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Original Articles

Functional Analysis of the Protein Encoded by the Virulence Gene *psvA* of *Pseudomonas syringae* pv. *eriobotryae*

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Summary : The *Pseudomonas syringae* pv. *eriobotryae* (*Pse*) virulence gene *psvA*, (2193 bp), has been isolated but not been functionally characterized. The *psvA* gene was divided into two parts; the N-terminal region (*psvAN*, nucleotides (nt) 1-1386), and the C-terminal region (*psvAC*, nt 1387-2193). Functional analysis of the proteins encoded by *psvAN* and *psvAC* was carried out. The PsvAC shows sequence similarity to the Ulp1 endopeptidase family, which includes small ubiquitin-like modifier (SUMO) proteases. A glutathione S-transferase-PsvAC (GST-PsvAC) fusion protein cleaved tomato SUMO (T-SUMO) *in vitro*, while GST alone did not. The enzymatic activity of GST-PsvAC was inhibited by the cysteine protease inhibitor *N*-ethylmaleimide, but not by the serine protease inhibitor phenylmethanesulfonyl fluoride. Moreover, substitution of cysteine with alanine at the predicted active site abolished PsvAC enzymatic activity. These results indicate that PsvAC acts as SUMO cysteine protease. PsvAN exhibited partial homology to effector proteins. Using a adenylate cyclase (Cya) reporter system, we showed that PsvA is translocated into plant cells. To confirm our previous report that PsvA is associated with the outer membrane, a *psvAN*-chloramphenicol acetyltransferase (CAT) fusion gene (*psvAN-CAT*) was constructed and transformed into *Pse* PE0. The PsvAN-CAT fusion protein was detected in the outer membrane and cytoplasmic fractions by Western blot analysis using an anti-CAT antibody, whereas CAT was detected only in the cytoplasmic fraction. Furthermore, an N-terminal deletion mutant of PsvAN-CAT was not detected in the outer membrane fraction. These results suggested that PsvAN might exert its function as an outer membrane protein.

Key words : *Pseudomonas syringae* pv. *eriobotryae*, Virulence gene *psvA*, SUMO protease, Subcellular localization.

Introduction

Bacterial canker, caused by *Pseudomonas syringae* pv. *eriobotryae* (*Pse*), is a serious disease of loquat trees (*Eriobotrya japonica* Lindl.) in Japan. This disease has also been reported in Australia and the United States (Lai *et al.* 1972 ; Wimalajeewa *et al.* 1978). *Pse* NAE6 harbors the 25, 52, and 60 Mdal plasmids, of which the 52 Mdal plasmid contains the virulence gene (Kamiunten 1995). The plasmid-encoded virulence gene *psvA* has been isolated and sequenced (Kamiunten 1999). The deduced PsvA protein comprised 731 amino acids and at the time had no significant similarity to any other known proteins.

Recently, we found that the C-terminal region of the PsvA protein shares significant homology with a small ubiquitin-like modifier (SUMO) protease belonging to the Ulp1 endopeptidase family. SUMO belongs to the growing family of ubiquitin-related proteins involved in posttranslational protein modification. It is present in all eukaryotic kingdoms and is highly conserved from yeast to humans. SUMO proteins covalently attach to and detach from other proteins in cells to modify their function (Melchior *et al.* 2003 ; Hotson *et al.* 2004 ; Mudgett 2005). SUMO proteases have two enzymatic activities ; a hydrolase activity that cleaves the C-terminal Gly-Gly residues

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from immature SUMOs to produce the mature forms, and an isopeptidase activity that deconjugates SUMO from SUMO-modified target proteins.

Type III effector proteins in plant pathogenic bacteria such as XopD (Hotson *et al.* 2003), AvrXv4 (Roden *et al.* 2004), and AvrBsT (Orth *et al.* 2000) have SUMO-specific protease activity. It was reported that XopD from *Xanthomonas campestris* pv. *vesicatoria* (Xcv) has a sequence similar to that of PsvA (Noël *et al.* 2002). XopD, comprising 545 amino acids, has similarity to the last 300 amino acids of PsvA. Mutation of *xopD* did not affect Xcv virulence (Noël *et al.* 2002). However, the XopD protein delays disease development in tomato leaves (Kim JG *et al.* 2008). The essential difference between PsvA and XopD is that PsvA is directly concerned with pathogenicity, while XopD is not. Although some bacterial avirulence genes encode SUMO proteases, the bacterial virulence gene encoding the SUMO protease has not been identified. Therefore, functional analysis of PsvA might clarify a new mechanism of pathogenicity in plant pathogenic bacteria. In this study, the PsvA protein was divided into two parts ; the N-terminal region (PsvAN), comprising aa 1-462, and the C-terminal region (PsvAC), comprising aa 463-731. Functional analysis of each region was carried out.

