

Characterization of cyclophilin-encoding genes in *Phytophthora*

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Abstract Recent research has shown that cyclophilins, proteins that catalyze the isomerization of peptidyl–prolyl bonds, play a variety of important roles in infection, including facilitating host penetration and colonization and activating pathogen effector proteins within the host cytoplasm. In the current study, bioinformatic analysis of the genomes of three species of plant pathogens in the genus *Phytophthora* has revealed extensive synteny between the 20 or 21 members of the cyclophilin gene family. In *P. infestans*, extensive EST studies give evidence of the expression of 14 of the 21 genes. Sequences homologous to 12 of the 14 expressed *P. infestans* cyclophilins were isolated using PCR and gene-specific primers in the broad host range pathogen, *P. nicotianae*. Quantitative real-time PCR measurements of transcript levels in *P. nicotianae* at four stages of asexual development and during infection of resistant and susceptible tobacco plants gave evidence of expression of seven of the *P. nicotianae* homologs. The most abun-

dantly expressed gene, *PnCyPA*, has a lower mRNA level in zoospores compared to other stages of asexual development and its expression increases during infection of susceptible plants. Immunocytochemical studies indicate that *PnCyPA* occurs in the nucleus and cytoplasm of *P. nicotianae* cells and is secreted from germinated cysts.

Keywords Cyclophilins · Oomycetes · Peptidyl–prolyl isomerases · *Phytophthora*

Introduction

Phytophthora is a genus of Oomycetes that includes over 60 species of destructive plant pathogens. Species that extensively affect commercial crops include *P. sojae*, which causes stem and root rot of soybean, *P. infestans*, the causative agent of the Irish potato famine in the 1800s (Gregory 1983) and *P. nicotianae*, a species with a broad host range that is a major pathogen of tobacco and citrus. *P. palmivora*, *P. megakarya* and other *Phytophthora* species that cause leaf blight, bark canker, pod rot and root rot of cocoa collectively threaten the economies of cocoa growing countries, and are responsible for 10–30% losses in worldwide cocoa production (Evans and Prior 1987). Members of the genus *Phytophthora* also have huge environmental impacts. *P. cinnamomi* kills thousands of species of native plants in forest and heath ecosystems in southern Australia (Hardham 2005) and *P. ramorum* has decimated oak forests along the Pacific Coast of the United States (Rizzo et al. 2002).

The Oomycetes form one of a number of groups of protists classified within the Stramenopiles, an assemblage that includes the heterokont algae, the diatoms and the apicomplexan malarial parasites (Van de Peer et al. 1996). However, although phylogenetically distinct, species of *Phytophthora*

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share a range of characteristics with plant pathogenic fungi. Common features in both groups include hyphal growth, spore dispersal methods and modes of acquiring nutrients and also extend to the molecular level (reviewed in Latijnhouwers et al. 2003). Recently, a large-scale study comparing full-length cDNA sequences revealed that a number of expressed sequences showed considerable similarity between *Phytophthora* and plant pathogenic fungi, but had no homologs in animal pathogenic fungi (Win et al. 2006). As a specific example, *Phytophthora* polygalacturonases, which catalyze the breakdown of pectin in the host plant cell wall, were found to have greater sequence similarity to fungal homologs than to polygalacturonases in other organisms (Götesson et al. 2002). The determination of proteins known to constitute virulence factors in fungal phytopathogens may thus aid in the identification of virulence factors in *Phytophthora*.

One group of virulence factors that has recently been identified in plant pathogenic fungi is the cyclophilins (Viaud et al. 2002). These proteins are conserved in both prokaryotes and eukaryotes, with most organisms possessing multiple cyclophilin genes encoding proteins with different sub-cellular localizations. Cyclophilins (EC 5.2.1.8) are members of the peptidyl-prolyl isomerase (PPIase) family that catalyze the *cis/trans* isomerization of peptide bonds preceded by proline residues, a rate-limiting step in many protein folding processes (Kiefhaber et al. 1990). Other members of this family include the FK506-binding proteins (FKBPs) and the parvulin-like PPIases. These three classes of PPIases are structurally distinct, even at the catalytic site, with cyclophilins being characterized by a cyclosporin A (CsA)-binding hydrophobic pocket formed by an eight-stranded β -barrel, termed a cyclophilin domain (Ke et al. 1991; Mikol et al. 1993). Among the PPIases, cyclophilins and FKBPs are known as immunophilins due to their effects on the immune system. Indeed, cyclophilins were discovered independently of their enzymatic activity, being the cellular target of the immunosuppressive drug CsA with which they form complexes that disrupt the calcineurin signaling pathway required for T-cell activation (Handschumacher et al. 1984; Kallen et al. 1991).

Although the role of cyclophilins in mediating the immunosuppressive effects of CsA has been well characterized, their physiological functions have yet to be fully elucidated. Due to their PPIase activity, initial focus was on the role of cyclophilins as foldases and chaperones during protein synthesis (Baker et al. 1994; Ferrereira et al. 1996; Freeman et al. 1996). However, more recently, it has emerged that the isomerization of prolyl bonds catalyzed by cyclophilins is an important post-translational modification for regulating protein activity and may be a key factor in signal transduction pathways. For example, the activity of interleukin-2 tyrosine kinase, which is involved in T-cell activation, depends on the *cis/trans* conformation of a prolyl residue within its SH2

domain and is specifically regulated by the human cyclophilin, hCyPA (Brazin et al. 2002). It has also become apparent that cyclophilins have a role in mediating protein–protein interactions, with multi-domain cyclophilin proteins potentially interacting with several proteins simultaneously. For example, the AtCyp59 protein of *Arabidopsis thaliana*, which contains a cyclophilin-like domain, an RNA recognition motif domain and a highly charged C-terminal domain, has been found to interact not only with the C-terminal domain of the largest subunit of RNA polymerase II, thereby regulating its phosphorylation, but also with SR splicing factor proteins and RNA molecules, implying a role in the regulation of protein–protein interactions required for mRNA splicing (Gullerova et al. 2006).

Through these and other mechanisms, cyclophilins play diverse roles including folding of proteins during development (Colley et al. 1991), response to environmental stress (Godoy et al. 2000), transcriptional control of meiosis (Arevalo-Rodriguez and Heitman 2005) and regulation of apoptosis (Montague et al. 1997; Walter et al. 1998). In addition, a growing body of evidence shows that cyclophilins are involved in pathogenicity in both plants and animals. Of particular interest in the context of the present study is the discovery that hCyPA cyclophilin homologs from the rice blast fungus *Magnaporthe grisea* and the grey mould *Botrytis cinerea* act as virulence factors during host plant infection (Viaud et al. 2002, 2003).

In the present study, the genomes of *P. sojae*, *P. ramorum* and *P. infestans* were surveyed to identify cyclophilin-encoding genes. From this bioinformatic analysis, it was shown that the *Phytophthora* cyclophilin family typically consists of approximately 20 conserved members. Sequences from orthologs of 12 expressed cyclophilin-encoding genes were isolated from the broad host range pathogen, *P. nicotianae* and the transcript expression profile of each was assessed by quantitative real-time PCR (qPCR). Evidence of expression was obtained for seven genes, with the most abundant being *PnCyPA*, a human cyclophilin A homolog. *P. nicotianae* cyclophilins were further characterized by immunolocalization studies during asexual development. In addition to cytoplasmic and nuclear labeling in all cell types, evidence of cyclophilin secretion from germinated cysts was also obtained. Possible roles of cyclophilins in *Phytophthora* biology are discussed.

