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His-PiPE induced HR, browning and generation of AOS in potato tissues. The PiPE was produced in the germination fluid from Pi and was existing in the cell wall of Pi. The role of PiPE peptides in the induction of HR in an incompatible interaction between Pi and potato cells is a prerequisite for the AOS and HR induction.

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A Novel Elicitor (PiPE) from *Phytophthora Infestans* Induces Active Oxygen Species and the Hypersensitive Response in Potato

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I. Introduction

An incompatible interaction between plants and pathogens often leads to rapid and localized plant cell death, termed the hypersensitive response (HR), at the infection site. Induction of biochemical defense responses in the host cells (Ebel and Scheel, 1992, Furuichi, 1993, Xu and Heath, 1998) likely involves recognition events for both elicitor and suppressor molecules from the pathogen at the host plasma membrane (Ebel and Scheel, 1992, Furuichi and Tomiyama, 1980, Furuichi et al., 2008) . Subsequently Ca^{2+} influx increases, pH decreases in the cytoplasm, and the cytosolic kinases are activated in the plant cells (Ebel and Scheel, 1992) (Furuichi et al., 2008)Furuichi et al., 1997)(Hamel et al., 2011). Electrolyte leakage contributing to host cell death (Goodman, 1968, Pavlovkin and Novacky, 1986, Tomiyama et al., 1983), resulting from the activation of a K^+ efflux across the plasma membrane (Tomiyama et al., 1968, Tomiyama et al., 1983, Atkinson et al., 1985, Baker et al., 1987, Tomiyama and Okamoto, 1989). Active oxygen species (AOS) also are generated rapidly at the plasma membrane of host cells during the incompatible interaction (Doke, 1983, Doke and Miura, 1995). Many of these responses associated with HR are duplicated by treatment of tissues with factors derived from pathogenic pathogens termed elicitors (Keen, 1975, Scheel et al., 1999). Elicitors from the oomycete pathogens, *Phytophthora* include 1,3- and 1,6-ß-D-glucans (Ayers et al., 1976, Sharp et al., 1984), glycoproteins (Keenan et al., 1985, Parker et al., 1988), and arachidonic acid (Bostock et al., 1981). A family of extracellular proteins produced by *Phytophthora* species, termed elicitins, also induces defense responses in plant cells (Ricci et al., 1989). Elicitins are highly conserved, 10-kDa proteins that are produced by several *Phytophthora* and *Pythium spp*. (Kamoun et al., 1994, Pernollet et al., 1993, Ricci et al., 1989). However, a 13-mer oligopeptide within a 42 kDa glycoprotein secreted by *Phytophthora megasperma*, also caused ion fluxes across the plasma membrane, the oxidative burst and phytoalexin biosynthesis in parsley cells (Nurnberger et al., 1994, Sacks et al., 1995). From the findings, it was suggested in the present report that we isolated PiPE elicitor from the fungal cell wall fractions in the *Phytophthora*. We described previously the elicitor activity of a hyphal cell wall preparation (HW), from *Pi* (Furuichi and Suzuki, 1989). This HW has a protein content of about 22% (Ikeda and Furuichi, 1993). Treatment of the HW with pronase or trichloroacetic acid prior to its addition to potato tuber tissues reduced elicitor activity in a dose-dependent manner (Ikeda and Furuichi, 1993). Purification of the activity that induced HR and phytoalexin accumulation in the potato cells, correlated with proteinaceous materials. Response to a monoclonal Abs (Abs), selected from mice hybridoma immunized with the HW of *Pi*, was retained by the purified protein (Ikeda and Furuichi, 1993).

In this study, we have isolated the hyphal cell wall proteins and found that active elicitor activity correlated with a PiPE that also reacted with the anti-PiPE Abs. After determining a partial amino acid sequence of the purified protein(s); we used RT-PCR to generate a sequence corresponding to a gene encoding the putative elicitor peptide. We used a His-tagged expression system with the PiPE gene to generate a fusion protein that demonstrated elicitor activity. Using the cDNA sequence for the elicitor clone, we examined for the presence of homologue sequences in other *Phytophthora* spp.

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The reported nucleotide sequence appears in the DDBJ/EMBL/GenBank databases under the accession number AB051573.

II. Results

a) Purification of antigens that recognize the-PiPE monoclonal Abs

Elicitor activity and the level of extractable proteins from mycelia (race 0 of *Pi*) grown in liquid culture increased for three weeks. We used homogenates of 15-days cultures as the initial PiPE source. Fractionation of elicitor activity by anion exchange chromatography at pH 8.1 (Fig. 1A) with elution of a linear gradient of NaCl (0-0.5 M) resulted in seven protein peaks. The results of assaying each fraction for Abs-binding activity are illustrated in Figure 1B. The maximum Abs-binding activity was detected in fractions F17 to F20, eluted at 0.35 M NaCl.

b) Elicitor activity of the fractions recognized by anti-PiPE Abs

Treatment of tubers from the resistant cultivar Eniwa (R_1) with the elicitor preparation (containing proteins and carbohydrates) showed that it was more active than on tubers from the susceptible cv. Irish Cobbler (r-gene) (Fig. 3B). Thus the initial preparation possesses the same specificity as the *Pi* isolate from which the cell wall elicitor was derived. The elicitor activity in the concentrated samples of fractions F15 to F21 from anion exchange chromatography showed similar response being more active on cv. Eniwa than on cv. Irish Cobbler. The intensity of the symptoms of browning and cell death characteristic of HR was much higher in fractions F19 and F20 than in the other fractions. Fractions F17-F20 were pooled and the proteins separated by SDS-PAGE and silver-stained (Fig. 2B). Several peptide bands were detected in each fraction. The anti-PiPE Abs recognized one clear band of protein (47 kDa) in fractions F17 and F18, and three protein bands (47, 38, and 34 kDa) in fractions F19 and F20 (Fig. 2C). Because no elicitor activity was detected in F17 and F18, containing 47 kDa peptides, we concluded that elicitor activity resided with either or both of the 38 kDa and 34 kDa protein bands.

c) Elicitor activity of affinity-purified PiPE

Affinity-purified PiPE peptides by using the anti-PiPE-Abs from fractions F19 and F20 had higher elicitor activity on tuber tissue from the resistant potato cv. Eniwa than on the susceptible cv. Irish Cobbler. The affinity-purified proteins transiently enhanced the production of AOS in suspension cultures of both potato cultivars after 30 min returning to the control treatment level after 150 min. AOS generation was higher in cv. Rishiri than in cv. Mayqueen. The cv. Eniwa showed similar enhanced production of AOS in potato tuber disks, whilst cv. Irish Cobbler showed lesser enhancement of the AOS production.

