

# Functional Characterization of a Gene Family Encoding Polygalacturonases in *Phytophthora parasitica*

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*Phytophthora parasitica* is an oomycete plant pathogen that causes severe disease in a wide variety of plant species. In our previous study, we discovered a multigene family encoding endopolygalacturonases (endoPG) in *Phytophthora parasitica*. Here, we screened the genomic library of *Phytophthora parasitica* for the genes encoding endoPG named *pppg2* through *pppg10*, and analyzed their functions. Results obtained by real-time quantitative reverse transcriptase-polymerase chain reaction demonstrated that some of these genes are highly induced during plant infection, which suggests their important roles in the pathogenesis of *Phytophthora parasitica*. Analysis by in-gel activity assay of recombinant proteins obtained from *Pichia pastoris* indicated that each of these genes encodes a functional endoPG. Investigation of the function of *pppg* genes in planta by a *Potato virus X* agroinfection system in tobacco revealed that each *pppg* caused specific effects, varying from no symptoms to dwarfism, necrosis, leaf curl, silvery leaf, and cracks in leaf stalks. Appearance of these effects depends on the expression of a *pppg* protein with a normal active site in the apoplast. These results indicated that each *pppg* plays a distinct role in the decomposition of plant cell wall.

During plant infection, pathogens produce a diverse range of cell wall-degrading enzymes (Walton 1994), including cellulases, hemicellulases, and pectinases. The latter includes a group of enzymes that degrade pectin, a major component of the primary cell wall and middle lamella of plants. According to the mode of action, pectinase can be divided into three different groups: polygalacturonase (PG), pectate lyase, and pectin methyl esterase. PG can hydrolyze the  $\alpha(1\rightarrow4)$  glycosidic bonds of pectin homogalacturonans by releasing monomers in a progressive fashion from the nonreducing ends of the polymer (exopolygalacturonase, EC 3.2.1.67) or internally cleaving the backbone of polygalacturonan (endopolygalacturonase [endoPG], EC 3.2.1.15).

Targeted mutagenesis of PG-encoding genes has demonstrated that PG is a virulence factor of several plant pathogenic fungi. Disruption of *BcPG1* (*Botrytis cinerea* polygalacturonase 1) reduces the virulence of *Botrytis cinerea* on host plants

(ten Have et al. 1998). Mutation in the endoPG gene reduced the ability of *Alternaria citri* to cause black rot symptoms in citrus and the maceration of potato tissues (Isshiki et al. 2001). Gene replacement mutants of *Claviceps purpurea*, which lack endoPG activity, are nearly nonpathogenic in rye (Oeser et al. 2002). However, genetic evidence obtained by targeted inactivation does not always support an essential role of PG in fungal pathogenicity. Disruption of *enpg-1*, which encodes the major extracellular endoPG produced by *Cryphonectria parasitica* in culture, demonstrated that this endoPG is not required for the expression of *Cryphonectria parasitica* virulence in American chestnut stems (Gao et al. 1996). In *Cochliobolus carbonum*, targeted inactivation of both the exo- and endoPG genes resulted in a nearly complete loss of PG activity. However, the double mutant was still pathogenic in maize (Scott-Craig et al. 1998). Disruption of *pg5*, which encodes endoPG, has no detectable effect on the virulence of *Fusarium oxysporum* f. sp. *lycopersici* in tomato (Garcia-Maceira et al. 2001). A possible explanation for these results is the presence of multigene families of endoPG in fungi, each gene performing defined biological tasks in the infection process. Recent study indicated that *Botrytis cinerea*, the cause of gray mold, contains at least six PG-encoding genes that are differentially expressed during interaction with plants and when grown in different carbon sources (ten Have et al. 2001; Wubben et al. 2000). *BcPG* recombinant proteins heterologously expressed by *Pichia pastoris* differ in several aspects, including specific activity, substrate preference, and mode of action. In addition, analysis by infiltration of recombinant proteins indicated the differential activity of these *BcPG* in causing necrosis in plants (Kars et al. 2005). *Sclerotinia sclerotiorum* secretes a large set of endoPG that differ in their catalytic properties and expression patterns. Of these endoPG, *sspg1* (*Sclerotinia sclerotiorum* polygalacturonase 1), *sspg2*, and *sspg3* are up-regulated during colonization in healthy plant tissues, *sspg5* was induced in the final phase of maceration, and *sspg6* and *sspg7* were expressed constitutively throughout the infection process (Kasza et al. 2004; Li et al. 2004). As well, *Chondrostereum purpureum*, which causes silvery leaf disease on apple, contains a multigene family of endoPG and is the first basidiomycete reported to have an endoPG gene family (Williams et al. 2002).

In addition to their roles as cell wall-degrading enzymes, and thus virulence factors, endoPG function as avirulence determinants that elicit plant defense responses. CLPG1, an endoPG gene of *Colletotrichum lindemuthianum*, shows elicitor activity in tobacco (*Nicotiana tabacum*) that depends on a functional catalytic site (Boudart et al. 2003). Thus, CLPG1 might induce plant defense response through release of oligo-

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galacturonans (Ridley et al. 2001). BcPG1 of *Botrytis cinerea*, however, activates grapevine defense response, which is not related to its enzymatic activity (Poinsot et al. 2003).

Pathogens belonging to the genus *Phytophthora* can cause diseases in economically important crops such as potato, tomato, and soybean as well as valuable forest trees in North America and Europe. To unravel the mechanisms involved in the pathogenesis of *Phytophthora* spp., genes required for the degradation of the plant cell wall must be characterized. Recent studies have demonstrated that *ppg1* encodes an endoPG in *Phytophthora infestans* that causes late blight on potato and tomato (Torto et al. 2002). Also, *Phytophthora cinnamomi* contains a large endoPG gene family with 19 members (Götesson et al. 2002). Not much is known, however, about the proteins encoded by each of these genes. Previously, we found the expression of *pppg1*, which encodes endoPG in *Phytophthora parasitica*, induced during plant infection (Yan and Liou 2005). Furthermore, Southern hybridization results with *pppg1* as the probe suggested the presence of other sequences homologous to *pppg1* in the *Phytophthora parasitica* genome. In the present study, we cloned and analyzed the function of *pppg* genes and found that they encode functional endoPG and have distinct biological functions in planta.

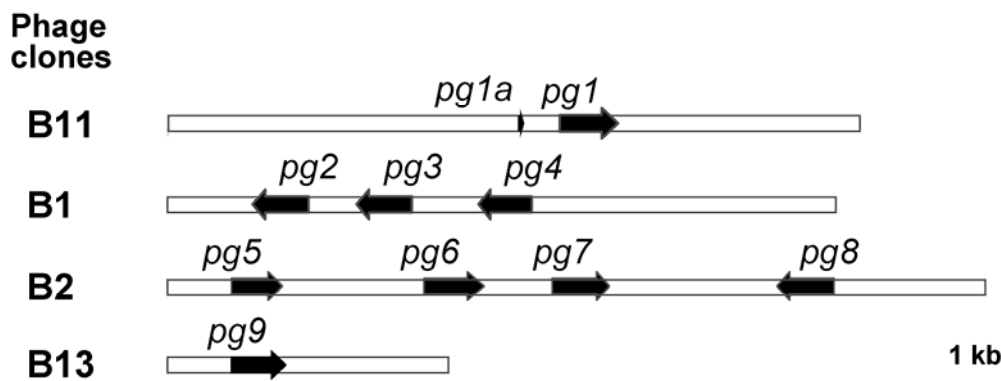
## RESULTS

### Isolation and sequence analysis of the endoPG family from *Phytophthora parasitica*.

Although our previous study suggested the presence of genes homologous to *pppg1* in the *Phytophthora parasitica* genome

