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Fungal Genetics and Biology 42 (2005) 339–350
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Cloning and analysis of *pppg1*, an inducible endopolygalacturonase gene from the oomycete plant pathogen *Phytophthora parasitica*

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Received 27 September 2004; accepted 6 January 2005

Abstract

Phytophthora parasitica is an oomycete plant pathogen that causes severe disease in a wide variety of crops. Here, we report the isolation of a gene, named *pppg1*, which encodes an extracellular endopolygalacturonase in *P. parasitica*. Both cDNA and a genomic clone were isolated and sequenced. The *pppg1* gene showed standard characteristics with respect to core promoter and intron sequences of *Phytophthora*. The predicted protein of *pppg1* has a calculated molecular mass of 39.7 kDa and a p*I* value of 5.2, and contains a putative signal peptide of 20 amino acid residues on the N-terminus. The deduced amino acid sequence is highly conserved with those of other *Phytophthora* and fungal endopolygalacturonases. Analysis by reverse transcription followed by real-time quantitative polymerase chain reaction showed that transcription of *pppg1* was repressed by glucose, but induced by pectin in the culture. Moreover, *pppg1* is highly expressed during interaction of *P. parasitica* with the host plant, suggesting its involvement in the process of host infection. Heterologous expression of *pppg1* in *Pichia pastoris* produced proteins with molecular mass ranging from 75 to 200 kDa, very likely due to differential glycosylation by the yeast. Deglycosylation of the recombinant protein resulted in a complete loss of the endopolygalacturonase activity.

Keywords: *Phytophthora parasitica*; Polygalacturonase; Glycosylation

1. Introduction

Plant pathogens produce a diverse range of cell wall degradation enzymes to facilitate invasion of the host tissue (Walton, 1994). Degradation of host tissues generally begins with secretion of pectic enzymes that hydrolyze pectin, the major component of the primary plant cell wall and middle lamella. Polygalacturonase (PG) is the pectic enzyme that hydrolyzes polygalacturonan, the key component of the pectin. Depending on the mode of action, PGs are further classified into endoPG and exoPG, although some enzymes are known to exhibit both endo- and exoPG activities (Cooper et al., 1978). EndoPG (EC 3.2.1.15) cleaves the backbone of polygalacturonan internally, whereas exoPG (EC 3.2.1.67) hydrolyzes monomers processively from the nonreducing end of the substrate. It has long been proposed that endoPGs play an important role in fungal pathogenicity. In *Aspergillus flavus*, endoPGs exist in multiple forms and virulence of pathogens has been related to specific PG isozymes (Brown et al., 1992; Cleveland and Cotty, 1991). In *Mycocentrospora acerina*, virulence of pathogen strains toward the hosts was correlated with total PG activity secreted by the fungus (Le-Cam et al., 1994). *Phomopsis cucurbitae* contains a set of PG isozymes which plays an important role in

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doi:10.1016/j.fgb.2005.01.003

post harvest decay of cantaloupe fruit caused by this fungus (Zhang et al., 1997). Furthermore, molecular genetic studies have demonstrated in several cases that endoPG is a virulence factor for plant pathogenic fungi. Disruption of one of the six endoPG genes caused a reduction in virulence of *Botrytis cinerea* on host plants (ten Have et al., 1998). Gene replacement mutants of *Claviceps purpuea* lacking endoPG activity are nearly nonpathogenic on rye (Oeser et al., 2002). Genetic evidence obtained by gene disruption or replacement experiments, however, does not always support an essential role of endoPGs in fungal pathogenicity. Targeted inactivation of both the endo- and exoPG genes had no detectable effect on the pathogenicity of *Cochliobolus carbonum* on maize (Scott-Craig et al., 1990, 1998). Inactivation of an endoPG gene did not result in a reduction in the pathogenicity of *Cryphonectria parasitica* on American chestnut stems (Gao et al., 1996). Disruption of the *Fusarium oxysporum* endoPG gene *pg5* did not alter the virulence of the fungus toward tomato plants (Garcia-Maceira et al., 2001).

In addition to their role as cell wall degrading enzymes and thus virulence factors, endoPGs also function as avirulence determinants through release of oligogalacturonides that can act as elicitors of the plant defense response (Ryan and Farmer, 1991). In *B. cinerea*, it was further demonstrated that BcPG1, which encodes an endoPG, activates grapevine defense response which is not related to its enzymatic activity (Poinssot et al., 2003). Besides, endoPGs are known to interact with polygalacturonase-inhibiting proteins (PGIPs), which are plant defense proteins present in the cell wall of all plants examined thus far. PGIPs inhibit specifically endoPGs of fungi, but not those of plant or bacteria. Inhibition of endoPGs may result in the accumulation of oligogalacturonides, and thereby enhance plant defense response (Cervone et al., 1989).

Phytophthora represents a large group of important and devastating oomycete pathogens, which cause diseases not only of the economically important crops such as potato, tomato, and soybean, but also of valuable forest trees in North America and Europe. Owing to similarities with fungi in morphology and growth patterns, oomycetes have long been considered a class within the kingdom Fungi. Analyses based on phenotypic characteristics and 18S rDNA sequence comparisons, however, indicated that they are phylogenetically distinct from true fungi, with the heterokont golden-brown algae being the closest relatives (Baldauf et al., 2000). Oomycetes differ from fungi in several aspects, including: (1) oomycetes do not synthesize sterol; (2) cell wall of oomycetes contains only a small amount (or even none) of chitin, which is a major constituent of fungal cell walls; (3) oomycetes synthesize lysine by the α,ε-diaminopimelic acid pathway, rather than the α-aminoadipic acid pathway used by fungi (Erwin and Ribeiro, 1996). Despite of distinction in taxonomy and physiology, fungi and oomycetes share characteristics in regard to strategies of infection and modes of interaction with host plants (Huitema et al., 2004; Latijnhouwers et al., 2003; Tyler, 2001). Previous studies have demonstrated that, like true fungi, *Phytophthora* species produce and secrete pectic enzymes in culture (Cole, 1970; Yuan and Tseng, 1980). Not much is known, however about the genes that encode these enzymes in *Phytophthora*, except for recent studies performed in two species. In *Phytophthora infestans*, which causes late blight in potato and tomato, the *pipg1* gene was found to encode an extracellular endoPG (Torto et al., 2002). In *Phytophthora cinnamomi*, a large PG gene family with 19 members was identified (Götesson et al., 2002). To extend our knowledge about endoPGs of *Phytophthora*, we have isolated and characterized *pppg1*, an endoPG gene from *P. parasitica* Dastur (=*Phytophthora nicotianae* Breda de Haan), which causes root rot, foot rot, leaf blight, and fruit rot in a wide variety of crops (Erwin and Ribeiro, 1996).

