Immunological Detection of *endo*Polygalacturonase Secretion by *Fusarium oxysporum* in Plant Tissue and Sequencing of Its Encoding Gene*

Tsutomu ARIE**, Satyanarayana GOUTHU**, Satoshi SHIMAZAKI***,
Takashi KAMAKURA**, Makoto KIMURA**, Mitsuko INOUE†,
Koji TAKIO**, Akira OZAKI**, Katsuyoshi YONEYAMA†
and Isamu YAMAGUCHI**

Abstract

Fusarium oxysporum, a soilborne phytopathogenic fungus, produces *endopolygalacturonase* (PG) which has been suggested to be related to disease development. A polyclonal antibody (PAb) APG1 was prepared against purified PG from *F. oxysporum* f. sp. *lycopersici* race 2. By direct tissue-blotted immunobinding assay (DT-IBA) with the antibody, PG production by the pathogen in the stem of the host plant was confirmed. Degenerate primers were designed based on partial amino acid residue information for the PG protein from the fungus: part of the PG-encoding gene was obtained by PCR. Using TAIL-PCR, the complete gene encoding PG was cloned and sequenced. The structural gene comprises 1318 bp coding for 371 amino acids with a putative signal peptide of 22 amino acids, and the open reading frame is interrupted by four introns of 47, 51, 50, and 54 bp. The deduced amino acid sequence of the mature protein showed 82.9, 29.0, 27.6, 14.2, and 26.9% homology with those of *F. moniliforme* PG, *Cochliobolus carbonum* PGNI, *Aspergillus niger* PG, *A. oryzae* PG, and *Sclerotinia sclerotiorum* PGI, respectively.

(Received June 12, 1997 ; Accepted October 16, 1997)

Key words : antibody, DT-IBA, pectin degradation, *Fusarium oxysporum* f. sp. *lycopersici*, *endopolygalacturonase* (PG), nucleotide sequence.

INTRODUCTION

Fusarium oxysporum Schlechtend.: Fr. is one of the most economically important soilborne pathogenic fungi in both temperate and tropical regions. The failure to control the disease despite the use of many conventional methods such as chemical fungicides, biocontrol agents, soil amendments, and resistant cultivars could be due to the poor understanding of the mechanism of disease development and the physiology and biochemistry of the pathogen.

Three stages have been proposed in *Fusarium* disease development⁴⁾. First, the pathogen invades roots of host plants. Second, the mycelia elongates through host cells to reach the stem vessels. During these two stages, cellular degradative enzymes are excreted⁴⁾. The third stage is the appearance of symptoms on host plants.

Some enzymes that digest vessel cell walls and/or toxins that kill cells have been reported to play important roles during the third stage⁴⁾.

The pectinolytic *endopolygalacturonases* (PGs, poly [1,4- α -D-galacturonide] glycanohydrolase, EC 3.2.1.15) have been reported as pathogenesis-related proteins which degrade plant cell wall structure, including vessel cell walls. Many species of plant pathogenic fungi and bacteria utilize the enzyme(s) in their offensive arsenal to penetrate and colonize plant tissues during infection¹²⁾.

Enzymatic analysis has been the usual strategy to study the taxonomy and evolution of pathogenic types such as formae speciales (ff. sp.) or races. However, isoenzymatic analysis of *F. oxysporum* has thus far afforded no clear relationship between isozyme polymorphism and pathogenic forms. We analyzed PG by isoelectric focusing (IEF) and found no differences

* This study was supported in part by a Grant for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan and a grant for the "Biodesign Research Group" from The Institute of Physical and Chemical Research (RIKEN).

** The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako 351-0198, Japan 理化学研究所

*** Present address : Kyowa Seed Co., 377 Ozawa, Chonan, Chosei-gun, Chiba 297-0142, Japan 現在 : 協和種苗 (株)

† Faculty of Agriculture, Meiji University, 1-1-1 Higashi-Mita, Tama, Kawasaki 214-0033, Japan 明治大学農学部

between ff. sp. *lycopersici* (Sacc.) W.C. Snyder & Hans. and *cucumerinum* Owen in PG isozyme patterns³⁾.

Recently, a PG protein was purified from *F. oxysporum* f. sp. *lycopersici*. Its isoelectric focusing showed at least 4 isoenzymes²⁰⁾. Di Pietro and Roncero (1996) also reported four forms of the protein, PG1 and three *N*-glycosylated isoforms⁶⁾. According to them, *N*-glycosylation was essential for the secretion of PG. Further, they detected PG1 protein in the stems of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* whereas a pectate lyase was observed in roots during early stages of infection.

PG genes have been recently cloned and characterized from several species of fungi, including *Cochliobolus carbonum* Nelson²²⁾, *Aspergillus niger* van Tieghem^{5,6,21)}, *A. oryzae* (Ahlburg) Cohn¹⁰⁾, *A. flavus* Link ex Fries²²⁾, *F. moniliforme* Sheld.⁵⁾, and *Sclerotinia sclerotiorum* (Lib.) de Bary in Vergh¹⁷⁾.

DNA sequence data of PG gene of *F. oxysporum* could clarify the importance of the gene as a symptom development-related factor and its role in the determination of host and/or symptom induction.