d) The effect of His-Strboh1 for the activation of AOS in potato plasma membrane

To examine the effect of His-Strboh1 to AOS generation in potato cells, entire sequence of Strboh1 was expressed with the *E. coli* (BL21pLysS) containing a C-terminal 6His-tag. Affinity purification yielded approximately 110 kDa translation product and His-Strboh1 was confirmed by SDS-PAGE and immunoblot analysis by using His-Abs. The effect of His-Strboh1 on the activity of AOS generation was measured by using luciferase subustrate (CLA) in potato microsomal fraction (cv Eniwa). Because it was reported that activated NADPH oxidase was localized in plasma membrane in mammalian cells, AOS measurements were performed with microsomal fraction prepared from potato tuber tissue. *E. coli* expressed His-Strboh1 (1.2μM) was treated to potato microsomal fraction and measured its chemiluminescence. In potato microsomal fraction treated His-Strboh1, AOS generation was transiently increased 5 to 10 min after the treatment, whereas treatment of Tiron (1, 2-dihydroxy-3, 5-benzenedisulfonic acid disodium salt) which was a scavenger of AOS inhibited the generation. Ten min after AOS generation was declined to the basal level in the microsomal fraction. These results suggested that His-Strboh1 expressed in E.coli was activated after the treatment to microsomal fraction.

Expression vector containing a N-terminal 6His-tag construct was employed for the production of His-Strboh1. Affinity purification yielded approximately 120 kDa product and the production of His-Strboh1 was confirmed by immunoblot analysis by using His-Abs. The effect of insect cells expressed His-Strboh1 on the generation of AOS was performed. The AOS generation was transiently increased up to 10 min after treatment. However, AOS generation was increased until 40 min, and the peak of AOS generation was 7 times higher than with the fusion from *E. coli.* These results suggested that glycosylation of His-Strboh1 had effect on the generation of AOS in membrane fraction of potato.

e) Immunochemical analysis of germination fluid using anti-PiPE Abs

Immunoblotting of the germination fluid from zoospores of *Pi* with anti-PiPE Abs, detected proteins of 47 and 38 kDa (Fig. 3D, E). We have reported that the germination fluid from race 0 caused a typical HR response and browning in the potato tuber disks of cv. Rishiri (Furuichi et al. 1979). Thus one protein of a size associated with elicitor activity from mycelial extracts, the 38 kDa, was detected in the germination fluids. We have tested the localization of PiPE antigens by using PiPE Abs and by using electron microscope of germinating zoospores (Fig. 3D). Figure 3D shows the germinative zoospores at five hour after the germination(x 14800). The results show PiPE existed on the surfes of the cell wall of *Pi* by using the rhodamine anti-PiPE Abs. (Fig.3Eb).

f) Peptide mapping of the PiPE isolated from HW and cloning of a partial cDNA fragment encoding PiPE

We had examined if the PiPE protein is a glycoprotein or not, by using SDS-PAGE and the stain by periodic acid-Shiff's reagent. Digestion of the purified PiPE with *Staphylococcus aureus* V8 protease, to generate peptides for amino acid sequencing, generated two major bands. A sequence of 20 amino acids determined from the N-terminus of the native peptide was identical to the sequence of one of the V8 protease-digested fragments (Table 1).

We designed oligonucleotides based on the N-terminus amino acid sequence (Table1) as PCR primers. We derived cDNA from RNA extracted from a freshly harvested mycelium (for 10 days) of *Pi* race 0 and used this material as template in PCR. A 674-bp PCR product was obtained, cloned and sequenced (Fig. 4A).

Searches using the Blast program (Stephen et al. 1997) revealed significant similarity to fructose 1, 6 bisphosphate aldolase (FBA) genes, with the highest similarity to yeast FBA (Fig. 4B). A database survey of Blast searches against *Pi* ESTs in DDBJ revealed a full length EST of 674 bp to correspond to the cDNA sequence. Our finding that a protein with homology to a FBA produced by *Pi* has elicitor activity is novel because the PiPE is secreted by the germinating spores also and is detected in extracts from the mycelia.

g) Southern blot analysis of the PiPE

Southern blot hybridization was used to determine the number of copies of the cloned PiPE gene sequences in the genomic DNA of *Pi,* races 0 and 1. Using a probe containing a 674-bp fragment of the PiPE open reading frame from *Pi,* four *Pst* I fragments and five *Hind* III fragments from the *Pi* race and two *Pst* I fragments and three *Hind* III fragments, four *Pst* I fragments and five *Hind* III fragments were detected in the race of *Pi*, and two *Pst* I fragments and three *Hind* III fragments were detected in *Pi* 831 (Fig. 5) were detected from *Pi*831 (Fig. 5). Because there is one *Pst* I site and no *Hind* III site in the cloned partial cDNA (674 bp) coding the PiPE (Fig. 4B), at least two copies of the PiPE gene occur in the *Pi* genome.

h) Elicitor activity and generation of AOS in functional analysis of the tagged PiPE

We generated a 6His-PiPE to demonstrate that the protein produced from the gene had elicitor activity. A His-PiPE band (27.5 kDa) was observed after SDS-PAGE and CBB-staining (Fig. 6A). A protein band of this size, was not obtained when the His- control from the plasmid vector without insert cDNA was analyzed (Fig. 6A, lane 1). The purified His-PiPE was recognized by both the anti-His Abs and anti-PiPE-monoclonal Abs (Fig. 6B).

The His-PiPE had stronger elicitor activity on Eniwa (R_1-gene) tuber cells than on Irish Cobbler (r-gene) suspension cultured cells responded with a stronger oxidative burst than those of cv. Irish Cobbler after exposure to the His-PiPE.

i) Presence of PiPE genes in different Phytophthora species

To investigate whether sequences encoding PiPE are present in other *Phytophthora* isolates, RT-PCR was performed. The primers indicated in Table 1 was used with cDNA derived from total RNA from freshly harvested mycelium of *Pi,* DN101 (race 0) and E003 (race 0), *Pi* 831 and St401 (race 1); *P. megasperma; P. nicotianae; P. cryptogea;* and *P. capsici* as templates. PCR products of 674-bp were generated from all tested samples (Fig. 8A). These RT-PCR products all hybridized with the probe from race 0 (Fig. 7B).

