Characterization and Evolutionary Analysis of a Large Polygalacturonase Gene Family in the Oomycete Plant Pathogen *Phytophthora cinnamomi*

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Polygalacturonases (PGs) are secreted by fungal pathogens during saprophytic and parasitic growth, and their degradation of pectin in the plant cell wall is believed to play a major role in tissue invasion and maceration. In this study, PG activity was demonstrated in culture filtrates of the oomycete plant pathogen, *Phytophthora cinnamomi*. A *P. cinnamomi* *pg* gene fragment amplified using degenerate primers based on conserved regions in fungal and plant PGs was used to isolate 17 complete *P. cinnamomi* *pg* genes and pseudogenes from a genomic library and partial sequence for another two genes. Gel blotting of genomic DNA indicated that there may be even more *pg* genes in the *P. cinnamomi* genome. *P. cinnamomi* *pg* gene sequences were expressed in PG-deficient yeast and found to confer PG activity, thereby confirming their functional identity. The predicted mature *P. cinnamomi* PGs fall into subgroups that exhibit large differences in the extent of *N*-glycosylation, isoelectric points, and *N*- and *C*-terminal structure. Evidence for birth-and-death and reticulate evolution in the *P. cinnamomi* *pg* gene family was obtained, and some codons for surface exposed residues in the *P. cinnamomi* PGs were shown to have been subject to diversifying selection. Contrary to accepted phylogenies for other proteins, phylogenetic analysis of the *P. cinnamomi* PGs revealed a closer relationship with PGs from true fungi than with those from plants.

Plant cell walls are a key factor in plant-pathogen interactions. They can constitute barriers to penetration and colonization by the pathogen, and they are also involved in defense responses of the host plant (Esquerré-Tugayé et al. 2000). In the primary cell wall, cellulose microfibrils are noncovalently bound to hemicelluloses, and the cellulose-hemicellulose network is embedded in a pectin gel (Carpita and Gibeau 1993). Pectin is also the main component of the middle lamella, which holds neighboring plant cells together. Filamentous plant pathogens penetrate host cell walls through enzymatic action and mechanical pressure (Mendgen and Deising 1993). Most research on cell wall-degrading enzymes (CWDE) produced by fungal pathogens has concentrated on pectinases (Annis and Goodwin 1997). Enzymatic depolymerization of pectin weakens the cell wall, exposes other cell wall components to degradative enzymes, and provides a carbon source for the pathogen. Pectinases are the only CWDE capable of tissue

maceration by disrupting the middle lamella and are often the first CWDE secreted by invading plant pathogenic fungi (Cooper 1983).

Endopolygalacturonases (endoPGs; EC 3.2.1.15) and exoPGs (EC 3.2.1.67) are pectinases that degrade the galacturonan backbone of pectin molecules. ExoPGs release monomers in a processive fashion from the nonreducing end of the substrate polymer, whereas endoPGs cleave the polygalacturonan backbone of the pectin molecule internally. Some endoPGs are hardly processive, while others have a high likelihood of releasing monomers in a processive fashion from the reducing end of the degradation product after the initial attack. These latter PGs are sometimes referred to as endo/exoPGs.

Plant polygalacturonase-inhibiting proteins (PGIPs) are present in the cell walls of many host plants (Darvill et al. 1994) and are effective against some fungal PGs (Cook et al. 1999; Desiderio et al. 1997; Sharrock and Labavitch 1994; Stotz et al. 1994) but ineffective against bacterial or plant PGs (Cervone et al. 1990). The differential inhibition of PGs by various PGIPs and evidence for adaptive evolution of PGIPs and PGs have been suggested to reflect an evolutionary arms race between PGIPs and PGs in coevolving hosts and pathogens (Stotz et al. 2000). PGIPs may also increase the half-life of oligogalacturonides that elicit plant defenses (Cervone et al. 1989).

Gene inactivation experiments have shown in several cases that pectinases are virulence factors for plant pathogenic fungi. The combined disruption of two pectate lyase genes in *Nectria haematococca* reduced virulence, although disruption of either gene alone did not (Rogers et al. 2000). The endoPG encoded by *pecA* in *Aspergillus flavus* contributes to invasion and spread of the pathogen in cotton bolls (Shieh et al. 1997), and the endoPG gene *Bcpg1* of *Botrytis cinerea* is required for full virulence, although mutants remain pathogenic (ten Have et al. 1998). In other experiments, pectinase gene inactivation failed to show a requirement for pectinases in pathogenesis, but the activity of enzymes encoded by nondisrupted pectinase genes is presumed to be sufficient for wild-type virulence in these cases (Bowen et al. 1995; Gao et al. 1996; García-Maceira et al. 2000; Scott-Craig et al. 1998).

Our understanding of the role of PGs and other aspects of the biochemistry and molecular biology of plant invasion by filamentous pathogens is based on studies of a small number of true fungi. The oomycetes are another group of filamentous plant pathogens that have a funguslike lifestyle but distinct phylogenetic affinities (Sogin and Silberman 1998). Oomycetes are responsible for many serious plant diseases. Members of one oomycete genus include *Phytophthora infestans*, which

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causes late blight of potato, and *P. cinnamomi*, which causes extensive losses in agriculture and widespread degradation in natural plant communities (Schumann 1991). Knowledge of the molecular mechanisms underlying *Phytophthora* species growth and pathogenicity is limited and is not in proportion to the economic and ecological importance of this group of pathogens.

There are some indications that PG activity is a pathogenicity factor in *Phytophthora* species required for host invasion and perhaps in the production of nutrients (Sanchez et al. 1994). Little is known, however, about the genes that encode *Phytophthora* PG enzymes apart from the recent isolation of a *P. infestans* cDNA clone encoding a *pg* gene (Torto et al. 2002), and nothing is known of the possible inhibition of oomycete PGs by plant PGIPs. In order to advance the understanding of the molecular biology of *Phytophthora* species growth and pathogenicity, we have isolated and characterized a significant portion of a large family of PG-encoding genes from *P. cinnamomi*.

RESULTS

Detection of PG activity in *P. cinnamomi* culture filtrates.

PG activity in *P. cinnamomi* culture filtrate precipitates was demonstrated using ruthenium red gel staining to detect degradation of the substrate polygalacturonate and the complementary 2-cyanoacetamide assay to detect production of reducing groups. In the 2-cyanoacetamide assay, an average of 0.22 ± 0.02 μmol reducing groups per minute per mg of total protein was produced (data not shown).

Isolation and sequencing of *P. cinnamomi* *pg* genes.

Degenerate oligonucleotide primers were designed using conserved regions of reported fungal and plant endoPG genes. A polymerase chain reaction (PCR) amplification product of the expected length was sequenced using the degenerate primers, and a database search confirmed that it was homologous to fungal and plant PG genes.

A radiolabeled probe was made from the *P. cinnamomi* *pg* PCR product and used to screen a *P. cinnamomi* genomic library in λEMBL3 . In the first round of screening, 60 positive plaques were detected. From 13 purified genomic clones, des-

ignated GC1, GC3 to GC7, GC9 to GC11, GC33, GC37, GC52, and GC60, 19 *Pcpg* gene sequences designated *Pcpg1* to *Pcpg19* were identified and sequenced, and the sequences were submitted to GenBank (accession numbers AF398105, AF398936 to AF398948). Seventeen complete genes were isolated. Three of these sequences appeared to be pseudogenes: *Pcpg5* contained an internal stop codon, *Pcpg18* contained a frameshift mutation, and *Pcpg13* was truncated and lacked the 5' coding region (Table 1). Otherwise, these three genes were similar to structurally normal *Pcpg* genes and the putative inactivating mutations might therefore be recent. The 5' coding and upstream regions of the two remaining genes, *Pcpg11* and *Pcpg17*, were not recovered in the genomic clones analyzed.

In a gel blot of *SaI*-digested *P. cinnamomi* genomic DNA probed with *Pcpg1*, 18 bands were observed (data not shown). The 13 genomic clones analyzed contained a total of 10 complete and 4 incomplete genomic *SaI* fragments that hybridized with the *Pcpg1* probe. This means that at least four genomic *SaI* fragments that hybridize with *Pcpg1* have not been isolated. The fact that only a single clone was isolated for 8 of the 19 *P. cinnamomi* *pg* genes also indicates, on statistical grounds, that the *P. cinnamomi* genomic library has not been sampled completely.

Genomic organization of the *Pcpg* multigene family.