2. Materials and methods

2.1. Strain of P. parasitica and culture conditions

Phytophthora parasitica strain used in this study (isolate 98151) was provided by Dr. P.J. Ann of Taiwan Agricultural Research Institute, and stored on sterile distilled water as described by Liou et al. (2002). For preparation of DNA, one culture block was transferred into a sterile 250-ml flask containing 100 ml of 5% V8 juice medium (5% Campbell's V-8 juice and 0.02% $CaCO_3$) and incubated at 25 °C in the dark for 7 days. To evaluate the effect of pectin on the expression of *pppg1*, *P. parasitica* was grown on a minimal medium (0.05% yeast extract, 0.05% KH_2PO_4, 0.025% $MgSO_4 \cdot 7H_2O$, and 0.1% asparagine, pH 7.0) (Kamoun et al., 1993) containing 2.5% glucose as the carbon source for 7 days at 25 °C. Then, the mycelia were washed and transferred to the same minimal medium amended with different concentrations of pectin for another 24 h. Following harvest by filtration, the mycelium was frozen instantly in liquid nitrogen, lyophilized, and stored at −80 °C until use.

2.2. Construction of P. parasitica genomic library

DNA was extracted from mycelia of *P. parasitica* using DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland). Genomic library was constructed using Lambda FIX II (Stratagene, La Jolla, CA) as the vector. Following digestion with *Bam*HI and separation by sucrose density gradient centrifugation, DNA fragments with size ranging from 9 to 23 kb were collected by ethanol

precipitation. Ligation of the DNA with the Lambda FIX II vector, in vitro packaging, and transfection of the phage particles into *Escherichia coli* strain XL1-Blue MRA(P_2) were performed according to instructions provided by the manufacturer. Titer of the original genomic library was estimated to be 5×10^5 pfu.

2.3. Isolation and characterization of pppg1

The genomic library was screened using partial sequence of *pppg1* as the probe. Partial sequence of *pppg1* was obtained by genomic PCR, using primers pipgF2 (5′-CTC GCC CTT GTG GCG GCC AT-3′) and pipgR2 (5′-AAC GGG CTG GGT TGG CTA CAA-3′), which were designed based on the sequence of *pipg1* (Torto et al., 2002). The reaction (50 μl) consisted of 500 ng of genomic DNA, 1.25 μM of oligonucleotide primers, 0.2 mM dNTP, 1× PCR buffer, and 2 U of DyNazyme II DNA polymerase (Finnzymes, Espoo, Finland). PCR was performed by denaturation at 94 °C for 5 min, followed by 25 cycles of 95 °C/30 s; 54 °C/30 s; and 72 °C/1 min, and a final extension of 10 min at 72 °C in a Thermocycler (GeneAmp PCR System 2400, Perkin-Elmer, Foster City, CA). After separation of the amplified product on a 1% agarose gel, DNA bands of expected size were collected from the gel using the Gel-M (Viogene, Sunnyvale, CA) and cloned into pGem T-easy (Promega, Madison, WI). Nucleotide sequences of the recombinant clones were determined on both strands of DNA using the BigDye terminator cycle sequencing ready reaction kit and an ABI Prism 310 Genetic Analyzer apparatus (Applied Biosystems, Foster City, CA). Sequence was analyzed using programs in the GCG software package (Genetics Computer Group, Wisconsin Package Version 10.0). Nucleotide and amino acid sequence homology searches were performed with the NCBI-BLAST program. Multiple sequence alignment was carried out using Clustal X (Thompson et al., 1994). PG sequences of *Phytophthora ramorum* and *Phytophthora sojae* were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). For simplicity, only number in the assigned name of each gene was shown. Phylogenetic analyses were done using programs in PHYLIP version 3.57c (Felsenstein, 1993). Genetic distances between pairs of amino acid sequences were calculated using PROTDIST (Dayhoff PAM). Phylogenetic trees were constructed by a distance method (Neighbor-Joining) using the original data set and 100 bootstrap data sets generated by the program SEQBOOT from the original set.

Library screening, subcloning, and Southern blot analysis were performed as described in standard protocols (Sambrook and Russell, 2002). Partial *pppg1* DNA fragment obtained by PCR was labeled by using a non-isotopic digoxigenin (DIG) PCR labeling kit (Roche, Mannheim, Germany) and used as a probe to screen the genomic library. Hybridization signal was detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche) according to instructions of the manufacturer. CDP-star (New England BioLabs, Beverly, MA) was used as the substrate for alkaline phosphatase. To confirm the presence of the *pppg1* gene, lambda DNA of positive clones was isolated by using Qiagne Lambda Midi Kit (Qiagen), digested with *Bam*HI, *Eco*RI, or *Hin*dIII, and analyzed by Southern hybridization. Subsequently, insert DNA of a representative phage clone, pFIX-Bb, was subcloned into pCCBAC *Hin*dIII Cloning-Ready Vector (Epicentri, Madison, WI), and subjected to sequence analysis on both DNA strands as described previously.

2.4. Rapid amplification of cDNA ends

Total RNA was prepared from mycelium of *P. parasitica* using Plant Total RNA Extraction Kit (Viogene). Further isolation of poly(A)$^+$ RNA was performed using Dynabeads mRNA Purification Kit (Dynal, Oslo, Norway). First-strand cDNAs were synthesized with the reverse transcriptase PowerScript (Clontech, Palo Alto, CA), using poly(A)$^+$ RNA as the template and oligonucleotides T_{25}VN (V: A, C, or G; N: A, T, C, or G) as the primer. Rapid amplification of cDNA ends (RACE) were performed using the SMART RACE cDNA amplification Kit (Clontech). Gene-specific primers were designed based on partial sequence of *pppg1* obtained by genomic PCR: 5′-CTT TGA TCC ACG TCG TCT-3′ for 5′-RACE and 5′-ATG AAG CTC TTC AAG TCC ACT CTC GCC ATT-3′ for 3′-RACE. Following separation by agarose gel electrophoresis, DNA fragments of interest were eluted from the gel and cloned into pGem T-easy (Promega) for sequence analysis.

2.5. Inoculation experiment

After germination, seedlings of tomato (*Lycopersicon esculentum* Mill.) cv. Summer sweet (Known-You Seed, Taiwan) were grown at 28 °C with a 16-h-light/8-h-dark cycle. Detached tomato leaves obtained from the third- to fourth-leaf stage were placed in petri dishes containing 1.5% (w/v) water agar and inoculated with 10^5 zoospores (in 1 ml) of *P. parasitica*. Control leaves were mock-inoculated with distilled water. Petri dishes with inoculated leaves were kept in the dark at 25 °C for 5 days. Poly(A)$^+$ RNA was isolated from the inoculated tomato leaves using Plant Total RNA Extraction Kit (Viogene) and Dynabeads mRNA Purification Kit (Dynal) according to procedures provided by the manufacturer. To avoid contamination by genomic DNA, poly(A)$^+$ RNA samples were treated with RNase-free DNase I (0.5 U/μl; Roche) at 37 °C for 1 h.

