African Journal of Biotechnology Vol. 11(10), pp. 2477-2489, 2 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2043
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Expression and functional analysis of polygalacturonase gene *Pcipg*5 from the plant pathogen *Phytophthora capsici*

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Accepted 4 November, 2011

As one of the cell wall-degrading enzymes, polygalacturonase involves in pathogenicity and virulence in a number of host pathogen interactions. In the polygalacturonase multigene family of *Phytophthora capsici*, *Pcipg*5 was screened from the genomic library. The *Pcipg*5 gene contains three putative active sites (179Asp, 200Asp, 201Asp) and one potential *N*-glycosylation site (252Asn). PCIPG5, four active-site mutagenesis proteins and *N*-glycosylation mutation were expressed in *Pichia pastoris* and purified by Ni-NTA Purification System. *Pcipg*5 functions were determined by Western blot, RT-PCR and Northern blot. Transmission electron microscopy discovery individually in treated pepper leaves was used to determine the virulence of PCIPG5. The results show that *Pcipg*5 can encode a pathogenesis-related protein during *P. capsici* infection of pepper leaves and degrade cell walls to produce necrotic lesions in treated pepper leaves. Meanwhile, the *N*-glycosylation mutagenesis protein decreased the activity compared with PCIPG5. It indicates that the existence *N*-glycosylation site in PCIPG5 plays a partial role in the activity of this enzyme.

Key words: *Phytophthora capsici*, *Pcipg*5, host-pathogen interactions, *N*-glycosylation.

INTRODUCTION

Plant pathogens can produce a diverse range of cell wall-degrading enzymes (CWDEs) such as polygalacturonases (PGs) (EC 3. 2. 1. 15), pectate lyases (PLs), pectin methylesterases (PMEs), and cellulases (Walton, 1994) in the course of host pathogen interactions. After pathogens breaching the cell wall barrier, these enzymes soften plant tissues and cause localized cell death. PGs are pectic enzymes that hydrolyze polygalacturonan and involve in pathogenicity and virulence in a number of host pathogen interactions (Collmer and Keen, 1986).

Straminopiles are fungus-like eukaryotic micro-organisms related to true fungi (Baldauf et al., 2000), and some of them represent major threat to agriculture and natural ecosystems. Members of these pathogens often cause many serious plant diseases (Lamour and Hausbeck, 2001). *Phytophthora capsici* is a straminopilous plant pathogen that induces severe disease in several economically important plant species (Hartman and Huang, 1993). It can cause one of the most widespread and destructive soilborne diseases. Previous studies demonstrated that *Phytophthora* species secreted a set of pectic enzymes under suitable culture conditions (Yuan and Tseng, 1980), but knowledge of the molecular mechanisms of *Phytophthora* species pathogenicity related to pectic enzymes is limited. Recently, many *pg* genes have been cloned from straminipilous pathogens (Torto et al., 2002; Götesson et al., 2002; Wu et al., 2008). Moreover, the size of *pg* multigene family has been suggested to vary corresponding to the specific interaction with plants.

Molecular studies have found that most proteins are encoded by different genes in multigene family whose members share functional motifs or domains. In most families, all members have different patterns of expression. Recent studies have identified few *pg* genes from

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Table 1. Primers and their sequences used in gene isolation, expression, site-directed mutation, and RT-PCR.

Applications of primers	Sequences (5'-3')
P710F	ACGG(A/C/G/T)CA(A/G)GG(A/C/T)GC(T/C)T (A/G)(G/T)TACTGG
P1150R	GATGATGGTCTTGATGCGGA
*pcipg*5F(A)	TATA*GAATTC*CACCACCACCACCACCACGACGACGACGACAAGACACCCATGATCCGTCAGGC
*pcipg*5R(B)	TATA*GCGGCCGC*TTAGCACTTGACAGTGCTGG
179-F	AAGAACACAGAAGGCTTCGACCTG
179-R	CAGGTCGAAGCCTTCTGTGTTCTT
200-F	TACAACCAGGAAGACTGTTTGGCA
200-R	TGCCAAACAGTCTTCCTGGTTGTA
201-F	TACAACCAGGATAACTGTTTGGCA
201-R	TGCCAAACAGTTATCCTGGTTGTA
pcipg5-N252Q-F	ACCATCGTCCAGAGCACCAACGGC
pcipg5-N252Q-R	GCCGTTGGTGCTCTGGACGATGGT
RT-5F	GGGTCCGGTACTCTTGACGGTCAG
RT-5R	CACGATGGCGTTCTTGACGTTGCT

The letters in bracket represent degenerate sites; the italic letters represent the recognition sites of restriction enzymes *Eco*R I and *No*t I respectively; the bold letters represent the affixation C-terminal His_6 tag; letters underlined represent the sequence after mutation.

Phytophthora species (Jarvis et al., 1981; Torto et al., 2002). In addition, a large *pg* gene family with 19 members in *P. cinnamomi* was identified, and phylogenetic analysis of this straminopilous pathogen's PGs revealed a closer relationship with PGs from true fungi than with those from plants (Götesson et al., 2002). Additionally, Wu et al. have analyzed the functional characterization of a *pg* gene family in *P. parasitica* (2008). However, little is known about the role of *pg* genes of *P. capsici* in pathogenicity.

Glycosylation is often presented in most of the enzymes which secreted from microorganism (Koseki et al., 2006; Schäffer and Messner, 2001; David and Virginia, 2007; Wu et al., 2008). Fungal glycoside hydrolases and other enzymes belonging to different protein families are often glycosylated, carrying both *O*-linked and *N*-linked glycans (Gusakov et al., 2008). Glycosylation sites play important roles in a variety of biological and physical properties, including the folding, secretion, solubility, and a stability of proteins (Rademacher et al., 1988; Varki, 1993). The glycosylation sites of the various CWDEs may play important roles in pathogenesis based on their dramatic effect on the activity of proteins (Haltiwanger and Lowe, 2004). The deglycosylations of *Aapergillus* and *P. parasitica* polygalacturonases by *N*-Glycosidase F were to result in reduction in molecular mass followed by complete inactivation of the polygalacturonase enzymes activity (Stratilová et al., 1998; Yan and Liou, 2005). *N*-glycosylation sites are occurred at asparagine residues in the consensus sequence of Asn-X-Ser/Thr. The *Pcipg* genes had differences in the number of *N*-glycosylation sites and different levels on each site. The various *N*-glycosylation may influence the biological function of polygalacturonase genes in *P. capsici*.

In the multigene families of endoPG in fungi, each gene performing may define biological tasks in the infection process (Wu et al., 2008). Sun et al. (2009) characterized the function of *Pcipg*2 (Genbank No. DQ415987) which represented endoPG activity and was required for virulence. So we designed experiments to prove that PCIPG5 was involved in cell wall degradation during *P. capsici* infection of pepper. We investigated expression levels of the *Pcipg*5 and its product in pepper leaves inoculated with *P. capsici* strain SD33. We also studied alterations of wild protein activity and resulting virulence on pepper leaves by using site-directed mutagenesis to analyze the three strictly conserved active-site Asp residues and *N*-glycosylation site within *Pcipg*5. We determined that *Pcipg*5 participated in the infection process and the development of *Phytophthora* blight in pepper.