Pcpg1 to *Pcpg13* were organized in three clusters (Fig. 1A). The physical location within the *P. cinnamomi* genome of these three clusters relative to one another or to the other six genes sequenced (*Pcpg14* to *Pcpg19*) is not known. A 12-kb contig of subclones derived from overlapping genomic clones 7 and 37 containing cluster I (*Pcpg1* to *Pcpg6*) was sequenced completely. The genomic clones containing clusters II (*Pcpg7* to *Pcpg11*) and III (*Pcpg12* and *Pcpg13*) were sequenced only to the extent necessary to determine the sequence, order, and orientation of the *pg* genes. Cluster I had a higher density of *pg* genes than clusters II and III (Fig 1A). Although the intergenic regions in clusters II and III were not sequenced completely, no sequence was found to indicate that other genes were interspersed among the *pg* genes.

In three instances (*Pcpg2* and *Pcpg3*, *Pcpg7* and *Pcpg8*, and *Pcpg12* and the truncated *Pcpg13*), pairs of adjacent genes

Table 1. Theoretical pI values and structural features of the *Phytophthora cinnamomi* polygalacturonases

	Theoretical pI value		Repeat sequence in N-terminal extension	C-terminal disulphide bridge	Potentially highly glycosylated	Probably nonfunctional ^a
	Including N-terminal extension	Excluding N-terminal extension				
PcPG1	NA ^b	5.1 ^{*c}	NA	+	No	No
PcPG2	NA	9.2*	NA	+	No	No
PcPG3	NA	9.2*	NA	+	No	No
PcPG4	NA	4.0*	NA	+	No	No
PcPG5	NA	4.7*	NA	+	No	Yes
PcPG6	NA	5.1*	NA	+	No	No
PcPG7	8.6*	9.3	D(P/Q)TQQQ	—	No	No
PcPG8	6.8*	8.8	D(P/Q)TQQQ	—	No	No
PcPG9	6.9*	7.0	D(P/Q)TQQQ	—	No	No
PcPG10	7.0*	8.8	D(P/Q)TQQQ	—	No	No
PcPG11	NA	NA	Not retrieved	+	No	No
PcPG12	5.1	7.7*	NPGQN	+	Yes	No
PcPG13	NA	NA	Truncated	+	Yes	Yes
PcPG14	5.0	7.7*	NPGQN	+	Yes	No
PcPG15	5.1	8.1*	NPGQN	+	Yes	Yes
PcPG16	6.2*	6.5	NPGQN	+	Yes	No
PcPG17	8.8*	8.7	NPGQN	+	Yes	No
PcPG18	7.7*	8.6	NPGQN	+	Yes	Yes
PcPG19	6.8*	8.5	D(P/Q)TQQQ	—	No	No

^a Nonfunctional proteins are included for comparison purposes.

^b NA = not applicable.

^c * indicates most probable mature protein.

were greater than 97% identical. The tandemly repeated genes *Pcpg12* and *Pcpg13* were also greater than 99% identical to *Pcpg14* and *Pcpg15*, respectively, and *Pcpg17* and *Pcpg18* were 93% identical to each other. Given that physically linked *P. cinnamomi* *pg* genes were in several instances very similar and that some of the physically linked genes were organized in widely spaced clusters (Fig. 1A), it is possible that *Pcpg14* and *Pcpg15* formed part of gene cluster III and that *Pcpg17* and *Pcpg18* were clustered, even though these four genes were recovered as single gene clones. Alternatively, since *P. cinnamomi* is a diploid organism, *Pcpg17* and *Pcpg18* may be alleles, and similarly, *Pcpg14* and *Pcpg15* may be allelic to each other or to *Pcpg12* or *Pcpg13*.

The sequences flanking these genes were examined to see if any further insight could be gained about their physical relationships. The downstream flanking regions of *Pcpg17* and *Pcpg18* were dissimilar (Fig. 1B), suggesting that they are unlikely to be alleles. The sequences downstream of *Pcpg12* and *Pcpg14* were almost identical, as were those downstream of *Pcpg13* and *Pcpg15*, but these two pairs of downstream regions were distinct from one another (Fig. 1C). A gene encoding a protein of unknown function, homologous to one found in several other organisms, including *Arabidopsis thaliana* (accession number AAD41975), was found downstream of *Pcpg13* and *Pcpg15* (Fig. 1C). Taken alone, these data would suggest that *Pcpg12* might be allelic to *Pcpg14* and that *Pcpg13* might be allelic to *Pcpg15*. *Pcpg14* and *Pcpg15* might, therefore, be physically linked members of a complex locus allelic to the complex locus carrying *Pcpg12* and *Pcpg13*. However, in the region immediately upstream of the start codon, *Pcpg12*, *Pcpg14*, and *Pcpg15* had 224 bp of almost identical sequence, but extended similarity was evident only between *Pcpg14* and *Pcpg15* (Fig. 1C). Given that the 5' coding region of *Pcpg13* has been deleted (Fig. 1C), it is possible that the intergenic region between *Pcpg12* and *Pcpg13*, which encompasses their upstream flanking regions, has been altered extensively by sequence rearrangement, making a definitive conclusion regarding possible allelism difficult.

A short (56 to 88 bp) intron in a conserved position was predicted in nine of the *P. cinnamomi* *pg* sequences by the presence of consensus splice donor and acceptor sites (Fig. 2). The incomplete *Pcpg17* sequence and the structurally similar pseudogene *Pcpg18* both contained an additional intron at identical positions. The upstream regions of the *P. cinnamomi* *pg* genes contained a motif (GCTCATYYNCAWTTT) that is also present at the transcription start points of a number of oomycete genes whose transcription start points have been determined (Pieterse et al. 1994). Putative polyadenylation signals (AATAAA) were found downstream of the stop codon in *Pcpg8*, *Pcpg9*, *Pcpg10*, *Pcpg12*, and *Pcpg14*. It was not possible to identify polyadenylation signals in the other genes.

Analysis of deduced amino acid sequences.

Amino acid sequences were deduced from the predicted open reading frames. The predicted unprocessed proteins varied in length from 361 to 454 amino acid residues (Fig. 2); the variation in length being due to N-terminal extensions ranging from approximately 15 to 92 amino acid residues in length (Fig. 3).

PGs have four highly conserved domains in the catalytic site and seven amino acid residues within these domains are strictly conserved in all reported PGs. Numbered according to their positions in PcPG4, these residues are N¹⁷⁸XD¹⁸⁰, D²⁰¹D²⁰², H²²³G²²⁴, and K²⁶¹. R²⁵⁹ is also conserved in all PGs except insect PGs, which have a conservative substitution to H at this site. These eight residues were conserved in all *P. cinnamomi* PGs except PcPG15 which had D²⁹¹A²⁹² instead of

D²⁹¹D²⁹² in the second conserved region. The *P. cinnamomi* PGs also have two conserved residues adjacent to the active site, exemplified by PcPG4 D¹⁸³ (equivalent to *Aspergillus niger* PGII D¹⁸³), which is conserved in all fungal PGs, and by Y²⁹³ (equivalent to *A. niger* PGII Y²⁹¹), which is conserved in all PGs. These residues have been shown to be involved in substrate binding in *A. niger* PGII (Pagès et al. 2000).

All of the complete *P. cinnamomi* PGs contained a signal peptide predicted according to the method of Nielsen and associates (1997) (Fig. 2). Putative monobasic (arginine) propeptide cleavage sites were present in all of the complete *P. cinnamomi* PGs except the hypothetical amino acid sequence corresponding to the pseudogene *Pcpg18*, which contained a dibasic (arginine-arginine) site (Figs. 2 and 3), suggesting possible similarity to dibasic propeptide cleavage sites found in some fungal PGs. A consensus sequence consisting of proline at position -4, amino acids with aliphatic side chains or methionine

Fig. 1. *Phytophthora cinnamomi* polygalacturonase gene clusters. **A**, *P. cinnamomi* polygalacturonase (*Pcpg*) genes 1 to 13 were organized in three clusters (I to III). The genomic clones (GCs) from which the genes were isolated are depicted below black arrows representing the relative location of the *Pcpg* genes and indicating their direction of transcription. The 5' region of *Pcpg11* was not contained within the genomic clones. *Pcpg13* was a truncated gene that lacked the 5' coding regions. **B**, Downstream regions of *Pcpg17* and *Pcpg18*. Patterns indicate sequence similarity. **C**, Sequenced portions of GC 1, GCs 6 and 60 and GC 11 showing the sequence context of genes *Pcpg12* to *Pcpg14*. Patterns indicate sequence identity. DNA gel blotting showed that the region between *Pcpg12* and *Pcpg13* that was not sequenced (thin line) did not contain *pg* coding sequence. The truncated *Pcpg13* gene is flanked on the 5' side by sequence not found in the upstream regions of *Pcpg12*, *Pcpg14*, and *Pcpg15*. White arrows indicate a gene of unknown function.

at positions -3 and -2, and an arginine at position -1 relative to the potential cleavage site was observed (Fig. 3).