III. Discussion

a) Immunochemical analysis of the localization of PiPE Abs in potato cells

Based on this similarity, we assume that fructose 1,6-bisphosphate aldolase peptides (FBA) may exist in the cell wall, and may be an elicitor of *Pi* against potato cells. Though FBA may exist in the cytosol of *Pi*, it could also be expressed and translocated into the cell wall of the germinating tube during the infection process. Secretion peptide from FBA was produced after germination of *Pi* spoors. Aldolases are known to be glycoproteins.

We propose a model for the elicitation of HR mediated by recognization of the PiPE by the StCDPK (a Ca^{2+} dependent protein kinase) in the potato cell plasma membrane (Furuichi et al., 1997). It was suggested that the PiPE binds to a 6H-StCDPK2 from a resistant cv. Rishiri (R_1), based on an ELISA assay using a monoclonal Abs of PiPE. It remains to be clarified what domain of the PiPE interacts with the CDPK of the host cells.

The isolated PiPE is showing the activity for AOS generation and tissue browning; it induced HR and AOS generation in cv. Rishiri, a resistant potato (R_1-gene), but induced only AOS generation in cv. Irish Cobbler, a susceptible potato (r-gene).

During the infection process, the PiPE might be produced by the oomycete directly from the germ tubes and infection hyphae. It may be recognized specifically by CDPK of potato cells, causing some conformational change in the structure of the kinase domain, which would result in induction of CDPK activation within several minutes. It is possible that as a consequence of the PiPE binding, the catalytic domain of CDPK is now accessible to the substrates for the kinase in potato cells. However, the activation pattern of the kinase is different from the suppressor treatment of *Pi* which results in activation of the CDPK within 5-10 minutes.

The glycoprotein has 65% homology to FBA from yeast, which has a elicitor activity like as fungal cell

wall elicitor of *Pi*. As shown by Figure 3D, 3E PiPE was secreted from fungal cell wall surfes and that PiPE was detective in the germination fluids by using immunochemical methods.

It was reported that ectopic expression of a heterologous CDPK (AK1-6H, an Arabidopsis calcium dependent protein kinase) in tomato protoplasts enhanced plasma membrane–associated NADPH oxidase activity (Tena et al., 2011). We examined the effect of CDPK Abs(Furuichi and Yokokawa, 2008), recognizing kinase domain-III, to AOS generation in potato microsomal fraction. It was observed that treatment of CDPK Abs to the microsomal fraction of potato, which was added with expressed His-Strboh1 protein from insect cells, inhibited approximately 50 % of AOS generation. It was suggested that CDPK kinase play an important role in the NADPH oxydase activation in potato microsome.

In the Cf9-Avr9 gene-for-gene interaction, a 68 to 70 kDa CDPK is activated in the plasma membrane fraction of tobacco cell cultures. The reported inhibitor studies were consistent with the evidence that CDPK was located upstream in the signal pathway that leads to the induction of AOS generation (Romeis et al., 2000), and it is in accordance with the results of quantitative RT-PCR in this study. Furthermore, arachidonic acid, an elicitor of Pi, induces activation of 78 kDa protein kinase C-like enzyme in potato tubers in calcium-dependent manner (Tena et al., 2011). These lines of evidence suggest that the NADPH oxidase is activated by the regulation of CDPK. Despite the importance of *Phytophthora* species as devastating plant pathogens, the basis of it specificity on potato cultivars is not resolved although elicitor active fractions have been isolated (Furuichi and Yokokawa, 2008, Furuichi and Suzuki, 1990, Kamoun et al., 1998, Joosten et al., 1999) The studies in the present results suggest that the PiPE with a sequence resembling that of fructose- 1, 6-bisphosphate aldolase could be functioning as an avirulence factor. This protein incited browning and AOS production to a greater extent on the resistant cultivars than the susceptible cultivars.

However all four (two race 0, avirulent and two race 1, virulent) of the *Phytophthora* species tested, possessed sequences that hybridized to the PiPE gene. Since the PiPE was recognized by anti-HW Abs, this PiPE was considered to exist in the cell wall of Pi cells. We observed that FITC-labeled Abs bound to the surfes of germinated spores of Pi by using fluorescent microscopy (×800) (unpublished data).

It was reported that ectopic expression of a heterologous CDPK in tomato protoplasts enhanced plasma membrane–associated NADPH oxidase activity (Tena et al., 2011, Furuichi and Yokokawa, 2010). We examined the effect of CDPK Abs, recognizing kinase domain-III, to AOS generation in potato microsomal fraction (Furuichi and Yokokawa, 2008). It was observed that treatment of CDPK Abs to the microsomal fraction of potato, which was added with expressed His-Enrboh1 protein from insect cells, inhibited approximately 50 % of AOS generation. It was suggested that CDPK kinase play an important role in the NADPH oxidase activation in potato plasma membrane. In the Cf9-Avr9 gene-for-gene interaction, a 68 to 70 kDa CDPK is activated in the plasma membrane fraction of tobacco cell cultures. The reported inhibitor studies were consistent with the evidence that CDPK was located upstream in the signal pathway that leads to the induction of AOS generation (Tena et al., 2011), and it is in accordance with the results of quantitative RT-PCR in this study. Furthermore, arachidonic acid, an elicitor of *Pi*, induces activation of 78 kDa protein kinase C-like enzyme in potato tubers in calcium-dependent manner (Subramaniam et al. 1997). These lines of evidence suggest that the NADPH oxidase is activated by the regulation of CDPK(Furuichi et al., 2012).

IV. Materials and Methods

a) Phytophthora strain and culture conditions

Phytophthora infestans (Mont.) de Bary isolates DN101(presented by R.Bostock) and E003 (race 0), and St 401 (race 0) and *P*831 (race 1.2.3.4.5, presented by Tooley, Cornell University) were maintained on rye agar medium supplemented with 2% sucrose and 0.2% bacto yeast extract at 18℃ in the dark. Other *Phytophthora* species were maintained in the dark. For liquid culture of the oomycete. The mycelia were grown in the dark at 18℃ for 2-3 weeks on the synthetic medium as described by Furuichi and Suzuki (1990). The mycelial mat was harvested by gentle filtration, washed, and frozen at –20℃. Zoospores from the mycelial mat of PI and the germination fluid were prepared using the methods reported previously (Doke and Tomiyama, 1977).

b) Extraction and purification of HW

Extractions of the HW were performed at 0 to 4℃ either in an ice bath or cold room. The frozen mycelial mats of *Pi* (race 0), DN101 were ground in liquid nitrogen to a powder that was homogenized in five volumes of 0.2 M phosphate buffer (pH 7.2) containing 1 M NaCl and phenylmethylsulfonyl fluoride (PMSF) in a final concentration of 25 mM. The homogenate was sonicated for 3 min and centrifuged a 20,000 x *g* for 20 min at 4℃. The supernatant was dialyzed overnight against cold 25 mM Tris-HCl, pH 8.1 (Spectra/Por molecular porous membrane, Lincoln, molecular weight cut-off 1,000). The proteins in the crude dialyzed extract were purified by FPLC-anion exchange chromatography on a column (4.6 mm—100 mm, 1.7 ml, PoRos QE/M; PerSeptive Biosystems, Tokyo) equilibrated in the same buffer. The flow rate was 5 ml/min. Proteins were eluted

with a linear gradient of 0 to 0.5 M NaCl in 25 mM Tris-HCl, pH 8.1. The column eluate was monitored at 280 nm and recovered in fractions of 1 ml.