MATERIALS AND METHODS

Isolation *Pcipg*5 and sequence analysis

A highly virulent *P. capsici* strain SD33 with high PGs activity was selected and the genomic library was constructed as described previously (Sun et al., 2009). Two degenerate primers were used to screen *Pcipg* genes from the genomic library. The primers were P 7 1 0F and P1150R (Table 1), which were designed based on the conserved sequences of other *pg* genes (Wu et al., 2008; Esquerré-Tugayé et al., 2000; Yan and Liou, 2005). *Pcipg* genes were screened from the genomic library with minor adjustment of the PCR reaction systems and parameters (Sun et al., 2009).

To verify the *pg* gene amino acid sequence, sequence data was analyzed using the GCG software package. Nucleotide and amino acid sequence homology searches were performed using the NCBI-BLAST program (http://www.ncbi.nlm.nih.gov/). Available complete

PGs amino acid sequences including oomycete pathogens, fungi, plants and bacteria were carried out using ClustalX (version 1.81) (Thompson et al., 1997) and GeneDoc (version 2.6.002) (Nicholas et al., 1997). The *pg* sequences of *P. ramorum* and *P. sojae* were downloaded from the Joint Genome Institute (http://www.jgi.doe.gov/). Genes for hypothetical proteins analysis was performed using the online open reading frame finder (http:// ncbi.nlm.nih.gov/projectes/gorf/).

RT-PCR and Northern blot analysis of *Pcipg*5 expression

To extract total RNA, pepper leaves treated with zoospore suspensions were collected at one day intervals from 1 to 7 dpi and ground in liquid nitrogen. Total RNA was extracted using an RNeasy plant minikit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Reverse transcription was performed using an Omniscript RT kit (Qiagen). Synthesized cDNA was used for PCR, and the primers of RT-5F and RT-5R (Table 1) were designed from nonconservative regions of *Pcipg*5. 18s RNA of pepper was used as an internal control. The PCR reactions and parameters were as previously described with minor adjustment (Fonseca et al., 2005). The PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. Results were obtained from three repeated experiments. cDNA from strain SD33 was used as a positive control. cDNA from fresh pepper leaves and RNA from fresh pepper leaves were used as negative controls.

Northern blot was performed according to the procedure of the Roche DIG DNA labeling kit (Sambrook et al., 1989), in which a nonconserved fragment was amplified using primers of RT-5F and RT-5R, and then used to synthesize a probe using the kit following the manufacturer's protocol. RNA from strain SD33 served as a positive control. RNA of fresh pepper leaves served as a negative control.

Western blot analysis

PCIPG5 was used to prepare antibodies in New Zealand white rabbits according to standard protocols (Kothari et al., 2006). The antibody purification and antiserum titer determination were performed as described previously (Yang et al., 2007). Zoospore suspensions of SD33 were used to inoculate pepper leaves (cultivar Kexing 3) as described by Sun et al. (2008). Diseased leaves were collected at one day intervals from 1 to 7 days post-inoculation (dpi), and the crude leaf protein was extracted by the method of Joubert et al. (2007). Western blot analysis was conducted as previously described (Sun et al., 2009). Purified PCIPG5 was used as a positive control, and the crude protein from fresh leaves inoculated with sterile distilled water was used as a negative control. Signals were detected by enhancing chemiluminescence (Cell Signaling Technology, Beverly, MA, USA).

Expression of *Pcipg*5 in *Pichia pastoris* and purification

*Pcipg*5 was expressed using an EasySelect *Pichia* Expression kit (Invitrogen, Carlsbad, CA 92008, USA). To obtain the mature protein, the *Pcipg*5 cDNA was used as template with primers *pcipg*5F and *pcipg*5R (Table 1) corresponding to the predicted *Pcipg*5 of amino acid residues 21–362. PCR parameters were as follows: 94°C for 4 min, 35 cycles of 94°C for 1 min, 67.3°C for 30 s, 72°C 1 min, and a final extension at 72°C for 10 min. The PCR products were cloned into the pEasy-T3 vector to generate pEasy/*Pcipg*5 plasmid. The *Pcipg*5 was excised as an *EcoR* I/*Not* I fragment from pEasy-*Pcipg*5 and then subcloned into pPIC9K to generate expression vector pPIC9K/*Pcipg*5. The constructed plasmid pPIC9K/*Pcipg*5 was linearized with *Stu* I and transformed into the *P. pastoris* GS115 to express. The protein was purified from culture supernatants after incubation for 7 days using a HisTrap HP column (Amersham Biosciences) and was named PCIPG5. Polyhistidine-containing recombinant proteins of PCIPG5 (Invitrogen) were purified by the Ni-NTA Purification System (Sun et al., 2009). Product was analyzed by SDS-PAGE.

Site-directed mutagenesis of *Pcipg*5, expression and purification

Based on the alignment of all *pg* genes as previously described, three Asp residues (179D, 200D, and 201D) were presumed to form the putative active site in *Pcipg*5. In order to further investigate *Pcipg*5 function, active-site-directed mutagenesis was conducted. Singly mutated (179D→D179E, 200D→D200E, 201D→D201N) and simultaneous mutations (179D 200D 201D→D179E D200E D201N) were performed by overlap PCR. The primers used for mutation were A and B (Table 1), flanking the complete *Pcipg*5. The recombinant plasmid pPIC9K/*Pcipg*5 containing the full-length *Pcipg*5 was used as the template. The internal overlapping primers were 179-F and 179-R for 179D-D179E, 200-F and 200-R for 200D-D200E, 201-F and 201-R for 200D-D200N (Table 1), whereby the mismatched bases in each primer are underlined. To obtain the products AC and DB, PCR reaction systems and parameters were minor adjusted on the previously described (Horton and Pease, 1991). Overlap extension was performed using the flanking primers to amplify the recombinant products as well as the non-productive strands. The products of the overlap extension with a C-terminal His_6 tag were purified. The procedures for obtaining D200E and D201N were similar to that of D179E. As a result, three plasmids (pPIC9K/*Pcipg*5-179M, pPIC9K/*Pcipg*5-200M and pPIC9K/*Pcipg*5-201M) were obtained. After three Asp residues were successfully simultaneously mutated and sequenced, the plasmid pPIC9K/ *Pcipg*5-179-200-201M was obtained. Mutant proteins were purified as described above. The mutant proteins were designated PCIPG5/D179M, PCIPG5/D200M, PCIPG5/D201M and PCIPG5/ D179-200-201M, respectively. The products were collected and analyzed by SDS-PAGE.

The amino acid sequence of *Pcipg*5 reveals one potential consensus *N*-glycosylation site at N(252)STN. The mutant named N252Q was constructed with mutagenic primers (pcipg5-N252Q-F and pcipg5-N252Q-R (Table 1)) using the recombinant plasmid pPIC9K/*Pcipg*5 containing the full-length *Pcipg*5. The mutated protein was designated PCIPG5/N252M. The empty vector pPIC9K served as a negative control, and the wild protein served as a positive control. The product was analyzed by SDS-PAGE. The proteins were expressed and purified as described above. Activity of PCIPG5 and mutated proteins assay was performed according to previously described (Jia et al., 2009).