(Yan and Liou 2005), attempts to screen the genomic library with *pppg1* used as a probe did not reveal clones representing genes other than *pppg1*. As an alternative, we used the degenerated primers conPGF1 and conPGR1 for genomic polymerase chain reaction (PCR). Amplicons of the expected size were cloned, and analyses of sequences of 16 independent clones revealed seven distinct sequences homologous to endoPG genes, including two (*con1* and *con7*) that deviate significantly from *pppg1*. Southern blot analysis with *con1* and *con7* used as probes revealed the presence of multiple bands (data not shown), indicating the existence of a multigene family of endoPG in *Phytophthora parasitica*. So, with *con1* and *con7* used as probes to screen the genomic library of *Phytophthora parasitica*, we obtained seven phage clones; three clones contained putative endoPG genes (*pppg2-pppg9*), as shown in Figure 1, and the other four contained sequences overlapping with the aforementioned clones or *pppg1* and, thus, are not described here. Of these putative endoPG genes, *pppg6* is interrupted by one intron and the others (*pppg2*, *pppg3*, *pppg4*, *pppg5*, *pppg7*, *pppg8*, and *pppg9*) are intronless. In addition, a new gene was cloned by rapid amplification of cDNA ends (RACE) in a separate study and was named *pppg10*.

Except for *pppg9*, which has 512 residues, the genes contained from 358 to 390 amino-acid residues (Table 1). Analysis of the deduced amino-acid sequences predicted the existence of a signal peptide at the N terminus and an active consensus site (CXGGHGXSIGSVG) (Henrissat 1991) in each protein (Fig. S1). *pppg9* is distinguished from the other genes in having an additional N-terminal domain of about 150 amino acids immediately following the signal peptide. Search of the putative N-



**Fig. 1.** Distribution of endopolygalacturonase genes of *Phytophthora parasitica* in the phage clones. Arrows indicate the localization of each gene, including *pppg1* (AY697926), *pppg2* (EU041943), *pppg3* (EU041944), *pppg4* (EU041945), *pppg5* (EU041946), *pppg6* (EU041947), *pppg7* (EU041948), *pppg8* (EU041949), and *pppg9* (EU041950).

**Table 1.** Characteristics of proteins encoded by the endopolygalacturonases genes of *Phytophthora parasitica*

Gene	Accession number	Nucleotides	Amino acids/ cleavage site <sup>a</sup>	pI	Mol. mass <sup>b</sup> X/Y (kDa)	N-glycosylation site <sup>c</sup>	Group <sup>d</sup>
<i>pppg1</i> <sup>e</sup>	AY697926	1,176	391/20 to 21	5.2	40/75 to 200	11	I
<i>pppg2</i>	EU041943	1,143	380/20 to 21	8.9	37/43	1	III
<i>pppg3</i>	EU041944	1,143	380/20 to 21	9.3	37/-	1	-
<i>pppg4</i>	EU041945	1,089	362/20 to 21	6.2	35/43	0	II
<i>pppg5</i>	EU041946	1,083	360/20 to 21	5.9	35/43 to 50	2	IV
<i>pppg6</i>	EU041947	1,092	363/20 to 21	5.2	36/45 to 55	0	II
<i>pppg7</i>	EU041948	1,077	358/17 to 18	4.1	35/55 to 70	1	I
<i>pppg8</i>	EU041949	1,086	361/20 to 21	9.5	35/40	0	III
<i>pppg9</i>	EU041950	1,539	512/19 to 20	4.0	50/-	2	-
<i>pppg10</i>	EU041951	1,176	391/20 to 21	5.0	39/43	9	III

<sup>a</sup> Location of the cleavage site of the signal peptide that was predicted with use of SignalP 3.0 (Bendtsen et al. 2004).

<sup>b</sup> Molecular mass was estimated according to the deduced amino acid sequence (X) or mobility of the recombinant protein obtained from yeast on a sodium dodecyl sulfate polyacrylamide gel (Y).

<sup>c</sup> Putative N-linked glycosylation site was predicted by use of PROSCAN.

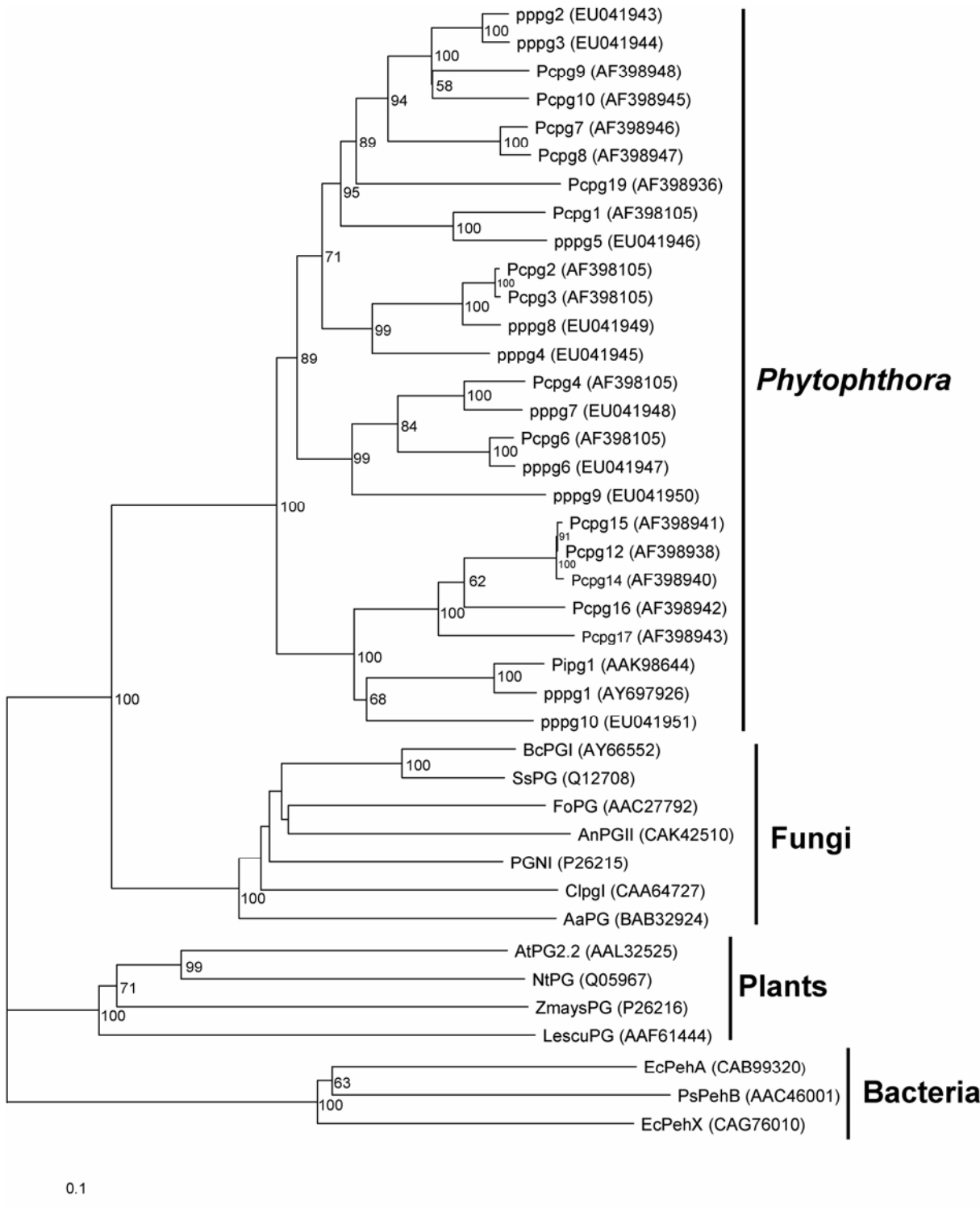
<sup>d</sup> Grouping was assigned according to the results obtained from agroinfection assays. - indicates that these genes were not analyzed in this experiment.