HW were isolated and purified after the homogenization and FPLC-anion exchange chromatography.

c) Protein Measurement

The protein contents of samples were measured according to Lowry et al. (1951) using bovine serum albumin (BSA) as the standard protein. Absorbance was measured at 595 nm.

d) Monoclonal Abs of the PiPE

The Abs used were monoclonal anti-PiPE Abs generated by mouse hybridomas as described in Ikeda and Furuichi (1993). PiPE was isolated from the *Pi* homogenate as reported previously (Furuichi and Suzuki, 1990).

Enzyme-linked immunosorbent assay (ELISA) was performed following the procedure described by McLaughlin et al. (1989). The wells of microtiter plates (Dynatech, Tokyo) were coated with the Abs diluted at 1/1,000 with 1% bovine serum albumin in phosphate-buffered saline (PBS). Immunoglobulin-alkaline phosphatase conjugates from rabbit were used as secondary Abs at 1/2,000 dilution. Absorbance due to alkaline phosphatase was measured with a Microplate Reader (Bio-Rad, Tokyo) at 595 nm.

e) Biological Assay

Induction of HR by the elicitor was assayed by using microscopic observation. 1) The cessation of cytoplasmic streaming, 2) Loss of stain ability by neutral red, and 3) Loss of ability for plasmolysis, of potato tuber tissue at 12 h after treatment.

The parenchymatous tissues of potato tuber from cvs. Eniwa (R_1) and Irish Cobbler (r) were aged for 16 h at 18℃ prior to being treated with 30 μl of the PiPE (1 mg ml^{-1} distilled water), which were isolated from fungal mat, and fractionated by FPLC then various FPLC fractions were used. The materials in the FLPC fractions were concentrated with a Centricon-30 micro concentrator (Amicon, Tokyo) (600 μg ml^{-1}). State replicates of studies here.

The cystospores were germinated by shaking in a flask with $CaCl_2$ (10^{-4} M).

f) Measurement of AOS generation from suspension of potato cells

A luciferase substrate, (CLA) was used to measure the concentration of AOS produced by suspension cultured potato cells. .Suspension-cultured cells (5 ml of 3- to 4-day-old cells of potato cv. Eniwa (R1-gene) and cv. Irish Cobbler (r-gene) were treated with His-tagged PiPE (500 μl of 600 μg ml^{-1}). At each time point, 500 μl of the treated suspension cells were centrifuged for 15 sec at room temperature to collect the supernatant. The supernatant (25 μl) was added to a 15 ml sample tube containing 426 μl of 39 mM HEPES (pH 7.0), 5 μl of 10 mM MgCl2 or of 10 mM CaCl2, 5 μl of 10 mM EGTA, 1.5 μl of 10 mM guanosine 5´ triphosphate (GTP)-λ-S and 15 μl of 500 μl CLA(Cypridina Luciferin Analog; 2 Methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one). CLA was added to the tube last. The sample was then incubated at 37°C for 3 min. Radiated light was measured for 15 sec just after adding 23 μl of 3.3 mM NADPH (Luminescence Reader, Atto, Tokyo).

g) SDS-PAGE and immuno blotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis as described previously (Laemmli, 1970) with a 12.5% acrylamide separation gel and 4.5% acrylamide stacking gel. Following electrophoresis, gels were silver-stained following the previously reported method or the proteins were transferred as described by Towbin et al. (1979) onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, pore size 0.45 μM, Millipore, Tokyo) using a MilliBlot™-SDS System (Millipore) (Stephen et al.) at 2 mA per cm^3 for 30 min. The PVDF membrane was incubated for 1 h at 25℃ in the blocking buffer (10 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 5% skimmed milk). Membranes were washed twice in TBS-Tween 20 for 5 min, incubated for 1 h at room temperature in the primary HW-Abs diluted at 1/1,000 with TBS. After washing a third time in TBS-Tween 20 for 10 min, membranes were incubated for 1 h at room temperature in rabbit anti-mouse IgG conjugates with alkaline phosphatase (Bio-Rad, Tokyo, Japan) diluted at 1/2,000 with TBS. After three washes in TBS-Tween-20, each for 10 min detection of the antigen-Abs complexes was carried out with alkaline phosphatase color reagent (Bio-Rad, Tokyo, Japan).The reactive membranes were majored by using typhoon (GE-Science,Tokyo,Japan). Controls were recorded by using without anti-PiPE-Abs and with control antisera. The experiments were determined by using 3 times measurements. The membrane with antigen-Abs complexes were recording by using the typhoon gel scanner.

h) Peptide mapping by protease

PiPE protein was digested by *Staphylococcus aureus* V8 protease as described by Cleveland et al. (1977). Briefly, protein bands from an SDS gel, stained after SDS-PAGE with Coomassie brilliant blue, were digested by *the* V8 protease without prior elution, by placing gel slices containing these bands in the sample wells of a second SDS gel, then overlaying each slice with the V8 protease. Digestion proceeded directly in the stacking gel during the subsequent electrophoresis.

i) N-terminal amino acid sequencing

For N-terminal amino acid sequence determination, PiPE peptides were concentrated with a Centricon-30 micro concentrator in a final concentration

of 100 pmol and transferred to a PVDF membrane as described previously (Southerton et al., 1993). Automated Edman degradation of the PiPE peptides was performed with a Shimadzu PPSQ-21 sequencer (Shimadzu, Kyoto) using the reagents and method of the manufacturer.