Materials and methods

Bacterial strains and growth conditions

Pse PE0 (nalidixic acid- and rifampicin-resistant), a derivative of the wild-type strain NAE6, does not contain the virulence plasmid containing the *psvA* gene. *Pse* and *E. coli* strains were cultured in YP medium (Kamiunten 1990) at 25°C and 37°C, respectively. Antibiotics were used at the following final concentrations : tetracycline, 30 μg/ml ; ampicillin, 50 μg/ml ; chloramphenicol, 50 μg/ml ; nalidixic acid, 50 μg/ml ; kanamycin, 50 μg/ml and rifampicin, 50 μg/ml.

Cloning, expression, and purification of T7-tomato SUMO-HA (T7-T-SUMO-HA) fusion protein

Total RNA was extracted from tomato leaf tissue using the SV Total RNA Isolation System (Promega), and mRNA was purified using the Oligotex-dT30 Super mRNA Purification Kit (Takara). RT-PCR was performed using the PrimeSTAR RT-PCR Kit (Takara). The 315-bp cDNA fragment of the T-SUMO gene (Hanania *et al.* 1999) was amplified using the following oligonucleotide primers : forward, 5'-GCG CCATGGCTGCTAGCGGCGGCAC, (NcoI site underlined) ; reverse, 5'-TCCCCCGGGAAAATTAGA GAAACAA (*SmaI* site underlined). The PCR product was cloned into pIVEX 2.5d to introduce a hemagglutinin tag (HA-tag) into the C-terminus of T-SUMO. The resulting plasmid was designated pIVEX 2.5d-T-SUMO. The *T-SUMO-HA* gene was amplified from pIVEX 2.5d-T-SUMO by PCR using primers with added *Bam*HI restriction sites, then digested with *Bam*HI, and cloned into pET3a to produce plasmid pET3a-T7-T-SUMO-HA. pET3a was used to generate an N-terminal T7-tag fusion protein. A schematic representation of the fusion protein T7-T-SUMO-HA is shown in Fig. 1A. The insert sequence was confirmed by DNA sequencing. The resultant plasmid was transformed into *E. coli* strain Origami (DE3) pLysS for protein expression. *E. coli* (pET3a-T7-T-SUMO-HA) was cultured in 400 ml of YP medium containing 50 μg/ml ampicillin until the OD_{600} reached 0.6. Protein expression was induced by the addition of IPTG to a final concentration of 0.4 mM and 2-3 h of additional incubation. Cells were harvested by centrifugation at 4,400 $\times$ *g* for 10 min at 4°C and the bacterial pellet was resuspended in 10 ml buffer A (10 mM KH_2PO_4, 1 mM EDTA, pH 7.0). Lysozyme was added to a final concentration of 50 μg/ml and the suspension was incubated at 4°C for 30 min. The cells were sonicated on ice and insoluble proteins were recovered by centrifugation at 12,000 $\times$ *g* for 20 min at 4°C. The pellet was resuspended in 10 ml buffer A containing 1% Triton X-100 and centrifuged at 25,000 $\times$ *g* for 20 min. The pelleted inclusion bodies were washed twice with the same buffer and twice with distilled water. The pellet was suspended in 3 ml solubilization buffer (6 M guanidine hydrochloride, 50 mM Tris pH 8.5, 10 mM EDTA) and incubated for 1 h at 37°C. The solubilized protein was dialyzed against two 1-liter changes of 50 mM Tris-HCl, pH 8.0, for a minimum of 12 h. The dialyzed solution was lyophilized, suspended in 50 mM Tris-HCl (pH 8.0), and used as a substrate for SUMO protease assays.

Cloning, expression, and purification of glutathione S-transferase-PsvAC (GST-PsvAC) fusion protein

The *psvAC* gene region was amplified with the forward primer (5'-CGGGATCCAGTGATTACGGCCGC, *Bam*HI site underlined) and the reverse primer (5'-CGGGATCCGGACGATTGCCCTGT, *Bam*HI site underlined), and cloned into the *Bam*HI site of pGEX-6P-1. The resulting plasmid pGEX-6P-1-GST-PsvAC was transformed into *E. coli* BL21. The sequence of insert was checked by sequencing. A schematic diagram of the GST-PsvAC fusion protein is shown in Fig. 1A. The transformed cells were grown in YP medium overnight and diluted 50-fold in 400 ml YP

containing ampicillin. The culture was grown until the OD_{600} reached 0.4-0.6 and protein expression was induced for 3 h with 0.4 mM IPTG. Induced cells were harvested by centrifugation and suspended in 20 ml of PBS (phosphate-buffered saline : 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). Cells were disrupted by sonication on ice. The lysate was centrifuged at 15,000 × *g* for 15 min and the supernatant was filtered through 0.45-μm filters. Fusion protein in the supernatant was purified using a glutathione Sepharose 4B column (Pharmacia) according to the manufacturer's instructions. GST-PsvAC fusion protein was eluted from the column with glutathione elution buffer. The eluted fusion protein was dialyzed for 12 h against a dialysis buffer containing 50 mM Tris-HCl, pH 7.5, and 1 mM DTT.