2.6. Real-time quantitative reverse transcription PCR (RT-PCR) analysis

Reverse transcription of poly(A)$^+$ RNA was performed with PowerScript reverse transcriptase (Clontech), using 5′-T_{25}VN-3′ as the primer. One microliter of the reverse transcription mixture was submitted to online quantitative PCR with the LightCycler system using FastStart DNA Master SYBR Green I kit (Roche). Oligonucleotide primers for quantitative PCR were designed using an on-line design tool: http://www.genscript.com/ssl-bin/app/primer. The PCR was performed in a volume of 20 μl containing 2 μl of LightCycler FastStart DNA Master SYBR Green I, 3 mM $MgCl_2$, and 1 μM primers. The instrument settings were: initial enzyme activation at 95 °C for 10 min, followed by 45 cycles of 95 °C/10 s; 60 °C/5 s; and 72 °C/10 s. The specific primers used were as follows: for the *pppg1* gene, forward primer 5′-CCA GTC TGG TCA ACA TCA CC-3′ and reverse primer 5′-GCA CTT GCC AAT GTT CGT AG-3′; for the actin gene, forward primer 5′-ACT CTG GTG ATG GTG T-3′ and reverse primer 5′-CGA GTA ACC ACG CTC C-3′. Actin was used as an internal control and the relative quantification for *pppg1* was corrected to the actin mRNA values. Amplification of specific transcripts was confirmed by melting curve analysis provided by the LightCycler system at the end of each run. PCR specificity and product length were further checked by agarose gel electrophoresis and ethidium bromide staining to verify that the primer pairs amplified a single product of the predicted size.

2.7. Expression of pppg1 in Pichia pastoris

The *pppg1* protein was expressed by using an EasySelect *Pichia* Expression Kit purchased from Invitrogen (Carlsbad, CA). The *pppg1* cDNA was used as a template for PCR to obtain a DNA fragment corresponding to the predicted *pppg1* from amino acid residues 21–391. Primers used were: ExpGF (5′-ATC GAT GGA CGA CGT GGA TCA AAG TGA-3′) and ExpGR (5′-TCT AGA CCG CAC TGC ACG TTG CTC GGT C-3′). The resulting PCR fragment was cloned into pGEM T-easy vector to generate the pGTPG1 plasmid. The *pppg1* gene was excised as a *Cla*I/*Xba*I fragment from pGTPG1 and subcloned into pPIC-alphaC with C-terminal His_6 tag to generate the expression vector pPIC-EXPG, which was then transformed into the *Pi. pastoris* X-33 strain using *Pichia* EasyComp transformation kit (Invitrogen). Transformants were selected on plates with YPDS medium and zeocin (100 μg/ml). To induce expression of the recombinant protein, colonies of *Pi. pastoris* were inoculated in BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.04% biotin, 1% glycerol, and 100 μg/ml zeocin) and grown with shaking (225 rpm) at 30 °C for 24 h. Afterwards, the overnight culture was resuspended in BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.04% biotin, 0.3 mM sodium molybdate, and 0.5% methanol) and grown for another 2 days. For rapid screening of colonies which expressed endoPG activity, total proteins present in BMGY or BMMY were precipitated by trichloroacetic acid (TCA) and analyzed by in-gel PG activity assay as shown below. The expressed *pppg1* protein was purified from the cultural medium by affinity chromatography using His-Trap HP column (Amersham–Pharmacia Biotech, Uppsala, Sweden) under native condition.

2.8. Analysis of endoPG activity

Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Deglycosylation of the *pppg1* recombinant protein was performed as described by Caprari et al. (1993). Twenty micrograms of protein was resuspended in 20 μl denaturing buffer (0.5% SDS and 1% β-mercaptoethanol) and denatured at 100 °C for 10 min. Subsequently, the denatured protein was subjected to deglycosylation with 500 U Endo H (New England BioLabs) in 50 mM sodium citrate (pH 5.5) at 37 °C for 4 h, followed by in-gel endoPG activity assay according to the method of McKeon (1988). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a Hoefer Mighty Small SE245 Unit (Hoefer Pharmacia Biotech, San Francisco, CA). Proteins were detected by staining with Coomassie brilliant blue R-250. For in-gel assay of endoPG activity, the polyacrylamide gel was washed twice in the following solutions: (1) 0.01 M Tris, pH 8.3, (2) 20% isopropanol, and (3) 10 mM sodium acetate, pH 4.5. Afterwards, it was incubated in solution A (50 mM sodium acetate, pH 4.5, and 10 mM EDTA) for 16 h and then solution B (0.01 M Tris, pH 8.3) for 30 min. The activity bands were visualized by staining the gel with 0.05% ruthenium red (Sigma–Aldrich, St. Louis, MO) in 0.01 M Tris, pH 8.3.

3. Results

3.1. Isolation and characterization of the pppg1 gene

Genomic PCR primed with oligonucleotides pipgF2 and pipgR2 amplified a DNA fragment of the expected size (data not shown). Sequence analysis and database search confirmed that it corresponded to partial sequence of a gene homologous to *pipg1*, the endoPG gene identified in *P. infestans*. The cloned DNA fragment was labeled with DIG and used as a probe to screen the

genomic library of *P. parasitica* in Lambda FIX II. In the first round of screening, 17 positive plaques were detected. Further characterization of these clones by restriction and Southern hybridization analyses indicated that three clones (pFIX-Bb, pFIX-Bc, and pFIX-Bd) contained sequences corresponding to the same genomic region. The remaining 14 clones, in contrast, showed no hybridization signal and thus were neglected in the following study. Full-length sequence of the insert DNA of a representative clone, pFIX-Bb, was determined. Blast search of the sequence encompassing this genomic fragment, which is 14.5-kb in length, indicated that it contained two genes in opposite directions: an endo-PG gene (named *pppg1*) and a downstream transducin-like gene, with an intergenic region of 3.3 kb. Besides, a 129-nt sequence corresponding to nucleotide positions 1160–1288 (Fig. 1) of *pppg1* was also found upstream of the *pppg1* gene.