Materials and methods

Analysis of *P. sojae*, *P. ramorum* and *P. infestans* cyclophilin sequences

Cyclophilin genes were identified in the v1.1 draft genome sequences of *P. sojae* and *P. ramorum* available

at <http://genome.jgi-psf.org> and in the *P. infestans* genome available at <http://www.broad.mit.edu/tools/data/seq.html>. The cyclophilin genes were identified using the keyword ‘cyclophilin’ in the search interface or by performing tBLASTn (Altschul et al. 1997) searches using the amino acid sequence of the archetypal cyclophilin, hCyPA (GenBank accession number P62937) and its *P. nicotianae* ortholog, PnCyPA (WS64; GenBank accession number CF891681) (Shan et al. 2004). Predicted cyclophilin sequences were aligned with ClustalW using the BLOSUM62 matrix (Higgins et al. 1994). Sequences were corrected to optimize amino acid alignments between homologs by changing intron–exon boundaries or by extending coding regions. The *P. capsici* homolog of Pr20, Ps20 and Pi20 was identified by BLAST of the v1.0 draft genome available at <http://shake.jgi-psf.org/Phyca1/>.

Identification of cyclophilin homologs in *P. nicotianae*

Genomic sequences from *P. sojae*, *P. ramorum* and *P. infestans* were aligned using the ClustalW multiple alignment program (Higgins et al. 1994) and regions that were conserved among orthologs but not in other members of the family were identified. These were used to design gene-specific primers to amplify cyclophilin sequences from *P. nicotianae* genomic DNA. Sequencing of PCR fragments at the Australian Genome Research Facility, Brisbane, Queensland confirmed that the amplified sequences (GenBank accession numbers FJ687591–FJ687600) originated from orthologs of the cyclophilin genes identified in the other *Phytophthora* members.

Sequencing of *PnCyPA* and *PnCyPB* genomic regions

Two of the *P. nicotianae* cyclophilin orthologs had previously been identified through an EST study of genes that were differentially up-regulated in germinated cysts of *P. nicotianae* (Shan et al. 2004). These cyclophilin-encoding sequences, WS64 and WS220 (GenBank accession numbers CF891681 and CF891689), correspond to *PnCyPA* and *PnCyPB*, respectively. *PnCyPA* and *PnCyPB* cDNAs were isolated from a Uni-ZAP XR cDNA library (Stratagene, La Jolla, CA) constructed from germinated cyst mRNA. The cDNAs were sequenced and PCR products amplified from clones acquired from this library were used to screen a *P. nicotianae* genomic BAC library (Shan and Hardham 2004). Full-length genomic sequences were obtained from positive BAC clones using gene-specific primers (GenBank accession numbers FJ438470 and FJ438471).

Analysis of *PnCyPA* and *PnCyPB* 5' ends

Cyclophilin cDNA clones were amplified from pBluescript KS vectors using T3 forward and gene-specific reverse

primers targeting the 5' end of *PnCyPA* and *PnCyPB*. PCR products were purified using the Promega Wizard SV Gel and PCR clean-up system (Promega Inc.), cloned into the pGEM T-Easy plasmid (Promega Inc.) and sequenced. 5' RACE was also conducted for *PnCyPA* using RNA isolated from vegetative hyphae. Primers specific for the long potential transcript of *PnCyPA* were also used in RT-PCR experiments, in which cDNAs from a wide range of developmental stages were used as the template.

DNA sequence analysis

Sequence runs obtained from cDNAs and cyclophilin positive BAC clones were edited and assembled into contigs using the Lasergene software suite (DNASTar, Madison, WI, USA) and processed into FASTA files. The sequences of the assembled contigs were aligned against *P. nicotianae* cDNA sequences to detect the presence of introns with ClustalW. DNA sequences were translated into protein sequences using the translation tool available at http://mol-biol.ru/eng/scripts/01_13.html. Genes were predicted from genomic sequences using FGENESH (Softberry Inc., Mount Kisco, NY, USA), with diatom-specific parameters and GENSCAN (Chesnick et al. 2000). To analyze promoter and coding regions, BIOEDIT v 7.0.5.3 (Hall 1999) was used to search for a *Phytophthora*-specific 19-nucleotide consensus sequence (McLeod et al. 2004). The cellular location of proteins was predicted according to localization motifs identified using TargetP 1.1 (Emanuelsson et al. 2000) and SignalP 3.0 (Bendtsen et al. 2004) was used to predict potential signal sequence motifs and cleavage sites. Theoretical molecular weights and isoelectric points (pIs) of the predicted proteins were calculated by Scansite (<http://scansite.mit.edu/proteincalc.html>).

Domains were identified using the National Centre for Biotechnology Information (NCBI) Conserved Domain Database BLAST server (Marchler-Bauer et al. 2005) with the Conserved Domain Architectural Retrieval Tool (CDART) (Geer et al. 2002). To determine the conservation of residues known to be important for contact of hCyPA with CsA and those which contact calcineurin in the hCyPA–CsA complex (He et al. 2004), *Phytophthora* cyclophilin sequences were aligned against hCyPA residues with ClustalW using the BLOSUM62 matrix. Alignments produced were manually trimmed to include only the cyclophilin domain using the Jalview multiple alignment editor (Clamp et al. 2004). Protein sequences of cyclophilins from a wide range of organisms were retrieved from NCBI (Supplementary Table 1). These sequences were trimmed using the program ParseClustal courtesy of Dr A. Dickerman (Virginia Bioinformatics Institute, Virginia Tech) and were used to generate a Neighbor-Joining phylogram with 1,000 bootstrap replicates using the PHYLIP

v3.66 suite of programs (Felsenstein 1989). Cladograms generated from this analysis were drawn using TreeViewX (Page 1996). The identities and similarities of the proteins were calculated using the Matrix Global Alignment Tool (MatGAT) v2.02 (Campanella et al. 2003) using a BLOSUM50 substitution matrix.

Phytophthora nicotianae isolate and culture conditions

P. nicotianae isolate H1111 (ATCC MYA-141), originally isolated by Dr D. Guest (University of Sydney), was used in all experiments. Cultures were maintained on V8 nutrient agar and V8 broth as described previously (Shan et al. 2004). To prepare vegetative hyphae samples, six to seven $5 \times 5 \text{ mm}^2$ of mycelium cut from the edge of a colony growing on V8 nutrient agar were placed in Petri dishes with approximately 30 ml of V8 broth and grown for 3 days at 25°C in the dark. The V8 broth was changed daily to maintain vegetative growth. Agar plugs were removed from mycelium after removal of liquid by filtration. Sporulating hyphae were initially prepared as for vegetative hyphae, but after 3 days in the dark, the cultures were transferred to the light for at least 14 days at 23°C without changing the media. Zoospore release from sporulating hyphae was induced as described (Mitchell and Hardham 1999). For preparation of germinated cysts, zoospores were vortexed to induce encystment and germinated in 2.5% v/v V8 juice (Campbell's Soups Aust. Pty. Ltd.) before harvesting by centrifugation at $700 \times g$ for 3 min. For DNA or RNA isolations, samples were frozen in liquid nitrogen and stored at -80°C .