In PcPG7 to PcPG10 and PcPG19 this consensus sequence was found upstream of the N-terminal extension (Fig. 3), suggesting retention of the N-terminal extension in the mature proteins, as is the case with *A. niger* PGD, a fungal PG with an N-terminal extension (Pařenicová et al. 2000b). In PcPG12 and PcPG14 to PcPG18, the consensus occurs downstream of the extension (Fig. 3), but in PcPG16, the conserved arginine is replaced with glutamic acid and in PcPG17 and PcPG18, by ly-

sine. This suggests that the N-terminal extensions may be lost in PcPG12, PcPG14, and PcPG15, but may be retained in PcPG16 to PcPG18, unless the lysine residues in PcPG17 and PcPG18 function in monobasic cleavage. PcPG16 to PcPG18 have nonconsensus arginine residues located upstream of the N-terminal extension that might act as alternative propeptide cleavage sites, although some of these may be too close to the predicted signal peptide cleavage sites to enable the *P. cinnamomi* maturase to engage these PcPGs (Fig. 3). PcPG16 also has a nonconsensus arginine residue just upstream of the mu-

Fig. 2. Structure of the *Phytophthora cinnamomi* polygalacturonase proteins. Predicted introns (I), nonsense (STOP), and frameshift (FS) mutations in the original DNA sequence, length of unprocessed protein in amino acids (pre), length of mature protein (mat), signal peptides (dark grey), N-terminal extensions (hatched), conserved cysteines (C), potential N-glycosylation sites (*), predicted monobasic propeptide cleavage sites (R or K) and predicted propeptides (light grey) are indicated. A dibasic site (RR) was found in PcPG18. Predicted signal peptides were 20-amino acid residues long, except the signal peptides of PcPG12, PcPG14 to PcPG16, and PcPG18, which were 22 residues long. The 5' region of *Pcpg11* and the region of *Pcpg17* encoding the signal peptide of PcPG17 were not retrieved. Protein structures corresponding to presumably non-functional *Pcpg5*, *Pcpg13*, *Pcpg15*, and *Pcpg18* are included for comparison purposes.

tated consensus sequence. If the *P. cinnamomi* maturase is able to cleave at this position, it would remove most of the N-terminal extension (Fig. 3).

Apart from the N-terminal extensions, the *P. cinnamomi* PGs were structurally similar. Except for proteins encoded by almost identical genes (PcPG2 and PcPG3, PcPG7 and PcPG8, and PcPG12 to PcPG15), pairwise sequence identity varied between 55 and 89%. The N-terminal extensions, on the other hand, were divided into two almost unrelated subgroups (Fig. 3, Table 1). The central region of the extension of PcPG10 contained glutamine-rich repeats (DQTQQQ), and downstream of these repeats, PcPG10 had a serine- and threonine-rich sequence. The extensions of PcPG7, PcPG8, PcPG9, and PcPG19 were similar to the extension of PcPG10, but contained large gaps compared to PcPG10. The extensions of the second subgroup, PcPG12 and PcPG14 to PcPG18, contained a different repeat (NPGQN) (Fig. 3, Table 1).

There was also a striking division of the *P. cinnamomi* PGs into two subgroups based on the number of potential *N*-glycosylation sites. PcPG1 to PcPG10, PcPG19, and the known part of PcPG11 contained no or few *N*-glycosylation sites, whereas PcPG12 and PcPG14 to PcPG18 carried 10 to 14 *N*-glycosylation sites, 8 of them conserved (Fig. 2, Table 1). The truncated *Pcpg13* gene encoded six of these conserved sites.

All *P. cinnamomi* PGs, except the partially recovered PcPG11 and the truncated PcPG13, had two conserved cysteines at the N-terminus, and all had two conserved cysteines in the central region (Fig. 2). PcPG1 to PcPG6 and PcPG11 to PcPG18 also had two more conserved cysteines at the C-terminus. Five of the six *P. cinnamomi* PGs characterized by few glycosylation sites and extensions containing glutamine-rich repeats lacked both of these cysteines and showed divergent C-terminal sequences compared to the other *P. cinnamomi* PGs (Fig. 2, Table 1).

The pI values of the inferred mature proteins, predicted using the method of Bjellqvist and associates (1993), varied between 4.0 and 9.2, the majority having neutral or slightly basic pI values (6.8 to 8.8) (Table 1). The distribution of pI values did not follow the division of the PGs into classes observed in the analysis of N-terminal structure and *N*-glycosylation pattern or the physical clustering. For example, the PcPGs with the highest and lowest predicted pI were encoded by the adjacent *Pcpg3* and *Pcpg4* genes in cluster I.

Functional analysis of *P. cinnamomi* PGs.

Pcpg genes were expressed in yeast and were shown to confer PG activity, confirming their functional identity as polygalacturonase genes. Preliminary experiments using reverse transcription (RT)-PCR detected expression of *Pcpg* genes in vitro and in planta. A detailed study of the activity and biochemical

properties of yeast-expressed PcPGs and a detailed analysis of *Pcpg* gene expression patterns are currently underway.

Phylogenetic analysis.

A phylogenetic tree based on an alignment of the *P. cinnamomi* PGs that excluded the variable N-terminal extensions was constructed (Fig. 4). Phylogenetically, the *P. cinnamomi* PGs fall into three clusters that correlate with the physical clustering (Fig. 1) and with the separation into different classes found in the analysis of glycosylation patterns and N- and C-terminal structure (Fig. 2). A phylogenetic analysis of the relationship between the *P. cinnamomi* PGs and the *P. infestans* PG PiPG1 (Torto et al. 2002) showed that PiPG1 is most homologous to the subgroup PcPG12 to PcPG18.

A multiple sequence alignment was made using reported PG sequences from bacteria, fungi, insects and plants, and five *P. cinnamomi* PGs representing the subgroups discernible in the *P. cinnamomi* PG phylogeny (Fig. 4). Sequences were chosen so that the monophyletic groups identified in the plant PG (Torki et al. 2000) and fungal endoPG (Wubben et al. 1999) phylogenies were represented and N- and C-terminal extensions were excluded. An unrooted phylogenetic tree was obtained that positioned the *P. cinnamomi* PGs closer to fungal and insect endoPGs than to plant and bacterial PGs or fungal exoPGs (Fig. 5).

Reticulate evolution.

Homologous genes often exchange genetic information by recombination or gene conversion leading to a patchwork of shared sequence polymorphisms between different members of a gene family. This phenomenon has been termed reticulate evolution. Analysis of shared polymorphisms in the *Pcpg* genes using Sawyer's runs test implemented in GENECONV 1.81 (discussed below) provided strong support for reticulate evolution in some *Pcpg* genes. In 10 instances, stretches of polymorphisms shared by pairs of *Pcpg* genes had global *P* values (discussed below) of less than 0.05, and 31 such fragments had pairwise *P* values less than 0.01. Randomized data (10 sample DNA alignments arising from permutation of the polymorphic sites) yielded no fragments with global *P* values less than 0.05 and, in no case, more than four fragments with pairwise *P* values less than 0.01.

Two genes in cluster I have undoubtedly been affected by reticulate evolution. A 473-bp-long fragment in *Pcpg4* (positions 331 to 803 in the analyzed *Pcpg* gene alignment) had an almost exact match in *Pcpg5* (global *P* value of less than 0.0001), while the flanking regions were only moderately similar. Two shorter fragments (positions 431 to 578 in *Pcpg1* and *Pcpg2* and positions 419 to 560 in *Pcpg7* and *Pcpg10*) had global *P* values of less than 0.001. It is also highly likely that a gene conversion or

Fig. 3. Alignment showing the N-terminal regions of the *Phytophthora cinnamomi* polygalacturonases. Regions of the unprocessed proteins upstream of the first conserved cysteine residue are shown. Predicted signal peptides are indicated in bold and italics. The consensus sequence preceding putative pro-peptide cleavage sites is underlined and arginine residues are in bold. Repeats are indicated with boxes.

recombination event has affected the 3' regions of *Pcpg16* and the ancestor of *Pcpg17* and *Pcpg18*; the global *P* value was less than 0.005 for positions 541 to 990 in *Pcpg16* and *Pcpg18* and was less than 0.01 for a slightly shorter fragment shared by *Pcpg16* and *Pcpg17* (positions 598 to 929).