The full-length *psvA* gene was amplified by PCR, introduced into pGEX-6P-1, and transformed into *E. coli* BL21(DE3). The GST-PsvA fusion protein was expressed in an inclusion body. We attempted to refold GST-PsvA using conventional refolding methods. However, we could not obtain soluble GST-PsvA, and therefore the full-length PsvA protein could not be used for the protease assay.

Site-directed mutagenesis of *psvAC*

psvAC was recloned into the *Bam*HI site of pUC19, and the resulting plasmid was designated pUC19-PsvAC. Site-directed mutagenesis of the cysteine residue at the predicted protease active site was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The mutagenic primers 5'-GACGGTTATTCTGCCGGCGATCATGTG-3' and its complement were used to replace the codon for cysteine with the codon for alanine (tgc replacing gcc). The resulting mutant plasmid was designated pUC19-psvACAla, and the sequence of the insert was checked to ensure no other mutations had been introduced during amplification. The *psvACAla* gene was subcloned into the *Bam*HI site of pGEX-6p-1 and transformed into *E. coli* BL21. A schematic representation of the fusion protein GST-PsvACAla is shown in Fig. 1A. The mutated protein, GST-PsvACAla, was purified by the same procedure as the GST-PsvAC protein.

***In vitro* protease assays**

The purified T7-T-SUMO-HA protein (0.1-0.4 μg) was mixed with the purified GST-PsvAC (1.0-1.5 μg), GST (1.0-2 μg), or buffer alone (50 mM Tris-HCl, pH 7.5) and incubated at 25°C for 1-12 h in 5-10 μl reaction buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM DTT. After incubation, the proteins were subjected to SDS-PAGE, and immunoblotted with an anti-T7 monoclonal antibody (Novagen) and a peroxidase-conjugated anti-mouse secondary antibody. The immunoblotted proteins were detected by the ECL method. For the protease inhibition assay, GST-PsvAC protein was preincubated for 20 min at 25°C with the cysteine protease inhibitor N-ethylmaleimide (NEM) (5 mM) or the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 mM). GST-PsvAC treated with protease inhibitor was tested for *in vitro* proteolytic activity as described above.

Adenylate cyclase (Cya) assays

The vector pLAFR3 was used for the expression of Cya fusion proteins. pLAFR3-psvA-Cya was constructed in two steps. First, a fragment was obtained by *Eco*RI-*Hin*dIII digestion of pARO-HA-'Cya, kindly provided by Dr. Mukaihara (Murata *et al.* 2006), and cloned into the same sites of pLAFR3 to generate pLAFR3-Cya. In a second step, the *psvA* gene was amplified by PCR using the forward primer (5'-CGGATCCAATAGCGAGAAAAACTGGCC, *Bam*HI site underlined) and the reverse primer (5'-CGGGATCCCAACTTCCACCACTTGCTTT), digested with *Bam*HI, and ligated into the *Bam*HI site of pLAFR3-Cya to generate pLAFR3-psvA-Cya. The *psvA-Cya* fusion gene was checked by nucleotide sequence analysis. The resulting plasmids were mobilized by triparental mating into *Pse* PE0 and *Pse* PE0 hrpL::Km, in which a kanamycin resistance gene was inserted into the *hrpL* gene.

To determine Cya enzyme activity *in planta, Pse* strains were infiltrated into loquat leaves at an OD_{600} of 0.5-0.6 in distilled water (DW). After 24 h, leaf samples (150 mg) were collected from the infiltrated areas in microfuge tubes and immediately frozen in liquid nitrogen. The frozen samples were ground by shaking with 5mm zirconia/silica beads and resuspended in 500 μl of Biotrak assay buffer. After boiling for 5 min, cell debris was removed by two rounds of centrifugation for 10 min. The supernatants were stored at -80°C until assayed.

To determine Cya enzyme activity *in vitro*, *Pse* strains were grown in YP medium to an OD_{600} of 0.5-0.6. The cultures were centrifuged, and the pellets were washed twice with DW and resuspended in sonication buffer (20 mM Tris-HCl [pH8.0], 10 mM $MgCl_2$). The cells were lysed by sonication. The lysate were centrifuged 4600 × g for 10min. An aliquot of 20 μl was suspended in 180 μl of reaction buffer (50 mM Tris-HCl, pH 8 ; 6 mM $MgCl_2$; 0.12 mM $CaCl_2$; 0.1 mg/ml bovine serum albumin ; 2 mM ATP ; and

0.1 μM bovine calmodulin, when required). The reaction was allowed to proceed for 10 min at 30°C and was stopped by boiling for 5 min. The samples were stored at -80°C until assayed.

cAMP and protein in bacteria or leaf samples were quantified by using cAMP Biotrak Enzyme immunoassay System (Amersham) and Bio-Rad Protein Assay Kit (Bio-Rad), respectively, according to the manufacturer's directions.