<sup>e</sup> Yan and Liou (2005).

linked glycosylation site indicated that the endoPG genes may contain one (*pppg2*, *pppg3*, and *pppg7*), two (*pppg5* and *pppg9*), or nine (*pppg10*) N-glycosylation sites, and some (*pppg4*, *pppg6*, and *pppg8*) contained no site (Table 1). In addition, *pppg2* and *pppg3* contained polyglutamine regions near the N terminus.

To examine the relation between the endoPG genes of *Phytophthora parasitica* and those from other organisms, we constructed a phylogenetic tree on the basis of multiple sequence

alignment of the endoPG genes from *Phytophthora parasitica*, *Phytophthora infestans* (Torto et al. 2002), *Phytophthora cinnamomi* (Götesson et al. 2002), fungi, plants, and bacteria (Fig. 2). The endoPG of *Phytophthora* spp., fungi, plants, and bacteria are separated into distinct groups, with *Phytophthora* endoPG closer to fungal endoPG than to plant and bacterial endoPG. Furthermore, endoPG from *Phytophthora* spp. were clustered into two groups: one of genes with 0, 1, or 2 N-glycosylation sites and the other of genes, including *pppg1* and



**Fig. 2.** Phylogenetic analysis of the polygalacturonase genes. The phylogram was generated by the neighbor-joining method as implemented in PAUP\* 4.0 beta. Numbers beside each node indicate bootstrap values as a percentage of 1,000 replicates. The accession number of each gene is shown in the parentheses. Exopolygalacturonases from bacteria were used as the outgroup.

*pppg10*, with multiple N-glycosylation sites. Some genes from the two *Phytophthora* spp. appearing in pairs were *Pcpg1* and *pppg5*, *Pcpg2/Pcpg3* and *pppg8*, *Pcpg4* and *pppg7*, and *Pcpg6* and *pppg6*.

### Gene expression.

To determine whether the endoPG genes are expressed by *Phytophthora parasitica* during infection of its host plant, detached tomato leaves were inoculated with zoospores of *Phytophthora parasitica* for quantification of gene expression by real time quantitative reverse transcriptase (qRT)-PCR. Raw data obtained from qRT-PCR were normalized with that of Ubc and WS21, which encode ubiquitin-conjugating enzyme and 40S ribosomal protein, respectively. As compared with the expression at the stage of germinated cysts, at 12 h postinoculation (hpi), the expression of *pppg4*, *pppg6*, and *pppg7* was upregulated, peaking at 48 hpi and declining at 72 hpi (Fig. 3A, Table S1). The expression of *pppg1*, known to be induced in planta (Yan and Liou 2005), displayed a similar expression pattern. The expression of *pppg2* and *pppg10* in the germinated cysts was too low to be used as a reference but was verified by RT-PCR and agarose gel electrophoresis (Fig. 3B). The expression of the other genes, *pppg3*, *pppg5*, *pppg8*, and *pppg9*, was not detectable in germinated cysts or in diseased plants, even though cDNA clones of *pppg3*, *pppg8*, and *pppg9* were obtained by RACE with RNA prepared from inoculated plants (data not shown).

### In-gel activity assay of recombinant proteins obtained from the yeast *Pichia pastoris*.

To determine whether endoPG genes encode proteins with enzymatic activity, recombinant proteins for *pppg2*, *pppg4*, *pppg5*, *pppg6*, *pppg7*, *pppg8*, and *pppg10* were expressed in *Pichia pastoris* and then underwent in-gel activity assay. *pppg3* was not analyzed due to its 96% identity with the coding sequence of *pppg2*, and *pppg9* will be investigated in a separate study. Western blot analysis with anti-6xHis-tag antibody used as the primary antibody revealed the recombinant proteins of *pppg2*, *pppg4*, *pppg8*, and *pppg10* as single bands and those of *pppg5*, *pppg6*, and *pppg7* as double bands, ranging from 37 to 70 kDa (Fig. 4A), slightly larger than the sizes based solely on deduced amino-acid sequences (Table 1). Further analysis by in-gel activity assay revealed the activity band of each recombinant protein with a size similar to that shown by Western blot analysis (Fig. 4B). The recombinant protein of *pppg1* was included in this experiment as a control (Fig. 4A and B, lane 1). Although *pppg1* and *pppg10* contain multiple putative N-glycosylation sites (Table 1), the recombinant protein of *pppg10* appeared as a condensed band of approximately 43 kDa. As well, although no N-linked glycosylation site was identified in *pppg4*, *pppg6*, and *pppg8*, the protein sizes were larger than those of deduced amino acid sequences.

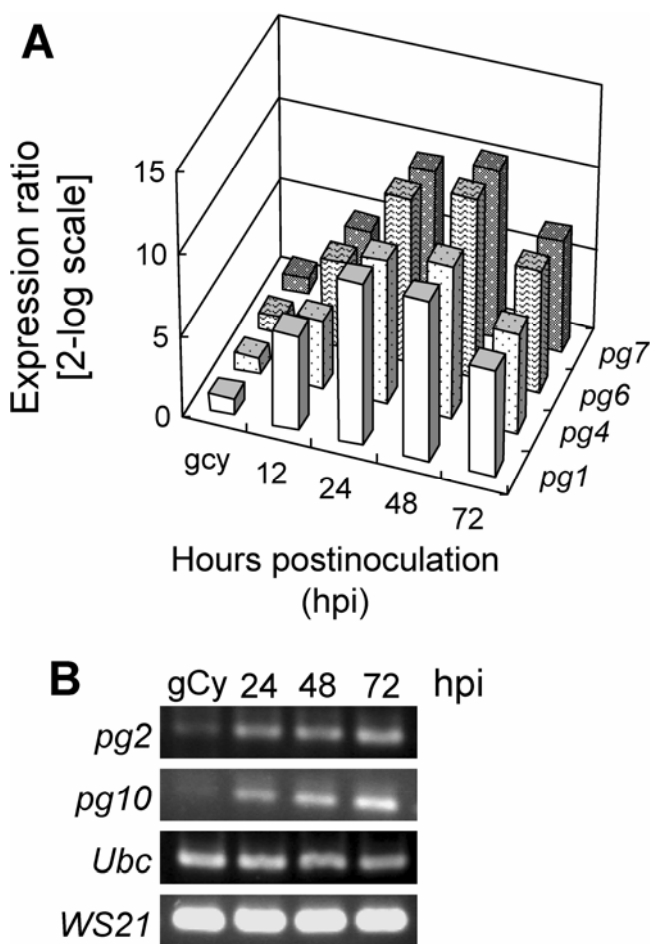
### Functional analysis of the endoPG genes by *Potato virus X* (PVX) agroinfection assay.