j) Fungal RNA preparation and RT-PCR

Total RNA from freshly harvested *Pi* mycelia was isolated using the guanidine hydrochloride extraction method reported by (Logemann et al., 1987). Amplification of cDNA with the degenerate primers shown in Table 1 was carried out using Ready-To-Go™ RT-PCR beads (Amersham-Pharmacia, Tokyo) according to the methods of the manufacturer. Reverse transcription was carried out by adding 20 ng to 2 μg of total RNA and a final concentration of 1 pM oligo d (T)18 primer to dissolved beads in DEPC-treated water. The incubation conditions were as follows: 30 min, 72℃; 5 min, 95℃. After that, the degenerate primer indicated in Table 1 was added for PCR. The incubation conditions were as follows: 4 min, 94℃; 35 cycles (40 min, 94℃; 1 min, 50℃; 1.5 min, 72℃); 7 min, 72℃.

k) Genomic DNA preparation and Southern blot hybridization

Genomic DNA from freshly harvested *Pi* mycelia was isolated using reported methods (Ausubel et al., 1987; Sambrook et al., 1989). DNA was treated with RNase and digested with *Hind*III or *Pst*I. Approximately 3 μg of digested DNA was electrophoresed on a 1% agarose gel. Alkaline DNA was transferred to a nylon membrane (Hybond N^+, Amersham-Pharmacia, Tokyo), and Southern blot hybridizations were performed at 55°C as reported (Ausubel et al., 1987). Probes for hybridization were synthesized by PCR using the primers described in Table 1 and comprised the nucleotide sequences (674-bp fragment — (Fig. 9). Probes were labeled using a AlkPhos Direct labeling and detection system (Amersham, Tokyo) according to the supplier's instructions. Membranes were washed twice at 65°C for 10 min in the primary wash buffer, then washed in secondary wash buffer (50 mM Tris base, 100 mM NaCl and 2 mM $MgCl_2$). Positive cDNA clones were detected using the CDP-*Star* chemiluminescent detection reagent according to the manufacturer's instructions.

l) Expression of recombinant PiPE

The *E. coli,* BL21 pLysS cells, harboring apCR T7/CT TOPO plasmid vector (Invitrogen) containing the insert cDNA the 674 base pairs. His tag added to C terminus. They were added to 10 ml LB medium. The 10 ml of LB medium was inoculated with 2 ml of the E. coli cultured, and then cultured for 4 h at 37℃ with shaking. Isopropyl-λ-D-thiogalactopyranoside was added to a final concentration of 0.6 mM and cultured for 24 h. The protein was harvested. Cells were harvested by centrifugation at 4000 rpm for 10 min at 4℃, and the pellet was resuspended in guanidinium lysis buffer (pH 7.8) and slowly shaken for 7 min before sonicated at ice-water temperature. The insoluble debris was removed by centrifugation at 6500 rpm for 15 min at 4℃. The supernatant was collected and stored at 4℃ for subsequent His-tag purification by following the Xpress™ System protocol as described by Invitrogen. The polyhistidine-tagged fusion protein was loaded onto a ProBond™ histidine-bind resin column equilibrated with lysate buffer. The column was washed with denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8). Then, the column was washed with denaturing wash buffer, ranging at pH 6.0 and pH 5.3. Finally, the protein was eluted with denaturing elution buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 4.0). The elute was dialyzed against 10 mM Tris-HCl, pH 8.0, and 0.1 % Triton X-100 overnight at 4℃ to remove urea. Each samples were stocked at -30℃What about the germination fluid also contains those PiPE. Affinity purified PiPE fraction were contained in a germination flud.

m) Purification of recombinant protein

For purification of His-fusion protein, ProBond™ Protein Purification kit (Invitrogen) was used. The polyhistidine-tagged fusion protein was loaded onto a ProBond™ histidine-bind resin column equilibrated with lysate buffer. The column was washed with 8 ml of denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8). Then, the column was washed with 8 ml of denaturing buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride) pH 6.0 and pH 5.3 successively. Finally, the protein was eluted with 5 ml of denaturing elution buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 4.0). the elute was dialyzed against 10 mM Tris-HCl, pH 8.0, 0.1 % Triton X-100 overnight at 4 ℃ to remove urea. During this time, the dialysis buffer was replaced 4 times. The purified protein concentration was determined using the BCA protein assay kit (Pierce) with bovine serum albumin (BSA) as standard.

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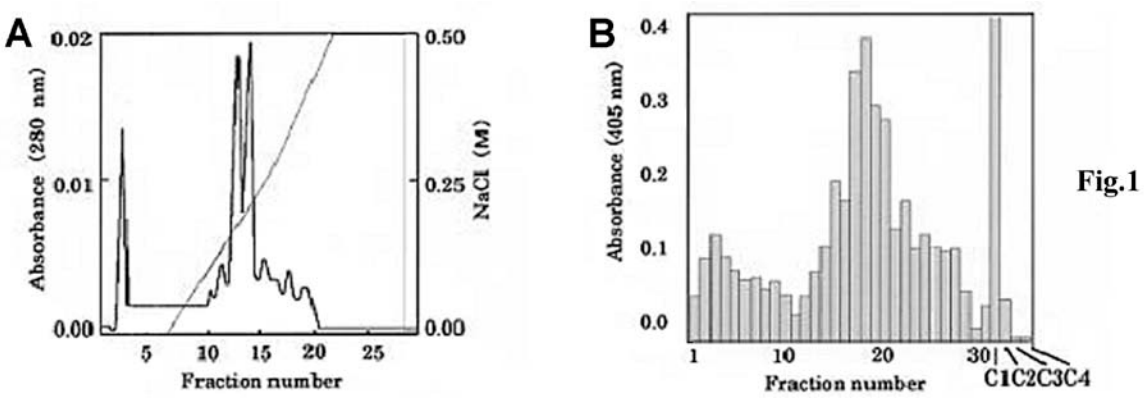

Fig.1

Figure 1 : Chromatogram of the crude extract prepared from Phytophthora infestans (Pi) (race 0) by FPLC-anion exchange chromatography (POROS QE/M column) at pH 8.1

A, Absorbance was monitored at 280 nm. The flow rate was 5 ml/min, and 1-ml fractions were collected after elution with a NaCl gradient. B, ELISA reactions using Abs against FPLC fractions of crude extract prepared from *Pi* (race 0). C1:Crude extract prepared from race 0. C2: Elution buffer used for FPLC, C3: C1-treated with the first Abs (Abs only), C4: C1-treated with second Abs (anti-mouse IgG) only. Data are the average of four replications. A 405 nm on the axis was the value of ELISA.