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-886 catcattgaggacgctgactgtgcgtacaagtggagcagcaccgatggctccgcgcaccgcagccaaagatcgtggatccgcaacgaacctctacgacgt
-786 tgtggtgaacccggacgtggtgtccaactgggaattcaataacatcgccgtgaacgccacgaacgtcggtcaatgcaatggcggaccgagcaacatacag
-686 tgctaatgcaacgaaccccttcgagcatgtatccatgggtttttgccgtaaacaatcatgccatgggtgtccaaattccgggccaaagaggggctaaag
-586 aattttcgcattggccaacggaacacaacgaactggtggcaataagaaatgaaaaagaattgtcaatctcttggctcctaaaggatgattcatattttcc
-486 tagtacatccgtgaagcaacgtggagcgcaagaacattgtgcctagtaaatgaacctatctacatacgacggacggtgacggttccactggagccggggc
-386 atccgctgtttgttttggtgcccgagattcgtatggtgtcgtacgttgaagggtctcaaagactctactgacattgctattgtccccacctcacgaacgt
-286 cgcgacgacgggacaatcatcatgttcacgaccaggacgactggtttagacggtccgaccttcaaaaagcaacctcgtcgccgtcccgttaacaagctga
-186 gtatttaggccacgaatttttgtccgatttgctgtatcccatgtggaaaacaggcaagtggcgtgacggtaatattgaccataaaccttgtgagttaccta
 -86 ctagtactttaatactcgttttaattcattctatcaccgagaaaataatcaggataggccccgtgataccaacgcgggctcagctcATTCTTCAAATTAC
  15 CTCGTGGTAAGCAAGAGCCCATTTCCATTGCAAGCGTCTTCACACCAACAATGAAGCTCTTCAAGTCCACTCTCGCCATTCTCGCCCTCGTGGCTGCTAT
   1                                                  M K L F K S T L A I L A L V A A M
 115 GGTCAACGGAGACGACGTGGATCAAAGTGAAGTCGTCCAACAGTCTCAAGAAAACCAGGACTACGAGGTGGGGCAGGTTTCTCCTGCAAGTCAGGTTGGT
  18 V N G D D V D Q S E V V Q Q S Q E N Q D Y E V G Q V S P A S Q V G
 215 CAAGATTCTCCGTACCAGGTGGACCAGGACGACACCTGCACGCTAACCGGCAACTACACAGAGGGCACCGATGTCTCGCAATGCAGCACCATTGTCATCG
  51 Q D S P Y Q V D Q D D T C T L T G N Y T E G T D V S Q C S T I V I D
 315 ACTCGTTGTCGGTGCCTGCTGGTGTAATGCTTGATCTTACCAATGTCACGGACGGTGCCAACATCAAGTTCCAGGGAACGTCGACCTTCGGGCCGAAGGT
  85 S L S V P A G V M L D L T N V T D G A N I K F Q G T S T F G P K
 415 AAGAACGCACAATTTATCTCTAATATAGCGTCTAACTCTTATCGCGTAGCTGTGGGCAGGTCCGTTTATTAAGCTCGCGGGAAATGACCTCACTGTGACC
 117                                                  L W A G P F I K L A G N D L T V T
 515 GGGCCTGGCACGCTCGATGGCCAGGGCGCGTGGTACTGGCCGCAGGGTCAGAACATCACTCGTCCGGTGTTCTTCCGCCTCAGCAGAGTTGCCAACTCGA
 134 G P G T L D G Q G A W Y W P Q G Q N I T R P V F F R L S R V A N S K
 615 AGCTGTCGGGCTTCACCATTAAGAACATGCCGTTCCGCACCTTCAGCATCCTCAGTTCAAACTACACGACGATCTCTGAGCTCACGATCGACTCTCGTGC
 168 L S G F T I K N M P F R T F S I L S S N Y T T I S E L T I D S R A
 715 AGGCAACGGCAGCGCCAAGAACACGGACGGCTTCGACCTGAGCAGAAACAACCACGTGACCATCACTAACAACCGAGTGTACAACCAGGACGACTGCCTG
 201 G N G S A K N T D G F D L S R N N H V T I T N N R V Y N Q D D C L
 815 GCCATGCAGTCCAGCTCGAACACGGTCTTTAGCAACAACTACTGCAGCGGCTCGCACGGTATTTCCATCGGCTCGCTCGGTGGCCCAGAGCAGAACGTCA
 234 A M Q S S S N T V F S N N Y C S G S H G I S I G S L G G P E Q N V N
 915 ATACGACCGTCTCTGGTCTGCTGGTCAAGGACAACACGATCGTGAACAGCACAAACGGCATCCGTATCAAGACCATCATTGGCCTCAAGGGACTGGTGAC
 268 T T V S G L L V K D N T I V N S T N G I R I K T I I G L K G L V T
1015 CAACGTCACCTACACCAACAACAGGCTTGTCAACGTCAAGCACGCTATTGTCATGCACTCGGACTACAACAAAACCAAGGGCGGGTACTCCGGCATCCCC
 301 N V T Y T N N R L V N V K H A I V M H S D Y N K T K G G Y S G I P
1115 TCCAGTCTGGTCAACATCACCAACATCAAGATCGACGGCCTTTTCGGCTCGGCCACGAACCTGTACGATATTGTAGCCAACCCGGCCGTGGTGTCCAACT
 334 S S L V N I T N I K I D G L F G S A T N L Y D I V A N P A V V S N W
1215 GGGAGTTCAAGAACGTCGCGGTTAACGCTACGAACATTGGCAAGTGCCAGGGCGGACCGAGCAACGTGCAGTGCTAAGGAGGAGTAGAGTTTTTATGCCG
 368 E F K N V A V N A T N I G K C Q G G P S N V Q C *
1315 TATACACAACATGATTGTTACGCGTGGGGCATCTGTTCCACTTCAacttgtgacttgatttttgtaatgcatggggatcggatcttgaaaggtcattaac
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Fig. 1. Nucleotide and deduced amino acid sequences of the *pppg1* gene (GenBank Accession No. AY697926). Transcription start site of the *pppg1* gene is marked with a triangle and designated as +1. Nucleotide sequences upstream of the transcription start site and downstream of the polyadenylation site were in lower case. Intron was underlined. The putative TATA-box, core promoter sequence, and regulatory sequences were double underlined. The polyadenylation site is marked with a star. Deduced amino acid sequence is shown below the nucleotide sequence. The N-terminal signal peptide was in bold case. Putative N-glycosylation sites are underlined. The stop codon is marked with an asterisk.