The remaining probable instances of reticulate evolution detected by Sawyer's runs test were also intracluster events or events involving genes that were closely related in all regions and therefore possibly clustered in the genome. Intercluster exchange of genetic information between more divergent genes may be less frequent and such events may have become obscured by subsequent more frequent intracluster sequence exchange and other mutations and therefore more difficult to detect using the runs test, which requires pairwise fragments with relatively few mismatches. In order to detect more subtle evidence for reticulate evolution, patterns of shared parsimoniously informative sites in the *Pcpg* gene alignment were explored by generating phylogenetic profiles using PhylPro (discussed below). The phylogenetic profiles for the alignment with no genes excluded are shown in Figure 6A. Several instances of strong recombination signals indicate that reticulate evolution has contributed to the evolution of the *P. cinnamomi* gene family. Putative recombinant sequences were identified by excluding sequences from the alignment until the alignment contained only one sequence that had a distinct minimum, or minima, in its phylogenetic profile, at least in the region in which one or more strong recombination signals occurred. The other sequences in the alignment presumably reflected the an-

cestral sequences of the recombinant, at least in that region of the gene.

Strong recombination signals appeared in the phylogenetic profile of *Pcpg6* when compared with its close relatives in cluster I (*Pcpg4* and *Pcpg5*) and to the more distantly related *Pcpg9* and *Pcpg10* in cluster II (Fig. 6B). The region between the signals at positions 410 and 510 in Figure 6B contained fragments shared by *Pcpg6* and the genes in cluster II. Since PhylPro does not enable tests of significance to be carried out, *Pcpg6* and two genes representing the probable parental lineages (*Pcpg5* and *Pcpg10*) were analyzed using the maximum chi-squared method (Maynard Smith 1993), which identifies the most probable breakpoints and estimates the probability that the alignment has a mosaic structure. The breakpoints in *Pcpg6* identified using PhylPro were also identified by the maximum chi-squared method, and the mosaic structure of *Pcpg6* was statistically significant ($P < 0.0001$ for the upstream breakpoint and $P < 0.0005$ for the downstream breakpoint).

The same set of phylogenetic profiles (Fig. 6B) also suggested the presence of two recombination breakpoints in *Pcpg4*, indicated by the two signals at positions 52 and 190. A comparison of *Pcpg4* with *Pcpg6* and *Pcpg10* using the maximum chi-squared method indicated that *Pcpg4* may also have been affected by reticulate evolution involving clusters I and II, but the support was considerably weaker ($P < 0.05$).

Strong recombination signals also appeared at positions 633 and 798 in the phylogenetic profile of *Pcpg2* when compared with *Pcpg4* and *Pcpg5* in cluster I and with the more distantly related *Pcpg12* and *Pcpg14* in cluster III (Fig. 6C). Phylogenetic trees generated using parsimony showed that in the regions 1 to 632 and 799 to 990 of the DNA alignment, *Pcpg2* was closely related to *Pcpg4* to *Pcpg6* (data not shown). Trees for the region 633 to 798 suggested that *Pcpg2* was related to *Pcpg12* to *Pcpg18* (data not shown), but the bootstrap support was not significant. *Pcpg2* and genes representing the putative parental lineages were also analyzed using the maximum chi-squared method. The breakpoints shown in Figure 6C were detected, but they were not statistically significant.

Significance tests for recombination in *Pcpg2* conducted using phylogenetic trees or the maximum chi-squared method relied on the assumption that single clearcut recombination breakpoints had been detected. Detailed inspection of informative sites in an alignment comprising *Pcpg2*, *Pcpg4*, *Pcpg5*, *Pcpg12*, and *Pcpg14* confirmed that *Pcpg2* in some regions was closely related to genes in cluster III but that distinct boundaries between blocks with conflicting phylogenies were difficult to locate (Fig. 7). Therefore, *Pcpg2* and genes representing the putative parental lineages were analyzed using SiScan (discussed below), which provides a measure of the relatedness signals in different parts of the alignment and tests the significance of the signals without attempting to locate breakpoints. An analysis of *Pcpg2*, *Pcpg5*, and *Pcpg12* (Fig. 8) showed the presence of regions in *Pcpg2* significantly similar to *Pcpg12* (*Z* score > 3), and the highest scores were obtained in the region between the strongest recombination signals detected by PhylPro (Fig. 6C).

Additional putative recombination events were detected using PhylPro, but the conflicting phylogenetic signals suggested by the patterns of shared informative sites were, in these cases, not significantly strong according to the described tests. However, the instances of reticulate evolution detected using Sawyer's runs test were also detected using PhylPro and were shown to be significant using the maximum chi-squared method.

Identification of sites undergoing diversifying selection.

Comparison of nonsynonymous or amino acid replacing (K_a) and synonymous or silent (K_s) substitution rates provides a

Fig. 4. Phylogenetic tree of the *Phytophthora cinnamomi* polygalacturonases. All of the PcPGs except the truncated PcPG13 and the incomplete PcPG11 were included in the analysis. Putative signal peptides, propeptides, and N-terminal extensions were excluded from the protein sequence alignment. The recovered part of PcPG11 was most similar to the C-terminal regions of PcPG9 and PcPG10. Ellipses indicate physical clustering in the genome. PcPG14 and PcPG15 are shown as part of cluster III, since they are probably allelic or physically linked to PcPG12 and PcPG13. Proteins with N-terminal extensions containing glutamine-rich repeats (underlined with solid lines) and extensions containing the repeat NPGQN (underlined with broken lines), as well as proteins with heavy and light potential glycosylation patterns, are indicated. Bootstrap support values are indicated where bootstrap support was ≥ 50 .

means for detecting adaptive sequence evolution. A K_a/K_s value greater than one in pairwise comparisons of homologous nucleotide sequences provides stringent evidence for diversifying selection (Yang and Bielawski 2000). The region of the *P. cinnamomi* *pg* genes from the codon encoding the first conserved cysteine (Fig. 2) to the 3' end of the coding sequence was used in an analysis of K_a/K_s , while the variable 5' coding regions were excluded. In the instances when two or more *P. cinnamomi* *pg* genes were 99% identical, only one sequence was included in the analysis. Pairwise comparisons of *Pcpg1*, *Pcpg2*, *Pcpg4* to *Pcpg10*, *Pcpg12*, and *Pcpg16* to *Pcpg19* using the YN00 program did not provide evidence for diversifying selection, because the K_a/K_s values were not greater than one in any of the compari-

sons. However, diversifying selection in proteins usually affects only a few sites at a few time points (Yang et al. 2000). Selection at most sites is presumably neutral or conservative, and hence, the overall K_a/K_s is less than one or is not significantly greater than one, even though some amino acid residues subject to diversifying selection might be present.

In order to detect more subtle diversifying selection, the same set of *P. cinnamomi* *pg* genes was analyzed using maximum likelihood models of codon substitution that allow for heterogenous selection pressure along the protein. Of the models available in the CODONML program (Yang et al. 2000), M1, M2, M7, and M8 were implemented. M1 assumes two classes of sites, one with $K_a/K_s = 0$ (conserved sites), and one

Fig. 5. Phylogenetic tree of bacterial, plant, fungal, animal, and *Phytophthora cinnamomi* polygalacturonases (PGs). ExoPGs are underlined. Except for the *P. cinnamomi* PGs, the remaining sequences have been demonstrated or designated to be endoPGs or endo/exoPGs. Bootstrap support values are indicated for clades with bootstrap support of 50 or higher. Accession numbers (SWISS-PROT or TrEMBL): *A. thaliana* Pga1;2 O22818, *A. thaliana* Pga1;7, O22935; *A. thaliana* Pga1;8, O48576; *A. thaliana* Pga2;2, O65401; *A. niger* PG1, P26213; *A. tubingensis* PGX, Q00293; *B. cinerea* PG4, Q9Y7W0; *C. carbonum* PGX, Q00359; *C. purpurea* PG1, P78607; *C. japonica*, P43212; *E. carotovora* PehA, P26509; *E. chrysanthemi* PehX, P15922; *F. moniliforme*, Q07181; *F. oxysporum* PGX, O74255; *K. marxianus*, O13478; *L. esculentum* PG4, Q96488; *M. domestica*, P48978; *N. tabacum*, Q05967; *P. cochleariae*, O97400; *P. solanacearum*, Q53241; *S. cerevisiae*, P47180; *S. oryzae*, AF207068; *S. purpureum*, P79074; and *Z. mays*, P26216.

with $K_a/K_s = 1$ (neutral sites). M2 allows for diversifying selection by adding a class with K_a/K_s estimated from the data. M7 implements a discrete approximation to a beta distribution with ten classes in the interval $0 \leq K_a/K_s \leq 1$, and M8 adds an additional class to allow for sites with $K_a/K_s > 1$. M1 and M7 constitute null hypotheses compared with the more general M2 and M8, respectively. When a more general model indicates the presence of a class of sites with $K_a/K_s > 1$, comparison with its null hypothesis using a likelihood-ratio test constitutes a test for diversifying selection (Yang et al. 2000). M1 was rejected in favor of M2, which allows for positive selection (Table 2). M2 suggested the presence of a small proportion (1.6%) of amino acid residues subject to diversifying selection (Table 2). An empirical Bayes approach (Nielsen and Yang 1998) identified two residues with a probability <0.05 of not having been subject to diversifying selection and two sites with a probability <0.10 (Table 3). Similarly, M7 was rejected in favor of M8 (Table 2), which also suggests that some sites have been under diversifying selection pressure. The empirical Bayesian approach identified the same residues subject to diversifying selection that had been identified implementing M2 (Table 3).