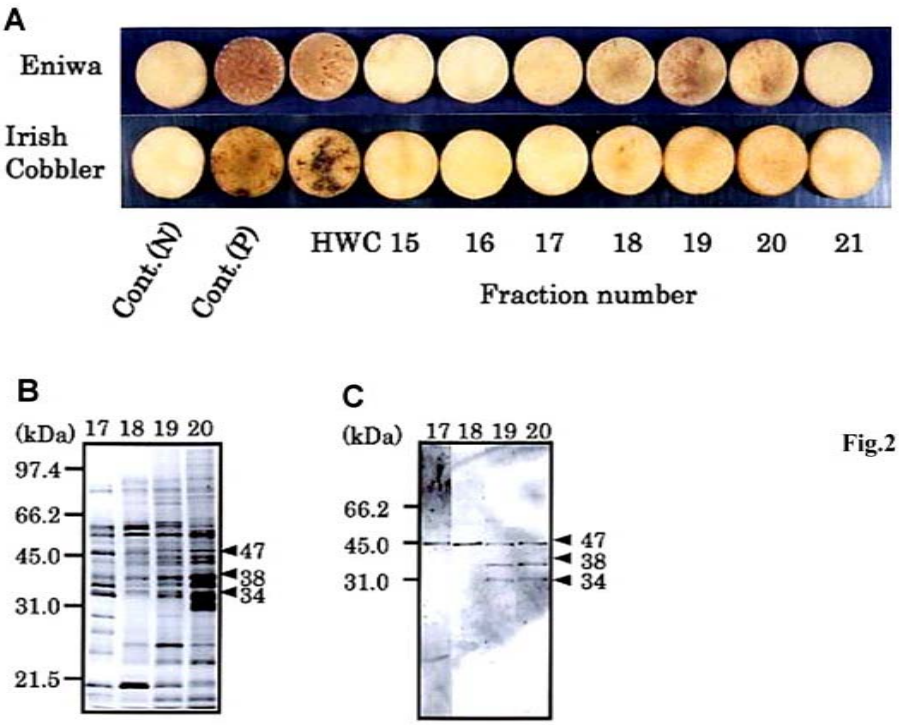

Fig.2

Figure 2 : Elicitor activity and peptide components of FPLC fractions

A, Elicitor activity as detected by browning after 30 min of treatment with preparations from *Pi* race 0. Each fraction (600 μg/ml -1protein) was applied to the cut surface. Cont.(N): water treatment, Cont.(P): the extract prepared, HW: wall elicitor (1mg/ml -1distilled water) prepared from the treatment. B, Silver staining of peptides obtained by SDS-PAGE of FPLC fractions extracted from mycelium of *Pi* (race 0). Fraction numbers are given above lanes. Molecular mass standards in kDa are designated. C, Western blot analysis of FPLC fractions with monoclonal Abs derived against HW. Samples and order of loading are identical to those in B.

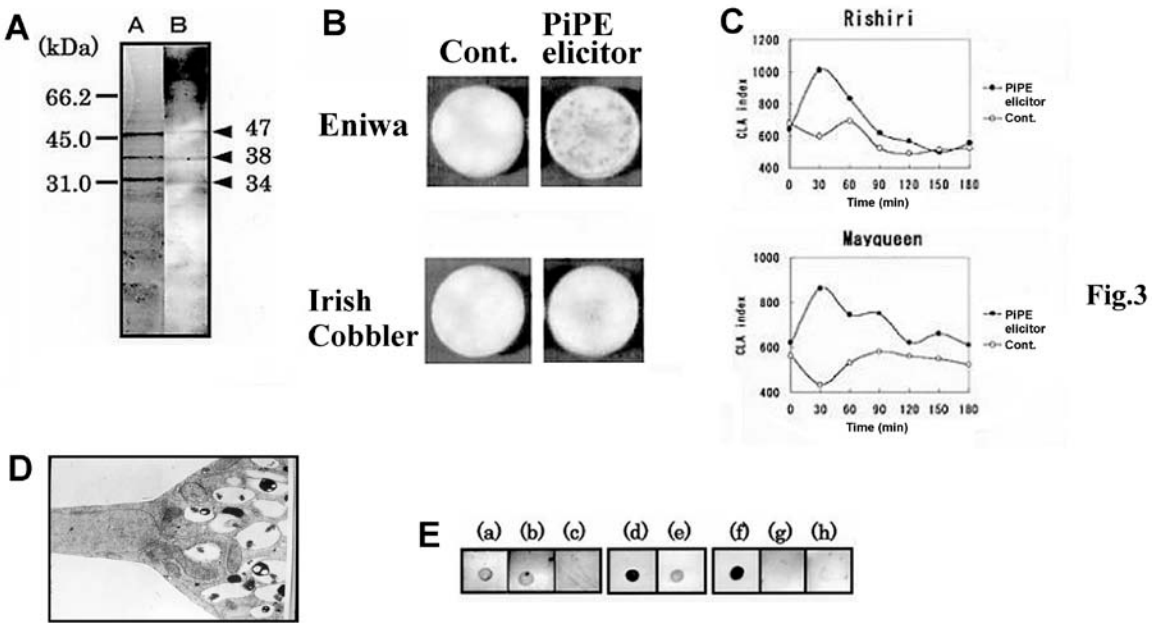

Fig.3

Figure 3 : Affinity purification of the Abs-binding protein

A, SDS-PAGE of affinity-purified Abs binding peptides. Gel was silver-stained. Molecular mass standards in kDa are given on the left. Western blot analysis of affinity-purified Abs-binding peptides. Molecular mass standards in kDa are given on the left. Germination fluid of *Pi* race 0, at 5h after shaking in flask. B, Elicitor activity of Abs binding protein on tuber tissues of potato and generation of active oxygen species in suspension culture cells of potato. Cvs. Eniwa (R_1) and Irish Cobbler (r) at 98 h after treatment. C, CLA index in culture cells of the resistance (R_1) and susceptible (r) potato cultivars was measured with a luminescence reader. The cultivar rishiri was used.

D, Electron microscopic observation of *Pi* (×14,800). Immunochemical assay of germination fluid of *Pi.* E, Immunochemical assay of the germination and culture-fluid of *Pi* (race 0). (a) Germination fluid. (b) The zoospore suspensions. (c) $CaCl_2$ as a control. (d) The culture fluid. (e) Rye medium as a control. (f) Homogenated soluble sample. Prepared from *Pi.* (g) The extract from *Pi* treated with the Abs 2A11. (h) The treatment with second Abs (anti-mouse immunogl obulin G).