To locate the 5′ and 3′ ends of the *pppg1* transcript, RACE was performed. The full-length cDNA contained 1359 bp nucleotides, including an open reading frame of 1176 bp. No AATAAA sequence typically preceding the polyadenylation site was found in the 3′ flanking region of *pppg1*. Comparison with the genomic sequence indicated that the *pppg1* gene contained an intron of 51 bp, which was demarcated by the highly conserved dinucleotides GT and AG on its 5′ and 3′ ends, respectively (Fig. 1). Recently, core promoter structure of some genes has been characterized in *P. infestans* (McLeod et al., 2004). Analysis of the sequence upstream of the *pppg1* transcription start site identified a similar core promoter sequence at positions −4 to 15, and a putative TATA-box at −72 to −81 (Fig. 1). Besides, three CREA-binding site consensus (5′-SYGGRG-3′), which is involved in the catabolite repression of pectinase in fungi, were found upstream of the *pppg1* transcription initiation site at positions −394, −461, and −851.

Open reading frame of *pppg1* encoded a polypeptide of 391 amino acid residues, with a predicted molecular mass of 39.7 kDa and an isoelectric point of 5.2. Analysis of the amino acid sequence predicted the existence of a signal peptide (amino acid residues 1–20) and 11 putative N-glycosylation sites (Fig. 1). A search of the protein database with the amino acid sequence of *pppg1* revealed a high degree of similarity to endoPGs from other *Phytophthora* and fungi, especially *pipg1* of *P. infestans*. The amino acid sequence identity of the entire coding region of *pppg1* is 91% with *pipg1* and varies from 71 to 36% with endoPGs of other *Phytophthora* and fungi. Multiple sequence alignment of the deduced *pppg1* amino acid sequence with those of other endoPGs indicated that identity was greatest at the putative catalytic domains (Fig. 2). A close match to the consensus fungal polygalacturonase active site, CXGGHGXS IGSVG, was found at amino acid residues 248–260 of *pppg1*, with a slight modification to CSGSHGISIGSLG. The RIK motif conserved in all polygalacturonase was located at positions 288–290. It has been demonstrated that the R and K residues of the RIK motif are involved in substrate binding in endoPGII of *Aspergillus niger* (Armand et al., 2000). The tyrosine residue shown to be indispensable for effective catalysis of endoPGII was also found at position 322 (Pagès et al., 2000). The putative monobasic (arginine) propeptide cleavage site found in some endoPGs of *P. cinnamomi*, however, was absent in *pppg1*. Major difference between *pppg1* and *pipg1* was located at the C-terminal region of the deduced amino acid sequence.

To examine the relationship of *pppg1* with other endoPGs, a phylogenetic tree was constructed based on an alignment of endoPG sequences from *Phytoph-*

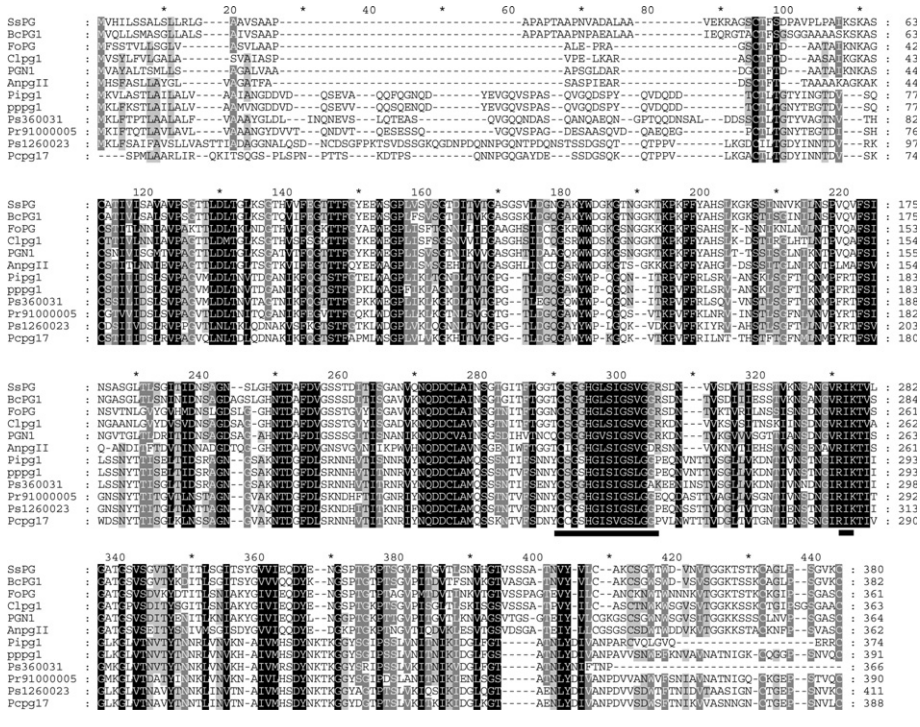

Fig. 2. Multiple sequence alignment of *pppg1* and other endopolygalacturonase. Sequences analyzed included: AnpgII (P26214, *A. niger*), BcPG1 (U68715, *B. cinerea*), PGN1 (M55979, *C. carbonum*), Clpg1 (X89370, *Co. lindemuthianum*), FoPG (AF078156, *F. oxysporum*), SsPG (Q12708, *Sclerotinia sclerotiorum*), *Pipg1* (AY052571, *P. infestans*), Ps360031 (ESTEXT_GENEWISE1.C_360031), Pr91000005 (FGENESH1_PG.C_SCAFFOLD_91000005), Ps1260023 (ESTEXT_GENEWISE1.C_1260023), *Pcpg*17 (AF398943), and *pppg1* (AY697926). Locations of the polygalacturonase active site and the RIK motif are underlined.

thora and fungi, with the variable N-terminal extensions excluded. The parsimony tree clearly separated the *Phytophthora* and fungal endoPGs into distinct groups (Fig. 3). Moreover, reminiscent of what has been demonstrated in *P. cinnamomi*, endoPGs of *Phytophthora* similarly fell into three clusters. *pppg1* together with *pipg1* were grouped into the same cluster as endoPGs of *P. cinnamomi* predicted to contain multiple glycosylation sites, including *Pcpg12*, *Pcpg14*, *Pcpg15*, *Pcpg16*, and *Pcpg17* (Götesson et al., 2002). Other members in this cluster included *Pr91000005* of *P. ramorum*, *Ps1260001*, *Ps1260023*, and *Ps360031* of *P. sojae*. Analyses of these sequences also revealed the presence of multiple N-glycosylation site consensus in each endoPG.

The copy number of *pppg1* was determined by Southern hybridization analysis of genomic DNA digested with three different restriction enzymes: *Bam*HI, *Hin*dIII, or *Xho*I. A single hybridization band was observed in all digests, indicating the presence of a single-copy *pppg1* in *P. parasitica* (Fig. 4A). However, when hybridization was carried out at 37 °C, multiple weak bands showed up, suggesting the presence of other sequences homologous to *pppg1* in the genome of *P. parasitica* (Fig. 4B).