Treatment of pepper leaves with PCIPG5 and five mutated proteins

Seedlings of pepper were maintained the fifth to sixth-leaf stage. PCIPG5 and the supernatant solution of five mutated proteins were individually dialyzed twice at 4°C for 24 h against 50 volumes of distilled water to filter out ions. Quantitative analysis of each protein was carried out as described by Bradford (1976). To evaluate the impact of the different proteins on leaves, the seedlings were spot-inoculated against the top side of the leaf with 2.5 µl (0.7 µg/µl) of each protein solution using a microsyringe (Sun et al., 2008). Distilled water and boiled proteins were served as negative controls. Zoospore suspensions (1×10^5 /ml) were served as a positive control. Each sample was infiltrated in two leaves per plant and experiments were performed with at least twice with fresh

batches of enzymes. The development of necrotic lesions on the leaves was determined daily for 7 days. Following inoculation, seedlings were maintained at 100% humidity at 28°C for 7 days. The percentage of leaf area with lesions was determined using Image J software (Developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Each treatment was repeated at least three times. Data was analyzed by analysis of variance (ANOVA) and Fisher's least significant difference test ($P<0.05$) (Statview v. 5. 0.1: SAS Institute, Cary, NC, USA). In order to determine PGs activity in pepper leaves treated with different proteins, treated leaves were collected daily from 1 to 7 days, and crude protein was extracted. PGs activity was determined as described earlier.

Transmission electron microscopy

Small pieces of Kexing 3 pepper leaves at the fifth to sixth-leaf stage were collected at one day intervals from 1 to 7 days after treatment with PCIPG5, five mutated proteins, heat-killed protein and distilled water in accordance with Wu et al. (2006) and prepared for electron microscopy as follows. Leaf pieces were immersed 2 h at 4°C in glutaraldehyde (2%) in sodium cacodylate (0.05 M, pH 7.2), and postfixed with OsO_4 (1%) in sodium cacodylate overnight. After dehydration with a graded ethanol series of 30%, 50%, 70%, and 95%, the material was embedded in resin using plastic molds. Ultrathin sections (0.1 µm) from selected Epon blocks were cut on a Reichert-Jung microtome, collected on nickel grids, and stained with uranyl acetate (2% in 50% acetone) 20 min, followed by lead citrate for 15 min. Sections were observed using a Philips Electron Microscope 301 (Eindhoven, The Netherland) and JEOL 1010 Transmission Electron Microscope (Tokyo, Japan).

RESULTS

Isolation and analysis of *Pcipg*5 structure

One complete sequence of *Pcipg*5 was identified (GenBank No. EF558847). A multiple sequence alignment of all the *Pcipg* genes reveals that *Pcipg*5 has higher similarity (91.69%) with *Pcipg*3 (GenBank No. DQ415988) than with any other *Pcipg* genes in genome sequences of *P. capsici* from JGI, but none was identical to *Pcipg*5. The open reading frame of *Pcipg*5 contains 1086 bp and encodes a polypeptide of 362 amino acid residues with a predicted molecular mass of 37 kDa. *Pcipg*5 contains a signal peptide of 20-amino acid residues, and has only one potential *N*-glycosylation site (N252), but does not exhibit an intron (Figure 1).

On the basis of the alignment of all reported amino acid sequences of *pg* genes, the amino acid sequence of *Pcipg*5 contains five strictly conserved sequence segments (WYW, RTFS, NTDGF, NQDDCLAMQSS, RIKT). *Pcipg*5 also appears to have strong similarity with PGs from bacteria, fungi and plants, particularly as it has the same domains as other PGs being included into the glycoside hyfrolase (GH) family 28 (Markovic and Janecek, 2001). *Pcipg*5 also has three conserved residues (Asp179, Asp200, and Asp201) adjacent to the active-site region in most of the PGs (Armand et al., 2000; Pouderoyen et al., 2003).

RT-PCR and Northern blot analysis of *Pcipg*5 expression in diseased pepper leaves

Pepper leaves treated with zoospore suspensions exhibited increasing severity of lesion symptoms from 1 to 7 dpi. Small water-soaked symptoms appeared on the leaves at 1 dpi, followed by the appearance of small lesion spots at 2 dpi, and more visible lesions from 3 to 5 dpi. The necrotic lesions expanded gradually and eventually resulted in partial leaf maceration around the inoculation sites at 7 dpi. *Pcipg*5 expression levels were lower observed from 1 to 2 dpi, reached the peak at 3 dpi, and decreased slightly from 4 to 7 dpi, which were consistent with RT-PCR and Northern blot (Figure 2A, B). The absence of any amplified product in RT-PCR using RNA excluded any DNA contamination.

Western blot analysis of *Pcipg*5 expression in diseased pepper leaves

We used antibody developed against recombinant protein PCIPG5 to detect the crude proteins extracted from diseased leaves at one day intervals from 1 to 7dpi. A special protein band (40 KDa) was detected each day after inoculation. This specific product in the treated leaves gradually increased in amount and correlated with increased disease severity from 1 to 7 dpi (Figure 2C).

However, the corresponding band was present in the positive control but absent in the negative control, and no additional protein bands were detected in various samples.

Expression of *Pcipg*5 and five mutations, purification

The apparent molecular weight of PCIPG5 was ca 40 kDa detected by SDS-PAGE within 1 to 7 days after methanol induction (Figure 3A) which was above the predicted molecular weight of 37 kDa. The shift of the molecular weight of PCIPG5 may be caused by one *N*-glycosylation site which was corresponding to the molecular weight of *N*-glycosylation mutated protein PCIPG5/N252M (ca 35KDa). However, fusion proteins of PGIPG5 and five mutations were purified to homogeneity (Figure 3B). The PG-1 of tomato was a dimmer of PG-2 during tomato fruit ripening. Another protein was expressed with the molecular weight of about 60 kDa during the expression of PCIPG5. The protein may be a dimer that was a higher yield than *Pcipg*5 (Tucker et al., 1980). Five mutations in the sequence of *Pcipg*5 were shown in supplementary data (Figures 7 to 11).