To examine the function of proteins encoded by the endoPG genes, we used PVX as a vector to systemically express the endoPG proteins in the tobacco *Nicotiana benthamiana*. The coding sequence downstream of the signal peptide of each endoPG gene was subcloned into pgR106:SP, in-frame with the signal peptide of tobacco PR1a, for secretion of the recombinant protein into the apoplast. Systemic leaves of the infiltrated plants were examined under white and UV light after infiltration. Furthermore, cross-sections of these leaves were examined for changes in morphology of cells. Effects caused by the recombinant proteins could be classified into the following four groups (Table 1).

Group I effects, with *pppg1* and *pppg7* expression, were no significant change in systemic leaves as compared with plants mock-inoculated or infiltrated with the empty vector (pgR106:SP) at 10 days postinfiltration (dpi), although a slight symptom of silvery leaf was observed with *pppg7* (Fig. 5A, 6A and B). Staining of leaves with ruthenium red revealed severely deformed mesophyll cells of *pppg7*-treated plants (Fig. 6C). Infiltration with pgR106:*pppg1* resulted in slight changes as compared with infiltration with the empty vector.

Group II effects, with *pppg4* and *pppg6* expression, were dwarf phenotypes (Fig. 5B). New leaves curled toward the back at 4 dpi, and their growth was slightly stunted. As the leaves expanded in size, they became heavily wrinkled and then showed necrotic spots with shot holes that appeared as spots of green fluorescence under UV light (Fig. 6A and B). Also, cross-wise cracks appeared on the leaf stalks (data not shown). These effects were predominant on the upper third and fourth leaves relative to the infiltrated ones on the test plants. Analysis of cross-sections of leaves treated with *pppg4* and *pppg6* showed deformed palisade and spongy mesophyll cells with barely any intercellular space left (Fig. 6C).

Group III, with *pppg2*, *pppg8*, and *pppg10* expression, were yellowing and dwarfism (Fig. 5B). Similar to group II effects, new leaves of plants treated with *pppg2*, *pppg8*, and *pppg10* curled up on emergence and leaf growth was stunted. As they



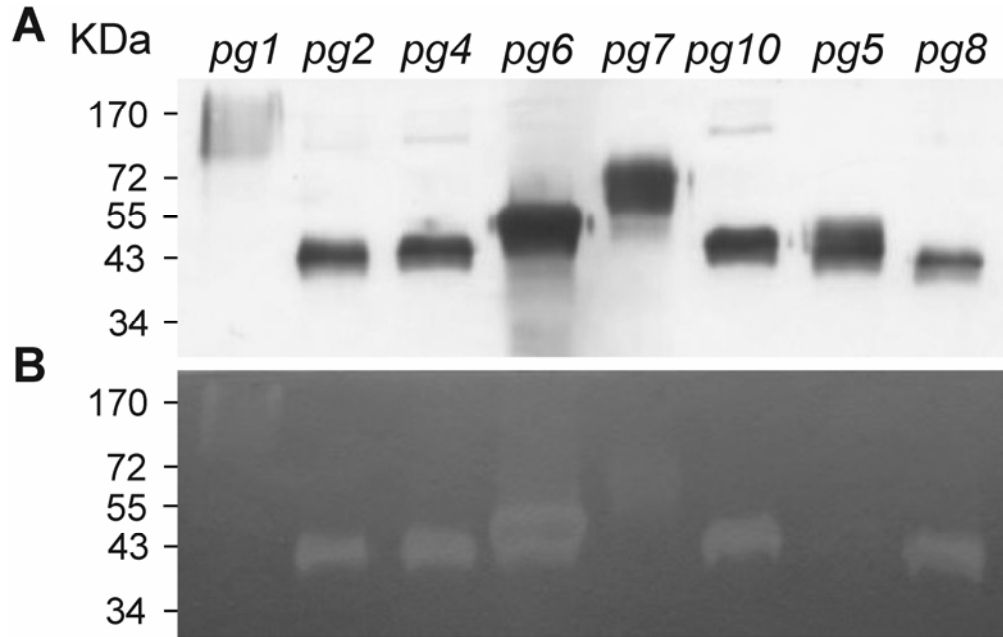
**Fig. 3.** Expression of *pppg* genes in tomato infected with *Phytophthora parasitica*. Following inoculation by zoospores, RNA was isolated from inoculated tomato leaves at the indicated times and was analyzed by **A**, real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and **B**, RT-PCR followed by agarose gel electrophoresis. Raw data obtained from qRT-PCR were normalized to that of WS21 and Ubc as the internal control. gCy = germinating cysts.

grew, the leaves became yellowed and wrinkled, with some necrosis visible under UV light (Fig. 6A and B). These effects were predominant on the upper third and fourth leaves of the test plants. On examination of leaf cross-sections, the morphology of the mesophyll was changed similar to that of plants treated with *pppg6*, with the disappearance of the intercellular space (Fig. 6C).

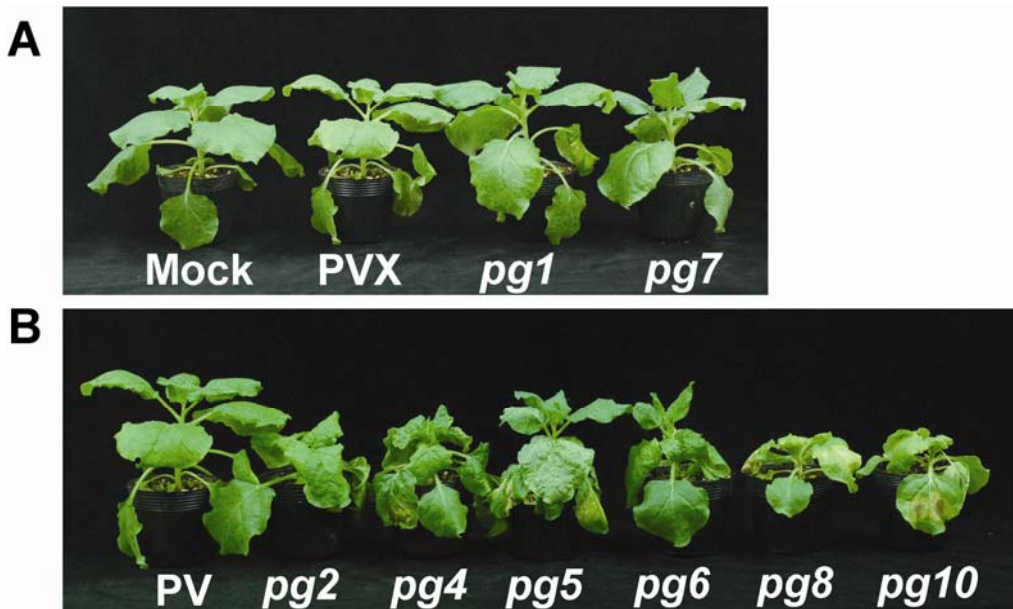
Group IV effects, with *pppg5* expression, were a phenotype of silvery leaf (Fig. 5B), with the mesophyll cells severely deformed (Fig. 6C) at 10 dpi. In addition, similar to group II effects, *pppg5* expression resulted in the appearance of cracks

on the leaf stalks of systemic leaves (Fig. 7A). Examination of the cross-sections showed cracked leaf stalks with more intercellular space than leaf stalks of plants infiltrated with the empty vector (Fig. 7B and C). Furthermore, epidermal cells were detached from the wax cuticle layer as compared with cells from plants infiltrated with the empty vector (Fig. 7D and E).