Two of the genes included in this analysis, *Pcpg5* and *Pcpg18*, appear to have only recently become pseudogenes and so were assumed to represent the sequence of their functional ancestors accurately. To check this assumption, the analysis was repeated excluding *Pcpg5* and *Pcpg18*, but the result was essentially the same.

It is not clear to what extent maximum-likelihood estimation under these models is affected by reticulate evolution (Yang and Bielawski, 2000). The analysis was, therefore, repeated using several tree topologies, but the result was essentially the same.

The sequences of the *P. cinnamomi* PGs suggest that they are structurally similar to *A. niger* PGII, whose crystal struc-

ture has been determined (Protein Data Bank ID 1CZF) (van Santen et al. 1999). The sites identified as undergoing diversifying selection in *P. cinnamomi* corresponded to residues 73, 86, 91, and 360 of *A. niger* PGII. A model of PcPG4 was constructed using the structures of *A. niger* PGII (van Santen et al. 1999) and *Erwinia carotovora* PehA (Pickersgill et al. 1998) as templates (Fig. 9A and B). The identified residues were predicted to be located in two separate regions of the protein. Three residues were located in two adjacent turns of the β -helix, one of them (L^{95}) close to the N-terminal end of the active site cleft, one (E^{90}) in an extended loop flanking the active site cleft, and one (E^{77}) on the opposite side of the β -helix to the active site cleft. According to the model, these three residues are all solvent exposed. The last eight residues of PcPG4, including the one remaining residue subject to diversifying selection (T^{360}) could not be modeled, but this residue is presumably shielding the C-terminal end of the core of the β -helix, as is the corresponding residue (S^{360}) in *A. niger* PGII (van Santen et al. 1999). PcPG4 T^{360} is also probably solvent exposed, as is *A. niger* PGII S^{360} . The high proportion of charged or polar residues occurring at positions 77, 90, and 360 (Table 4) is also consistent with these residues being solvent exposed. In contrast, about half of the *P. cinnamomi* PGs had a strongly hydrophobic residue (leucine) at position 95, and the remainder had polar or charged residues (Table 4).

DISCUSSION

Biological significance

of the *P. cinnamomi* pg multigene family.

Although oomycete pathogens, including species of *Phytophthora*, have been shown to produce pectinases (Cole 1970; Jarvis et al. 1981; Sanchez et al. 1994; Yoshikawa et al. 1977), little is known about these enzymes at the molecular and biochemical level. In this study, we have shown that *P. cinnamomi* contains a large pg multigene family comprising more than 19 pg genes. Other plant pathogens contain multiple pg genes and many PG isoforms, but the size of the gene family in *P. cinnamomi* is remarkable. Amongst the true fungi, *A. niger* has seven pg genes and at least nine PG isoforms (Bussink et al. 1992; Stratilová et al. 1993); *B. cinerea* has at least six genes and produces 13 isoforms (van der Cruyssen et al. 2001; Wubben et al. 1999); and *Sclerotinia sclerotiorum* has at least seven genes and secretes 14 PG isoforms (Fraissinet-Tachet et al. 1995; Martel et al. 1996). The large number of isoforms in *S. sclerotiorum* and *B. cinerea* could be due to the presence of additional pg genes or to posttranslational modifications. The latter situation is exemplified by *Fusarium moniliforme*, which has four PG isoforms encoded by one gene (Caprari et al. 1996).

The biological significance of multiple PGs in plant pathogens is not fully understood, although a number of possibilities have been proposed. The size of pg multigene families has been suggested to vary with the specificity of the interaction, so that pathogens with wide host ranges have large pg gene families and pathogens with narrow host ranges have few pg genes (Esquerré-Tugayé et al. 2000). In this context, a large pg gene family might have developed to enable the pathogen to successfully colonize hosts that differ in cell wall structure and composition and in response to the diversity of PGIPs and other defenses encountered in different hosts. The generalization holds true for *B. cinerea* and *S. sclerotiorum*, which have wide host ranges, and for *Colletotrichum lindemuthianum*, which is restricted to fabaceous hosts and has only two pg genes. However, it does not hold true for *Botrytis* species with a narrow host range. *Botrytis* species restricted to one host contain homologues to all members of the *B. cinerea* pg gene family (Wubben et al. 1999). Nor does it hold true in *Phytophthora* species. Consistent with the generalization,

Fig. 6. Phylogenetic profiles of the *Phytophthora cinnamomi* polygalacturonase (*Pcpg*) genes. The analyzed alignments were **A**, *Pcpg1*, *Pcpg2*, *Pcpg4* to *Pcpg6*, *Pcpg8* to *Pcpg10*, *Pcpg12*, and *Pcpg16* to *Pcpg19*; **B**, *Pcpg6* (bold), *Pcpg4*, *Pcpg5*, *Pcpg9*, and *Pcpg10*; and **C**, *Pcpg2* (bold), *Pcpg4*, *Pcpg5*, *Pcpg12*, and *Pcpg14*. Positions in the alignment are indicated on the x-axis; phylogenetic correlation values on the y-axis. Low correlation values near the ends of the alignments were caused by the small size of the upstream or downstream windows at these positions and are not significant.

P. cinnamomi has a large *pg* gene family and a very wide host range (Erwin and Ribeiro 1996), but gel blots of *P. infestans* genomic DNA probed with a *P. cinnamomi* *pg* gene fragment show a similarly large number of hybridizing bands (data not shown), suggesting that *P. infestans* has a similar number of *pg* genes to that in *P. cinnamomi*, even though *P. infestans* is restricted to solanaceous plants, mainly potato and tomato. The apparently large *pg* gene family of *P. infestans* could be inherited from an ancestor with a wide host range, and as in *P. cinnamomi*, not all the PG-like sequences may encode functional genes, but it is also possible that factors other than host range could have provided selective pressures leading to the evolution of large *pg* gene families.

The diversity of PG isoforms may reflect the complexity of the pectin molecules in plant cell walls, and the need for enzymes capable of cleaving the homogalacturonan backbone in a variety of structural contexts. PGs must be able to degrade plant cell walls of different composition and pH in different tissues and, during parasitic growth, may need to degrade homogalacturonan to completion without allowing intermediate degradation products to accumulate, since oligogalacturonides with a degree of polymerization of 8 to 15 have been shown to elicit plant defenses (Hahn et al. 1989). For these reasons, it seems likely that an array of PGs with a wide range of action patterns, specificities, and abilities to function in different environments would be advantageous to the microorganism.

The presence of temporal and spatial regulation of *pg* expression in many fungi supports the contention that enzyme specialization is an important feature of fungal pectinolytic systems. It has been proposed, for example, that constitutively secreted PGs in *S. sclerotiorum* and *B. cinerea* release monomers that activate the expression of other *pg* genes, because

several other members of the gene family in these pathogens are induced by galacturonic acid (Fraissinet-Tachet and Fevre 1996; Wubben et al. 2000). *S. sclerotiorum* PG isoforms are sequentially secreted in cultures grown on polygalacturonate medium, suggesting that PG isoforms specific for progressively shorter oligogalacturonides are expressed as degradation of the substrate proceeds (Martel et al. 1998). It is possible that the products released at each stage of the degradation of the pectin polymer, starting with the oligomers produced by early secreted endoPGs, effect the successive induction and repression of *pg* genes encoding PGs with different biochemical properties, until degradation is complete (Martel et al. 1998). Based on the expression patterns, different *B. cinerea* *pg* genes are suggested to function in lesion expansion, nutrient provision, and infection in acidic environments (ten Have et al. 2001). These results support the hypothesis that constitutively produced enzymes release degradation products capable of inducing a complex pectinolytic machinery.

Diversity of *P. cinnamomi* PGs.

PG isoforms participating in an orchestrated attack on plant tissue would be expected to have become adapted to function in different environments and to display different substrate specificities and modes of action. Consistent with this idea, the extensively characterized *A. niger* PGs display considerable differences in biochemical attributes (Benen et al. 1999; Pařenicová et al. 1998, 2000b). Little biochemical characterization of other PGs has been performed, but some conclusions about the diversity of properties and roles of PG isoforms can be drawn from analysis of the primary sequences.