The activity of PCIPG5 and five mutated proteins

The results show that the activity rates among various

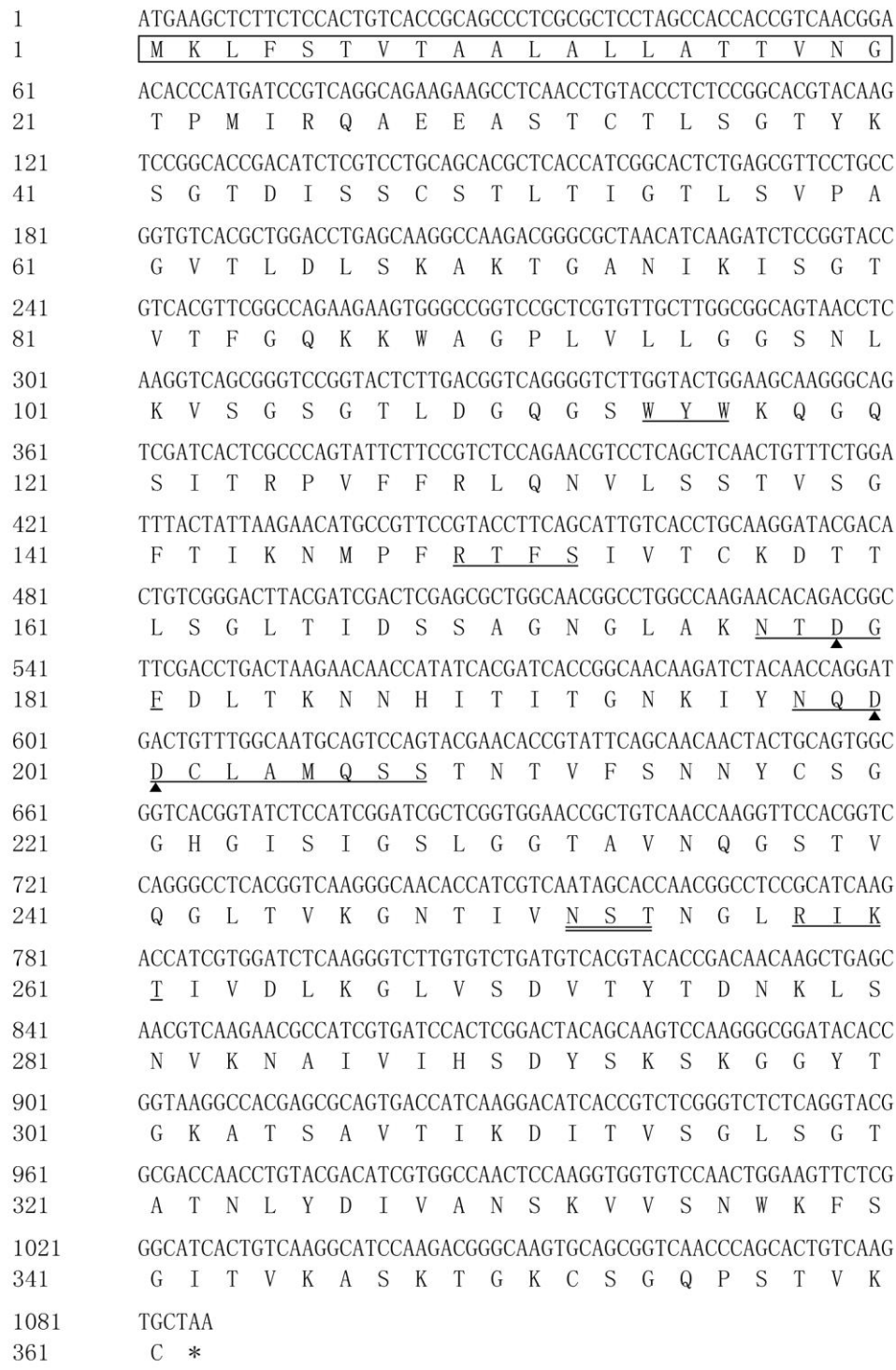

Figure 1. Deduction of the amino acid sequence of *Pcipg*5. Signal peptides represented in boxes. Three aspartic acid residues (Asp179, Asp200, and Asp201) regarded as the active-site components in PGs are indicated by arrowhead. The potential *N*-glycosylation site is double-underlined. Five strictly conserved sequence segments are underlined. Highly conserved domains in all reported PGs are underlined.

proteins were significantly different (P<0.05). The activity rate of PCIPG5 and PCIPG5/N252M was higher than that of four active-site-mutated proteins individually, and PCIPG5 activity levels were higher than that of PCIPG5/

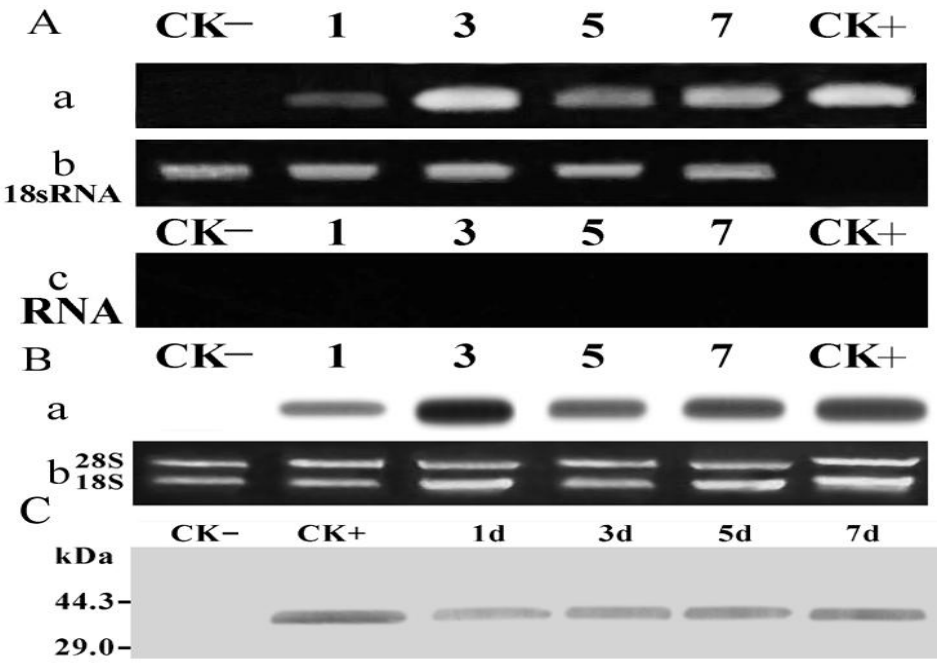

Figure 2. A. RT-PCR analysis expression of *Pcipg*5 after SD33 infection on pepper leaves. (a) CK-: cDNA from fresh pepper lanes, 1 to 7: cDNA from diseased leaves at one day intervals from 1 to 7 dpi, CK+: cDNA from SD33. (b) 18sRNA used as a loading control. (c) CK-: RNA from fresh pepper leaves, 1 to 7: RNA from diseased leaves at one day intervals from 1 to 7 dpi, CK+: RNA from SD33. M: molecular standard. The experiment was conducted three times with similar results. **B.** Northern blot analysis of *Pcipg*5 expression in infected leaves. (a) CK-: RNA of fresh pepper leaves, 1 to 7: RNA from infected leaves at one day intervals from 1 to 7 dpi, CK+: RNA of SD33. (b) 28S rRNA and 18S rRNA are also shown (lower panel). **C.** Western blot analysis of PCIPG5 expression level during *P. capsici* SD33 infected pepper leaves using antiserum. CK-: uninoculated plants (empty vector), CK+: PCIPG5 purification, 1 to 7d: Expression level of PCIPG5 at one day intervals from 1 to 7 dpi.

N252M. The activity level of PCIPG5 was affected by digesting *N*-glycosylation site, which the activity level PCIPG5/N252M was decreased 20% compared with PCIPG5 ($P<0.05$) (Figure 4A). And the relative activity rates of the four mutated proteins were varied from 0.12 to 0.15, and there was no significant difference among them ($P>0.05$) (data not shown). One unit of enzymatic activity was expressed by 1.0 nmoles of galacturonic acid produced×mg of /protein/h. The data on PG activity levels among various proteins was analyzed by analysis of variance (ANOVA) and Fisher's least significant difference test ($P<0.05$) (Statview v. 5. 0.1: SAS Institute, Cary, NC, USA).