This paper deals with production of an antibody against a PG protein of *F. oxysporum* f. sp. *lycopersici*, detection of PG production in plant tissues by DT-IBA, partial amino acid sequencing of the protein, sequencing of the complete gene, and comparison of the amino acid sequences.

MATERIALS AND METHODS

Fungal isolates and culture conditions *Fusarium oxysporum* f. sp. *lycopersici* race 2, 880621a-1 isolated in Imaichi, Tochigi was maintained on potato dextrose agar (PDA) medium.

To purify the endopolygalacturonase (PG) protein, an inducing medium made of 0.09% (w/v) $\text{NH}_4\text{H}_2\text{PO}_4$, 0.2% (w/v) $(\text{NH}_4)_2\text{HPO}_4$, 0.047% (w/v) MgSO_4 , 0.05% (w/v) KCl, and 1.0% (w/v) pectin (from citrus fruit, Sigma Chemical Co., St. Louis, MO, USA) was used²¹⁾ to culture *F. oxysporum* 880621a-1 at 25°C for 40 days.

PG purification and determination of PG activity PG was purified from the culture filtrate of *F. oxysporum* 880621a-1 grown on the inducing medium in three steps, as follows. Three liters of culture suspension was filtered through Toyo No. 2 paper to remove fungal matter. The filtrate was 40% saturated with ammonium sulfate and centrifuged at $10,000 \times g$ for 15 min to remove the precipitate. The supernatant was then 90% saturated with ammonium sulfate and centrifuged to collect the precipitate containing the PG protein. The precipitate was suspended in 0.1 M sodium acetate buffer (AcB, pH 4.0) and subjected to gel filtration on Sephadex G-25 Coarse (Pharmacia Biotech, Uppsala, Sweden) followed by ion exchange on SP Sephadex C-50 (Pharmacia Biotech). The active fraction was then concentrated by ultra filtration with Centriprep-3 (Amicon, Beverly, MA, USA) and gel filtrated again with

Sephadex G-75 (Pharmacia Biotech). All steps in the chromatography procedure were performed at room temperature. Protein concentrations were measured using Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol.

PG activity was monitored by measuring the release rate of reducing sugar in enzyme-substrate reaction mixture using a modified DNS test¹²⁾. Five-hundred μl of each fraction was mixed with 50 μl of the substrate solution, 0.5% (w/v) polygalacturonic acid in AcB. After 30 min incubation at 25°C, 0.2 μl of 6.85 M potassium ferricyanide was added, and the mixture was immediately put in a 95°C water bath for 30 min. The absorbance (600 nm/405 nm) was determined with a microtiter plate reader. The mixture without incubation at 25°C was used as a negative control.

Preparation of antibody against the purified PG

Polyclonal antibody against purified PG was raised in mouse following the usual method. One hundred μl of purified PG (320 μg protein/ml) was mixed with Freund's complete adjuvant or Freund's incomplete adjuvant (50% v/v) and injected to immunize female mice (BALB/c) intraperitoneally five times at 2-week intervals. Fourteen days after the final injection, 100 μl of purified PG was injected intravenously for boosting. Five days later, orbital blood samples were collected and centrifuged ($10,000 \times g$, 10 min) to get antisera. Western blotting analysis was performed to confirm the production of an antibody against PG. An SDS-PAGE gel with the PG sample was transblotted onto a PVDF membrane (BioRad) by semi-dry blotting electrophoresis (2 mA/cm², 45 min) following the manufacturer's protocol. The blots were recovered and treated with the antibody, secondary antibody (a mixture of biotinylated anti-mouse IgM-goat IgG and horseradish peroxidase avidin D), and the substrate solution (2.8 mM 4-chloro-1-naphthol in 0.02% hydrogen peroxide) for immunobinding assay²⁾.

DT-IBA Direct tissue-blotted immunobinding assay (DT-IBA)²⁾ was used to examine whether PG was produced in the stem tissues of tomato plants infected with *F. oxysporum* 880621a-1. From 40-day-old tomato plants (cv. Momotaro, Takii Seeds, Kyoto) with and without inoculation, vertical sections were prepared from stems of ca. 1 cm above the soil surface and put directly on a membrane saturated with Tris buffered saline (pH 7.0) and incubated for 15 min at room temperature. The membrane was recovered and treated with the PG-antibody as in the immunobinding assay procedure.

Partial amino acid sequencing Purified PG was reduced and S-carboxymethylated according to Hirs (1967)⁹⁾ and digested with *Achromobacter* Protease I provided by Dr. T. Masaki, Ibaraki Univ., Ami, Ibaraki¹⁴⁾. Peptides thus obtained were separated by RP-HPLC on a column of Superspher RP-Select B (2 $\times$ 125 mm, E. Merck, Darmstadt, Germany) using a linear

gradient of acetonitrile (0–36% in 24 min) in 0.1% TFA at a flow rate of 0.2 ml/min with model 1090M liquid chromatography (Hewlett-Packard Co., Waldbronn, Germany). Methionyl bonds in intact protein were cleaved with cyanogen bromide in 70% formic acid⁸⁾ and subjected to gel permeation chromatography on SynChropak GPC-100, -300, and -500 columns (4.6×250 mm each, MICRA Scientific, Lafayette, IN, USA) connected in series. The columns were pre-equilibrated with 10 mM phosphate (pH 6) containing 6 M guanidine hydrochloride and eluted at a flow rate of 0.17 ml/min. Isolated peptides were applied for automated Edman degradation with model 477A Protein Sequencer connected on-line to model 120A PTH Analyzer (PE Applied Biosystems, Foster City, CA, USA). The intact protein was also analyzed to obtain the amino (N)-terminal sequence.