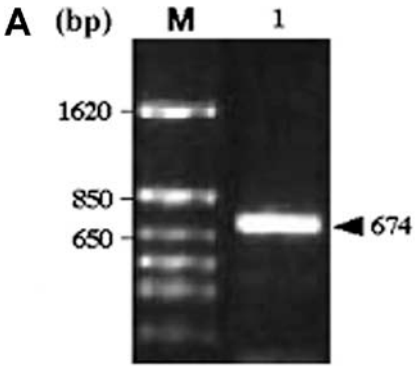

Fig.4

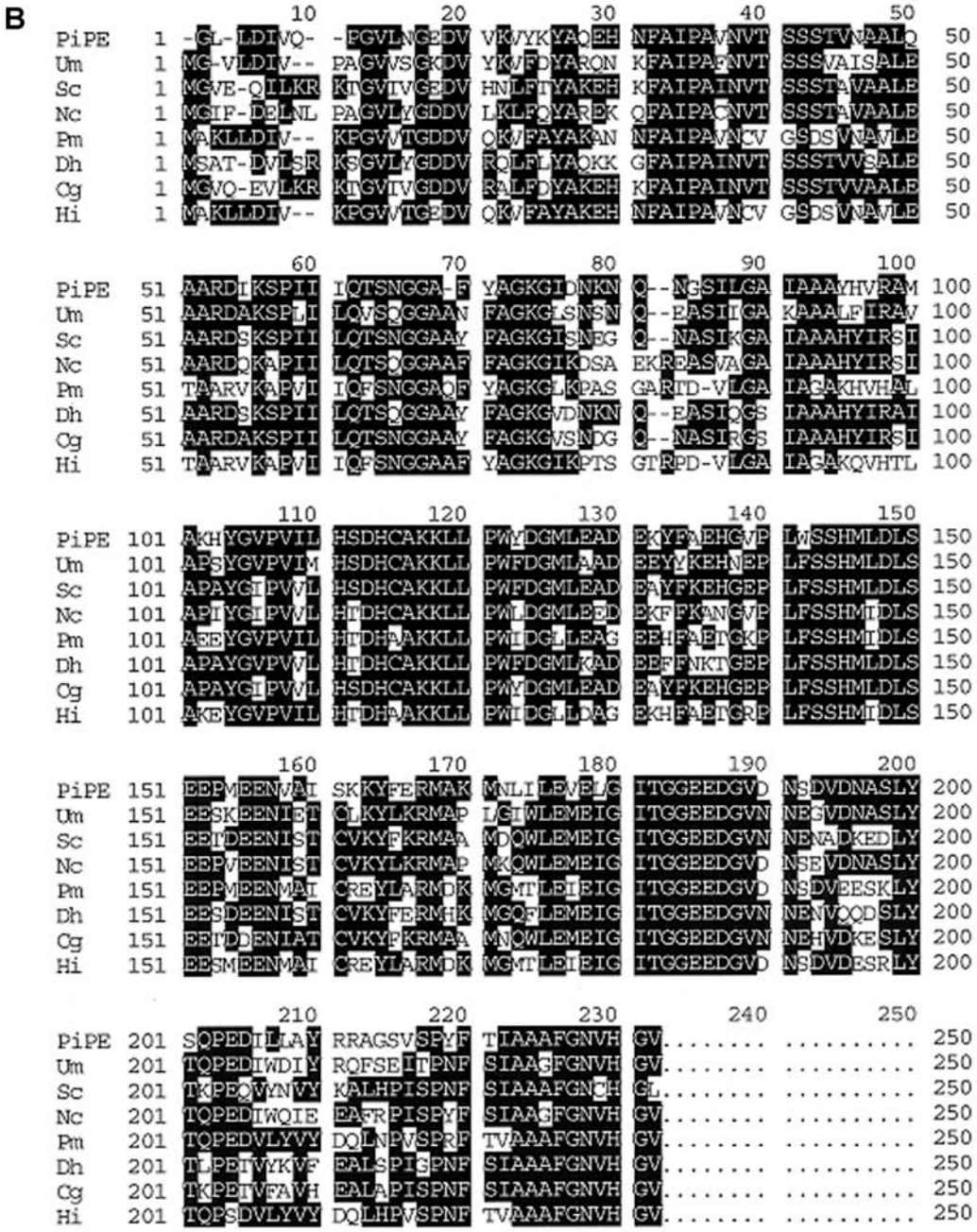

Fig.4

Figure 4 : Partial sequence alignment of the PiPE from Pi

A, Electrophoresis showed PiPE-gene (674 bp). B, Alignment analysis of PiPE-gene from *Pi*. It was shown that seven other FAB-genes were reported from fungus. Partial nucleotide sequence of the mRNA encoding the PiPE protein from *Pi* and deduced amino acid sequence. The arrowhead indicates predicted glycosylation sites. The alignments were made with the program DNAsys. Residue letters are indicated under the sequences. (PiPE: Peptide elicitor of *Pi*, Um: *Ustilago maydis*, Sc: *Saccharomyces cerevisiae*, Nc: *Neurospora crassa*, Pm: *Pasteurella multocida*, Dh: *Debaryomyces hansenii*, Cg: *Candida glabrata*, Hi: *Haemophilus influenzae).*

Genomic DNA (3 μg) of the Pi isolates Pi831 (race 1) (lanes 1 and 3) and DN101 (race 0) (lanes 2 and 4) and were digested with PstI (lanes 1 and 2) or HindIII (lanes 3 and 4) and hybridized with a probe containing a 674-bp fragment internal to the PiPE open reading frame from Pi isolate DN101. Numbers on the left are DNA size markers in bp.