The primary sequences indicate that the *P. cinnamomi* PGs are divided into subgroups with respect to potential glycosylation sites and N- and C-terminal structures (Table 1). Differential glycosylation of PGs is also present in *B. cinerea* (Wubben et al. 1999), but the division of the *P. cinnamomi* PGs into lightly or potentially heavily N-glycosylated isoforms is striking. Potential N-glycosylation sites are present on all faces of the potentially heavily glycosylated *P. cinnamomi* PGs but are absent from the active site (Fig. 9C and D). Possible functions of N-glycosylation include conferring stability (Wyss and Wagner 1996) or protease resistance (Rudd et al. 2001) to enzymes secreted into hostile environments. Deglycosylation under nondenaturing conditions of *A. niger* PGs led to complete inactivation, indicating that glycosylation may also be crucial for enzymatic activity (Stratilová et al. 1998). Interestingly, PiPG1 (Torto et al. 2002), which is similar to PcPG12 to

Fig. 7. Analysis of informative sites in *Pcp2*. Alignment of sequences of *Pcp2*, *Pcp4*, *Pcp5*, *Pcp12*, and *Pcp14*. Highlighted nucleotides indicate where *Pcp2* shares informative sites with genes representative of one or other of the putative ancestral lineages.

Fig. 8. Reticulate evolution affecting *Pcp2*. Changes in Z-scores for patterns for a set of sequences including *Pcp2* (putative recombinant), *Pcp5* and *Pcp12* (representing parental lineages), and a randomized outlier are indicated. Positions in the alignment are indicated on the x-axis; Z-scores on the y-axis. Bold line indicates Z-scores for the pattern ($Pcp2 = Pcp12 \neq Pcp5 \neq \text{outlier}$), thin line indicates Z-scores for the pattern ($Pcp2 = Pcp5 \neq Pcp12 \neq \text{outlier}$).

PcPG18, is also potentially heavily glycosylated, but most of the putative glycosylation sites are not conserved between *P. cinnamomi* and *P. infestans*.

Another structural feature that might reflect functional diversification in the *P. cinnamomi* PGs is the division into three classes with either no N-terminal extension or with one of two types of extension (Table 1). N-terminal extensions of PGs are diverse compared to the remainder of the protein. Although the *A. niger* PGD (Pařenicová et al. 2000b) and *B. cinerea* PG3 (Wubben et al. 1999) proteins are closely related phylogenetically (Wubben et al. 1999), the N-terminal extensions are dissimilar. Similarly, the two types of extensions in *P. cinnamomi* PGs are unrelated. *A. niger* PGD is an endo/exoPG with extreme processive behavior and is thought to be an oligogalacturonase that completely degrades oligomers released by endoPGs, rather than a PG that is involved in the initial attack on the cell wall (Pařenicová et al. 2000b). The mode of action and substrate specificity of *A. niger* PGD has been suggested to be defined by its extension (Pařenicová et al. 2000b). It has also been suggested that the unique C-terminal extension of *A. niger* rhamnogalacturonase B defines its substrate specificity and distinguishes it from other known rhamnogalacturonases (Suykerbuyk et al. 1997). The C-terminal extension of *A. niger* rhamnogalacturonase B and the N-terminal extensions of *P. cinnamomi* PGs all contain glutamine-containing repeat sequences, D(P/Q)TQQQ and NPGQN in *P. cinnamomi* and GEQ in *A. niger* rhamnogalacturonase B (Suykerbuyk et al. 1997), perhaps suggesting a role for glutamine residues in interaction with a particular feature of the pectin molecule.

The complete *P. cinnamomi* PGs had two conserved cysteines predicted to form a disulphide-bonded N-terminal structure capping the β -helical core as in *A. niger* PGII (van Santen et al. 1999). All reported eukaryotic PGs have at least one disulphide bridge in the C-terminal region, and PiPG, which appears truncated in the C-terminal region compared to fungal and *P. cinnamomi* PGs, seems to have regained two C-terminal cysteines through a frameshift and a point mutation, indicating that C-terminal disulphide bonding is probably important for PG stability and function. However, PcPG7 to PcPG10 and PcPG19 lack C-terminal cysteine residues (Table 1), but most of these are active when expressed in yeast, suggesting that they may have an alternative C-terminal capping structure.

All eight highly conserved amino acids in the catalytic site of the PG enzymes were conserved in all the PcPGs except for PcPG15, which had D²⁹¹A²⁹² instead of D²⁹¹D²⁹². The missing aspartic acid residue in PcPG15 is catalytically active. A conservative site-directed mutation of this residue in *A. niger* PGII (D to E) reduced the specific activity of the enzyme to approximately 0.5% of wild-type activity, and mutating the D residue to N reduced the specific activity to approximately 0.01% (Armand et al. 2000). It is, therefore, probable that *Pcpg15* does not encode a functional PG (Table 1).

The predicted pIs of the *P. cinnamomi* PGs vary widely. The pI may influence enzyme specificity for substrates with different charge (Bradford 1976). A basic pI may, for example, confer affinity for negatively charged polygalacturonate. Basic pI may also decrease protein mobility within the cell wall, leading to highly localized wall degradation, as observed upon secretion of the extremely basic polygalacturonate lyase by the biotroph *Uromyces viciae-fabae* (Mendgen et al. 1996).

Relationship of the *P. cinnamomi* PGs with PGs from other taxa.

It is currently not possible to resolve the eukaryotic phylogeny because of the rapid diversification that gave rise to most, or possibly all, eukaryotic phyla (Philippe et al. 2000). Analysis of small subunit rRNA, β -tubulin genes, and a large set of *P. infestans* esterases has suggested that heterokonts, including *Phytophthora*, are either equidistant to fungi and green algae/plants (Förster et al. 1990) or more closely related to green algae/plants than to fungi (Kamoun et al. 1999; Weerakoon et al. 1998). The present study extends the recent finding by Torto and associates (2002) that PiPG is more similar to fungal and insect endoPGs (Fig. 5) than to any other PG. Convergent evolution of the PGs of fungi and oomycetes or conservation of features of the ancestral protein are possible explanations for the phylogenetic affiliation of the *P. cinnamomi* PGs and fungal endoPGs. Plant pectic substrates and defenses against pathogen attack may provide similar selective pressures for microorganisms from different phyla, while plants are likely to have had different requirements in terms of the mode of action and substrate specificity of their own PGs. Another possible explanation is horizontal gene transfer between the fungal and heterokont lineages. Analysis of insertions and deletions in an alignment of all reported PG sequences showed that *P. cinnamomi* PGs and fungal endoPGs shared only one deletion not present in other PGs. However, other insertions and deletions and introns were not at conserved positions in *P. cinnamomi* PGs and fungal endoPGs. Furthermore, codon usage and GC content in the *P. cinnamomi* *pg* genes did not distinguish these genes from other reported *P. cinnamomi* genes. If oomycetes have acquired *pg* genes, the absence of evidence for horizontal gene transfer in the *P. cinnamomi* *pg* sequences might be due to adaptation of the sequences to the codon preferences and processing constraints of the *P. cinnamomi* genome.

Evolution of the *P. cinnamomi* *pg* multigene family.

The existence of pairs of almost identical genes in the *P. cinnamomi* *pg* gene family is likely to be due to recent duplication events. The presence of recently duplicated genes and genes that have acquired mutations leading to loss of function (Table 1) indicate that the gene family is undergoing a process of birth-and-death evolution (Nei and Hughes 1992). *Pg* genes that appear to be recently duplicated are also present in *S. sclerotiorum* (Fraissinet-Tachet et al. 1995) and in *Claviceps*

Table 2. Probabilities of various distributions of *Phytophthora cinnamomi* polygalacturonase sites with respect to K_a/K_s values

Model ^a	Proportion of sites in classes with $K_a/K_s > 1$	K_a/K_s	$\ln L^b$	χ^2^c	Degrees of freedom	Probability
M1	NA ^d	NA	-8475.422	NA	NA	NA
M2	0.016	6.17	-8463.203	24.438	2	< 0.01
M7	NA	NA	-7896.923	NA	NA	NA
M8	0.016	1.50	-7891.308	11.230	2	< 0.01

^a M1 = neutral ($K_a/K_s = 0$ or $K_a/K_s = 1$), M2 = selection (one class with $K_a/K_s \neq 1$ added to M1), M7 = discrete approximation to beta distribution with 10 classes in the interval $0 \leq K_a/K_s \leq 1$, M8 = M7 with an extra class added to allow for a class with $K_a/K_s > 1$. Program used for analysis: CODONML 3.0a (Yang et al., 2000).

^b Log likelihood.

^c Likelihood ratio test: $\chi^2 = 2(\ln L_{\text{alternative hypothesis}} - \ln L_{\text{null hypothesis}})$.

^d NA = not applicable.

purpurea (Tenberge et al. 1996), but the *pg* gene families of *A. niger* and *B. cinerea* seem to be evolutionarily stable, containing only functional *pg* genes and no pseudogenes (Benen et al. 1999; Pařenicová et al. 1998, 2000a, 2000b; Wubben et al. 1999).