Construction of the *psvAN*-chroramphenicol acetyl transferase (*psvAN-CAT*) fusion gene

To construct the *psvAN-CAT* fusion gene, *psvAN* was amplified by PCR using the forward primer (5'-CGGATCCAATAGCGAGAAAAACTGGCC, *Bam*HI site underlined) and the reverse primer (5'-CGGAATTCGCGGCCGTAATCACTCAGGG, *Eco*RI site underlined), and cloned into the *Bam*HI/*Eco*RI sites of pLAFR3. The resulting plasmid was designated pLAFR3-psvAN. The CAT gene was amplified by PCR from pHSG399 with primers (5'-CGGAATTCATGGAGAAAAAATC and 5'-CGGAATTCCAATAACTGCCTTAA, *Eco*RI sites underlined) and inserted into *Eco*RI sites of pLAFR3-psvAN. The resulting plasmid was designated pLAFR3-psvAN-CAT (Fig. 1 B) and the sequence of the insert was checked by sequencing. As a control, plasmid pLAFR3-CAT was constructed by inserting the PCR-amplified *CAT* fragment into the *Eco*RI site of pLAFR3 (Fig. 1 B).

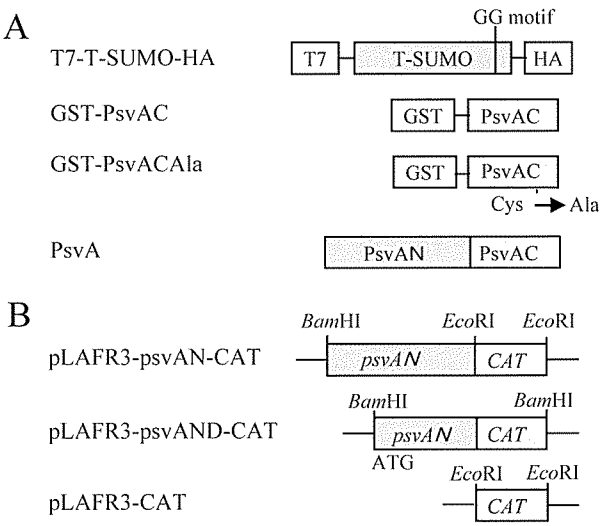

Fig. 1. Schematic representation of fusion proteins expressed in E. coli and fusion genes subcloned into pLAFR3.

A T7-T-SUMO-HA protein has a Gly-Gly (GG) motif that acts as a cleavage site for SUMO proteases. Fusion protein GST-PsvAC and mutant protein GST-PsvACAla were assayed for protease activity using T7-T-SUMO-HA protein as substrate. Cys→Ala indicates replacement of cysteine with alanine.

B *psvAN-CAT* and *psvAND-CAT*, which lacks the sequence encoding the N-terminal region of the *psvAN-CAT* fusion protein, were subcloned into plasmid pLAFR3. Constructed plasmids were introduced into *Pse* PE0 strains, and subcellular localizations of fusion gene products were analyzed by Western blotting using anti-CAT serum. pLAFR3-CAT was constructed as a control plasmid.

The N-terminal deletion mutant of pLAFR3-*psvAN-CAT* was generated by PCR using the primer pair 5'-CGGGATCCATGCCAGCGATTAAC and 5'-CGGGATCCCAATAACTGCCTTAA, (*Bam*HI sites underlined). The amplified DNA containing the initial ATG codon was digested with *Bam*HI and ligated into the *Bam*HI site of pLAFR3. The resulting plasmid, pLAFR3-psvAND-CAT, had a 459-bp deletion at the N-terminus of *psvAN-CAT* (Fig. 1 B). The sequence of insert was checked by sequencing.

Subcellular fractionation

Subcellular fractionation was carried out essentially as described previously (Kamiunten *et al.* 2004). Cultures of *Pse* PE0 strains harboring pLAFR3-psvAN-CAT, pLAFR3-CAT or pLAFR3-psvAND-CAT were grown overnight in 200 ml YP medium and harvested by centrifugation at 10,000 × *g* for 5 min. The supernatants were passed through a 0.2-μm-pore-size filter to remove the remaining bacteria. The supernatant proteins were precipitated with 50 % (w/v) $(NH_4)_2SO_4$, collected by centrifugation at 25,000 × *g* for 20 min, resuspended in 50 mM Tris-HCl (pH 8.0) and retained as the supernatant fraction. The pelleted cells were suspended in 10 ml buffer B (50 mM Tris-HCl pH 8.0, 20 % sucrose, 2 mM EDTA and 0.2 mg/ml lysozyme) and incubated at room temperature for 30 min. After centrifugation at 10,000 × *g* for 5 min, the cells were suspended in 0.01 M HEPES, pH 7.5, and disrupted by three passages through a French pressure cell. The remaining intact cells were removed by sedimentation at 10,000 × *g* for 5 min at 4°C. The supernatants were centrifuged at 200,000 × *g* for 1 h at 4°C. The resulting pellets containing the cell membranes were saved as the total membrane fractions. The supernatants were further centrifuged at 200,000 × *g* for 1 h at 4°C and saved as the cytoplasmic fractions.