Infection of tobacco seedlings

Seeds from susceptible (*N. tabacum* cv. Petit Gerard) and resistant (*N. tabacum* cv. NC2326) cultivars were sterilized in 6% hydrogen peroxide in 100% ethanol for 1 min before being washed in sterile water and germinated on sterile moist filter paper in sealed Petri dishes at 23°C for 14–20 days. For inoculation, 8 ml of zoospores at a concentration of 1×10^5 per ml were added to approximately 200 whole seedlings and incubated for 1 h. Seedlings were then removed, rinsed in water and incubated for 4–24 h after inoculation. Seedlings were then blotted on filter paper to remove excess liquid and frozen in liquid nitrogen.

Quantitative real-time PCR

RNA was isolated from vegetative hyphae, sporulating hyphae, zoospores, 4 h germinated cysts and infected tobacco seedlings, DNase-treated with RQ1 DNaseI (Promega, Madison, WI, USA) and then reverse transcribed using SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo(dT) primers (Invitrogen) to produce

cDNA according to the manufacturer's instructions. Genomic DNA contamination was checked by PCR of cDNA reactions which omitted reverse transcriptase. Levels of gene transcripts were quantified using a Rotor-Gene 3000 real-time cycler (Corbett Life Science, Mortlake, NSW) and FastStart SYBR Green Master Mix (Roche Applied Science, Mannheim, Germany), as described by the manufacturer. In general, transcript levels of three independent biological replicates were determined for each sample and four technical replicates were performed for each measurement. Minus reverse transcriptase controls were included for each cDNA. Relative levels of gene transcripts were computed using the qPCR Analysis Software, version 6.0 (Corbett Research) using the comparative quantitation method. *WS41* (GenBank accession number CF891677), a gene shown to be constitutively expressed throughout the *P. nicotianae* lifecycle stages tested (Shan et al. 2004; Yan and Liou 2006), was used as a normalizing reference gene. Cycling conditions were 40 cycles of 95°C for 15 s (initial step of 95°C for 6 min), 20 s at 55°C, 25 s at 72°C, with data acquired at 59°C. A melt curve (65–95°C) was carried out for each experiment to check for primer-dimer formation. Sample means were compared by analysis of variance with Tukey post hoc comparison with SPSS, version 16 for Windows (SPSS Inc., Chicago, IL).

Immunoblots and immunolocalization

Anti-hCyPA polyclonal antibodies (Calbiochem, La Jolla, CA) were used in immunoblotting and immunomicroscopy assays as described previously (Cope et al. 1996). For immunoblots, tissue from vegetative hyphae, sporulating hyphae and 4 h germinated cysts was ground in liquid nitrogen and soluble proteins extracted in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 8.0, 10% v/v glycerol buffer with protease inhibitors (Roche Applied Science). Samples were centrifuged for 10 min at $14,000 \times g$ to remove cellular debris. Zoospores, which lack cell walls, were not ground, but were vortexed vigorously in the presence of the extraction buffer. Proteins were separated on 12% SDS-PAGE gels and transferred to HybondC membranes (Amersham Corp., Arlington Heights, IL) using a MiniTrans Blot Apparatus (BioRad, Richmond, CA) according to manufacturer's instructions. Precision Plus Kaleidoscope molecular weight markers (BioRad, Hercules, CA) were included on each gel. Membranes were stained with Ponceau S to check protein transfer and loading levels. After blocking with 1% w/v bovine serum albumin (BSA) and 5% w/v skim milk powder in phosphate buffered saline (PBS; 2.7 mM KCl, 138 mM NaCl, 1.5 mM KH_2PO_4 , 20.4 mM NaH_2PO_4 , pH 7) for 2 h, membranes were incubated for 45 min at room temperature in primary antibody, rinsed three times for 5 min in PBT (PBS plus 0.5% v/v Tween 20) and then

incubated in sheep anti-rabbit antibody conjugated to alkaline phosphatase (Chemicon International, Temecula, CA, USA) for 1 h, rinsed and color developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as the color substrate.

For immunofluorescence microscopy, hyphae were fixed in 4% v/v formaldehyde in 50 mM Pipes buffer, pH 6.8 and embedded in OTC compound embedding medium (ProSci-Tech, Thuringowa, Qld) as previously described (Hardham 2001). Zoospores and 4 h germinated cysts were fixed, embedded in butyl-methyl methacrylate and sectioned using an Ultracut microtome (Reichert-Jung, Cambridge Instruments, GmbH, Nussloch, Germany) as described in Hardham (2001). Methacrylate resin was etched in acetone and sections were blocked for up to 3 h in 1% w/v BSA and 0.1% v/v fish scale gelatin in PBS. In the case of germinated cyst sections, this was followed by blocking for 90 min in 1% pectin (after filtration through a 0.2 µm filter). Blocking solutions were removed by pipetting and sections were incubated in primary anti-hCyPA antibodies for 1 h at 37°C, rinsed three times and then incubated in sheep anti-rabbit antibodies conjugated to fluorescein isothiocyanate (Chemicon International, Temecula, CA, USA) for 1 h at 37°C. Slides were then rinsed and mounted in a glycerol-based mounting medium. Sections were viewed using an Axioplan microscope (Zeiss, Germany) and photographed with a digital camera (Princeton Instruments, Inc., Trenton, NJ, USA). In control experiments, the primary antibody to hCyPA was omitted or replaced by a range of other polyclonal antibodies.

For immunogold labeling, zoospores and 3 h germinated cysts were embedded in Lowicryl K4M (Lowi, Waldkraiburg, Germany) as previously described (Hardham 2001). Lowicryl ultrathin sections were blocked with 1% w/v BSA plus 0.1% v/v fish scale gelatin in PBS for 1 h and then incubated with the anti-hCyPA rabbit antibody diluted in 1% w/v BSA plus 0.1% v/v fish scale gelatin for 1 h at room temperature. Sections were rinsed three times in PBT and incubated with goat anti-rabbit conjugated to 10-nm gold (Amersham Biosciences, Birminghamshire, UK) for 1 h at room temperature. Sections were counterstained with uranyl acetate and lead citrate and examined with a Hitachi H7100FA transmission electron microscope equipped with a digital camera. Image processing was performed using Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA) and Adobe Illustrator CS2 (Adobe Systems, San Jose, CA, USA). Immunogold labeling was quantified by counting numbers of gold particles in selected regions of the cells. Surface areas were determined using ImageJ (NIH, Bethesda, MD, USA). For assessment of secreted cyclophilins, gold particles within 0.25 µm of the plasma membrane were counted.

Results

Bioinformatic analysis of cyclophilins in *P. ramorum*, *P. sojae* and *P. infestans*

Database searches of the *P. sojae*, *P. ramorum* and *P. infestans* genomes identified 20 cyclophilin sequences in *P. sojae* and 21 in *P. ramorum* and *P. infestans*. Of the JGI-predicted gene models, only 14 of the *P. sojae* models and five of the *P. ramorum* models were predicted to encode full-length proteins. By manually comparing the sequences of *P. sojae*, *P. ramorum* and *P. infestans*, coding regions of several of the incomplete predicted gene models were extended into full-length open reading frames (ORFs). Gene models were assigned the numbers as shown in Supplementary Tables 2, 3 and 4 which also include the lengths of the final predicted polypeptides, their estimated molecular weights, the number of predicted introns for each gene and the TargetP predicted localizations for proteins with full N-terminal sequences. The availability of corresponding EST sequences provided evidence for the expression of eight of the 20 *P. sojae* cyclophilins and 14 of the 21 *P. infestans* cyclophilins. No EST data were available for the *P. ramorum* homologs of these genes.