Fungal genomic DNA isolation and manipulation

Fungal genomic DNA (gDNA) of *F. oxysporum* 880621a-1 was purified by a miniprep procedure²³⁾. DNA gel blots on positively charged Nylon membrane (Boehringer Mannheim Biochemica, Mannheim, Germany) were prepared by alkaline transblotting, hybridized with a probe prepared using DIG DNA Labeling and Detection Kit Nonradioactive (Boehringer), and detected using the same kit.

Amplification of PG gene by PCR and Thermal Asymmetric Interlaced (TAIL)-PCR Degenerate primers (Table 1) for PCR were designed based on the amino acid sequence data. Primers were dissolved (100 μ M) in water and stored at -20°C . Each PCR reaction mixture contained about 20 ng of gDNA of *F. oxysporum* 880621a-1 in 50 μ l reaction buffer [1×PCR Buffer II (PE Applied Biosystems), 0.2 mM dNTPs, 2.5 mM MgCl_2 , 2 μ M of each primer, and 0.025 U AmpliTaq Gold (PE Applied Biosystems)]. Denaturation at 95°C for 12 min was followed by 35 cycles of 94°C for 1 min, 55°C for 30 sec, and 72°C for 1 min. Reactions were held at 4°C after a final extension at 72°C for 10 min. For analysis of PCR

products, 10 μ l of each reaction mixture was subjected to agarose gel (1.2%) electrophoresis in TAE buffer.

A schematic explanation of TAIL-PCR is shown in Fig. 1. Arbitrary degenerate (AD) primers (Table 1) were prepared according to Liu and Whittier (1995)¹³⁾ and Arie *et al.* (1997)¹⁾. Primary TAIL-PCR¹³⁾ reaction mixtures (final volume 25 μ l) contained 1×PCR Buffer II, 2.5 mM MgCl_2 , 0.2 mM each dNTPs, 0.4 μ M specific primer, 5 μ M AD primer (Table 1), 0.016 U AmpliTaq (PE Applied Biosystems), and approximately 20 ng genomic DNA of *F. oxysporum* 880621a-1. The secondary amplification reactions (final volume 50 μ l) contained the same components as the primary reactions except that 0.2 μ M of a nested specific primer (Table 1), 4 μ M of the same AD primer as used in the primary reaction and 0.012 U AmpliTaq were used. The template was 1 μ l of a 1 : 50 dilution of the primary reaction mixture. Thermal conditions for TAIL-PCR were described by Liu and Whittier (1995)¹³⁾. For analysis of TAIL-PCR products, 10 μ l of each reaction was subjected to agarose gel (1.2%) electrophoresis in TAE buffer.

Cloning, sequencing, and analysis of PCR products PCR products were cloned into vector pCR2.1 following recommendations of the supplier (Invitrogen Co., San Diego, CA). TAIL-PCR products were also cloned into pCR2.1 vector directly from the reaction. Clones were selected by colony hybridization using the cloned partial PG gene (amplified with degenerate primers) as a probe. Clones were purified on QIAGEN-Tip 100 columns (Qiagen Inc., Chatsworth, CA, USA) following the manufacturer's recommendation, and the inserts were sequenced using the M13 Reverse or M13 (-40) Forward primers. DNA sequences were determined using Cy5 AutoRead Sequencing Kit (Pharmacia Biotech) and ALFexpress DNA sequencer (Pharmacia Biotech). Sequences were assembled and analyzed using the GeneWorks program (IntelliGenetics, Inc., Mountain View, CA, USA) and Genetyx-Max program (Software

Table 1. Primers used for PCR

Name	Nucleotide sequence ^{a)}	Corresponding genomic location
PG1	5'-GAYAAAYGAYTTYRAYCCBATYG-3'	bp 336-357, degenerate primer designed based on amino acid sequence
PG2	5'-CANGTRTTNGTVGGRTARTTRC-3'	bp 1308-1287, degenerate primer
PG3	5'-AATGCCAAGAGCGGTAGCTTG-3'	bp 674-695 at the nested distal 5' end of the fragment amplified with PG1+PG2
PG4	5'-CTCACTGTTAACAATCTGAG-3'	bp 931-912 at the nested distal 3' end of the fragment amplified with PG1+PG2
PG5	5'-ACAGTGAGAAATGGATGCC-3'	bp 924-941 at the nested distal 5' end of the fragment amplified with PG1+PG2
PG6	5'-GTGAACTGCCTGTGATGTCG-3'	bp 623-604 at the nested distal 3' end of the fragment amplified with PG1+PG2
N1	5'-TGYTGYWSNCARTTYGG-3'	AD primer for TAIL-PCR
TP1	5'-NGTCGASWGANAWGAA-3'	AD primer for TAIL-PCR

a) Abbreviations: Y, C and T; R, A and G; W, A and T; S, G and C; B, C, G, and T; V, A, C, and G; N, A, C, G, and T.