For isolation of inner and outer membrane fractions, bacteria were grown in 400 ml YP medium and harvested by centrifugation as described above. The cells were suspended in 20 ml buffer C (50 mM Tris-HCl pH 8.0, 20 % sucrose, 0.2 mM dithiothreitol, and 1 mg/ml DNase I) and disrupted by three passages through a French pressure cell. The disrupted-cell suspension was treated with 0.2 mg/ml lysozyme for 30 min, diluted with two volumes 50 mM Tris-HCl (pH 8.0), and centrifuged at 10,000 × *g* for 5 min at 4°C.

The supernatants were supplemented with KCl to a final concentration of 0.2 M and centrifuged at 260,000 × *g* for 2 h. The resulting pellets containing the total membranes were suspended in 1 ml buffer D (50 mM Tris-HCl pH 8.0, 20 % sucrose, 1 mM EDTA, and 0.2 mM dithiothreitol). To separate the inner and outer membranes, the samples were layered on a 25-60 % (w/v) sucrose gradient and centrifuged at 125,000 × *g* for 24 h at 4°C. Fractions (0.5 ml) were collected and their absorbances were measured at 280 nm. The protein content of each fraction was determined using the BCA Protein Assay Kit (Pierce). The lighter and heavier peak fractions contained the inner and outer membranes, respectively.

Purity of the isolated membrane fractions and Western blotting

To determine the purity and cross-contamination of the separated fractions, activities of NADH oxidase (an inner membrane marker), malate dehydrogenase (a cytoplasmic marker), alkaline phosphatase (a periplasmic marker) and 2-keto-3-deoxy-octonate (KDO) (an outer membrane marker) were assayed using published procedures (Osborn *et al.* 1972 ; De Maagd *et al.* 1986 ; Karkhanis *et al.* 1978). The subcellular fractionated samples (10 μg protein) were separated by 15 % SDS-PAGE and analyzed by ECL Western blotting using anti-CAT serum (Sigma).

Results and Discussion

PsvAC has sequence homology to SUMO proteases

Recently, we found that PsvAC has homology with SUMO proteases, such as Ulp1 (Li *et al.* 1999) and XopD (MEROPS Protease Database http://merops.sanger.ac.uk/). A multiple sequence alignment of the peptidase unit responsible for enzyme activity is shown in Fig. 2. Like Ulp1 and XopD, the putative active site residues (His, Asp, Gln and Cys) are conserved in PsvAC, suggesting that PsvAC might have SUMO protease activity.

PsvAC has SUMO protease activity

To determine whether PsvAC has SUMO-specific protease activity *in vitro*, the purified GST-PsvAC was incubated with T7-T-SUMO-HA, subjected to SDS-PAGE, and immunoblotted with an anti-T7 antibody. A shift in mobility of T7-T-SUMO-HA was used to assess hydrolase activity. GST-PsvAC was able to cleave the T7-T-SUMO-HA fusion protein (Fig. 3A, lane 2), while buffer and GST could not (Fig. 3A, lane 1,3). These results suggest that PsvAC has SUMO protease activity.

We then investigated the effect of different protease inhibitors on the enzymatic activity of GST-PsvAC. As shown in Fig. 3B, the SUMO protease activity of GST-PsvAC was inhibited by the cysteine protease inhibitor NEM (Fig. 3B, lane 2), but not by the serine protease inhibitor PMSF (Fig. 3B, lane 3). PsvAC shows homology cysteine proteases belonging to the Ulp1 endopeptidase family, which contain four putative active-site amino acids (Fig. 2). To investigate the functional role of the active site cysteine residue in PsvAC, we used site-directed mutagenesis to replace the cysteine with an alanine residue. As shown in Fig. 3C, mutation of the cysteine residue resulted in loss of protease activity. These observations indicate that *psvAC* encodes an active SUMO cysteine protease.

In this experiment, the T-SUMO protein was used as substrate to test the C-terminal hydrolase activity of PsvA. Only two plant *SUMO* genes are present in the sequence databases ; one from tomato and one from *Arabidopsis*. In the future, however, we will have to use the SUMO protein isolated from loquat, which is the natural host of *Pse.* In plants, SUMO has been linked to suppression of induction of defense re-

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PsvAC:512  PELPQVTETSWLLDGHLHAYTNDLARRLQEESNAHLLHFADSQIVTMLNSED-EAQRNVA   570
XopD :328  PELPPVRATSWLLDGHLRAYTDDLARRLRGEPNAHLLHFADSQVVTMLSSAD-PDQQARA   386
Ulp1 :439  -DFKTLAPRRWLNDTIIEFFM-----KYIEKSTPNTVAF-NSFFYTNLSERGYQGVRRWM   491
                    ** *                          *  *   * *