Analysis of the protein sequences showed that in the majority of the predicted proteins, the only recognized motif was a single cyclophilin-like domain. In addition, there were five multi-domain cyclophilins in all three species (Fig. 1). With the exception of Ps20, Pr20 and Pi20, the predicted multi-domain cyclophilin sequences displayed domain organizations similar to those of known proteins from other organisms. Among the multi-domain cyclophilins with known domain structures, Ps13, Pr13 and Pi13 each possess a WD40 domain which coordinates protein–protein interactions in multi-protein complexes (Li and Roberts 2001). Ps18, Pr18 and Pi18 possess U-box domains, which are modified DNA-binding, ring finger domains (Aravind and Koonin 2000) and Ps10, Pr10 and Pi10 have RNA recognition motifs, which bind to RNA (Birney et al. 1993). Ps11, Pr11 and Pi11 represent members of the FK506- and cyclosporin-binding protein (FCBP) dual-family immunophilins which possess not only a cyclophilin, but also an FKBP immunophilin domain (Adams et al. 2005). CDART analysis of the NCBI Entrez Protein Database showed that eukaryotic proteins with this unusual domain organization have previously been identified in the apicomplexan parasites *Toxoplasma gondii* and *Theileria parva*.

In addition to these proteins, the JGI-predicted Pi9-2 protein was modeled to possess a dehydratase domain, which is involved in removal of oxygen and hydrogen from organic compounds. However, this gene model is most likely incorrect, since it possesses introns that are much larger than those normally found in *Phytophthora* genes

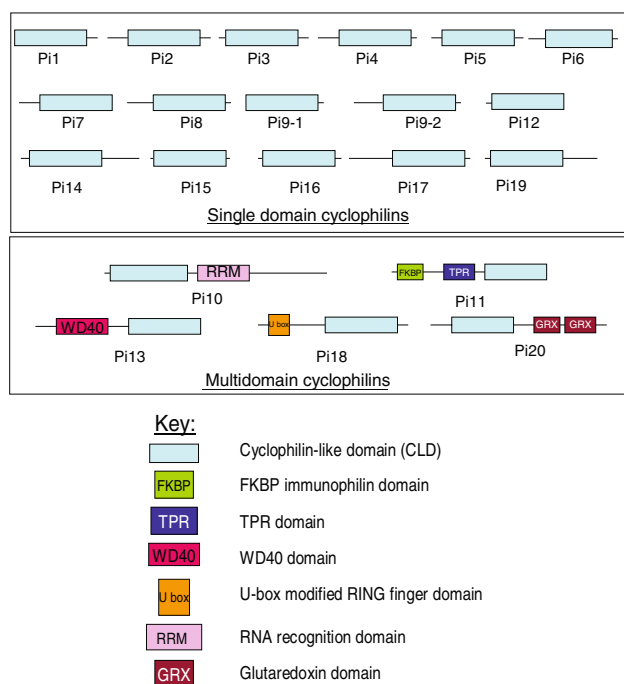


Fig. 1 Domain structures of single- and multi-domain cyclophilins from *P. infestans*. Orthologs of each of these proteins possessing similar domain architectures were found in *P. sojae* and *P. ramorum*

(Kamoun 2003). Further, the corresponding gene models in *P. sojae* (Ps9) and *P. ramorum* (Pr9) encode single-domain cyclophilins with an adjacent dehydratase gene. In addition, Pi9-1, which differs from Pi9-2 by 34 out of its 171 amino acids in the cyclophilin-like domain and which appears to have arisen from a gene duplication event, is predicted to be a single-domain protein.

In contrast to members of the other multi-domain cyclophilins in *Phytophthora* species, *Ps20*, *Pr20* and *Pi20* were predicted to encode multi-domain proteins with a domain structure not previously described. *Ps20*, *Pr20* and *Pi20* are unusual multi-domain proteins, containing a divergent cyclophilin domain and glutaredoxin electron transport domains. A gene with this structure is also present in the *P. capsici* genome and, in this study, the *P. nicotianae* homolog has been partially sequenced. Sequence comparisons of the cyclophilin-like domains between *Ps20*, *Pr20*, *Pi20* and hCyPA showed that residues implicated in PPIase activity, corresponding to R55, F60, W121 and H126 in hCyPA (Kallen et al. 1991; Zydowsky et al. 1992), were only partially conserved in *Ps20*, *Pr20* and *Pi20*, indicating that these proteins may not have PPIase activity (Supplementary Fig. 1). By contrast, these residues were fully conserved in seven of the *P. sojae*, *P. ramorum* and *P. infestans* sequences, suggesting that these 21 proteins are functional PPIases. The multiple sequence alignment also showed that, consistent with findings on cyclophilins of other organisms, such as *Plasmodium falciparum* (Bell

et al. 2006), residues required for CsA-binding were more conserved than residues required for binding to calcineurin in the presence of CsA. The alignment also revealed the insertion of several amino acids in the linker region between the $\alpha 1$ helix and $\beta 3$ sheet in most of the *Phytophthora* proteins compared to hCyPA (boxed region in Supplementary Fig. 1).

Phylogenetic analysis of cyclophilins in *P. ramorum*, *P. sojae* and *P. infestans*

Construction of a Neighbor-Joining tree (Supplementary Fig. 2) revealed that there was clear orthology between most of the *P. sojae*, *P. ramorum* and *P. infestans* proteins. This agrees with a previous study (Tyler et al. 2006) showing that a large proportion of genes from *P. sojae* and *P. ramorum* are orthologous. This strong orthology appears to extend to other members of *Phytophthora*, as supported by the isolation of sequences corresponding to 12 *P. nicotianae* orthologs of the 14 expressed *P. infestans* cyclophilin genes as described below.

Orthologous relationships were also supported through assessment of the synteny of flanking genomic regions and, in the case of multi-domain cyclophilins, similar domain architectures. The grouping of orthologs on the cladogram was further established by the clustering of members from other species with known orthologous relationships rather than according to species of origin. For instance, *Ps13*, *Pr13* and *Pi13* clustered together with HAL53 (human), SpCYP9 (*Schizosaccharomyces pombe*), dmCG3511 (*Drosophila melanogaster*) and AtCYP71 (*A. thaliana*) which are established orthologs possessing N-terminal WD40 motifs (Pemberton and Kay 2005). However, the two-domain FCBP proteins did not cluster according to their domain structure. In addition, the phylogenetic analysis also revealed that *Phytophthora* cyclophilins are divergent from fungal hCyPA homologs, including the virulence factors BCPI and MgCYP1, which cluster together in a separate clade.

Phylogenetic analysis also showed that, consistent with previous findings (Chou and Gasser 1997), many of the cyclophilin proteins grouped together according to their putative localizations. For example, the hCyPA homologs, *Ps1*, *Pr1*, *Pi1* and PnCyPA, clustered with cytosolic cyclophilins from other species, while *Ps2*, *Pr2*, *Pi2* and PnCyPB clustered with proteins targeted to the secretory pathway.

Analysis of cyclophilin EST data

Expression profiles of *P. sojae* and *P. infestans* cyclophilins were studied by compiling and analyzing cyclophilin EST data from the PFGD and BROAD institute databases (Supplementary Tables 5 and 6). These analyses showed that the

majority of *Phytophthora* cyclophilins, including the orthologs of *PnCyPA* (*Ps1* and *Pi1*) and *PnCyPB* (*Ps2* and *Pi2*) are expressed in a wide range of developmental stages. In contrast, ESTs for some of the cyclophilins identified to date came only from specific stages or from material grown under specific conditions. For instance, *Pi14* has been detected only during mating and *Ps11* was detected only during infection.