Fig. 1. Recovery of sequences of *endopolygalacturonase* (PG) by PCR and TAIL-PCR. (a) PCR with genomic DNA and primers PG1+2 (Table 1) to get the 973 bp fragment of PG-encoding gene. (b) TAIL-PCR with genomic DNA and primers PG3+TP1 (Table 1) generated a product that was used as a template in a second reaction with primer TP1 and nested primer PG5 (Table 1) to yield a 3' fragment of 489 bp. (c) To obtain a sequence on the opposite side of the 973 bp fragment, TAIL-PCR was done with genomic DNA and primers PG4+N1 (Table 1), which generated a product that was used as template in a second reaction with N1 and nested primer PG6 (Table 1), yielding a fragment of 994 bp. From the sequence data, we knew that the 994 bp fragment was amplified with primers PG6 + PG6, suggesting that PG6 instead of N1 worked accidentally as an AD primer on the 5' end in the second reaction (indicated with ? in the figure).

Development Co., Shibuya, Tokyo). BLAST⁷⁾ searches were done using the NCBI/GenBank databases.

RESULTS AND DISCUSSION

PG was purified from 5500 ml of culture filtrate of *F. oxysporum* 880621a-1. The final 500 μ l fraction obtained through Sephadex G-75 containing 320 μ g protein/ml was determined carrying 117.8 U/mg specific activity to release reducing sugar from polygalacturonic acid. The purification factor was 46.0 fold that of the original solution.

Polyclonal antibody designated as PAb APG1 raised against the purified PG reacted positively with the PG protein of $\sim$ 40 kD in Western analysis of the PG protein (data not shown). Positive reaction with the antigenic solution was observed up to a 1000 fold dilution of the antibody by immunobinding assay. Its specificity toward *F. oxysporum* PG protein was apparent by its non-reaction with all fractions obtained during the PG purifying procedure except for those containing PG-protein, and by its lack of cross reaction with the juice of tomato stem tissues and fruit.

By DT-IBA with PAb APG1, the stem sections of tomato plants infected with *F. oxysporum* 880621a-1 showed positive reaction (Fig. 2). Blue color was observed at the vascular system of the stem which confirms secretion of PG by *F. oxysporum* in the vessels of the host plant. This corresponds with Di Pietro's (1996) finding by isoenzymatic analysis⁶⁾.

Through amino acid sequencing of Protease I-digested

Fig. 2. DT-IBA of stems of tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici* race 2 880621a-1 with a polyclonal antibody APG1 raised against the purified *endopolygalacturonase* protein. D: stem sections from infected tomato plants with *F. oxysporum*; H: stem sections from tomato plants without infection. Positive reaction was observed at the vascular system of the infected samples.

PG protein 12 fragments (total 148 residues) were obtained, which helped us to design degenerate primers. N-terminal amino-acid sequence was confirmed by sequencing of the undigested PG protein. In Fig. 3, N-terminal sequence is indicated by an underline and the partial amino acid sequences with bold characters.

Degenerate primers PG1+2 (Table 1), designed using partial amino acid sequence data, were used for PCR with genomic DNA of *F. oxysporum* 880621a-1 as a template. Single unique DNA fragment of 973 bp, encod-

ing a part of the PG gene was amplified (Fig. 1). Cloning and sequencing of this product revealed that it was homologous to a part of the previously reported PG gene of *F. moniliforme*⁵⁾.

To clone the adjacent fragments to either side of the

973 bp fragment, we performed TAIL-PCR (Fig. 1). In the primary TAIL-PCR reaction which was aimed to amplify sequences 5' of the 973 bp fragment, AD primer N1 and the fragment-specific primer PG4 (Table 1) were used with genomic DNA. Products of this reaction were

Fig. 3 (1)

Fig. 3 (2)

Fig. 3. Nucleotide sequence of the *endopolygalacturonase* gene from *Fusarium oxysporum* f. sp. *lycopersici* race 2 880621a-1 and the predicted amino acid sequence of the encoded protein. Introns are shown with small letters. The amino acid sequence is shown in single-letter code below the corresponding codons. Determined amino acid sequences are indicated with boldfaces. The putative CAAT and TATA boxes in the promoter regions are single and double underlined, respectively. Putative N-glycosylation sites are underlined (dashed). The priming sites of PG1-6 primers are indicated with arrows.

used as templates in a second reaction with the same AD primer (N1) and a nested 973 bp fragment-specific primer (PG6, Table 1), yielding a major product of 994 bp. From the sequence data, we knew that the 994 bp fragment was amplified with primers PG6+PG6, suggesting that PG6 instead of N1 worked accidentally as an AD primer on the 5' in the second reaction (Fig. 1).

An additional pair of reactions, designed to amplify sequences 3' of the 973 bp fragment, used AD primer TP1 (Table 1) and the fragment-specific primer PG3 (Table 1) for the first reaction and TP1 and specific primer PG5 (Table 1) for the second reaction, yielding a fragment of 489 bp. The 5' end of the 994 bp fragment and the 3' end of the 489 bp fragment were sequenced. Thus a total of 1783 bp sequence data was obtained (Fig. 3). The nucleotide sequence data presented will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Database with the following accession number: AB000124 (submitted on Dec. 27, 1996).