PsvAC:571  LRRLVGDAVNPAPPIAFMPINRDNVHWSLLVVDRRDNHSPAAYHYDSMGTPHPH------   624
XopD :387  QRLLAGDDI---PPIVFLPINQPNAHWSLLVVDRRNKDAVAAYHYDSMAQKDPQ------   437
Ulp1 :492  KRKKTQIDK---LDKIFTPINLNQSHWALGIIDLKKK---TIGYVDSLSNG-PNAMSFAI   544
            *             * ***   ** *  *           **     *

PsvAC:625  -----QHWHAQMAAWRLGLDASQVYKMPTAIQPDGYSCGDHVLTGIEVLA             669
XopD :438  -----QRYLADMAAYHLGLDYQQTHEMPIAIQSDGYSCGDHVLTGIEVLA             482
Ulp1 :545  LTDLQKYVMEESKHTIGEDFDLIH-LDCPQQPNGYDCGIYVCMNTLYGS              592
              *          * *          ** ** **
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Fig. 2. Amino acid sequence alignment of peptidase units of PsvA, XopD from *Xanthomonas campestris*, and Ulp1 from *Saccharomyces cerevisiae*. Asterisks indicate identical residues. Putative active sites are shown in bold.

sponses by ethylene-inducing xylanase (Hanania *et al.* 1999). SUMO is also associated with abiotic stress responses (Kurepa *et al.* 2003), abscisic acid signaling (Lois *et al.* 2003), and flowering time (Murtas *et al.* 2003). To elucidate the role of the SUMO protease in plant pathogenesis, it is necessary to investigate SUMO target proteins. How SUMO protease contributes to the virulence of *P. s. eriobotryae* remains to be determined.

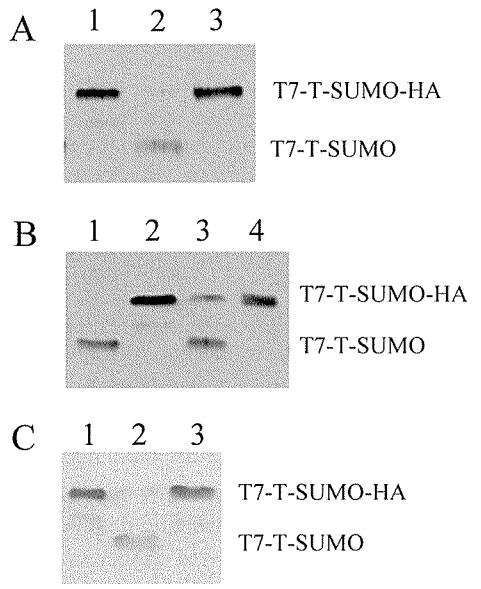

Fig. 3. Enzymatic activity of GST-PsvAC *in vitro*.

A T7-T-SUMO-HA was incubated with buffer (*lane 1*), GST-PsvAC (*lane 2*), or GST (*lane 3*). After separation by 15% SDS-PAGE, samples were analyzed by Western blotting using anti-T7 antibody.

B Effects of protease inhibitors on hydrolysis of GST-PsvAC. T7-T-SUMO-HA incubated with untreated GST-PsvAC (*lane 1*) or with GST-PsvAC pretreated with NEM (*lane 2*), PMSF (*lane 3*), or buffer (*lane 4*). Mixtures were subjected to immunoblot analysis as above.

C Protease activity of mutant GST-PsvACala with a cysteine to alanine substitution in the putative active site. T7-T-SUMO-HA was incubated with buffer (*lane 1*), GST-PsvAC (*lane 2*), or GST-PsvACala (*lane 3*). Mixtures were subjected to immunoblot analysis as above.

PsvA is translocated into plant cells

We have previously reported that the N-terminal region (aa 1-97) has 40 % sequence similarity to the avirulence A protein from *P. s.* pv. *glycinea* (Kamiunten 1999). We repeated the homology search of PsvAN using BLAST. We noticed that the N-terminal sequence (aa 1-65) of PsvAN also shows 40 % identity to the type III effector HopAT1 (Chang *et al.* 2005) from *P. s.* pv. *phaseolicola*. Furthermore, PsvAN showed partial homology to the effectors HsvG and HsvB of *Pantoea agglomerans* (Nissan *et al.* 2006). These results suggested that PsvAN might have a characteristic of type III secretion signal and structural feature of type III effector. Therefore, we examined whether PsvA is translocated into plant cells using the adenylate cyclase (Cya) translocation assay (Casper-Lindley *et al.* 2002). Protein extracts isolated from strains PE0 (pLAFR3-psvA-Cya) and PE0 hrpL::Km (pLAFR3-psvA-Cya) had little endogenous Cya activities. However, addition of calmodulin to the protein extracts from both strains restored higher levels of enzyme activity, indicating that the PsvA-Cya fusion proteins were equally expressed in the two strains and the expressed proteins possessed cyclase activity (Table 1). To assess the translocation of the fusion proteins into host cells, cAMP levels were determined 24 h after infiltration. Plant tissue infiltrated with PE0 (pLAFR3-psvA-Cya) had significantly higher cAMP levels than plant tissue infiltrated with the *hrpL* mutant strain PE0 hrpL::Km (pLAFR3-psvA-Cya). Regulation of the T3SS (type III secretion system) is known to depend on the sigma factor HrpL (Xiao *et al.* 1994). These results suggested that PsvA-Cya was translocated into plant cells by the T3SS.