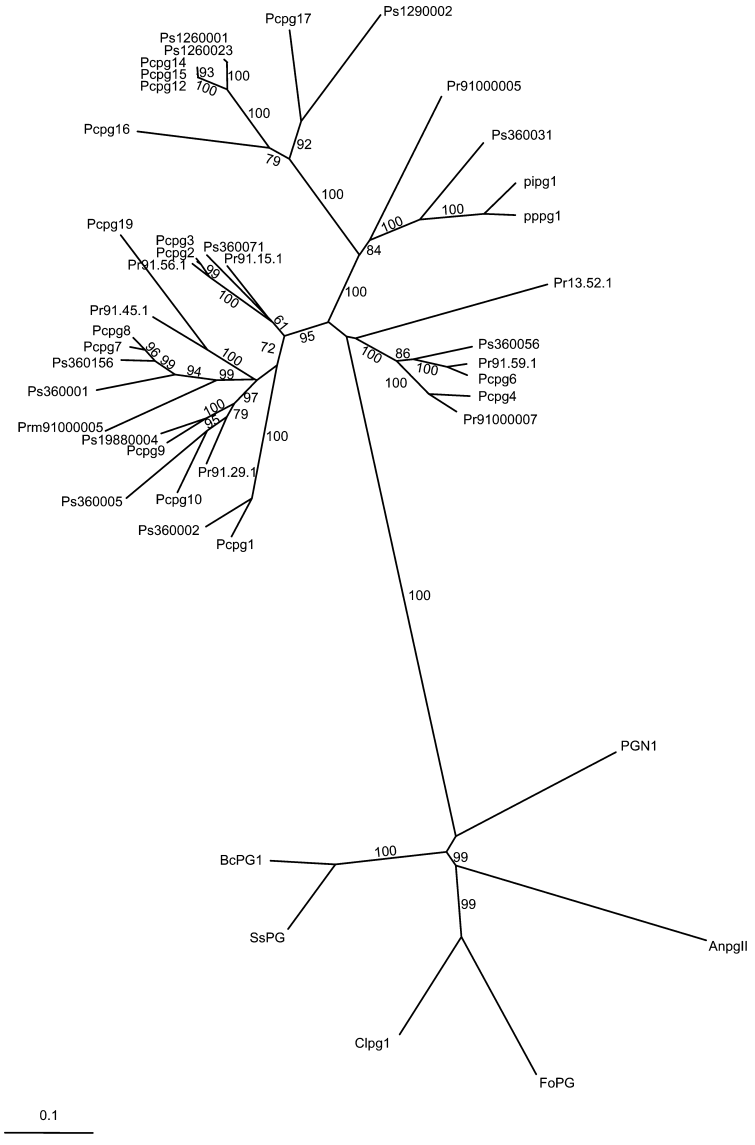

Fig. 3. Phylogenetic analysis of *pppg1* and endopolygalacturonase from fungi and other *Phytophthora*. Accession numbers of fungal endoPGs were the same as those shown in Fig. 2. Accession numbers of endoPGs from *Phytophthora cinnamomi*: AF398105 (*Pcpg1*, *Pcpg2*, *Pcpg3*, *Pcpg4*, and *Pcpg6*), AF398946 (*Pcpg7*), AF398947 (*Pcpg8*), AF398948 (*Pcpg9*), AF398945 (*Pcpg10*), AF398937 (*Pcpg11*), AF398938 (*Pcpg12*), AF398940 (*Pcpg14*), AF398941 (*Pcpg15*), AF398942 (*Pcpg16*), AF398943 (*Pcpg17*), and AF398936 (*Pcpg19*). Sequences of *P. ramorum* and *P. sojae* were obtained from the website of US Department of Energy Joint Genome Institute. For simplicity, only number in the assigned name of each gene was shown. The phylogenetic tree was constructed using Neighbor-Joining in PHYLIP with 100 bootstrap resamplings. Bootstrap values supported in more than 60 of the trees are indicated.

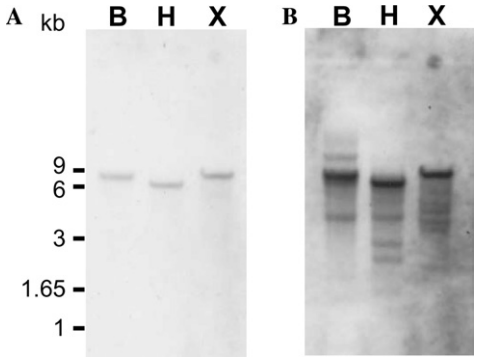

Fig. 4. Southern blot analysis of *P. parasitica* DNA. Genomic DNA of *P. parasitica* was digested with *Bam*HI (B), *Hin*dIII (H), or *Xho*I (X) and subjected to Southern hybridization using DIG-labeled *pppg1* DNA fragment as the probe. Hybridization was performed according to the standard protocol (A) or at 37 °C (B). Positions of the size marker (kb) are indicated on the left.

3.2. Expression of pppg1 in culture and in diseased tomato plants

To investigate the effect of different carbon source on the expression of *pppg1*, *P. parasitica* was grown on a minimal medium containing 2.5% glucose for 7 days at 25 °C, and then shifted to a minimal medium amended with 0.1, 0.25, or 0.5% pectin, respectively, for 24 h. The relative mRNA level of *pppg1* in each treatment was measured by real-time quantitative RT-PCR and normalized with the actin message (Table 1). With medium amended with 2.5% glucose, the concentration of *pppg1* transcript was pretty low. Upon transfer to the medium supplemented with 0.1% pectin, the mRNA level of *pppg1* increased by 5.8-fold relative to the mRNA level displayed with 2.5% glucose (Fig. 5). Increase in the

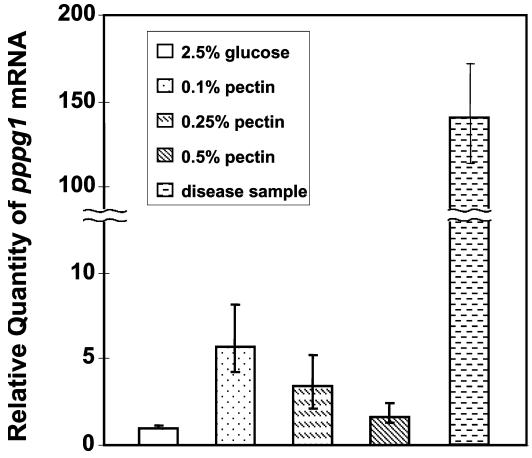

Fig. 5. Quantitation of the *pppg1* transcript by real-time quantitative RT-PCR. *P. parasitica* was grown in the minimal medium amended with 2.5% glucose for 7 days, and then shifted to the same minimal medium amended with 0.1, 0.25, or 0.5% pectin for 24 h. For the *in planta* experiment, detached tomato leaves were inoculated with zoospores of *P. parasitica* and incubated at 25 °C for 5 days. The amount of *pppg1* transcript was measured by real-time quantitative RT-PCR and normalized to actin mRNA. Results of three experiments are presented as means ± SE