The codon usage frequencies were A: 26.01%, G: 23.11%, C: 25.46%, and T: 25.41% in the region.

The coding region consisted of 1318 bp was interrupted by four putative introns of 47, 51, 50, and 54 bp (Fig. 3), which are at identical positions to that of *F. moniliforme* PG gene⁵⁾. The existence of the third intron was confirmed basing on the amino acid sequence data

(Fig. 3). The coding sequence comprised 371 amino acids with a putative signal peptide of 22 amino acids (Met-Arg). The molecular mass was calculated at 41,132.17 and 38,825.34 with and without the 22 amino acids, respectively. The size was in good agreement with 40 kD size determined by SDS-PAGE²⁰⁾. The 5' region of the sequence contained a TATA motif (TATAAAA) at position -106 and a potential CAAT box (CAAT) at position -155 bp. The sequence for initiation of translation (ACAATGG) was the same as that of *F. moniliforme* and highly homologous to the consensus sequence reported for higher eukaryotes¹¹⁾.

The PG sequence was compared with other fungal PG sequences: PG from *F. moniliforme*⁵⁾, PGNI from *C. carbonum*¹⁹⁾, PG from *A. niger*¹⁸⁾, PG from *A. oryzae*¹⁰⁾, and PGI from *S. sclerotiorum*¹⁶⁾ (Fig. 4). The deduced amino acid sequences are of similar sizes (from 362 to 380 aa). The *F. oxysporum* f. sp. *lycopersici* PG aa sequence showed 82.9, 29.0, 27.6, 14.2, and 26.9% identity to those of *F. moniliforme* PG, *C. carbonum* PGNI, *A. niger* PG, *A. oryzae* PG, and *S. sclerotiorum* PGI, respectively.

The sequence of the amino acid residues of the N-terminal of the PG shown in Fig. 3 was the same as that of the reported 11-residue sequence of the PGI, Asp-Pro-Cys-Ser-Val-Thr-Asp-Tyl-Ser-Gly-Leu⁹⁾.

Foly	MVRNI..AIAALLPAAFASTLPKRDPCSVTDYSGLATAVS.....	38
Fmon	MVRNIVSRQLCSQLFALPSSSLQERDPCSVTEYSGLATAVS.....	
Ccar	MVAYALTSMGLGTTDLDTGLKSGATVLSAGALVAAAPSGLDARDG.CTF..	
Anig	MHSFASLLAGTTDLDTGLTSGTKVYGLAASATLASASPIEAR.GSCTF..	
Aory	MQLLQSSVIAATV.GAALVAAPVELKARDSCFTSADAKSG.....	
Sscl	MVHILSSALLRLGA AVSAAPAPAPTAAPNVADALAAVEKRAGSCTFSD	
Foly	SCTNIVLNGFQVPTGKALDLSKLKDGATVTFKGKTTFA	76
Fmon	SCKNIVLNGFQVPTGKQLDLSSLQNDSTVTFKGTTFA	
Ccar	.TDAATAIKNKASCSNIVISGMTVPA.....TFQGTTFG	
Anig	.KTAAAKAGKAGCSTITLDNIEVPA.....IFEGTTTFD	
Aory	.KT.....SCSTITLSNIEVPAGETLDTGLNDGTTVIFSGETTFG	
Sscl	PAVPLPAIKSKASCATIVISAVAVPSGTTDLDTGLKSGTHVVFEGTTTFG	
Foly	TTADNDFDPIVISGNGITITGASGHVIDGNGPAYWDGEGSNKNDNPKPDH	126
Fmon	TTADNDFNPIVISGNSNITITGASGHVIDGNGQAYWDGKGSNSNSNQKPDH	
Ccar	YK.EWEGPLISVSGTNIKVV GASGHTIDAAGQKWWDGKGSNG.GKTKPKF	
Anig	YE.EWAGPLISM S G K D I T V T G A S G H L I N C D G A R W W D G K G T S . . G K K K P K F	
Aory	YK.EWEGPLISVSGTNIKVQQASGAKIDGDSRWWDGKGGNG.GKTKPKF	
Sscl	TE.EWSGPLVSVSGTDITVTGASGSVLDGNGALYWDGKG TNG.GKTKPKF	
Foly	FIVVKKTTGNSKITNLNIQNWPVHCFDITGSSQLTISGLILDNRLGDKPN	176
Fmon	FIVVQKTTGNSKITNLNIQNWPVHCFDITGSSQLTISGLILDNRLGDKPN	
Ccar	FYAHSLTT..SSISGLNIKNTPVQAFSINGVTGLTLDRITIDNSAGDS..	
Anig	FYAHGLDS..SSITGLNIKNTPLMAFSVQADD.ITLTDITINNADGDT..	
Aory	FYAHKLDS..SSITGLQIYNTPVQGF SIQSDN.LNITDVTIDNSAG.T..	
Sscl	FYAHSLK.GKSSINNVKILNSPVQVFSINSASGLTSLSGITIDNSAGN...	
Foly	AKSGSLPAAHNSDGF DISSSDHVTLDNIHVYNQDDCVAVTSGTNIIVSNM	226
Fmon	AKSGSLPAAHNTDGF DISSSDHVTLDNNHVYNQDDCVAVTSGTNIIVSNM	
Ccar	AGAHNTDAFDIGSSSGITISNANIKNQDDCVAINSGSDIHVTNC	
Anig	L.GGHNTDAFDVGNSVG VNI IKPWVHNQDDCLAINSGENIWFTSG	
Aory	AEGHNTDAFDVGSSTYINIDGATVYNQDDCLAINSGSHITFTNG	
Sscl	SL..GHNTDAFDVGSSTDITISGANVQNQDDCLAINSGTGITFTGG	
Foly	YCSGGHGLSIGSVGGKSNNVVNGVQFLDSQIVNSENGCRIKSNSGTGTGTI	276
Fmon	YCSGGHGLSIGSVGGKSDNVVDGVQFLSSQVVNSQNGCRIKSNSGATGTI	
Ccar	QCSGGHGV SIGSVGGRKDN TVKGVVVS GTTIANSDNGVRIKTISGATGSV	
Anig	TCIGGHGLSIGSVGGRSNNVVKNV TIEHSTVSNSENAVRIKT VSGATGSV	
Aory	YCDGGHGLSIGSVGGRSDNTVEDVTISNSKVVNSQNGVRIKT VYDATGTV	
Sscl	TCSGGHGLSIGSVGGRSDNVVSDV IIGSSTVKNSANGVRIKT VSGATGSV	
Foly	ANVTYQNI SLTNISKYGV DVQ QDYLN GGP TGKPTNGVKISGIKFIKVTGT	326
Fmon	NNVTYQNI ALTNISTYGV DVQ QDYLN GGP TGKPTNGVKISNIKFIKVTGT	
Ccar	SDITYENITLKNIAKYGIVIEQDYLN GGP TGKPTTGVPITGVTLKNVAGS	
Anig	SEITYSNIVMSGISDYGVVIQDYEDGKPTGKPTNGVTITDVKLESVTGT	
Aory	SNVKFEDITLSGITKYGLIVEQDYENGSP TGTPTNGIKVSDITFDKVTGT	
Sscl	SGVTYKDITLSGITSYGVVIEQDYENGSP TGTGKPTSGVPITGVTL SNVHGT	
Foly	VA.SSAQDWYILCGDGSCSGFTFSGNAITGGGKTSS.C.NYPTNT.CPS	371
Fmon	VA.SSAQDWFILCGDGSCSGFTFSGNAITGGGKTSS.C.NYPTNT.CPS	
Ccar	VT.GSGTEIYVLCGKGSCSGWNWSGV SITGGKKSSS.CLNVP SGASC	
Anig	VD.SKATDIYLLCGSGSCSDWTWDDVKVTGGKKSTA.CKNYPSVASC	
Aory	VVESDATDIYILCGSGSCTGLTWSG.SITGG.KTSSKCE NVSTGASC	
Sscl	VS.SSATNVYVLC AK..CSGWTWDVN.VTGG.KTSTKCAGLP SGVKC	