To determine whether these effects were due to the enzymatic activity of endoPG, two types of mutants were constructed for each *pppg* gene: i) *SPd*, with the signal peptide of PR1a deleted to prevent secretion of the recombinant proteins into the apoplast fluid, and ii) *D/E*, with two aspartic acid resi-



**Fig. 4.** Analysis of the *pppg* recombinant proteins heterologously expressed in *Pichia pastoris*. Recombinant proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by **A**, Western blot analysis with anti-6xHis-tag antibody used as the primary antibody or **B**, in-gel polygalacturonase activity assay with polygalacturonic acid used as the substrate.



**Fig. 5.** Symptoms caused by the systemic expression of *pppg* genes in *Nicotiana benthamiana* with *Potato virus X* (PVX) used as the vector. *N. benthamiana* plants were infiltrated with a cell suspension of *Agrobacterium tumefaciens* harboring pgR106:SP (PVX; the empty vector) or pgR106:*pppg* as specified. Mock indicates plants were infiltrated with the solution MMA (10 mM morpholineethanesulfonic acid, pH 5.6, 10 mM MgCl<sub>2</sub>, 200 μM acetosyringone), used to resuspend the bacteria. The plants were photographed 10 days postinfiltration. **A**, group I and **B**, groups II to IV.

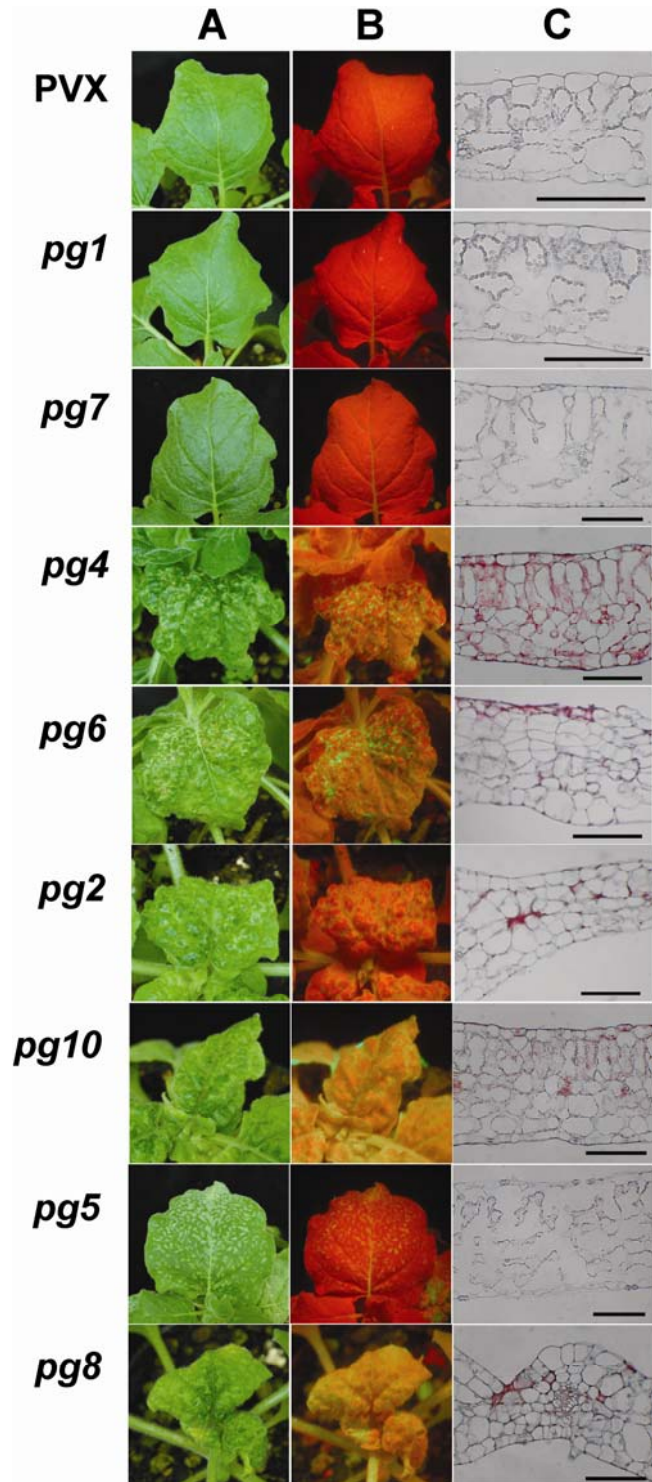
dues essential for the enzymatic activity of endoPG replaced with glutamic acids (Armand et al. 2000). *Nicotiana benthamiana* infiltrated with either SPd or D/E mutants of any *pppg* gene developed and grew normally like those treated with the empty vector, which indicates that the aforementioned effects indeed resulted from the enzymatic activity of the endoPG present in the apoplast (Fig. 8).

## DISCUSSION

The plant cell wall provides a physical barrier to infection by pathogens. To invade the host, pathogens need to secrete pectinases, which degrade pectins embedded in the plant cell wall. Although great interest in pectinases of *Phytophthora* spp. arose from the early 1970s (Cole 1970), our knowledge about these proteins is limited. Only a small number of genes encoding pectinases in *Phytophthora* spp. have been characterized in any detail (Götesson et al. 2002; Torto et al. 2002; Yan and Liou 2005). In this study, we cloned and analyzed the function of nine endoPG genes from *Phytophthora parasitica*. Together with *pppg1* reported previously by our laboratory (Yan and Liou 2005), 10 endoPG genes have been identified in this pathogen. Phylogenetic analysis indicated that endoPG of *Phytophthora* spp. form a cluster distinct from those of fungi and plants. Furthermore, endoPG of *Phytophthora* spp. are grouped into two clades, one heavily and the other lightly glycosylated. A multigene family of PG with 19 members has been identified in *P. cinnamomi* (Götesson et al. 2002). In the phylogenetic tree, some *pppg* genes are closely related to certain *Pcpg* genes of *Phytophthora cinnamomi*, which suggests that they may have evolved prior to their speciation and are thus orthologs. In contrast, as was found with some *Pcpg* genes (*Pcpg7* to *Pcpg8*, *Pcpg2* to *Pcpg3*, and *Pcpg12* to *Pcpg15*), *pppg2* is tightly related to *pppg3*, very likely because of a recent gene duplication, a phenomenon also found in other secretory proteins of *Phytophthora* spp. (Jiang et al. 2006). In addition, no gene closely related to *pppg9* or *pppg10* was identified in *Phytophthora cinnamomi*, although their respective homologs were found in *Phytophthora ramorum* by database search (data not shown). Together, these results support the divergence of endoPG genes before species separation within the genus *Phytophthora*, and moreover, the duplication of certain endoPG genes could still take place after the speciation event, with the formation of large endoPG gene families in these pathogens.