Development of disease symptoms treated with PCIPG5 and five mutated proteins

The presence of lesions in the positive control leaves (inoculated with zoospore suspensions) was regarded as a typical disease symptom. Disease assessment was conducted from 1 to 7 dpi. In contrast to the typical blight symptoms developed, the necrotic lesions developed slowly in leaves treated with PCIPG5. Visible symptoms did not appear until 5 dpi, but did not show typical of *Phytophthora* blight symptoms as observed in the positive control. The mean lesion area in leaves treated with PCIPG5 and PCIPG5/N252M was 1.31 cm^2 and 1.04 cm^2 respectively, distinctly smaller than those of the positive control (2.23 cm^2) in treated leaves (Figure 5a). The mean lesion area in the positive control was significantly different from that of treated with PCIPG5 or PCIPG5/N252M ($P<0.05$), but the mean lesion area in leaves treated with PCIPG5 was close to that of PCIPG5/N252M treated leaves ($P>0.05$) (data not shown). However, leaves exhibited no necrotic lesions in leaves treated with four active site mutated proteins and negative controls, respectively (Figure 5b).

PGs activity in pepper leaves treated with PCIPG5 and five mutated proteins

PGs activity was low in leaves treated with PCIPG5 or PCIPG5/N252M at 1 dpi and then began to increase at 2 dpi, which was consistent with the slight symptoms

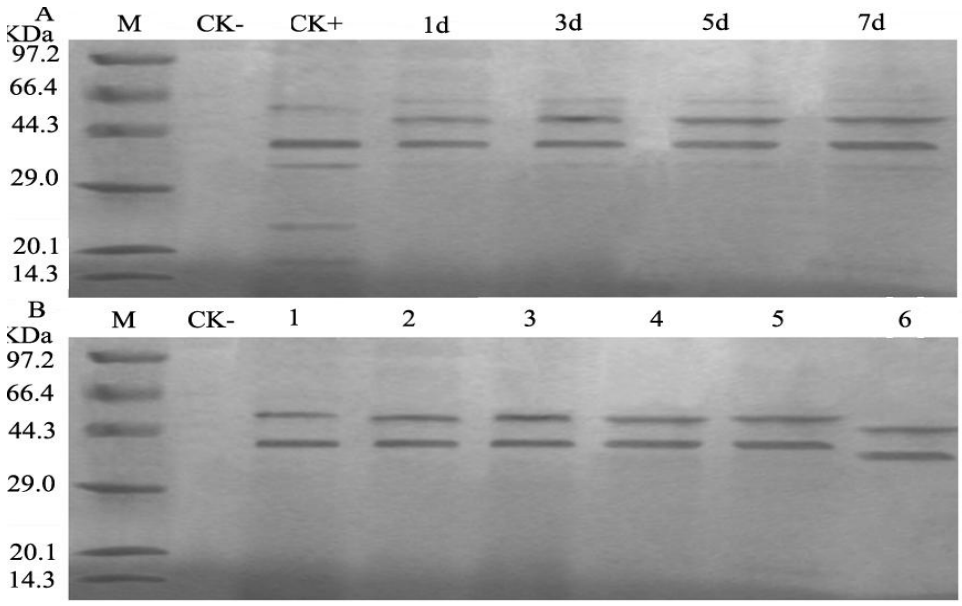

Figure 3. A. SDS-PAGE analysis of recombinant protein expression in *Pi*. *Pastoris* GS115. CK-: pPIC9k (empty vector), CK+: zoospore suspension expression after induction, 1 to 7d: recombinant fusion protein expression from 1 to 7d after induction. M: Low molecular weight marker. B. SDS-PAGE analysis of purified fusion protein. Lane 1: PCIPG5 purification, lane 2: PCIPG5/D179M purification, lane 3: PCIPG5/D200M purification, lane 4: PCIPG5/D201M purification, lane 5: PCIPG5/D179-200-201M purification, lane 6: PCIPG5/N252M purification. M: marker. CK-: pPIC9k (empty vector).

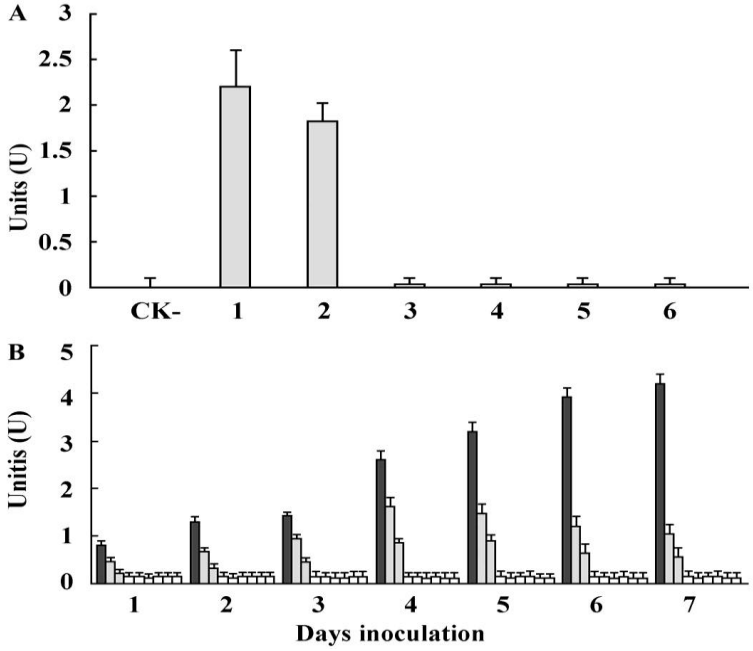

Figure 4. A. Purified fusion protein activity analysis. CK-: wild protein heat inactivation, 1: PCIPG5, 2: PCIPG5/N252M 3: PCIPG5/D179M, 4: PCIPG5/D200M, 5: PCIPG5/D201M, 6: PCIPG5/D179-200-201M. **B.** PG activity trends in crude protein extracted from pepper leaves infiltrated with single constructs or combinations from 1 to 7 days. Each column group represents zoospore suspensions (positive control), PCIPG5, PCIPG5/N252M, PCIPG5/D179M, PCIPG5/D200M, PCIPG5/D201M, PCIPG5/D179-200-201M, heat-killed wild protein (negative control), and distilled water (negative control), respectively.