Fig. 4. Comparison of the deduced amino acid sequence. The deduced amino acid sequence of the PG of *Fusarium oxysporum* f. sp. *lycopersici* race 2 880621a-1 (Foly) was compared with the PG of *F. moniliforme* (Fmon) (Caprari *et al.*, 1993), PGNI of *Cochliobolus carbonum* (Ccar) (Scott-Craig *et al.*, 1990), PG of *Aspergillus niger* (Anig) (Ruttkovski *et al.*, 1991), PG of *A. oryzae* (Aory) (Kitamoto *et al.*, 1993), and PGI of *Sclerotinia sclerotiorum* (Sscl) (Reymond *et al.*, 1994). Gaps were introduced into the sequences to achieve optimal alignment and are represented by dots. Amino acid residues identical to the *F. oxysporum* PG are shown in bold letters.

Kitamoto *et al.* (1993)¹⁰ reported the presence of three regions with conserved amino acid sequences for various PGs. Caprari *et al.* (1993)⁵ reported a fourth region. We could confirm these sequences (Asn¹⁸⁷-Ser¹⁹⁶, Asn²⁰⁸-

Ala²¹⁴, Gly²³¹-Ser²³⁸, and Ser²⁶⁰-Ser²⁶⁸) in *F. oxysporum*, and additionally found a region at Gln²⁹⁸-Ile³¹⁵ that is highly maintained among the species (Fig. 4). This suggests that these domains possibly are of some enzymatic

Fig. 5. Genomic Southern hybridization of digested genomic DNA of *Fusarium oxysporum* f. sp. *lycopersici* race 2 880621a-1. gDNA was digested with *Eco*RI (no site on PG encoding region), *Bam*HI (no site), *Ban*II (one site), and *Hinc*II (one site), respectively, and separated in a 0.8% agarose gel to blot and probed with the cloned 973 bp fragment amplified with PG1+PG2 primers. Lane 1: λ HindIII marker; Lane 2: gDNA/*Eco*RI; Lane 3: gDNA/*Bam*HI; Lane 4: gDNA/*Ban*II, Lane 5: gDNA/*Hinc*II. Note that just one band was detected from the gDNA digested with *Eco*RI and *Bam*HI, and two bands were from the gDNA digested with *Ban*II and *Hinc*II.

importance such as catalysis or substrate binding.