P. nicotianae cyclophilin expression during asexual development and infection

P. nicotianae homologs of cyclophilin genes known from the EST data to be expressed in *P. infestans* were amplified from *P. nicotianae* genomic DNA. To do this, gene-specific primers based on the cyclophilin gene sequences in *P. infestans*, *P. sojae* and *P. ramorum* were used and *P. nicotianae* homologs to 12 of the 14 expressed *P. infestans* cyclophilins were obtained. The expression profiles of the *P. nicotianae* cyclophilins during four stages of asexual development, namely vegetative hyphae, sporulating hyphae, zoospores and germinated cysts, as well as in susceptible and resistant tobacco plants, 4 and 24 h post

infection, were assessed by qPCR. The results of this experiment provided evidence for the expression of seven out of the 12 genes in the cDNAs tested. In the qPCR data, levels of *P. nicotianae* cyclophilin transcripts are calculated relative to those of the normalizing gene, *WS41*, a *P. nicotianae* gene that is constitutively expressed at moderate levels throughout development (Shan et al. 2004; Yan and Liou 2006). The results in Fig. 2a show that during the asexual developmental stages tested, *PnCyPA* and *PnCyPB*, were the two most abundantly expressed cyclophilin genes. Analysis of variance of the transcript levels revealed that *PnCyPA* is significantly down-regulated in zoospores ($P < 0.05$) compared to vegetative hyphae, sporulating hyphae and germinated cysts. The transcript levels of *PnCyPB* were not statistically different between the different stages.

Levels of expression of the *P. nicotianae* cyclophilin genes were also determined during infection of susceptible and resistant tobacco seedlings (Fig. 2b). qPCR analysis showed that of the three most abundantly expressed genes during infection, *PnCyPA*, *PnCyPB* and *Pn6*, only *PnCyPA* levels were up-regulated between 4 and 24 h after inoculation and only in susceptible plants ($P < 0.05$).

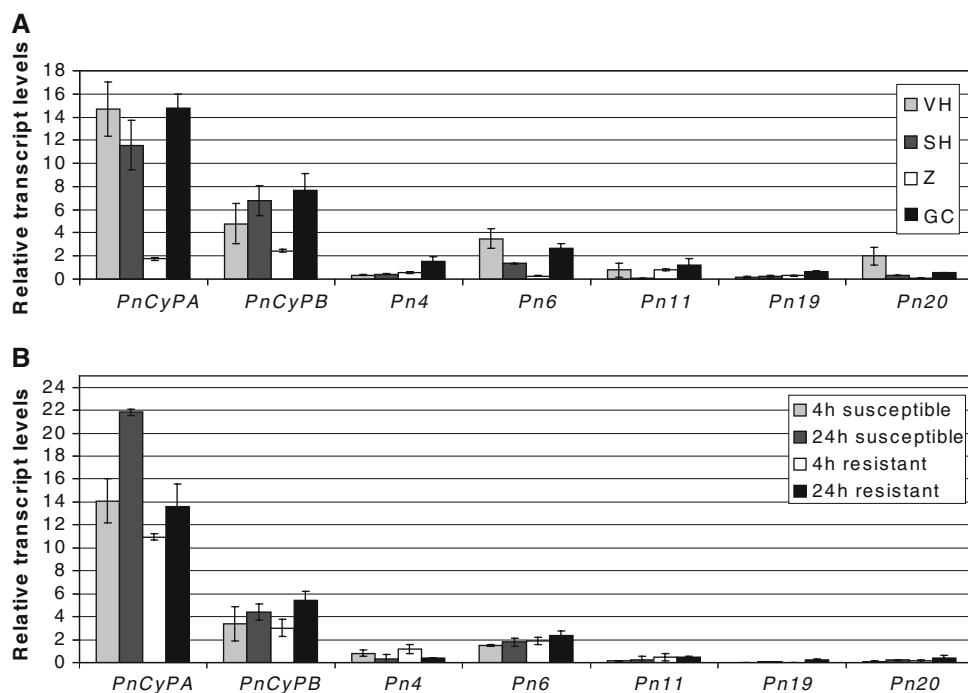


Fig. 2 Relative transcript levels of *P. nicotianae* cyclophilin transcripts as measured by qPCR. **a** Relative transcript levels of cyclophilins in *P. nicotianae* during key stages in asexual growth in vitro: vegetative hyphae (VH), sporulating hyphae (SH), zoospores (Z) and 4 h germinated cysts (GC). **b** Relative transcript levels of cyclophilins during *P. nicotianae* infection of susceptible and resistant tobacco seedlings at 4 and 24 h post inoculation. Data for *Pn9*, *Pn10*, *Pn12*,

Pn16 and *Pn18* were omitted from the graph, since qPCR provided no evidence for the expression of these genes. Values are shown relative to levels of the constitutively expressed gene, *WS41* and represent the mean \pm SEM. Analysis of variance indicates that *PnCyPA* levels are significantly higher in susceptible plants 24 h after inoculation than in the other infected samples ($P < 0.05$)

Gene structure of *PnCypA* and *PnCypB* from *P. nicotianae*

The structure of the two most abundantly expressed cyclophilin genes in *P. nicotianae*, *PnCypA* and *PnCypB*, was determined by isolating genomic BAC clones containing *PnCypA* and *PnCypB* sequences from a *P. nicotianae* genomic BAC library (Shan and Hardham 2004). Sequencing of the genomic *PnCypA* gene revealed that this gene was a homolog of the human *hCypA* gene and had two in-frame potential translational start sites (Fig. 3a). The longer ORF was predicted to encode a 235 amino acid cyclophilin with a signal peptide that is cleaved to produce a 22.3 kDa mature peptide with a pI of 9.06, while the shorter ORF was predicted to encode a 171 amino acid, 18.3 kDa proteins with a pI of 7.69. Since *hCypA* homologs of several organisms produce two different polypeptide products from the same reading frame, sequences upstream of both ATGs were examined and experiments to amplify the 5' ends of *PnCypA* transcripts from vegetative hyphae RNA and from cDNAs isolated from a wide range of developmental stages were performed. Sequence analysis showed that the start codons of both putative short and long ORFs were downstream of a 19-nucleotide *Phytophthora*-specific promoter motif normally found 30–80 bp upstream of the translational start sites of *Phytophthora* genes (McLeod et al. 2004). However, 5' extension analysis and RT-PCR using primers that would amplify the 5' end of the long ORF provided no evidence for expression of the long ORF. This indicates that, in contrast to the genes of several fungal *hCypA* homologs, the gene encoding the *hCypA* homolog in *P. nicotianae* is unlikely to encode two forms of cyclophilin, at least in the developmental stages assessed in our study.

In the case of *PnCypB*, comparisons between the genomic and cDNA sequences revealed the presence of an 82 bp intron within the gene. *PnCypB* was found to encode a 206 amino acid human cyclophilin B homolog with a predicted N-terminal cleavable 22 amino acid signal sequence. Promoter analysis showed that the start codon for *PnCypB* was 44 bp downstream of the 19-nucleotide *Phytophthora*-specific promoter motif (McLeod et al. 2004) (Fig. 3b). The *PnCypB* pre-protein was predicted to have a pI of 7.89 and a molecular weight of 22.1 kDa, while the mature protein was predicted to have a pI of 7.31 and a molecular weight of 19.7 kDa. 5' extension of *PnCypB* showed that the full-length transcript with signal peptide is expressed.