Localization of CAT and PsvA-CAT proteins in *Pse*

In a previous paper (Kamiunten *et al.* 2004), we reported that PsvA was detected by immunoblotting in the outer membrane fraction of *Pse*, but not detected in the supernatant fraction. The quantity of PsvA protein secreted into culture medium might be very low

Table 1. Calmodulin-dependent adenylate cyclase activity of PsvA-Cya protein expressed *in vitro* and *in planta*

Strains	*in vitro* Cya activity (nmol cAMP/mg protein)[a)]		*in planta* Cya activity (nmol cAMP/mg protein)[a)]
	−Calmodulin	+ Calmodulin	Time (24 h after infiltraion)
PE0(pLAFR3-psvA-Cya)	8.60±0.99	4800±711.81	276.67±44.97
PE0 hrpL::Km(pLAFR3-psvA-Cya)	3.70±0.86	4800±1019.80	0.17±0.02

a) Data are mean±SD of 3 replicates.

Table 2. Activities of the marker enzymes in the cell fractions

Fractions	Activities of maker enzymes (Δ A340 or Δ 420/min per mg protein)	
P. s. pv. *eriobotryae* PE0 (pLAFR3-CAT)		
Total membrane	Malate dehydrogenase	0.073 ± 0.022
	NADH oxidase	0.226 ± 0.015
	Alkaline phosphatase	0.014 ± 0.004
Cytoplasm	Malate dehydrogenase	0.123 ± 0.026
	NADH oxidase	0.037 ± 0.008
	Alkaline phosphatase	0.012 ± 0.006
P. s. pv. *eriobotryae* PE0 (pLAFR3-psvAN-CAT)		
Total membrane	Malate dehydrogenase	0.042 ± 0.016
	NADH oxidase	0.383 ± 0.014
	Alkaline phosphatase	0.022 ± 0.009
Cytoplasm	Malate dehydrogenase	0.172 ± 0.033
	NADH oxidase	0.064 ± 0.006
	Alkaline phosphatase	0.008 ± 0.005

NADH, nicotinamide adenine dinuleotide
Data are mean ± SD (standard deviation) of three experiments

and PsvA might not be detected in the supernatant fraction by our immunoblot assay. To confirm that PsvA is associated with the outer membrane, we determined the subcellular localization of PsvAN. The C-terminal SUMO protease region (PsvAC) was replaced with a CAT protein, a cytoplasmic bacterial enzyme. Sequence analysis confirmed that the constructed *psvAN-CAT* fusion gene comprised the correct open reading frame (data not shown). Two newly constructed plasmids pLAFR3-psvAN-CAT and pLAFR3-CAT were introduced into *Pse* PE0. We obtained total membrane, cytoplasmic, and supernatant fractions from the transformed strains by differential centrifugation. The identity and purity of each fraction were determined by assaying activities of appropriate marker enzymes (Table2). The total membrane and cytoplasmic fraction showed the highest activity of their respective marker enzymes, but little activity for the other enzymes tested. These assays demonstrated that each fraction was not heavily cross-contaminated. The proteins from each fraction were subjected to Western blot analysis using anti-CAT serum (Fig. 4). For *Pse* PE0 (pLAFR3-CAT), a positive signal was only detected in the cytoplasmic fraction and not in the total membrane and supernatant fractions. However, for PE0 (pLAFR3-psvAN-CAT), the signal was detected in the total membrane fraction as well as in the cytoplasmic fraction. PsvA-CAT fusion protein could not be detected in the supernatant fraction. The total membranes were then separated into inner and outer membrane fractions by sucrose-gradient centrifugation to determine more precisely the subcellular location of PsvAN-CAT. To assess the purity of the obtained fractions, we measured the KDO content and the activity of NADH oxidase (Table3). The inner membrane fractions were enriched in NADH oxidase and deficient in KDO, and the opposite was true for the outer membrane fractions, indicating efficient separation of the inner and outer membranes. The fractions were analyzed by SDS-PAGE and Western blotting and probed with an anti-CAT antibody. The signal was only detected in the outer-membrane fraction of *Pse* PE0 (pLAFR3-psvAN-CAT), while no signal was detected in the inner and outer membrane fractions of *Pse* PE0 (pLAFR3-CAT) (Fig. 5). We then constructed an N-terminal deletion mutant of the *psvAN-CAT* gene and examined the subcellular localization of its gene product in *Pse* PE0. As shown in Fig. 5, no signal was detected in outer membrane fraction. These results indicated that the N-terminal region (aa 1-153) of PsvA plays an important role in its outer-membrane localization.