Table 1
Relative quantitation of *pppg1* expression using *actin* as a control

Treatment	(1) *pppg1* C_T	(2) Actin C_T	(1) − (2) ΔC_T	$\Delta\Delta C_T$	*pppg1* Rel. to actin
Glucose	30.08	14.89	15.19		
	32.11	16.76	15.35		
	31.56	16.54	15.02		
Average			15.19 ± 0.17	0.00 ± 0.17	1(0.9–1.1)
Pectin 0.1%	30.30	17.19	13.11		
	29.96	17.27	12.69		
	29.64	17.50	12.14		
Average			12.65 ± 0.49	−2.54 ± 0.49	5.8(4.2–8.1)
Pectin 0.25%	29.74	16.72	13.02		
	29.68	16.58	13.10		
	30.90	16.79	14.11		
Average			13.41 ± 0.61	−1.78 ± 0.61	3.4(2.2–5.2)
Pectin 0.5%	31.43	16.59	14.84		
	30.66	16.70	13.96		
	31.19	16.81	14.38		
Average			14.39 ± 0.44	−0.79 ± 0.44	1.7(1.3–2.4)
Disease sample	31.53	23.15	8.38		
	33.58	25.52	8.06		
	32.98	25.19	7.79		
Average			8.08 ± 0.30	−7.11 ± 0.30	138.1(112.6–169.5)

Note. Reference for method of calculation: Livak and Schmittgen (2001).

concentration of pectin, however, did not induce expression of the *pppg1* gene to an even higher level than with 0.1% pectin (3.4- and 1.7-fold for 0.25 and 0.5% pectin, respectively).

To determine whether *pppg1* is expressed by *P. parasitica* upon infection of its host plants, detached leaves obtained from tomato seedlings were inoculated with zoospores. Small brown spots appeared on the surface of tomato leaves at 1 day post-inoculation (dpi) at 25 °C, followed by the appearance of water-soaked regions at 2 dpi. The necrotic lesions expanded gradually and eventually resulted in complete leaf maceration at 3–5 dpi. Samples were taken from mock-infected and infected plant material at 5 dpi for RNA isolation. Real-time quantitative RT-PCR was used to detect the presence of *pppg1* and actin transcript in the infected tomato leaves. As shown in Fig. 5, expression of *pppg1* was strongly induced upon infection of the tomato plants: 138.1-fold relative to the *pppg1* mRNA level displayed with 2.5% glucose. These results demonstrated that expression of *pppg1* was strongly up-regulated during interaction of *P. parasitica* with host plants.

3.3. Heterologous expression of pppg1 in yeast

Analysis of the deduced amino acid sequence predicted that *pppg1* encoded a secretory glycoprotein. To characterize the gene product, *pppg1* was expressed in the *Pi. pastoris* expression system. Recombinant *pppg1* proteins collected from the culture filtrate were analyzed by SDS–PAGE followed by in-gel PG activity assay. No endoPG activity was detectable while transformants were grown in BMGY (Fig. 6A, lane 1). After induction with methanol, *Pi. pastoris* transformed with pPIC-EXPG secreted recombinant *pppg1* proteins which showed enzymatic activity of polygalacturonase (Fig. 6A, lane 2). Surprisingly, the PG activity appeared as a broad band which stretched from more than 200 kDa down to 75 kDa, rather than a single, sharp band. Further purification of the recombinant protein by Ni^{2+}–NTA affinity chromatography generated the same broad band with high molecular mass either by activity staining (Fig. 6B, lane 1) or Coomassie blue staining (Fig. 6C, lane 1). Since the protein encoded by *pppg1* is presumably a secretory protein, which undergoes glycosylation after translation, it is not unexpected that the PG activity band should exhibit a molecular mass bigger than that estimated from the deduced amino acid sequence. It is intriguing, however, that PG activity showed up as a broad band even with very low concentration of the recombinant protein (data not shown). To find out whether the broad banding resulted from differential glycosylation of the recombinant *pppg1* protein, endoH was used to remove the glycosyl residues. As shown in Fig. 6, molecular mass of the recombinant protein reduced to about 48 kDa (Fig. 6C, lane 2), close to that predicted for the recombinant protein. In the mean time, the deglycosylated protein was found to completely lose its PG activity (Fig. 6B, lane 2). It is thus obvious that glycosylation is required for *pppg1* to function as a polygalacturonase, and glycosylation may occur in more than one form in the *pppg1* recombinant protein expressed in a heterologous system.

4. Discussion

To invade the hosts, plant pathogens secrete extracellular pectinases that degrade polymers embedded in the plant cell wall. Despite the long lasting interest in the pectinases of *Phytophthora*, not much is known about these enzymes at the molecular and biochemical level. In the present study, we describe the cloning and characterization of the *pppg1* gene, which encodes endoPG in

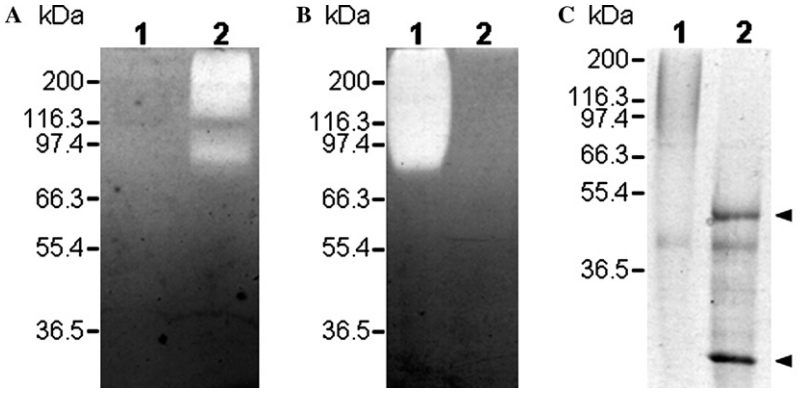

Fig. 6. Analysis of the His-tagged recombinant *pppg1* protein expressed in *Pi. pastoris*. Recombinant protein was analyzed by SDS–PAGE followed by in-gel endoPG activity assay (A and B) or Coomassie blue staining (C). (A) Crude extract of the cultural filtrate prior to (lane 1) and after induction with methanol (lane 2); (B and C) recombinant protein purified by His-Trap chromatography prior to (lane 1) and after endoH cleavage (lane 2). Arrows on the right side of (C) indicated positions of the deglycosylated *pppg1* recombinant protein (upper) and endoH (lower), respectively. Positions of the size marker are marked on the left of the gel.