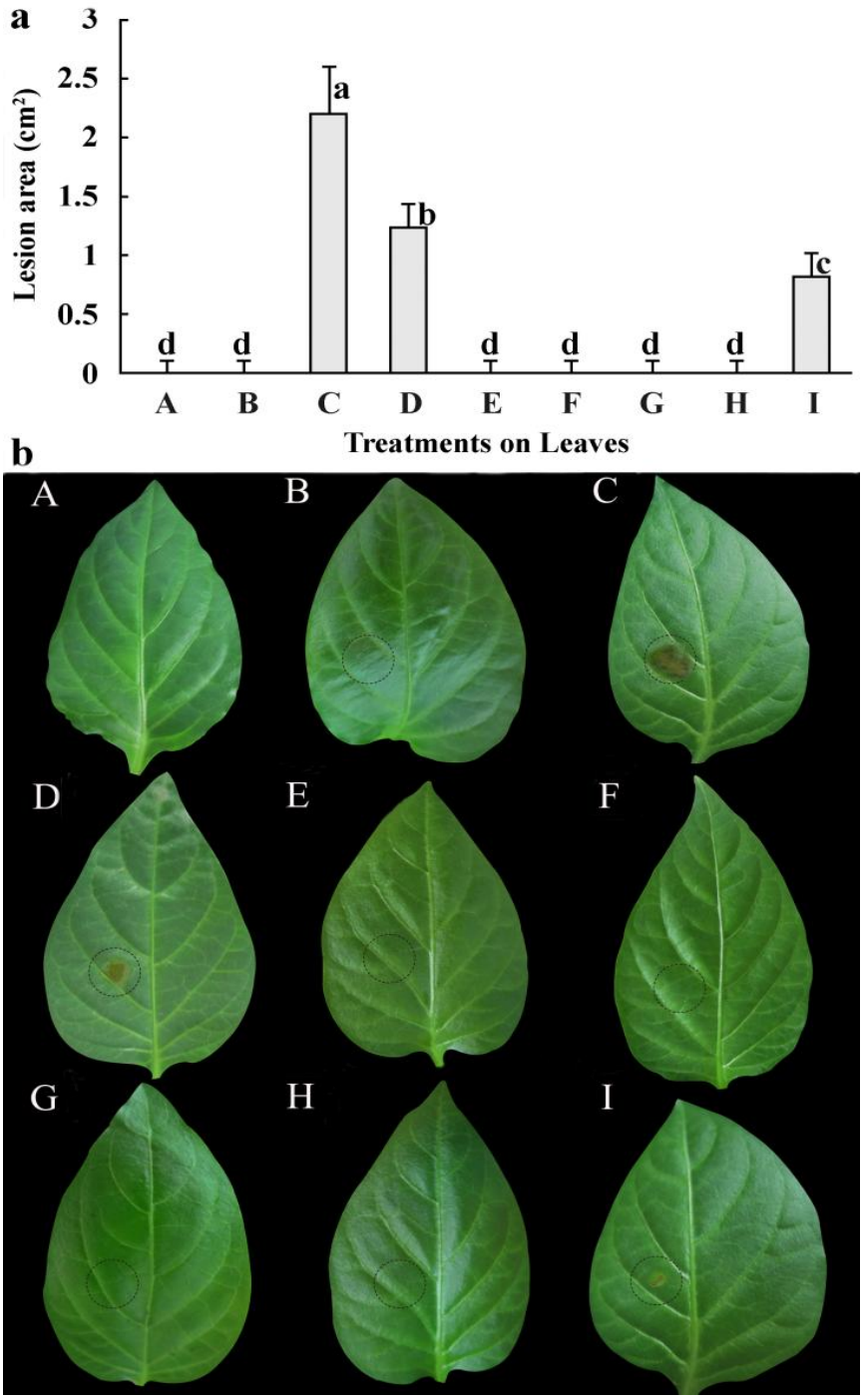

Figure 5. a. Bars represent the mean ± standard error of 14 leaves. **b.** Symptoms on *Capsicum annuum* L. leaves following inoculation with PCIPG5, five mutated proteins, zoospore suspensions (positive control), heat-killed wild protein and distilled water (negative control). Zones with lesions are marked with a dotted black circle. Pictures were taken five days after treatment. The letters A to I are distilled water, heat-killed wild protein, zoospore suspensions, PCIPG5, PCIPG5/D179M, PCIPG5/D200M, PCIPG5/D201M, PCIPG5/D179-200-201M, PCIPG5/N252M, and respectively.

(water-soaked regions) appearing on the leaves treated with PCIPG5 and PCIPG5/N252M, respectively. PGs activity then gradually increased and eventually showed definite activity peaks at 4 dpi. Moreover, the necrotic lesion severity in PCIPG5 or PCIPG5/N252M treated leaves gradually increased in parallel with PGs activity

trends from 1 to 7 dpi. In addition, PGs activity in the positive control was higher than that of PCIPG5 or PCIPG5/N252M treated leaves. PGs activity was very low in treated leaves with four active-site mutated proteins, heat-killed proteins and distilled water, which was consistent with the absence of symptoms in treated leaves. Moreover, PGs activity in PCIPG5/N252M treated leaves was somewhat low compared to that of in PCIPG5 treated leaves (Figure 4B).

The virulence determination of PCIPG5 and five mutation proteins

Ultrastructure of the cell walls from pepper leaves was impaired by PCIPG5, PCIPG5/N252M, and five mutated proteins. The cell walls of pepper leaves were slightly degraded and their plasma membrane was ruptured on the first day after treatment (dat) with PCIPG5 and PCIPG5/N252M, respectively. The extent of degradation was gradually increased from 1 to 3 dpi, and then became macerated enough to collapse the intercellular spaces on the surface of the leaves. The cell walls were disrupted at 5 dpi, and gradually deteriorated up to 7 dpi, finally collapsing and rupturing completely (Figure 6C and H). The cell walls were not affected by treatment with active-site mutated proteins. The protoplast, plasma membrane, and chloroplast were found intact without visible injury (Figure 6D to G). These results were similar to those of the negative control leaves (Figure 6A). Thus, the symptoms were recorded by electron micrograph at 7 dat.

DISCUSSION

Today, more and more evidences display that PGs degraded pectins in the plant cell wall during *Phytophthora* spp. pathogens infection of plants (Torto et al., 2002; Yan and Liou, 2005; Wu et al., 2008; Sun et al., 2009). Like *Phytophthora* spp. and many other plant pathogens, PGs gene family is greatly expanded in *P. capsici*. But the detailed expression about endoPGs genes of *P. capsici* impacting on pepper or other hosts was limited except that of *Pcipg*2 reported previously by our laboratory (Sun et al., 2009). In this study, we further characterized the expression patterns and function of *Pcipg*5 in terms of *P. capsici* infection of pepper.

In the study, results showed that *Pcipg*5 exhibited similar expression trends at various dpi in RT-PCR, Northern blot and Western blot analysis (Figure 2). *Pcipg*5 transcription levels displayed as an increased trend from 1 dpi to 3 dpi by RT-PCR and Northern blot. Furthermore, Western blot suggested that PCIPG5 was secreted during *P. capsici* infection, and that *Pcipg*5 might be involved in triggering pathogenesis-related response as the development of disease symptoms. Meanwhile, we speculated that other *Pcipg* genes in this family might also secrete pathogenesis-related related proteins during *P. capsici* infection. The roles of other *Pcipg* genes in the host-pathogen interaction remain an important target for further research.

In fact, PGs are a series isoenzymes secreted by *P. capsici* during infection on pepper. This may be the actual reason why PGs activity levels in the positive control was always higher than that in the PCIPG5 treatment leaves (Figure 4B). However, the PGs activity trend was always correlated with necrotic lesions development in treatment leaves. Conversely, PGs activity was considerably lower in four mutated proteins treatment leaves and corresponding to no necrotic lesions produced (Figures 4B, 5B6–9). Similar results were obtained from the mutations of *Pcipg*2 (Sun et al., 2009). Hence, *Pcipg*5 plays a role in pathogenicity as well as *Pcipg*2 in this gene family, and each of three conserved residues (Asp179, Asp200, and Asp201) in active site is involved in the activity of PCIPG5. Taken together, it is reasonable to expect that these three Asp residues might be main components in active site in most of the PGs of *Phytophthora* spp. These results provide insight into the role of *pg* genes from other straminopilous pathogens based on mutation of conserved Asp residues in the active site.