The organization of the *P. cinnamomi* *pg* genes suggests a possible mechanism for the initiation of gene family growth. The members of each pair of nearly identical genes are in inverted orientation with respect to one another. However, apart from three recent duplications, there is a tendency for repeated genes to be in direct orientation. If duplication involves production of inverted repeats, this might be an unstable arrangement, and the gene might need to undergo a second duplication, before an inverted copy is lost in order to achieve a net gain of one gene in direct orientation. Comparison of a segment of tomato chromosome 2 with syntenous segments of the *A. thaliana* genome showed several examples of conservation of gene order but not of gene orientation (Ku et al. 2000). A model was proposed in which gene duplication events give rise to inverted repeats, followed by loss of the original gene, leading to change in gene orientation (Ku et al. 2000). An inverted repeat duplication mechanism is consistent with the organization of the two almost identical *P. infestans* *ipiO* genes (Pieterse et al. 1994) (inverted orientation) and the more divergent *Phytophthora* elicitor genes (Duclos et al. 1998; Mao and Tyler 1996; Panabières et al. 1995) (direct orientation). However, the three *P. infestans* *ipiB* genes (96% identical) whose intergenic regions are still 98% identical, indicating recent duplication, are arranged in direct orientation.

After the initial generation of a direct tandem repeat through the mechanism suggested above, there is the potential for mispairing by strand slippage at meiosis and recombination to increase or decrease the gene number and to allow genetic exchange between nonorthologous genes, as has been postulated for many R gene families in plants (Ellis et al. 2000) and for *P. cinnamomi* *pg* genes in this study. Reticulate evolution may be an important mechanism in the evolution of the *P. cinnamomi* *pg* gene family, because it has the potential to accelerate the development of pathogen fitness, including the ability to avoid inhibition by PGIPs. The evolutionary importance of recombination has been extensively investigated in the tomato *Hcr9* genes (Parniske and Jones 1999), a family of resistance genes involved in an evolutionary arms race with fungal avirulence genes. A model has been presented in which sequence exchange is prevented from leading to excessive homogenization by physical and genetic separation of gene clusters, which suppresses mispairing and recombination. A rare ectopic recombination event was shown to have introduced a gene from one cluster of *Hcr9* genes to a distant, diverged cluster, and subsequent recombination with other genes in the recipient cluster has generated novel gene structures (Parniske and Jones 1999). This model may be applicable to the *P. cinnamomi* *pg* genes. Greater structural similarities and more frequent intercluster recombination events suggest that cluster I, cluster II, and *Pcpg19* may be in closer physical proximity to one another than to *Pcpg12* to *Pcpg18*. No whole gene seems to have been transferred between the lightly and heavily glycosylated subgroups, but the possible ectopic recombination event involving *Pcpg2* depicted in Figures 6C and 7 indicates that genetic exchange may occur between divergent *P. cinnamomi* *pg* gene subgroups to create novel combinations of structural features.

The *P. cinnamomi* *pg* sequences generated by gene duplications and modified by mutation and recombination may also have undergone adaptive evolution. The similarity between *P. cinnamomi* and fungal PGs (Fig. 5) and the fact that the residues possibly subject to diversifying selection are solvent exposed (Fig. 9) suggests that one source of the selective pressure behind the adaptive evolution of the *P. cinnamomi* PGs could be

interaction with PGIPs. A single amino acid substitution can confer a new PG target specificity upon closely related PGIPs (Leckie et al. 1999), indicating that small structural changes may confer great advantages in the evolutionary arms race. The residues subject to diversifying selection identified in fungal PGs (Stotz et al. 2000) and in this study are widely separated over the PG protein but are not situated in the catalytic site. This need not mean that PGIPs do not drive diversifying selection in PGs. It is not known which regions of PGs interact with PGIPs (Leckie et al. 1999), and some PGIPs have a noncompetitive mode of inhibition (Yao et al. 1995), consistent with interactions with PG residues outside the catalytic site. Moreover, an inhibitor binding outside the catalytic site might effect competitive inhibition indirectly by preventing substrate access.

None of the fungal residues likely to be subject to diversifying selection (Stotz et al. 2000) correspond to any of the residues identified in the present study. If certain regions of PGs from different taxa are consistently important in interactions with PGIPs, the sets of identified residues might be expected to overlap, but this does not seem to be the case. Similarly, entirely different sets of positively selected residues were identified in the analysis of two different selections of plant PGIPs, indicating that those residues in PGIPs that are both under diversifying selection and free to respond differ between groups of plants (Stotz et al. 2000).

Another possible diversifying selective pressure is proteolytic attack. *P. cinnamomi* PG residue 360 (Table 4) is probably part of the capping structure at the C-terminus of the PG protein. It is conceivable that the host secretes proteases aimed at the degradative enzymes of the pathogen and that the ends of the β -helix might be targets for attack. Variations in capping structure residues might protect a certain proportion of the PGs from proteolytic attack in any given situation.

This study has shown that *pg* genes form a major gene family in *P. cinnamomi* and indicates that the genes encode structurally diverse isoforms. Just as plants have responded to selection pressure imposed by plant pathogens by evolving large multigene families, it would seem that *P. cinnamomi* has responded to selective pressure imposed by plants by likewise evolving a large *pg* multigene family by similar mechanisms. Birth-and-death evolution, reticulate evolution, and adaptive evolution may have contributed to the development of the large *P. cinnamomi* *pg* gene family. PGs are important in saprophytic and parasitic growth and, therefore, have been the focus of much study in fungi. The *P. cinnamomi* *pg* gene family exhibits intriguing differences compared with true fungi and warrants further investigation. Future work will examine the features and functional abilities of *P. cinnamomi* PG proteins, the regulation of *P. cinnamomi* *pg* gene expression during plant infection, and the possible inhibition of *P. cinnamomi* PGs by plant PGIPs.

Table 3. Probabilities that amino acid sites in the *Phytophthora cinnamomi* polygalacturonases do not belong to the class of sites under diversifying selection pressure

Site ^a	Probability (P)	
	M2 ^b	M8
77 (73)	0.001	0.017
90 (86)	0.017	0.065
95 (91)	0.076	0.243
360 (360)	0.051	0.068

^a Position in the unprocessed PcPG4 protein is indicated and in brackets position of the corresponding residue in the unprocessed *Aspergillus niger* PGII is indicated.

^b M2 allows for diversifying selection by adding a class with K_a/K_s estimated from the data. M8 adds an additional class to allow for sites with $K_a/K_s > 1$.

Fig. 9. Amino acid residues in *Phytophthora cinnamomi* polygalacturonases (PGs) that have been subject to diversifying selection and position of *N*-glycosylation sites. Models of *P. cinnamomi* PGs were made using the crystal structures of *A. niger* endoPGII and *E. carotovora* PehB as templates. The PG molecule consists of β strands forming a β -helix. While most of the hinge regions that connect the β -strands of the β -helix are only a few residues long, some are elongated and form the active site cleft. **A**, Model of PcPG4 showing the active site cleft from the N-terminal end. The amino (N) and carboxyl (C) termini of the protein are indicated. **B**, Top view looking down into the active site cleft of PcPG4. The side chains of the residues identified as subject to diversifying selection are shown in yellow. Three of the residues (E⁷⁷, E⁹⁰, and L⁹⁵) are located on two adjacent turns of the β -helix and T³⁶⁰ is located at the C-terminus. T³⁶⁰ is not shown since the C-terminus could not be modeled. Catalytically important residues (Armand et al. 2000) are shown in red, and other residues that are conserved in PGs are shown in orange. **C**, and **D**, Similar view to A and B showing a model of PcPG16 with asparagine residues in the potential *N*-glycosylation sites of PcPG16 indicated in yellow.

MATERIALS AND METHODS

Strain of *P. cinnamomi* used and culture conditions.

A strain of *P. cinnamomi* (H1000, DAR 52646, ATCC 200982) isolated from a soil sample taken in the Brisbane Ranges, Victoria, Australia, was maintained on V8 agar (Hardham et al. 1991). Sterile 250 ml Erlenmeyer flasks containing 80 ml of V8 broth were inoculated with 10 small cubes of agar excised from the periphery of a *P. cinnamomi* colony after 3 days of growth. The cultures were incubated at 25°C until harvest of hyphae or collection of the growth medium.

Isolation of protein.