P. parasitica. Only one single copy of *pppg1* was lit up by standard Southern hybridization. Analysis performed at a lower stringency, however, suggested the possibility that *P. parasitica* may contain other sequences homologous to *pppg1*. Many fungi are known to contain multiple forms of endoPG due to the presence of multiple genes or post-translational modification of a single gene product (Caprari et al., 1993; Wubben et al., 1999). It has been proposed that multiple forms of endoPG may provide pathogens with increased efficiency of degradation, a wider host range, or an ability to obtain nutrients at various stages of their life cycles to ensure better chance of successful infection (Williams et al., 2002). The generations hold true for *P. cinnamomi*, which has a wide host range and contains a large family with 19 endoPG genes (Götesson et al., 2002). Studies in *P. infestans*, however, suggested the presence of multiple *pipg1*-like sequences in its genome, despite that host range of *P. infestans* is restricted to solanaceous plants, including mainly potato and tomato (Torto et al., 2002). Search of the database of US Department of Energy Joint Genome Institute also revealed the existence of multiple endoPG genes in *P. ramorum* and *P. sojae*, although these sequences were identified via annotation of EST/cDNA (and/or genomic) data and should be treated with caution. It is thus obvious that copy number of endoPG genes is not necessarily correlated with the host range of a pathogen. Factors other than host range may exert more impact on the evolution of the endoPG multigene families in *Phytophthora*.

Analyses of the deduced amino acid sequence indicated that *pppg1* contained all the signature sequence of endoPGs, with the highest degree of identity to *pipg1* of *P. infestans*, and to a lesser extent, *Ps360031* of *P. sojae* and *Pr9100005* of *P. ramorum*. Since our strategy to clone the endoPG genes of *P. parasitica* was designed based on the sequence of *pipg1*, it is possible that only sequences highly similar to *pipg1* would be identified. However, recent phylogenetic analyses based on mitochondrial and nuclear DNA sequences have demonstrated that, while *P. ramorum* (clade 8), *P. cinnamomi* (clade 7), and *P. sojae* (clade 7) were in different clades, both *P. parasitica* and *P. infestans* were grouped in clade 1 (Cooke et al., 2000; Kroon et al., 2004). Hence, the other explanation for the observed high degree of identity between *pppg1* and *pipg1* could be a closer phylogenetic relationship between *P. parasitica* and *P. infestans*. In this context, it is interesting to note that in no case were multiple endoPGs from a single *Phytophthora* species formed a monophyletic group of their own. In contrast, as shown in Fig. 3, endoPGs from multigene families of *P. cinnamomi*, *P. ramorum*, and *P. sojae* were split into different clusters. Furthermore, as in the case of *pppg1*, which was grouped closely with *pipg1*, *Ps360031*, and *Pr91000005*, endoPGs from different *Phytophthora* spp. might cluster tightly to form subgroups. This suggests that gene duplications of endoPGs might occur prior to divergence of *Phytophthora* species. Moreover, after evolution of new species, gene duplications of certain endoPGs could still happen in this organism and thereby led to the existing multigene families of endoPGs as seen in *P. cinnamomi* (Götesson et al., 2002), and very likely *P. ramorum* and *P. sojae* as well. Analysis of more endoPG genes from *P. infestans* and *P. parasitica* may help to confirm this possibility.

EndoPG genes clustered in the same group as *pppg1*, including *pipg1* and those from other *Phytophthora*, share the characteristic of containing multiple putative N-glycosylation sites. To verify that the protein encoded by *pppg1* contains enzymatic activity, *pppg1* was expressed in a yeast expression system. Analysis by in-gel activity staining indicated that there exist multiple forms of recombinant endoPG with different molecular masses. After deglycosylation by endoH cleavage, only a single protein band with expected molecular mass was visualized by Coomassie blue staining, although deglycosylation was accompanied by complete loss of the endoPG activity. It is thus obvious that glycosylation, even via a heterologous system, is indispensable for the *pppg1* protein to display its endoPG activity. In support of our result, it has been demonstrated previously that deglycosylation of *Aspergillus* polygalacturonase led to reduction in molecular mass followed by complete inactivation of the polygalacturonase enzyme activity (Stratilová et al., 1998). AnPGII of *A. niger* contains only one glycosylation site. However, a total of 11 putative glycosylation sites have been identified in the amino acid sequence of *pppg1*. Since differences in the number of glycosylation sites and/or different levels of branching on each site may account for the variation in characteristics of the enzyme, it is likely that more than one form of endoPGs may be produced via differential glycosylation (Yang et al., 1997). Indeed, recombinant *pppg1* proteins obtained from the yeast expression system were present in multiple forms. Site-directed mutagenesis analysis is now undertaken to identify the amino acid residues which are involved in the glycosylation of *pppg1*.

Expression of endoPG genes is generally subjected to regulation by the carbon source available. Previous analysis of the *pppg1* by Northern hybridization failed, however, due to the limiting amount of *pppg1* messages (data not shown). The sensitive real-time quantitative RT-PCR allowed us to demonstrate that, while repressed by glucose, expression of *pppg1* is moderately induced by pectin and highly induced in planta during infection of tomato plants by *P. parasitica*. It can be envisaged that a coordinated regulation of gene expression may occur in response to different carbon sources. The signaling pathway that controls expression of pectinase gene is currently unknown in *Phytophthora*, nor is information in regard to the *cis*-acting regulatory

sequences. However, it has been demonstrated in several fungal systems that glucose represses PG accumulation and CREA may contribute to catabolite repression of pectinase gene in fungi (Di Pietro and Roncero, 1996; Panozzo et al., 1998; Reymond-Cotton et al., 1996). In *Colletotrichum lindemuthianum*, sequence motifs TLE and PLEs were found to be required for the transcriptional activation of CLPG2 by pectin and during appressorium development (Herbert et al., 2002). Although *Phytophthora* is phylogenetically distinct from true fungi, sequence analysis revealed the presence of several CREA-binding site consensus upstream of the *pppg1* transcription initiation site. Besides, a sequence with strong homology to TLE (CATTCT) was also located at position −55. Further analyses are required to confirm importance of these sequences in the regulation of *pppg1* expression in *P. parasitica*.

Acknowledgments

This research was supported in part by grants from Nation Science Council, Taiwan. We thank Dr. P.J. Ann for providing *P. parasitica* isolate and the US Department of Energy Joint Genome Institute for providing access to sequence database.

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