putative conserved 16 nt core sequence (GCTCATTYYNCAWTTT) possibly used as an initiation of transcription point in oomycetes has been identified (Pieterse et al., 1994). Recently, homology to this conserved sequence has been identified in the upstream region of several cloned *Phytophthora* genes (Marshall et al., 2001; Panabieres et al., 1995). Analyses of the upstream region of all the *P. infestans* glucanase genes revealed regions with 56–81% similarity to this sequence (Table 5).

Putative cellular locations of *P. infestans* glucanase genes could be deduced from their predicted structural features (signal peptides, transmembrane helixes, and glycosylation sites). The presence of predicted signal peptides in PiEXO1 and PiENDO1 could result in transportation of the enzymes from the cytoplasm to plasma membranes (PiEXO1) or the extra-cellular and periplasmic space. The absence of signal peptides, and presence of peroxisome-targeting signals (PTSs) in PiEXO2, PiEXO3, and PiENDO2 (Table 4) leads to the conclusion that these proteins could possibly function in the cytoplasm within microbodies (peroxisomes). All three genes contain the A(R/H)L tripeptide characteristic of PTS1 signals (Gould et al., 1990; Johnson and Olsen, 2001). However, the significance of these signals needs further investigation since the tripeptide is conventionally located at the C-terminal in other eukaryotes, whereas PiEXO3 and PiENDO2 contain the consensus at the N-terminus. The presence of several predicted N-glycosylation sites in the proteins could lead to the formation of different isozymes with different functions and locations in development (Cid et al., 1995).

The *P. infestans* glucanases are developmentally regulated (Fig. 5), as is also true in plants and fungi (Cid et al., 1995; Simmons, 1994). They all have highest expression in mycelia indicating a possible role in nutrition or hyphal growth and branching. They were also all expressed in young hyphae (germinating sporangia) and some in germinating cysts, indicating a possible role in hyphal tip growth. The expression of all of the glucanases in sporangia (Fig. 5) can be expected, since glucanases would be required for the breakdown of the β-glucan rich sporangial wall (45% dry weight) during germination events, and because glucanases are required for the mobilization of the energy rich storage mycolaminarin (20% of the sporangial dry weight) during germination and survival (Wang and Bartnicki-Garcia, 1980). The lack of expression of most of the glucanases in zoospores would suggest that they are not required in this stage (Fig. 5). However, since zoospores are viewed as preprogrammed cells (Erwin et al., 1983), the glucanases could be transcribed and translated in the sporangium, organized into zoospores and have a function such as energy release. The glucanase genes are also expressed in the later stages of *P. infestans* host infection, indicating a possible role in nutrition or growth during pathogenesis (Fig. 6).

An interesting finding was that *Piexo3* had a larger transcript size in sporangia compared to mycelia

(Fig. 5c). The first possibility would be that this difference in transcript size is due to cross-hybridization to a homologous copy of *Piexo3* identified in Southern blot analyses using a full length gene probe (Fig. 3c) vs. a N-terminal probe (data not shown). However, these probes showed the same hybridization pattern in Northern blot analyses. Consequently, we were unable to determine if this difference in transcript size was due to cross hybridization to a homologous gene, or due to tissue-specific differential transcript processing. In barley (*Hordeum vulgare*) tissue-specific processing of a 1,3-β-glucanase transcripts at separate polyadenylation sites has been implicated as the cause of different transcript sizes of two mRNAs originating from the same gene. The functional significance of tissue specific polyadenylation sites has not been determined (Xu et al., 1994).

The role of the cloned *P. infestans* glucanases will be investigated further. One approach is via gene silencing, however this approach might not be informative because redundancy of these proteins is likely (Esteban and Aldana, 1999a; Esteban et al., 1999b; Kokani and Mrsa, 2001; Mouyna et al., 1998). Another approach is to determine the substrate specifications and localization of the cloned genes. We believe that understanding glucanases in *P. infestans* will help contribute to our understanding of the basic biology of oomycetes.

Acknowledgments

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