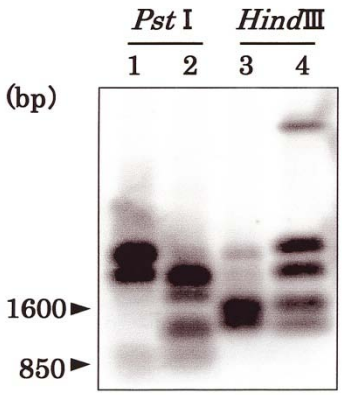

Fig.5

Figure 5 : Southern blot analysis of the PiPE gene from Pi

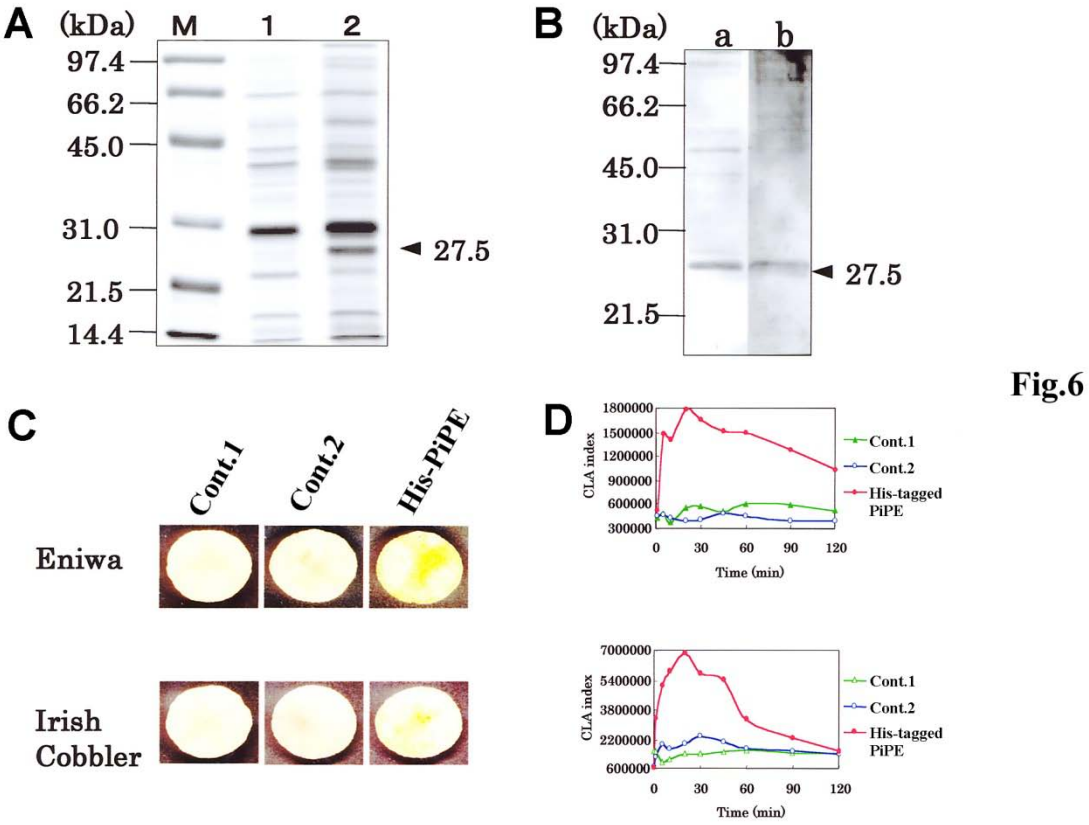

Fig.6

Figure 6 : Purification and immunodetection of the His-tagged PiPE protein

A. SDS-PAGE of the His-PiPE protein expressed in *E. coli*. Lane 1: control vector without PiPE gene sequence. Lane 2 : expressed proteins with the PiPE gene. B, Western immunoblot analysis of fusion proteins with anti-His Abs (lane a) and anti-HW-Abs (lane b). C, Elicitor assay of the affinity purified protein

expressed from *E. coli* on tuber tissue of potato cultivars. Control 1: water treatment. Control 2: Expressed fusion protein and purified from the plasmid vector alone, then applied onto the tuber disks. Fusion protein: His-PiPE. The photograph was taken 96 h after the treatment. **D,** Effect of the His-PiPE on the generation of active oxygen species in suspension cultured cells of potato cv. Rishiri (R_1-gene) and Mayqueen (r-gene). The CLA index was measured by a luminometer.

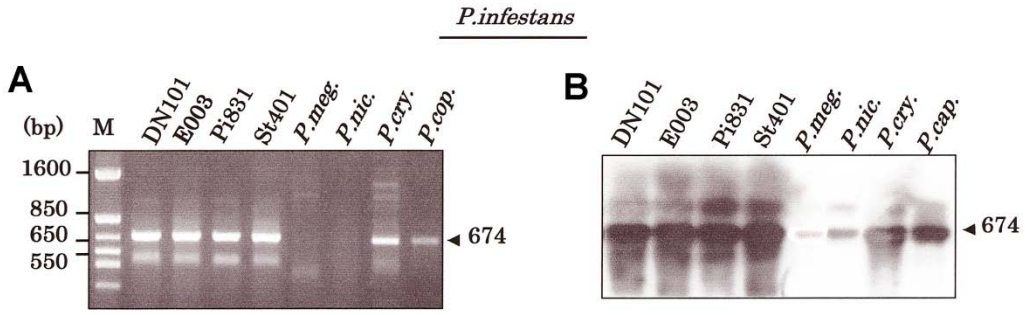

Fig.7

Figure 7 : Distribution of the PiPE genes in other *Phytophthora strains*

A, Agarose gel electrophoresis of the RT-PCR product. The templates were total RNA extracted from freshly harvested mycelial culture of (race 0) isolates, DN101 and E003, race 1 isolates Pi831 and St401; P. meg., *P. megasperma*; P. nic., *P. nicotianae*; P. cry., *P. cryptogea*; and P. cap., *P. capsici*. DNA size markers in bp are in the lane M. DNA size markers (in bp) are given in lane M. The experiments were repeated twice. The same results were obtained. **B,** RT-PCR clones were hybridized with a probe containing a 974-bp fragment internal to the PiPE open reading frame from DN101. The samples and order of loading are identical to those in (a). Agarose gel electrophoresis of cDNA encoding the protein moiety of the PiPE from Pi using RT-PCR, lane 1. Total RNA extracted from freshly harvested mycelial culture of *Pi* was used as the template with the primers indicated in Table 1. DNA size (bp) markers are in lane M. RT-PCR products were hybridized with a probe containing a 674-bp fragment internal to the PiPE open reading frame from isolate DN101. The samples and order of loading are identical to those in Figure 7A.

Table 1 : Amino acid sequences of peptides derived from the PiPE elicitor and deduced degenerate oligonucleotides used for PCR

Antigenic peptide	Sequence
PiPE N-terminus	
	[a] 1 — 21
	[c] M-G-L-L-D-I-V-Q-P-G-V-L-X[b]-G-E-D-V-V-X-V-Y
	5′ ATGGGNYTNYTNGAYATAGTG 3′
	5′ ATHGTICARCCIGGIGT 3′
PiPE digest	
	207 — 226
	L-G-S-V-S-P-Y-F-T-I-A-A-A-F-G-N-V-H-G-V
	3′ GGNATRAARTGSTARCGNCG 5′ 3′ CGNAARCCNTTRCANGTRCC 5′

[a] Numbers above ends of peptides refer to the coding sequence predicted in Fig. 5(b).

[b] X stands for unidentified amino acid.

[c] Arrows indicate sequences and orientation of the primer used for PCR.

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