The degradation degree of plant cell walls was sufficient to cause cell wall degradation which finally led to obvious lesions on pepper leaves (Figures 5B4 and 6C). The functions of PCIPG5 were similar to PCIPG2 encoded by *Pcipg*2 (Sun et al., 2009). On the other hand, the correlation of PCIPG5 activity with the extent of damage to the pepper cell walls suggested that PCIPG5 might be a pathogenicity factor during *P. capsici* infection of pepper plants, and further implied that *Pcipg*5 might be a gene with distinct functions for virulence in *Pcipg* multigene family in *P. capsici*.

The present study confirms that the PCIPG5 secreted by *P. capsici* supports degradation of the plant cell walls, as observed by TEM observations after treatment with PCIPG5. The function of a single gene in large multigene family might be complemented by other genes producing enzymes that exhibit similar activities (Wu et al., 1997). Some studies have found that the colonization hosts were associated with a series of CWDEs. Thus, PCIPG5 might help *P. capsici* colonization on the surfaces of pepper hosts, penetrate of the cell wall, and infect pepper leaves.

Various *N*-linked glycosylation structures are found in the *Pcipg* gene family through the *N*-linked glycosylation site prediction by the software of NetNGlyc 1.0 Server. *Pcipg*2 contains three *N*-glycosylation sites whereas *Pcipg*5 has only one potential *N*-glycosylation site. *N*-linked glycosylation does not occur at every potential site and the role played by glycosylation in different proteins is highly variable and depends on the individual protein (Escrevente et al., 2008). The amino acid sequence of *Pcipg*5 with *Pcipg*2 has 80.39% identity, and their nucleotide sequences have 78.79% identity. It is interesting

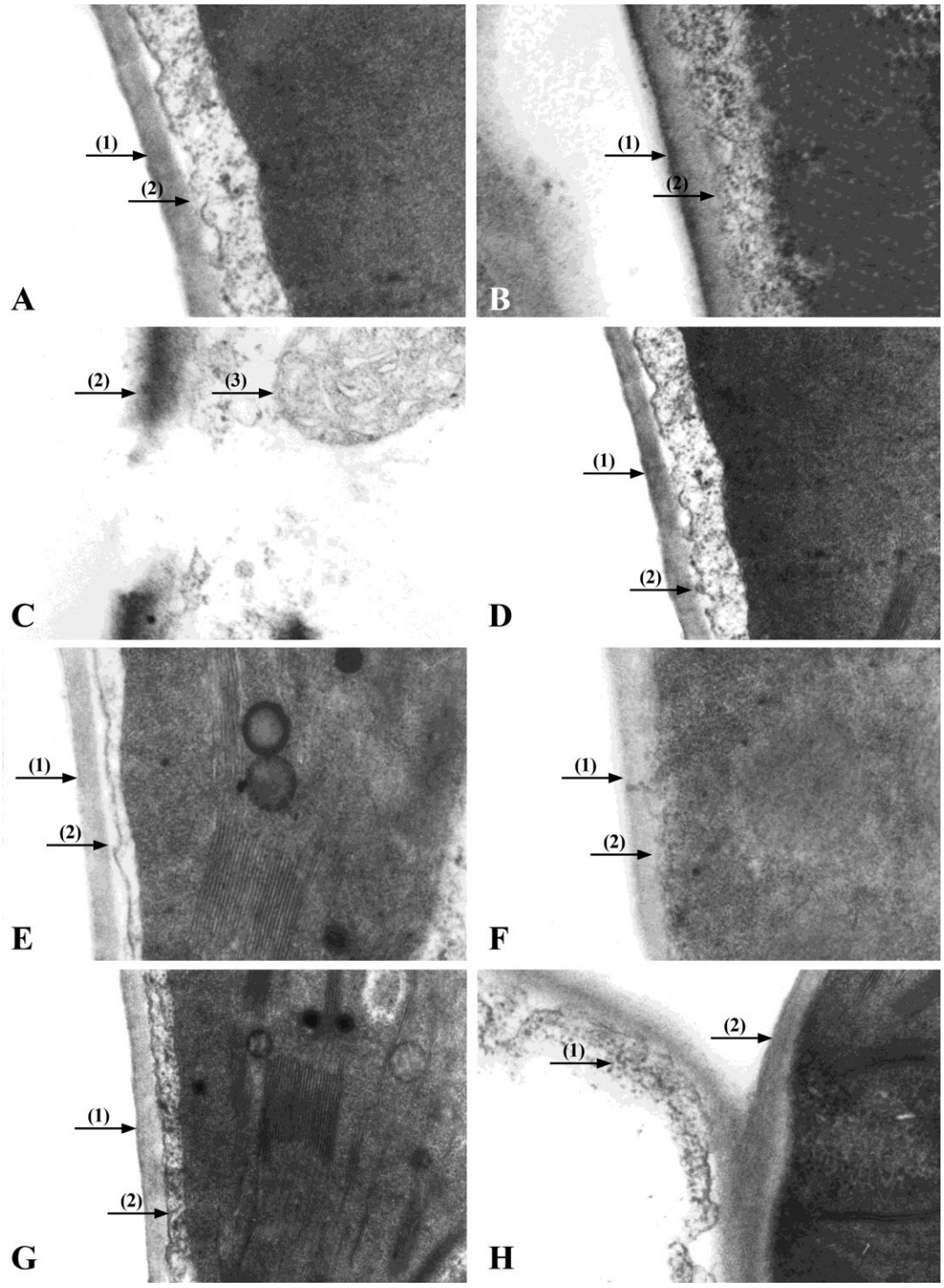

Figure 6. Electron micrographs showing dissolution of leaf cell walls after separate treatment at 7 dpi. A: distilled water (negative control), B: heat-killed protein, C: PCIPG5, D: PCIPG5/D179M, E: PCIPG5/D200M, F: PCIPG5/D201M, G: PCIPG5/D179-200-201M, H: PCIPG5/N252M. (1) Cell walls, (2) plasma membrane, (3) chloroplast membrane. Each experiment was repeated at least twice.

that these two genes have different *N*-glycosylation sites even though they are highly homologous.

In all eukaryotes, *N*-glycosylation is necessary for viability (Zhao et al., 2008). The wild-type NTPDase3 as one of family enzymes, which was glycosylated at N81, was susceptible to PNGase-F inactivation and underwent

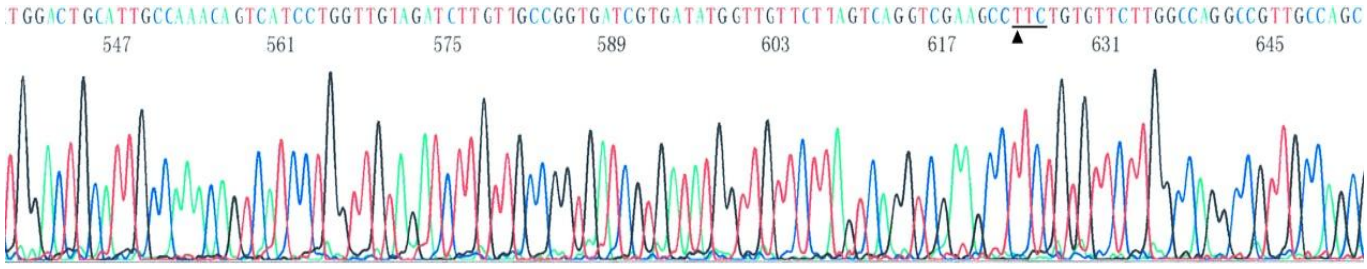

Figure 7. The mutation of 179D→D179E, the C was replaced by A at site 623, indicated by an arrowhead.