Three potential *N*-glycosylation sites (Asn-Xaa-Thr/Ser) were found (indicated with dashed underlines in Fig. 3). Although two of them (Asn²⁷⁸-Val-Thr²⁸⁰ and Asn²⁸⁸-Ile-Ser²⁹⁰) corresponded to those of *F. moniliforme* PG, the sites were not conserved among the species. The existence of these potential *N*-glycosylation sites is in agreement with the observation of PG1 and the other *N*-glycosylated isoforms⁹. There is no enzymatic information, such as its kinetics and substrate-specificity for the isoforms.

gDNA of *F. oxysporum* digested with *Eco*RI (having no site on the PG encoding region), *Bam*HI (no site), *Ban*II (having one site at 638 bp), and *Hinc*II (having one site at 920 bp) were probed with the pCR2.1 carrying the 973 bp PCR product amplified with PG1+PG2. Only one hybridizing band appeared in the *F. oxysporum* gDNA digested with *Eco*RI and *Bam*HI, respectively, and two bands in the gDNA digested with *Ban*II and *Hinc*II (Fig. 5). According to the results of genomic Southern hybridization, *F. oxysporum* f. sp. *lycopersici* has one PG-encoding gene on its genome.

The facts such as *F. oxysporum* being an inhabitant of the vessels²) and the specific induction of PG protein in the presence of pectin or plant tissue^{6,20}), coupled with our DT-IBA result showing PG protein secretion in the vessels of tomato stems, strongly suggest that PG has

some role in disease development.

To understand the infection mechanism of *F. oxysporum* clearly, pectinolytic enzymes have been the subject of enzymatic investigations. However, when and how the enzyme-coding genes are induced and the proteins secreted is still unclear, even in the recent isoenzymatic study by Di Pietro and Roncero (1996)⁹.

Our final goal is to know when PG is expressed, if it is essential to symptom development, and/or whether PG is the determining factor in the f. sp.-host specificity. Towards that purpose, the existence of a single copy of the PG gene presents good candidate for gene disruption, in order to evaluate its importance during symptom development. A genetical knockout study of the PG gene in *C. carbonum* has already disproven its role in causing disease on maize¹⁹). Still, studying the role of PG of *F. oxysporum* is worthwhile, considering the great emphasis given to this protein's role in tomato wilt disease by many workers.

Among the phytopathogenic species of *F. oxysporum*, many pathogenic formae speciales⁴) with high host specificity have been reported. Although IEF patterns showed no difference between the isoenzymes of *F. oxysporum* ff. sp. *lycopersici* and *cucumerinum*³), knowing the differences in the nucleotide and/or amino acid sequences between formae and whether the formae have different PG gene induction on different plants is still of interest.

In addition, many non-pathogenic *F. oxysporum* strains^{4,15}) have been reported and used as biological control agents. Do those non-pathogenic strains have a PG encoding gene? If they have, PG may have no role in the induction of symptoms or, the critical point in determining susceptibility to *F. oxysporum* occurs before the PG is secreted.

The authors gratefully acknowledge Mr. T. Shimizu, RIKEN for helping in DNA sequencing and growing plants.

Literature cited

1. Arie, T., Christiansen, S.K., Yoder, O.C. and Turgeon, B.G. (1997). Efficient cloning of Ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG box. *Fungal Genet. Biol.* 21: 118-130.
2. Arie, T., Hayashi, Y., Yoneyama, K., Nagatani, A., Furuya, M. and Yamaguchi, I. (1995). Detection of *Fusarium* spp. in plant with monoclonal antibody. *Ann. Phytopathol. Soc. Jpn.* 61: 311-317.
3. Arie, T., Shimazaki, S., Inoue, M., Yoneyama, K. and Yamaguchi, I. (1993). Polygalacturonase produced by *Fusarium oxysporum* f. sp. *lycopersici* 880621a-1 (Abstr. in Japanese). *Ann. Phytopathol. Soc. Jpn.* 59: 734.
4. Beckman, C.H. (1987). *The Nature of Wilt Diseases of Plants*, APS Press, St. Paul, Minnesota, pp. 145-148.
5. Caprari, C., Richter, A., Bergmann, C., Locicero, S., Cervone, F. and De Lorenzo, G. (1993). Cloning and characterization of a gene encoding the *endo*-polygalacturonase of *Fusarium moniliforme*. *Mycol.*