To determine whether these *pppg* genes are indeed expressed, qRT-PCR of RNA isolated from plants inoculated with zoospores of *Phytophthora parasitica* revealed expression of *pppg4*, *pppg6*, and *pppg7* similar to that of *pppg1*—up-regulated at 12 hpi and peaking at 48 hpi—which suggests their importance in the early phase of plant infection. The expression of other genes, however, was very low or even undetectable. The expression of *pppg2* and *pppg10* was detected in inoculated plants but was very low in germinated cysts, which indicates that their expression is induced in planta as well. In *Botrytis cinerea*, *Bcpg1* through *Bcpg6* are differentially expressed, depending on stage of infection and host (ten Have et al. 2001). Also, studies of *Sclerotinia sclerotiorum* revealed that different endoPG genes are expressed consecutively during pathogenesis: *pg1*, *pg2*, and *pg3* are expressed during invasion and colonization and *pg5* in the late stage of maceration, but *pg6* and *pg7* are constitutively expressed across the colonization process (Kasza et al. 2004). Like *Botrytis cinerea* and *Sclerotinia sclerotiorum*, *Phytophthora parasitica* can infect a large number of plants. Further experiments will help elucidate the detailed expression pattern of *pppg* genes in terms of host and stage of infection by *Phytophthora parasitica*.

The deduced amino-acid sequences in the active site of the 10 *pppg* genes are highly conserved. Analysis of the expression of these genes in *Pichia pastoris* followed by in-gel activity assay demonstrated that each of the recombinant proteins contained the enzymatic activity of endoPG. The activity of *pppg5* and *pppg7*, however, was weaker than that of other genes, which indicates distinct biochemical characteristics of



**Fig. 6.** A close-up of symptoms caused by the expression of *pppg* genes on leaves of *Nicotiana benthamiana*. *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* harboring pgR106:SP (PVX; the empty vector) or pgR106:*pppg* as specified. **A**, At 10 days postinfiltration, the plants were examined under white light, **B**, UV light, or **C**, cut into cross-sections and stained with ruthenium red. Bars correspond to 100  $\mu$ m.

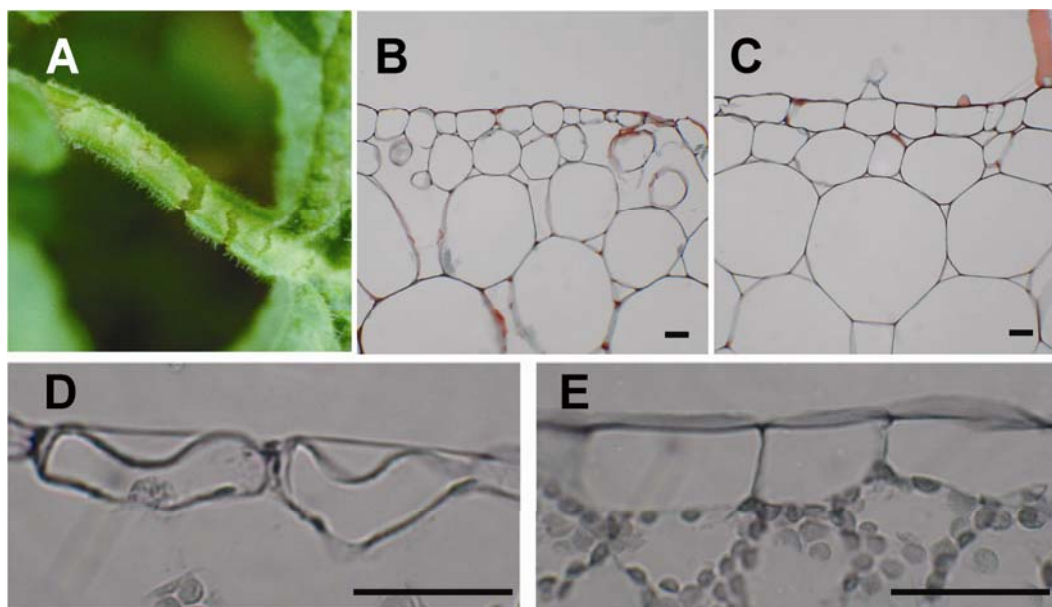
these two proteins, such as substrate preference and protein stability, than those of other genes. In *Botrytis cinerea*, polygalacturonate was shown to be a poor substrate for BcPG1 and BcPG4 as compared with BcPG2, BcPG3, and BcPG6 (Kars et al. 2005). Of special interest, although predicted to contain nine N-glycosylation sites and closely related to *pppg1*, *pppg10* appeared as a condensed protein band of 43 kDa, in sharp contrast to *pppg1*, with its 11 N-glycosylation sites and range in size from 75 to 200 kDa, very likely due to a differential glycosylation of the *pppg1* protein (Yan and Liou 2005). Some *pppg* genes (*pppg4*, *pppg6*, and *pppg8*) did not contain any N-glycosylation sites. However, their corresponding proteins appear to be larger than those of the deduced amino acid sequences. Thus, other types of post-translational modification might be required for the maturation of these proteins.

Like *Phytophthora parasitica* and *Phytophthora cinnamomi*, many plant pathogens are known to contain a multigene family of endoPG (Williams et al. 2002; Wubben et al. 1999). The number of endoPG genes may depend on the host range of a pathogen (Esquerré-Tugayé et al. 2000). Search of the Joint Genome Initiative (JGI) database (United States Department of Energy), however, suggested the presence of various endoPG homologs in the genome of *Phytophthora sojae*, which is known to have a narrow host range (Yan and Liou 2005). Thus, the number of endoPG genes is not related to host range. The composition of plant cell walls varies significantly among cell types and species (Vorwerk et al. 2004). For pathogens to succeed in infection, a profile of endoPG that function differently in plant tissues may be required to compensate for the complicated composition and structure of pectins in the cell walls. To address this possibility, we expressed the *pppg* genes ectopically in plants, using the binary vector pgR106 (Lu et al. 2003). The *pppg* genes were mobilized systemically along with the PVX vector, and their proteins were secreted into the apoplast of the infiltrated plants. Plant cell walls are highly hydrated, and proteins less than 50 kDa diffuse freely through a typical wall in less than 1 h (Baron-Epel et al. 1988). Thus, once secreted, the *pppg* proteins may have a very good chance to reach their targets in the middle lamella, which is essential for cell-cell adhesion, and thereby cause distinct effects, depending on how and where they worked. Effects caused by the *pppg*

proteins in tobacco were classified into four groups (Table 1). *pppg1* and *pppg7* (group I) failed to cause significant effects, perhaps because of their large molecular mass, greater than 50 kDa (Table 1; Fig. 4), which assumes that these two proteins were modified in a similar way in plants. Treatment with other *pppg* genes, in contrast, caused obvious effects in the plants, which appeared only when *pppg* proteins with normal active sites were secreted into the apoplast. Why these *pppg* genes functioned differently in planta, however, is not easy to explain. No relation was found between the effects and characteristics of the proteins, such as isoelectric point values, number of putative N-glycosylation sites, phylogeny, and locations of these genes in the genome of *Phytophthora parasitica*. In *Botrytis cinerea*, BcPG were found to differ in many aspects, including maximum activity,  $K_m$ , optimal pH, and mode of action, which underlie their differences in necrotizing activity (Kars et al. 2005). Analysis of the recombinant *pppg* proteins by in-gel activity showed the activity of *pppg5* and *pppg7* much weaker than that of others, which suggests differences in biochemical properties of the *pppg* proteins. Thus, the differential effects caused by each *pppg* might reflect the complicated interplay between the enzyme and the microenvironment it worked; the latter involved the composition and structure of the cell wall at a particular stage in leaf development. To our knowledge, this is the first demonstration that systemic expression of different endoPG genes from a pathogen caused distinct effects in plants.