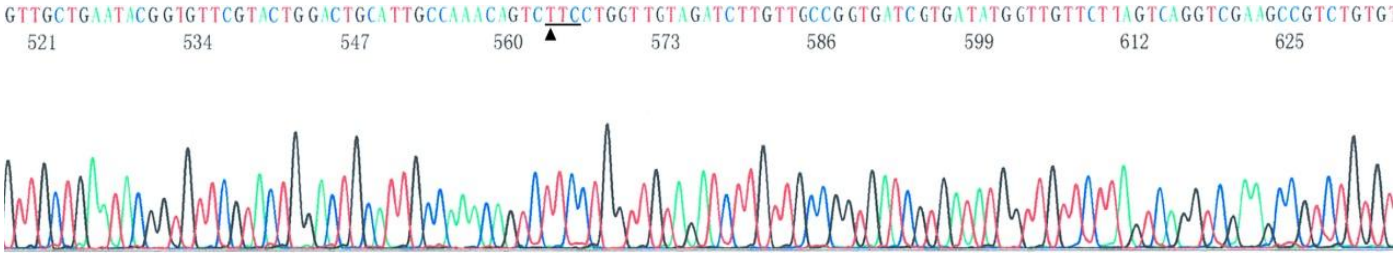

Figure 8. The mutation of 200D→D200E, the T was replaced by A at site 563, indicated by an arrowhead.

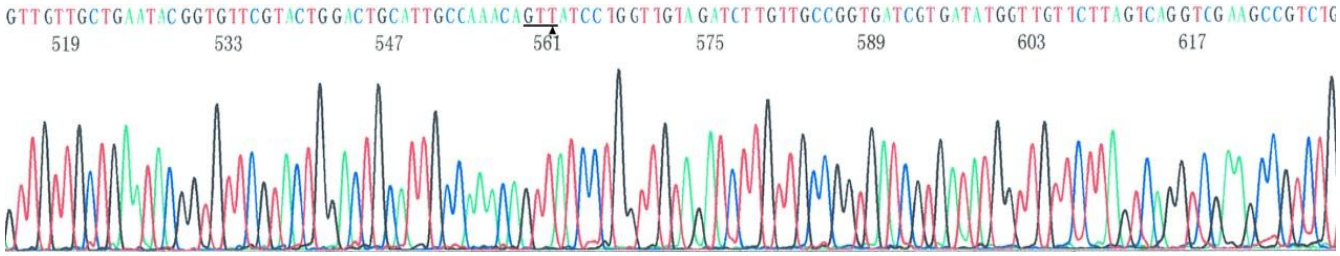

Figure 9. The mutation of 201D→D201N, the G was replaced by A at site 561, indicated by an arrowhead.

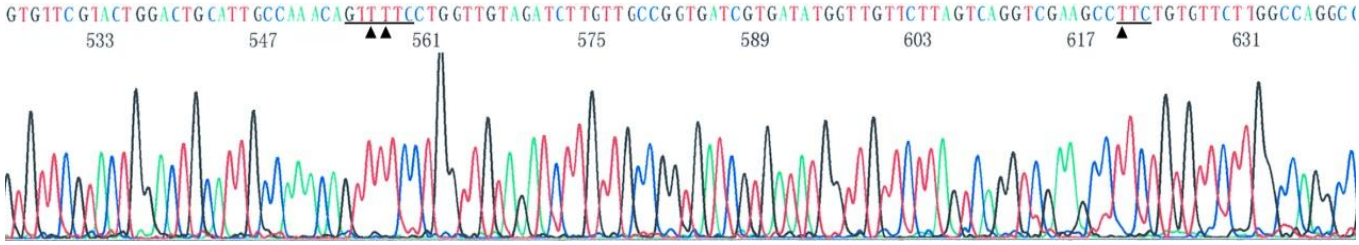

Figure 10. The mutation of 179D 200D 201D→D179E D200E D201N. The C was replaced by A at site 620, the T was replaced by A at site 557, and G was replaced by A at site 556. Each mutation is indicated by an arrowhead.

a 40% decrease in ATPase activity and a 30% decrease in ADPase activity after PNGase-F treatment (Murphy and Kirley, 2003). Deglycosylated PGs of *Aspergillus* spp. and *P. parasitica* which decreased completely inactive (Stratilová et al., 1998; Yan and Liou, 2005). In the study, the *Pcipg*5 had only one potential *N*-glycosylation site. PCIPG5/N252M activity was somewhat low compared with that of PCIPG5. This result indicates that the existence *N*-glycosylation site in PCIPG5 plays a partial role in the activity of this enzyme. From the structure-function point of view, in order to facilitate a protein function, the *N*-linked sugar chains have to keep some distance from the functional regions in the three-dimensional structure to avoid steric hindrance and minimize a disadvantageous obstruction against the domain movements (Yan et al., 1999). The position of

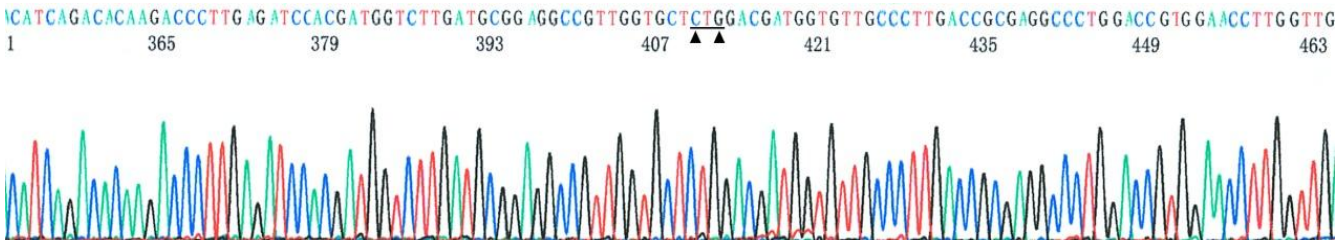

Figure 11. The mutation of 252N→N252Q, the T was replaced by G at site 410, and the A was replaced by C at site 412, indicated by an arrowhead.

one *N*-glycosylation site (N252) is not adjacent to strictly conserved residues at the potential active site at the gene level. Thus, it is possible that *N*-glycosylation portion of PCIPG5 is not directly involved in the catalytic functions of the active site.

It is unknown whether gene-for-gene interactions exist in *P. capsici*-pepper/other hosts systems, thus we should further explore the potential function of *Pcipg*5 as a virulence factor. In addition, our findings suggest that *Pcipg* gene family members might express and secrete virulence proteins during infection of pepper plants. The roles of these genes in host-pathogen interactions are important targets for further research. The future research could clarify the functions of *Pcipg*5 or other *Pg* genes in this gene family using RNA silencing or insertional mutagenesis and analyze various function of *N*-glycosylation in the multigene family by *N*-glycosylation mutation and other methods.

ACKNOWLEDGEMENTS

The authors thank P. W. Tooley for critically reading the manuscript and language corrections. This research was supported by 863 Program of the Ministry of Science and Technology of China (2006AA02Z198), National Basic Research and Development Program 973 of China (2009CB119000).

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