Immunoblot and immunolocalization of *P. nicotianae* cyclophilin

The distribution of cyclophilin proteins in *P. nicotianae* was investigated using a polyclonal antibody raised against *hCypA*. Recognition of *Phytophthora* proteins by this antibody was first assessed using immunoblots of proteins

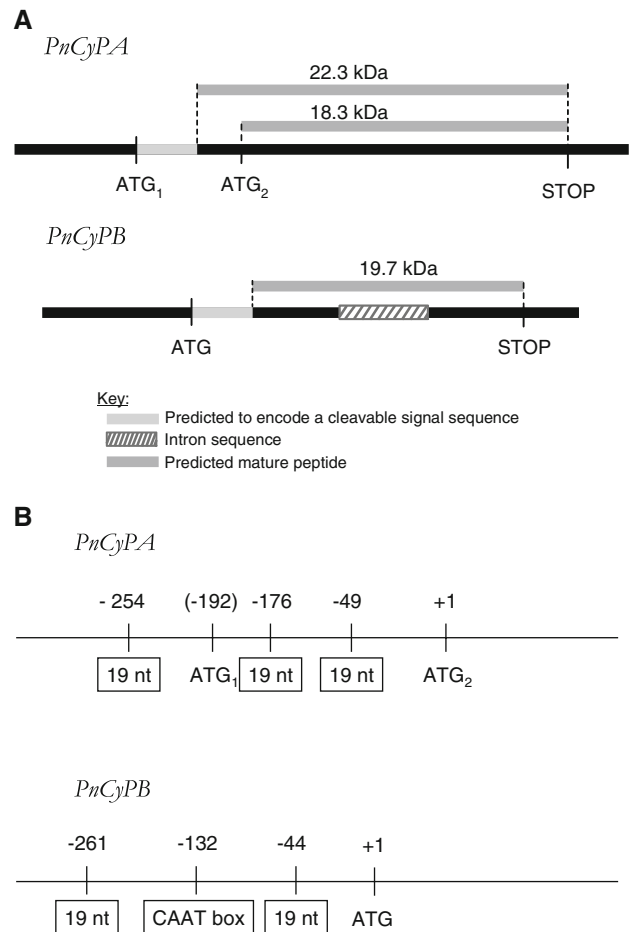


Fig. 3 Gene structures of *PnCypA* and *PnCypB*. **a** *PnCypA* and *PnCypB* genes with their predicted protein products. **b** Promoter motifs upstream of the putative start codons of *PnCypA* and *PnCypB*. The upstream putative translation start codon of a long putative *PnCypA* transcript is denoted as ATG₁, while the putative start codon of a short predicted transcript is denoted as ATG₂. The distances of potential promoter motifs and ATG₁ are shown in bp relative to ATG₂ for *PnCypA* and ATG for *PnCypB*

extracted from vegetative hyphae, sporulating hyphae, zoospores and 4 h germinated cysts (Fig. 4). The antibody recognized a protein with a molecular mass of 18 kDa in all four developmental stages. In *P. infestans*, *P. sojae* and *P. ramorum* genomes, the cyclophilin proteins homologous to *PnCypA* have the highest similarity to *hCypA* with approximately 71–72% identity to the *hCypA* sequence at the amino acid level. Similarly, the *PnCypA* gene encodes an 18 kDa homolog of *hCypA* with 72.3% amino acid identity to *hCypA*. Thus, it is likely that the protein recognized by the anti-*hCypA* antibody is the protein product of *PnCypA*.

Immunofluorescence labeling with the polyclonal anti-*hCypA* antibody showed punctate and diffuse cytoplasmic labeling of vegetative hyphae (Fig. 5a). Cytoplasmic labeling was also apparent in sporulating hyphae and sporangia (Fig. 5b). In zoospores, diffuse cytoplasmic labeling was

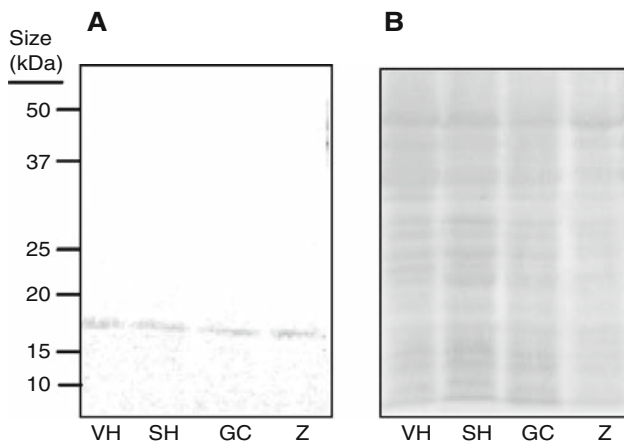


Fig. 4 Immunoblot of cyclophilins during *P. nicotianae* asexual stages using an antibody raised against hCyPA. **a** One prominent band of 18 kDa relative molecular weight was labeled by the anti-hCyPA antibody in protein extracts from vegetative hyphae (VH), sporulating hyphae (SH), 4 h germinated cysts (GC) and zoospores (Z). **b** Ponceau S stained membrane before labeling showing loading levels of protein extracts. Positions of molecular weight standards are shown on the left in kDa

accompanied in many cells by two or three bright spots of fluorescence, whose distribution varied from cell to cell (Fig. 5c). In 4 h germinated cysts, the antibody labeled the cytoplasm and cell surface of the entire germling (Fig. 5d). Controls included omission of the primary antibody and use of a range of polyclonal antibodies directed towards other antigens (data not shown). The results indicated that labeling with the anti-hCyPA antibody was specific, including the labeling at the surface of germinated cysts. Immunogold labeling of zoospores with the anti-hCyPA antibody showed gold particles in the cytoplasm and nucleus with no statistically significant difference between the labeling densities in these two compartments (Fig. 6). Labeling that might account for the punctate spots detected in zoospores using immunofluorescence microscopy was not observed. In germinated cysts, gold particles occurred in the cytoplasm and nucleus, however, a high density of gold particles was also detected on the cell surface, a result consistent with the immunofluorescence assays.