In response to infection by the systemic fungus *Chondrostereum purpureum*, apple tree shows the 'silvery leaf' symptom, which is due to airspaces caused by partial separation of the epidermal cells from the palisade cells (Spiers et al. 1987), and endoPG produced by *Chondrostereum purpureum* is believed to be responsible for the development of the symptom (Miyairi et al. 1977). In our study, the expression of *pppg5* also caused a phenotype of silvery leaf with epidermal cells detached from the wax cuticle layer (Fig. 7D), which provided the first direct evidence that expression of a single endoPG from a pathogen can cause silvery leaf in plants.

In addition to their role as cell wall-degrading enzymes, and thus virulence factors, some endoPG also function as avirulence factors that elicit the plant defense response (Boudart et



**Fig. 7.** Effect of *pppg5* in *Nicotiana benthamiana*. **A**, **B**, and **D**, Plants infected with *Agrobacterium tumefaciens* harboring pgR106:*pppg5* or **C**, and **E**, pgR106:SP (PVX; the empty vector). Ten days after infection, plants were examined under white light (**A**) and were cut into cross-sections and stained with ruthenium red for microscopy observation of the cortical region of a leaf stalk (**B** and **C**) or epidermal cells (**D** and **E**). Bars correspond to 25  $\mu$ m.

al. 2003; Poinssot et al. 2003). That *pppg* genes may cause plant defense response is now under investigation.

## MATERIALS AND METHODS

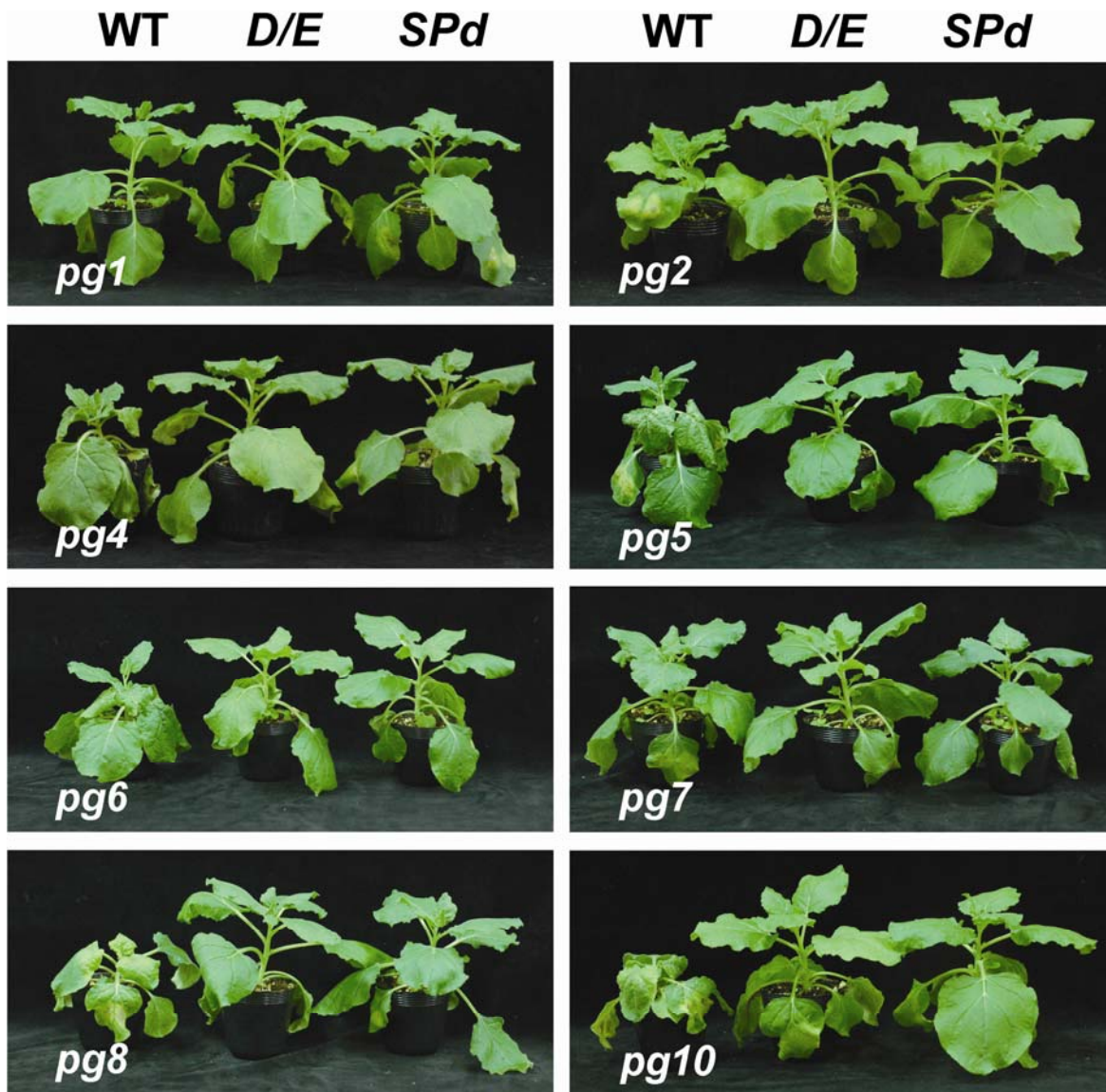
### *Phytophthora parasitica* strains and growth conditions.

*Phytophthora parasitica* 98151 (mating type A2) was provided by P. J. Ann (Taiwan Agricultural Research Institute, WuFeng, Taiwan) and was stored in sterile distilled water. For preparation of nucleic acids, one culture block of *Phytophthora parasitica* from 5% V8 juice agar (5% Campbell's V8 juice, 0.02% CaCO<sub>3</sub>, 2% agar) was transferred into a sterile 250-ml flask containing 100 ml of 5% V8 juice medium (5% Campbell's V8 juice and 0.02% CaCO<sub>3</sub>) and was incubated at 25°C in the dark for 7 days. Following harvest by filtration, the mycelia were frozen instantly in liquid nitrogen and were lyophilized and stored at -80°C until use.

### Isolation of genes encoding polygalacturonase in *Phytophthora parasitica* and sequence analysis.

DNA was extracted from mycelia of *Phytophthora parasitica* by use of DNeasy plant mini kit (Qiagen, Basel, Swit-

zerland). Two sets of degenerated primers, conPGF1 and conPGR1 (Table S2), were designed according to amino-acid sequences conserved among endoPG of *Phytophthora parasitica* (*pppg1*; Yan and Liou 2005), *Phytophthora cinnamomi* (*PcPG2*, *PcPG3*, and *PcPG9*; Götesson et al. 2002), and *Phytophthora ramorum* (gwEuk.91.29.1, gwEuk.91.45.1, and fgenesh1\_pm.C\_scaffold\_91000005; JGI database). Genomic PCR involved use of these primers, with the reaction mixture (50 µl) consisting of 500 ng of genomic DNA, 6 µM primers, 0.2 mM deoxynucleoside triphosphate, 1× PCR buffer, and 2 U DyNzyme II DNA polymerase (Finnzyme, Espoo, Finland) and denaturation at 96°C for 5 min, followed by 25 cycles of amplification (96°C for 20 s, 52°C for 20 s, 72°C for 20 s) and a final extension for 5 min at 72°C in a Thermocycler (GeneAmp PCR system 2400, Perkin-Elmer, Foster City, CA, U.S.A.). After analysis by agarose gel electrophoresis, amplicons of the expected size were cloned and analyzed. Sequences representing putative endoPG were used to screen a genomic library of *Phytophthora parasitica* as described previously (Yan and Liou 2005). In addition, *pppg10* was cloned by RACE as described by Yan and Liou (2005). Nucleotide and amino-acid sequence homology searches were performed with the National Center for



**Fig. 8.** Effect of *pppg* genes on *Nicotiana benthamiana* depends on the expression of *pppg* as secretory proteins with normal active sites. *N. benthamiana* plants were infiltrated with a cell suspension of *Agrobacterium tumefaciens* harboring pgR106:*pppg* (wt), pgR106:*pppg* with mutations in the putative active site (*D/E*), or pgR106:*pppg* without a signal peptide (*SPd*). Plants were photographed 10 days postinfiltration.