Four 80 ml *P. cinnamomi* liquid cultures were filtered through two layers of miracloth after 4 days of growth. The culture filtrates were pooled, and proteins were precipitated by gradual addition of (NH₄)₂SO₄ to a total concentration of 0.57 g per ml. The sample was left to precipitate overnight at 4°C and then centrifuged at 12,000 g for 10 min. The pellet was dissolved in 1 ml of 50 mM sodium acetate buffer (pH 4.2) containing 0.15 M NaCl. The sample was transferred to dialysis tubing and dialyzed in 1 l of acetate buffer for 24 h, with a change of buffer every 8 h. Protein concentrations were determined according to Bradford (1976) using Bio-Rad (Hercules, CA, U.S.A.) protein assay reagents and bovine serum albumin as the protein standard.

PG activity assays.

PG activity in *P. cinnamomi* culture filtrates was detected using the 2-cyanoacetamide assay described by Gross (1982) and the ruthenium red gel staining assay described by Collmer and associates (1988). Three replicates of each PG activity assay were performed.

Isolation of *P. cinnamomi* pg genes.

Using degenerate primers, a polygalacturonase (*pg*) gene fragment was amplified from *P. cinnamomi* genomic DNA isolated as described by Dudler (1990). The sequences of the oligonucleotide primers were: 5' GTIIAIAA(C/T)CA (A/G)GA-(C/T)GA(C/T)TG 3', 5' CCAI(C/G)T(C/T)TT(A/G)ATIC (G/T) IAIICC(A/G)TT 3'. The PCR program was: 94°C for 3 min, then 35 cycles of 96°C for 45 s, 50°C for 1 min, and 72°C for 1 min, and then one cycle of 72°C for 1 min.

A *P. cinnamomi* genomic DNA library in λ EMBL3 (Weerakoon et al. 1998) was screened with the *pg* gene fragment as a probe following standard protocols (Sambrook et al. 1989). λ EMBL3 phage were purified from plate lysates, and phage DNA was prepared using a small scale phage DNA isolation method (Sambrook et al. 1989). *Sal*I fragments of insert DNA were ligated to *Sal*I-digested pBluescript KS+ and used to transform *Escherichia coli* strain DH5 α . In some cases, subclones were also made using *Pst*I, *Hind*III, and *Xho*I when *Sal*I subclones were inconveniently small or in order to obtain overlapping subclones. Standard methods were used for subcloning, preparation of plasmid DNA, and DNA gel blot analysis (Sambrook et al. 1989).

Sequencing and sequence analysis.

Cycle sequencing was performed using BIG DYE chemistry (Perkin Elmer Applied Biosystems, Foster City, CA, U.S.A.). Large plasmid clones were sequenced using primer walking or nested deletions generated by an Erase-a-Base kit (Promega, Madison, WI, U.S.A.). Subclones were sequenced in both strands and ordered into contigs using restriction mapping of phage clones and comparison of overlapping plasmid subclones. Sequence data were analyzed using appropriate programs in the Wisconsin GCG Package vs. 8.1-UNIX (Genetics Computer Group, Madison, WI, U.S.A.). Protein database

searches were performed using the BLASTX program (Altschul et al. 1997).

Evolutionary analysis and protein structure modeling.

For phylogenetic analysis, deduced PG amino acid sequences were aligned using Pileup in the Wisconsin GCG Package v. 8.1-UNIX (Genetics Computer Group) and the alignments were adjusted manually. Highly variable regions in the amino and carboxyl termini were excluded. Phylogenetic analysis by weighted parsimony was performed using PAUP* v. 4.0b4a (Swofford 1998), and the PROTPARS matrix (Felsenstein 1993) was used to weight changes among amino acids. Bootstrap replicates (1,000) were performed.

Reticulate evolution was detected using Sawyer’s runs test (implemented in GENECONV 1.81) (Sawyer 1999) to analyze shared polymorphisms in a 990-basepair-long DNA alignment that excluded the variable 5’ regions of the genes upstream of the codon encoding the first conserved cysteine. Sawyer’s runs test searches an alignment and finds identical or almost identical fragments in pairs of sequences. In our analysis, mismatches were allowed (the GENECONV parameter gscale was set to 1). The significance of the hypothesis that a shared fragment arose through reticulate evolution is estimated by comparison with data sets obtained through random permutation of the columns of the real data set. The “pairwise” probability (*P*) value for a fragment is the proportion of the permuted alignments having a fragment as long or longer than the observed fragment in the same sequence pair. In a large alignment some fragments with low pairwise *P* values would be expected by chance. Therefore a “global” permutation *P* value for a certain fragment found in two sequences is calculated as the proportion of permuted alignments having a fragment as long or longer than the observed fragment in any of its sequence pairs. Global *P* values protect against the statistical artifact that some comparisons may produce fragments with seemingly significant pairwise *P* values by chance.

The same alignment was also explored for further evidence of reticulate evolution by analysis of parsimoniously informative sites (sites where at least two different nucleotides are present in at least two sequences each) using PhylPro (Weiller 1998). For each site in a given sequence of the alignment, PhylPro evaluates how well the pairwise distances between the sequence in question and the other sequences in the alignment correlate upstream and downstream of that site. For each nucleotide position, distance data were calculated by pairwise comparison from an upstream and a downstream window of parsimoniously informative positions. In our analysis, the window limits were set to 15 differences. The correlation between the two sets of distance data is called the phylogenetic correlation. The phylogenetic correlation data for each sequence in the alignment are then shown graphically as phylogenetic profiles. If the phylogenetic correlation for a sequence is very low at a certain site and stands out in the phylogenetic profile graph as a downward spike, this position is a likely site of past recombination in that sequence.

In the maximum chi-squared method (Maynard Smith 1992), implemented in START (Jolley et al. 2001), polymorphic sites (*n*) are defined as sites polymorphic in the putative recombinant and two parental sequences. The recombinant and the parents are compared, and in each comparison the number of differences (*s*) is determined. When an arbitrary cut is made at the *k*th site, the recombinant and each parent differ at *r/k* sites before the cut and (*s – r*)/(*n – k*) sites after the cut. For each site the value *c* is calculated as the value of χ^2 for a 2 × 2 matrix with the proportion of differences between each parent and the recombinant in each of the rows of the matrix, and the optimal recombination breakpoint occurs after the site with the highest *c* value. The significance of a division is tested by generating random sequence pairs of length *n* differing at *s* sites and comparing *c*_{max} for these sequences with *c*_{max} for the real data. The method is based on the assumption that the investigated region contains only one recombination breakpoint, and alignments containing more than one breakpoint are analyzed in steps.

The sister-scanning method, implemented in SiScan 2.0 (Gibbs et al. 2000), passes a sliding window over an alignment comprising a putative recombinant, two parental sequences, and an outlier. The outlier is a real sequence or a randomized sequence generated for each position of the window by randomizing the positions of the nucleotides within the window in one of the real sequences. Including a randomized outlier decreases the risk of false positives that are due to compositional similarity in the genes. At each position of the window, the number of sites that conform to each of the possible patterns of equality and inequality are counted. For each position of the window, four randomized sequences are generated by assigning a new nucleotide at each position chosen at random, without replacement, from the nucleotides that occur at that position in the real sequences, and the number of sites conforming to each possible pattern is counted. This is repeated 100 times to create a population of scores from randomized sequences, and a Z-score for each of the patterns is calculated. In our analysis, a window size of 150 sites, a step size of 20 sites, and an outlier generated by randomization from the putative recombinant were used.

To test for diversifying selection, the ratio between the number of nonsynonymous substitutions per nonsynonymous site (*K_a*) and the number of synonymous substitutions per synonymous site (*K_s*) in pairwise comparisons of *P. cinnamomi* pg sequences were estimated using the methods of Yang and Nielsen (2000) and Nei and Gojobori (1986), which are implemented in YN00 in the PAML package vs. 3.0a (Yang et al. 2000). To implement a more sensitive test for diversifying selection and to detect codons undergoing diversifying selection, the CODONML program in the PAML package version 3.0a (Yang et al. 2000) was used.

The SwissModel First Approach Mode and the Swiss-PdbViewer (Geux and Peitsch 1997; Peitsch 1995, 1996) were used to align *P. cinnamomi* PG sequences to the known structures of *A. niger* endoPGII and *E. carotovora* PehA and to view and analyze the models produced.

Table 4. Amino acid substitutions present at each of the sites apparently undergoing diversifying selection in the *Phytophthora cinnamomi* PGs

Site ^a	PcPG													
	1	2	4	5	6	7	8	9	10	12	16	17	18	19
77 (73)	T	K	E	V	V	E	E	Q	T	S	S	K	T	Q
90 (86)	S	A	E	S	S	E	E	V	A	D	S	S	S	N
95 (91)	S	L	L	L	L	R	Q	L	L	K	N	L	L	T
360 (360)	T	T	T	S	D	S	S	A	D	K	P	L	P	A

^a Position in the unprocessed PcPG4 protein; position of the corresponding residue in the unprocessed *Aspergillus niger* PGII as indicated in brackets.

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