Discussion

In this study, cyclophilin gene families in the genomes of three *Phytophthora* species were analyzed and found to consist of 20 or 21 members, including single- and multi-domain proteins. Phylogenetic analysis of the cyclophilin-like domains revealed that the *Phytophthora* cyclophilins generally cluster with cyclophilins from other organisms according to their cellular localizations and, for the multi-domain proteins, their domain structure. The exception to

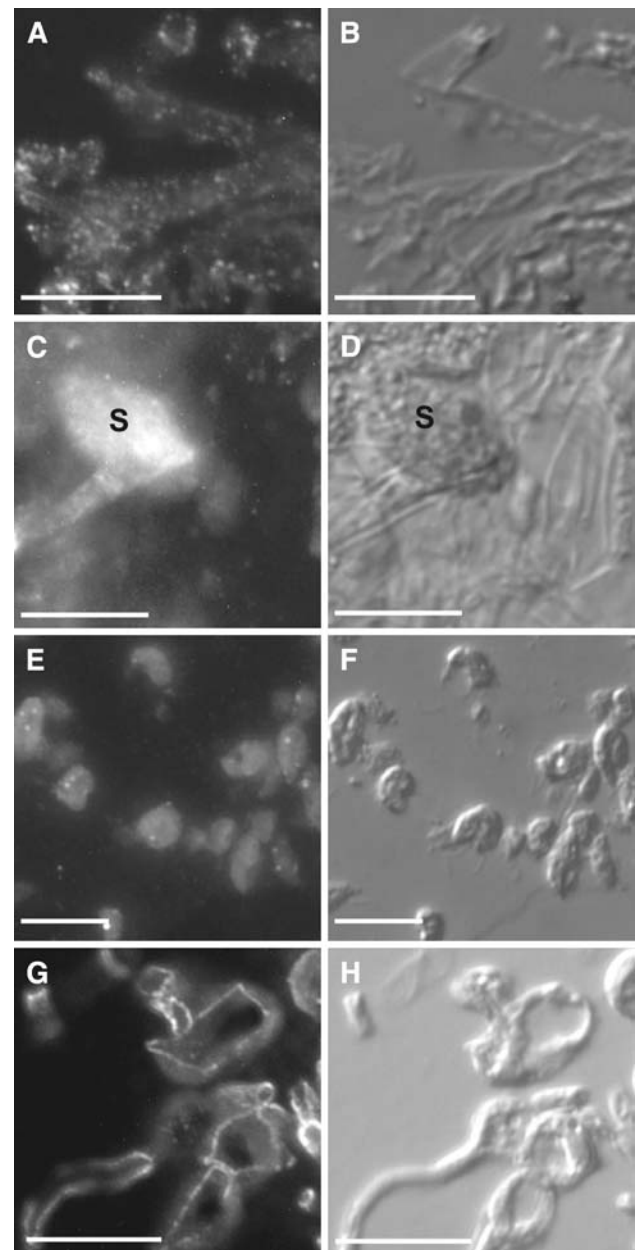


Fig. 5 Immunofluorescence localization of cyclophilins using anti-hCyPA antibodies in *P. nicotianae* **(a)** vegetative hyphae, **(c)** sporulating hyphae, **(e)** zoospores and **(g)** 4 h germinated cysts. DIC images of each sample are shown on the right **(b, d, g, h)**. In the germinated cyst sample **(g, h)**, cells below the cut surface of the section are not accessible to the antibody. S sporangia. Scale bar 50 μ m

this is the *Phytophthora* FCBP proteins, which do not cluster with the apicomplexan FCBP proteins, suggesting that the two groups of dual cyclophilin–FKBP proteins may have evolved independently after the divergence of the apicomplexan and the oomycete lineages within the Stramenopile assemblage. The fact that dual cyclophilin–FKBP proteins are not found in all apicomplexans (Adams et al. 2005) is consistent with this scenario although, of course, the genes may have been lost from some apicomplexan species.

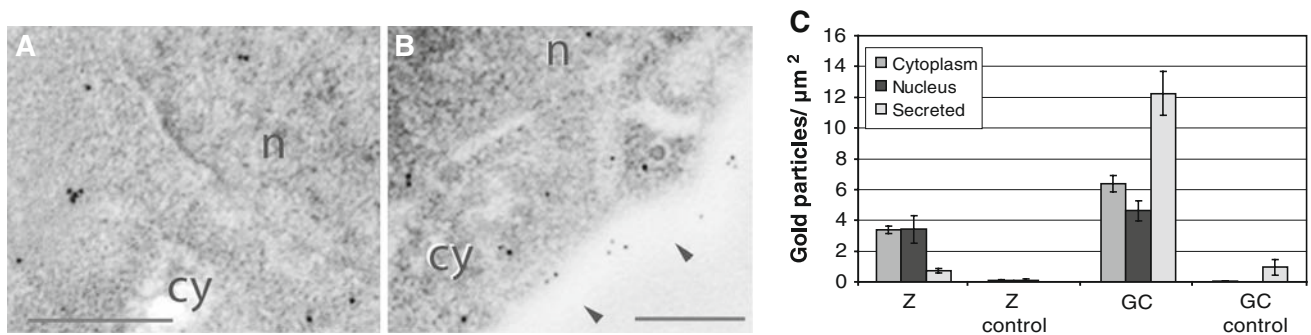


Fig. 6 Immunogold labeling of *P. nicotianae* zoospores and 3 h germinated cysts with the anti-hCyPA antibody. Micrographs show representative labeling of **a** zoospores and **b** germinated cysts. Cytoplasm (cy), nuclei (n) and the cyst surface are labeled. The arrowheads indicate the cyst cell wall, which is electron-lucent in this non-osmicated

material. **c** Labeling densities for the cytoplasm, nucleus and for a region 0.25 μm in width outside the plasma membrane were calculated in zoospores (Z) and germinated cysts (GC). Controls were labeled with secondary antibodies only. Scale bars 2 μm

The clustering of the *Phytophthora* cyclophilin proteins with orthologs in other organisms provides clues to potential roles of these proteins. For example, Ps10, Pr10 and Pi10, *Phytophthora* cyclophilins that possess an RNA recognition domain, cluster with and may have a role similar to other cyclophilins with RNA recognition domains, such as KIN241 of *Paramecium tetraurelia*, which is involved in transcriptional control, cell morphogenesis, cortical organization and nuclear reorganization (Krzywicka et al. 2001). Similarly, Pr18, Ps18 and Pi18 may have a specific role in the nucleus, as does their ortholog human cyclophilin 60 (Wang et al. 1996). Ps16, Pr16, Pi16, Pr14, Ps14 and Pi14, which group together with heat shock-associated cyclophilins, may be involved in stress responses (Pemberton and Kay 2005).

The cyclophilin-glutaredoxin multi-domain proteins identified in *P. ramorum* (Pr20), *P. sojae* (Ps20) and *P. infestans* (Pi20) also appears to be present in *P. capsici* and *P. nicotianae* (Pn20). The combination of cyclophilin and glutaredoxin domains has not been reported previously, but could contribute to pathogen survival under stressful conditions, including those encountered during the plant defence response. Glutaredoxin domains are involved in redox reactions and act to protect cells from oxidative damage (Grant 2001). The *P. infestans* EST database contains evidence of expression of both the glutaredoxin and the cyclophilin domains. Although *Pi20* ESTs occur in samples of vegetative hyphae and in mycelia growing under nutrient-stress, our qPCR analysis of *Pn20* indicates that it is expressed most highly in vegetative hyphae and gives no indication of enhanced expression in either sporulating hyphae (i.e. a situation of nutrient-stress) or during plant infection.

Sequences from 12 *P. nicotianae* cyclophilin-encoding genes were isolated and evidence for expression of seven was found in the cDNAs tested. EST data from *P. infestans* and *P. sojae* and the qPCR data from *P. nicotianae* indicate that *Phytophthora* cyclophilin genes are expressed under a

variety of conditions, although the expression of some members of the gene family occurs only under certain circumstances. Two of the five genes, for which there was no evidence of expression in *P. nicotianae*, had ESTs that appeared only in *P. infestans* cDNA libraries taken from situations not tested in the current study (e.g. mating, peroxide-treated mycelium and cleaving sporangia). Six of the *P. infestans* genes, for which there is EST evidence of expression, lack similar evidence from studies of *P. sojae*. The qPCR analysis of *P. nicotianae* cyclophilin genes revealed differences in expression levels during asexual development, with a general tendency for low levels of expression in zoospores, particularly in the three most abundantly expressed genes, *PnCyPA*, *PnCyPB* and *Pn6*. Early inhibitor experiments suggested that mRNA and protein synthesis were not required in zoospores (Penington et al. 1989), although more recent studies have shown that these processes do occur (Krämer et al. 1997; Škalamera et al. 2004). Nevertheless, it may be that the low cyclophilin transcription rates in zoospores reflect lower rates of protein synthesis in zoospores than at other times in the lifecycle.