- Res. 97: 497-505.
6. Di Pietro, A. and Roncero, M.I.G. (1996). *Endo*-polygalacturonase from *Fusarium oxysporum* f. sp. *lycopersici*: Purification, characterization, and production during infection of tomato plants. *Phytopathology* 86: 1324-1330.
 7. Gish, W. and States, D.J. (1993). Identification of protein coding regions by database similarity search. *Nat. Genet.* 3: 266-272.
 8. Gross, E. (1967). The cyanogen bromide reaction. *Methods Enzymol.* 11: 238-255.
 9. Hirs, C.H.W. (1967). Reduction and S-carboxymethylation of proteins. *Methods Enzymol.* 11: 199-203.
 10. Kitamoto, N., Kimura, T., Kito, Y., Ohmiya, K. and Tsukagoshi, N. (1993). Structural features of a polygalacturonase gene cloned from *Aspergillus oryzae* KBN616. *FEMS Microbiol. Lett.* 111: 37-41.
 11. Kozak, M. (1981). Composition and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* 12: 857-872.
 12. Kumar, C.R. and Subramanian, D. (1979). Studies on *Fusarium* wilt of cotton II. The role of pectolytic enzymes. *Phytopathol. Z.* 94: 343-356.
 13. Liu, Y.G. and Whittier, F. (1995). Thermal Asymmetric Interlaced PCR: Automatable amplification and sequencing of insert end fragment from P1 and YAC clones for chromosome walking. *Genomics* 25: 674-681.
 14. Masaki, T., Tanabe, M., Nakamura, K. and Soejima, M. (1981). Studies on a new proteolytic enzyme from *Achromobacter lyticus* M497-1, I. Purification and some enzymatic properties. *Biochim. Biophys. Acta* 660: 44-50.
 15. Ogawa, K. and Komada, H. (1985). Biological control of *Fusarium* wilt of sweet potato with cross-protection by nonpathogenic *Fusarium oxysporum*. In *Ecology and Management of Soilborne Plant Pathogens* (Parker, C.A. et al. eds.), Amer. Phytopathol. Soc., St. Paul, Minnesota, pp. 121-123.
 16. Reymond, P., Deleage, G., Rasclé, C. and Fevre, M. (1994). Cloning and sequence analysis of a polygalacturonase-encoding gene from the phytopathogenic fungus *Sclerotinia sclerotiorum*. *Gene* 146: 233-237.
 17. Reymond-Cotton, P., Fraissinet-Tachet, L. and Fevre, M. (1996). Expression of the *Sclerotinia sclerotiorum* polygalacturonase *pg1* gene: possible involvement of CREA in glucose catabolite repression. *Curr. Genet.* 30: 240-245.
 18. Ruttkowski, E., Khanh, N.Q., Wientjes, F.J. and Gottschalk, M. (1991). Characterization of a polygalacturonase gene of *Aspergillus niger* RM5344. *Mol. Microbiol.* 5: 1353-1361.
 19. Scott-Craig, J.S., Panaccione, D.G., Cervone, F. and Walton, D.J. (1990). *Endopolygalacturonase* is not required for pathogenicity of *Cochliobolus carbonum* on Maize. *Plant Cell* 2: 1191-1200.
 20. Shimazaki, S., Arie, T., Yoneyama, K., Takio, K. and Yamaguchi, I. (1994). Purification, amino acid analysis of polygalacturonase produced by *Fusarium oxysporum* f. sp. *lycopersici* race 2 and its antibody preparation. *Ann. Phytopathol. Soc. Jpn.* 60: 751 (Abstr. in Japanese).
 21. Szecsi, A. (1990). Analysis of pectic enzyme zymograms of *Fusarium* species. II. Comparison of polygalacturonase zymograms of *Fusarium culmorum* and *Fusarium graminearum*. *J. Phytopathol.* 130: 180-196.
 22. Whitehead, M.P., Shieh, M.T., Cleveland, T.E., Cary, J.W. and Dean, R.A. (1995). Isolation and characterization of polygalacturonase genes (*pecA* and *pecB*) from *Aspergillus flavus*. *Appl. Environ. Microbiol.* 61: 3316-3322.
 23. Wirsal, S., Turgeon, B.G. and Yoder, O.C. (1996). Detection of the *Cochliobolus heterostrophus* mating-type (*MAT*) locus promotes the function of *MAT*. *Curr. Genet.* 29: 241-249.

和 文 摘 要

有江 力・Satyanarayana GOUTHU・島崎 聡・鎌倉高志・木村 真・井上美津子・瀧尾擴士・尾崎 明・米山勝美・山口勇： *Fusarium oxysporum* の植物組織中におけるエンドポリガラクトナーゼの分泌の免疫学的観察と本酵素をコードする遺伝子の単離

土壌病原糸状菌 *Fusarium oxysporum* の産生するエンドポリガラクトナーゼ (PG) は、病徴発現において重要な役割を果たすといわれている。精製したトマト萎凋病菌 (*F. oxysporum* f. sp. *lycopersici* race 2) 由来の PG タンパク質を抗原としてポリクローナル抗体 APG1 を作製し、これを用いて direct tissue-immunobinding assay (DT-IBA) 法により、寄主植物の茎中で *F. oxysporum* f. sp. *lycopersici* が PG を産生していることを認めた。また、PG タンパク質のアミノ酸解析により得られた部分アミノ酸配列情報をもとにプライマーをデザインし、PCR と TAIL-PCR により、PG をコードする遺伝子の全長を含む 1783 bp の塩基配列を決定した。PG 遺伝子は、47, 51, 50, 54 bp の4つのイントロンを含む 1318 bp のコード領域からなり、371 アミノ酸残基をコードし、このうち、22 残基はシグナルペプチドであることが推測された。アミノ酸レベルで、*F. moniliforme* PG, *Cochliobolus carbonum* PGNI, *Aspergillus niger* PG, *A. oryzae* PG および *Sclerotinia sclerotiorum* PGI とそれぞれ、82.9%, 29.0%, 27.6%, 14.2%, 26.9% の相同性を示した。