Biotechnology Information BLAST program. Putative core activity sites and N-glycosylation sites were predicted by use of PROSCAN. The signal peptide of each endoPG gene was predicted with use of SignalP 3.0 (Bendtsen et al. 2004). The amino-acid sequences were aligned by use of the multiple alignment option in Clustal X. Phylogenetic trees were generated by neighbor-joining, as implemented in PAUP\* 4.0 Beta (Sinauer Associates, Sunderland, MA, U.S.A.) with the default parameters. Nodal support of the tree was estimated by bootstrapping, with 1,000 pseudoreplicate data sets.

#### Real-time qRT-PCR analysis.

Inoculation of detached tomato leaves with zoospore suspension of *Phytophthora parasitica* was performed as described (Yan and Liou 2006). Total RNA was isolated from the leaves with TriZol reagent (Invitrogen, Carlsbad, CA, U.S.A.), followed by treatment with RNase-free DNase I (0.5 U/μl; Roche, Mannheim, Germany) at 37°C for 1 h to remove genomic DNA. Reverse transcription involved use of PowerScript reverse transcriptase (Clontech, Palo Alto, CA, U.S.A.), with 5'-T<sub>25</sub>VN-3' (V: A, C, or G; N: A, C, G, or T) as the primer. After 10× dilution with double-distilled H<sub>2</sub>O, 1 μl of the reverse transcription mixture was submitted for online quantitative PCR with the LightCycler system by use of the FastStart DNA master SYBR green I kit (Roche). PCR was performed in a volume of 20 μl containing 2 μl LightCycler FastStart DNA master SYBR green I, 3 mM MgCl<sub>2</sub>, and 1 μM primers. The instrument settings were initial enzyme activation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 5 s, and 72°C for 10 s. Design of oligonucleotide primers for quantitative PCR involved use of the GenScript Real-Time Primer Design tool (Table S2). Crude data obtained from Lightcycler were normalized, with Ubc and WS21 used as internal controls, followed by a randomization test implemented in the relative expression software tool (REST) for significance (Yan and Liou 2006). All experiments involved use of RNAs prepared from at least two biological repeats.

#### Expression of recombinant protein in *Pichia pastoris* and in-gel PG activity assay.

A DNA fragment corresponding to the coding sequence of each gene that did not include the signal peptide was subcloned into pPIC-αC (Invitrogen) with the Myc epitope and polyhistidine tag fused in-frame to its C terminus. The expression of recombinant proteins with an EasySelect *Pichia* expression kit (Invitrogen) and subsequent in-gel PG activity assays were as described (Yan and Liou 2005), with polygalacturonic acid used as the substrate. Western blot analysis followed standard procedures with anti-6X-His-tag used as the primary antibody.

#### Construction of plasmids for PVX agroinfection.

The binary vector pgR106 (Lu et al. 2003) and the bacterial strain C58C1 were provided by D. C. Baulcombe (The Sainsbury Laboratory, John Innes Centre, U.K.). For construction of the test clones, the signal peptide of tobacco PR1a was inserted into the *Clai* and *NotI* sites of pgR106 and the resultant plasmid was named pgR106:SP. Subsequently, a DNA fragment corresponding to the coding sequence of each endoPG gene that did not include the signal peptide was subcloned in-frame with the tobacco signal peptide into pgR106:SP by use of the *NotI* site. The binary expression constructs (named pgR106:pppg) were introduced into *Agrobacterium tumefaciens* C58C1 by electroporation, and transformants were selected on Luria-Bertanl (LB) agar supplemented with 12.5 ppm tetracycline and 25 ppm kanamycin at 28°C. Plasmids obtained from the transformants were then tested by PCR for the presence of

endoPG insert and further checked by DNA sequencing. Mutagenesis at the active site of the *pppg* genes was as described (Higuchi et al. 1988), and the mutated sequences were confirmed by DNA sequencing.

#### PVX agroinfection assay.

*Nicotiana benthamiana* plants were grown on a peat moss mixture for 24 days in the greenhouse at 25°C with a photoperiod of 16 h. Three days before infiltration, *Agrobacterium tumefaciens* cells carrying pgR106:pppg were inoculated into LB broth supplemented with tetracycline and kanamycin at 28°C for 48 h. The resultant cultures were diluted 50× with the induction medium (90 mM sodium phosphate buffer, pH 7.0, 7 mM ammonium sulfate, 1.5 mM sodium citrate, 1 mM MgSO<sub>4</sub>, 0.2% glucose, 0.1 mM CaCl<sub>2</sub>, 10 mM morpholine-ethanesulfonic acid [MES], 20 μM acetosyringone, 25 ppm kanamycin, and 12.5 ppm tetracycline) and were grown for another 24 h at 28°C. Cells were harvested by centrifugation (8,000 rpm for 15 min at 4°C) and were resuspended with MMA (10 mM MES, pH 5.6, 10 mM MgCl<sub>2</sub>, 200 μM acetosyringone) to an optical density at 600 nm of 0.5. Infiltration involved use of a needleless 1-ml syringe placed against the lower side of the leaf. Experiments were performed at least three times for each construct, with each experiment involving at least three plants per construct.

#### Microscopy observation.

Leaves of *Nicotiana benthamiana* were cut into squares of approximately 1 cm<sup>2</sup> and were fixed in FAA solution (37% formaldehyde/glacial acetic acid/70% ethanol, 5:5:90) for 20 h at room temperature. The leaf samples were then dehydrated through graded ethanol series, were stained with safranin O, and were embedded in Steedman's wax, made with polyethylene glycol 400 (Sigma-Aldrich, St. Louis) and 1-hexadecanol (Sigma-Aldrich) at a ratio of 9:1. Sections of 8 μm were cut with a microtome and were stained with ruthenium red (0.05%, wt/vol) (Atkinson et al. 2002) and observed under a light microscope.

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## AUTHOR-RECOMMENDED INTERNET RESOURCES

- United States Department of Energy JGI database:  
[genome.jgi-psf.org/Phyra1\\_1](http://genome.jgi-psf.org/Phyra1_1)
- Pôle BioInformatique de Lyon Network Protein Sequence Analysis  
 PROSCAN: [npsa-pbil.ibcp.fr](http://npsa-pbil.ibcp.fr)
- GenScript Real-Time Primer Design website:  
[www.genscript.com/ssl-bin/app/primer](http://www.genscript.com/ssl-bin/app/primer)