Bioinformatic analysis shows that the *PnCyPA* gene is an ortholog of the archetypal human cyclophilin *hCyPA* and encodes an 18 kDa protein. The immunoblots of *P. nicotianae* proteins labeled with the anti-hCyPA antibody show reaction of this antibody only with an 18 kDa band. Together with the fact that the second most highly expressed gene, *PnCyPB*, encodes a 20 or 22 kDa protein (depending on the presence or absence of the secretion signal peptide), it is therefore most likely that the anti-hCyPA antibody reacts only with *PnCyPA*. The immunoblots gave little evidence of changes in *PnCyPA* levels during asexual development and did not reflect the lower *PnCyPA* transcripts levels in zoospores. This could be because higher levels of *PnCyPA* transcription and translation in these cells are accompanied by higher rates of protein turnover than occur in zoospores. Such a phenomenon has been observed

for the heat shock protein, human cyclophilin 40, which exhibits increased degradation following heat stress, but maintains a constant level of protein due to increased mRNA transcription (Mark et al. 2001).

Within the cytosol, PnCyPA is likely to chaperone or aid in the folding of cytoplasmic proteins. The punctate cytoplasmic labeling in *P. nicotianae* hyphae is similar to that seen in NIH 3T3, NRK and HeLa tissue culture cells, in which the hCyPA protein localizes to vesicles and the golgi apparatus, where it may serve a protein folding function through direct or indirect association with binding partners (Sarris et al. 1992). As observed in the current study of *P. nicotianae*, in mammalian and fungal cells, hCyPA and its orthologs have also been observed in the nucleus, despite the absence of a nuclear localization signal (Le Hir et al. 1995; Chiu et al. 2003; Arevalo-Rodriguez and Heitman 2005). In *Saccharomyces cerevisiae*, nuclear accumulation of Cpr1, the hCyPA homolog, regulates sporulation and is thought to occur by diffusion through nuclear pore complexes and retention through interaction with nuclear binding partners (Arevalo-Rodriguez and Heitman 2005). In *P. nicotianae*, the quantitative immunogold labeling showed that PnCyPA is present at similar levels in the nucleus and cytoplasm of zoospores and germinated cysts, indicating the absence of nuclear accumulation in these cells.

Although PnCyPB and other *Phytophthora* cyclophilins encode N-terminal signal sequences directing secretion, the immunoblots with the hCyPA antibodies only show recognition of a protein corresponding to the size of PnCyPA. Although the PnCyPA protein is predicted to be cytosolic, its orthologs, which also lack signal sequences, are secreted. For example, hCyPA is released from cells via a regulated vesicular pathway in response to stresses (Sherry et al. 1992; Suzuki et al. 2006). The immunolabeling with anti-hCyPA antibodies gives evidence of secretion of PnCyPA from *P. nicotianae* germinated cysts, but not from other stages of the lifecycle. The secreted cyclophilins are associated with the wall of the germinated cysts, raising the possibility that PnCyPA might be secreted by wall-less zoospores, but not retained at the cell surface. However, other proteins secreted by *Phytophthora* zoospores have been localized by immunofluorescence and immunogold labeling (Gubler and Hardham 1988; Hardham and Gubler 1990). Cyclophilin secretion by germinated cysts may facilitate folding of other secreted proteins, including those involved in host invasion, as occurs in *Mycoplasma pneumoniae*, where CsA inhibition of adhesion gives evidence that secreted cyclophilins aid folding of proline-rich adhesins and adhesin-related accessory proteins (Reddy et al. 1996). Both spore and hyphal adhesives have now been identified in *Phytophthora* (Gaulin et al. 2002; Robold and Hardham 2005), and future evaluation of cyclophilin regulation of their function may be instructive.

Recent studies of pathogen proteins that have been transported into the host cell cytoplasm have highlighted the role of host cyclophilins in the activation of pathogen effectors (Coaker et al. 2005) and it seems likely that secreted pathogen cyclophilins will be involved in the folding and activation of pathogen effectors that function in the apoplast. Whether or not this interaction between *Phytophthora* cyclophilins and effectors occurs via a previously identified motif similar to the short hCyPA-binding motif (Piotukh et al. 2005) remains to be determined. Bioinformatic screening of a selection of 22 *Phytophthora* effectors revealed that four proteins possessed this binding motif (P. Gan, unpublished observations).

There is growing evidence that pathogen cyclophilins, in particular homologs of hCyPA, play a role in the infection of both plant and animal hosts. In *M. grisea*, the hCyPA homolog, *MgCYP1*, is up-regulated during vegetative growth and pathogenesis and is required for penetration peg formation, appressorium turgor generation and sporulation (Viaud et al. 2002). In *B. cinerea*, the hCyPA homolog, *BCP1*, is up-regulated after host plant penetration and is required for penetration and in planta growth (Viaud et al. 2003). Up-regulation of PnCyPA during infection in susceptible tobacco plants is consistent with a role for this cyclophilin in infection. In the animal pathogenic fungi *Cryptococcus neoformans* and *Candida albicans*, and in the plant pathogen *Sclerotinia sclerotiorum*, hCyPA homologs are required for development and pathogenesis and may function through their regulation of the assembly or localization of calcineurin (Odom et al. 1997; Fox and Heitman 2002; Blankenship et al. 2003; Harel et al. 2006). In these pathogens, it is the hCyPA homologs that confer sensitivity to CsA and its analogs, and thus constitute a potential target for control (Cruz et al. 2000; Viaud et al. 2003). In *P. nicotianae*, however, CsA at concentrations of up to 100 µg/ml does not significantly inhibit growth under normal in vitro culture conditions (P. Gan, unpublished observations). This lack of sensitivity to CsA may be due to differences in PnCyPA protein sequence, which disrupt its interaction with CsA. The linker region between the $\alpha 1$ helix and $\beta 3$ sheet in cyclophilins shows considerable diversity in *Phytophthora* and in other organisms including plants, yeasts, invertebrates and mammals and may provide the structural variation bestowing specificity of interaction partners in vivo (Galat 1999). Although the amino acids required for CsA-binding are conserved in PnCyPA, structural variation in this linker region may prevent the interaction between CsA and PnCyPA, thus conferring resistance against CsA treatment. Alternatively, CsA may inhibit *P. nicotianae* growth only under specific conditions, such as in planta growth, as has been observed for *C. neoformans*, where the pathogen was only sensitive to CsA at the elevated temperatures encountered during host invasion (Odom et al. 1997).

As the cellular functions of cyclophilins continue to be elucidated, their key role not only in protein biosynthesis and maintenance of protein integrity, but also in the regulation of transcription, post-translational modification and protein activity is emerging. The current study has unraveled the composition of the cyclophilin gene family in *Phytophthora*, revealing the strong synteny between cyclophilin genes in different *Phytophthora* species and profiling their expression patterns during development and plant infection. These results provide a framework for future studies of the regulation and function of cyclophilins in *Phytophthora*.

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