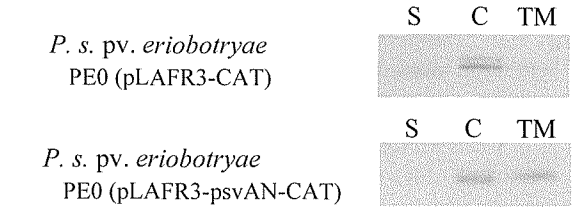

Fig. 4. Subcellular localization of CAT and PsvAN-CAT fusion protein in *Pse* PE0. Cells of *Pse* PE0 harboring pLAFR3-CAT or pLAFR3-psvAN-CAT were fractionated into supernatant (S), cytoplasmic (C), and total membrane (TM) fractions. Each fraction was subjected to immunoblot analysis using a CAT-specific antibody.

Table 3. NADH oxidase activity and KDO content in the inner and outer membrane fractions

Fractions	NADH oxidase (Δ A340/min per mg protein)	KDO (μg/ml)
P. s. pv. *eriobotryae* PE0 (pLAFR3-CAT)		
Inner membrane	0.257±0.062	8±0.816
Outer membrane	0.001±0.002	34±6.377
P. s. pv. *eriobotryae* PE0 (pLAFR3-psvAN-CAT)		
Inner membrane	0.214±0.041	13±2.160
Outer membrane	0.001±0.001	42±4.320

KDO, 2-keto-3-deoxy-octonate
Data are mean ± SD (standard deviation) of three experiments

It was reported that the effector protein SipB, one of the invasion proteins of *Salmonella enterica*, was localized to the bacterial outer membrane (Kim HG *et*

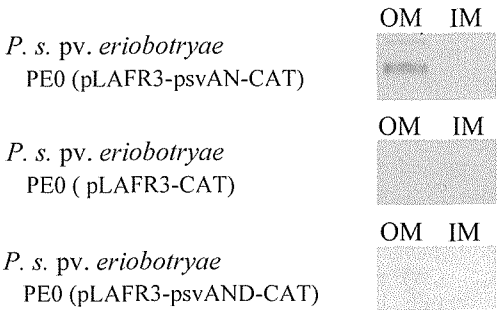

Fig. 5. Detection of the PsvAN-CAT and PsvAND-CAT fusion proteins in the outer membrane fraction. Cells of *Pse* PE0 harboring pLAFR3-psvAN-CAT, pLAFR3-psvAND-CAT, which lacks the N-terminal region of *psvAN-CAT* gene, or pLAFR3-CAT were fractionated into inner membrane (IM) and outer membrane (OM) fractions by sucrose density gradient centrifugation. Each fraction was then subjected to immunoblot analysis using a CAT-specific antibody.

al. 2008). They suggested that SipB, which was thought to be part of the translocon, was localized to the outer membrane after its secretion outside the cell. Although PsvA and SipB effectors seem to have different biological functions, we should examine whether PsvA shows similar intracellular behavior to SipB after translocation into the host cells.

Both PsvA and XopD possess SUMO protease activity in their C-terminal regions. However, PsvA is directly involved in the pathogenicity, whereas XopD is not. The structural and functional differences in the N-terminal regions of PsvA and XopD may be reflected in their pathogenic expression. The N-terminus of XopD is necessary for its subnuclear localization (Hotson *et al.* 2003). However, our results show that PsvAN plays an important role in its outer membrane localization, suggesting that the association of PsvA with the outer-membrane is important for its pathogenic expression.

In this study, we found that (1) PsvA is translocated into plant cells, (2) PsvAC has SUMO protease activity, and (3) PsvAN plays an important role in its outer-membrane localization. However, further investigations will be required to define the role and biological function of PsvA in the pathogenesis of *Pse.*

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ビワがんしゅ病細菌の病原性遺伝子*psvA*にコードされている蛋白質の機能解析

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キーワード：ビワがんしゅ病細菌，病原性遺伝子*psvA*，SUMOプロテアーゼ，細胞内局在性

要　約

ビワがんしゅ病細菌（*Pse*）の病原性遺伝子*psvA*が分離されたが，その機能は明らかにされていない．本論文では*psvA*をN末端領域（psvAN：1-1386塩基）とC末端領域（*psvAC*：1387-2193塩基）に分け，それぞれの領域にコードされている蛋白質の機能解析を行った．PsvACは低分子ユビキチン様修飾因子（SUMO）プロテアーゼを含むUlp1エンドペプチダーゼファミリーと相同性を示していた．glutathione S-transferase（GST）とPsvACの融合蛋白質（GST-PsvAC）はトマトSUMOを切断したが，GSTのみでは切断されなかった．GST-PsvACの酵素活性はセリンプロテアーゼ阻害剤のphenylmethanesulfonyl fluorideでは阻害されず，システインプロテアーゼ阻害剤の*N*-ethylmaleimideで阻害された．更に予想される活性部位のシステインをアラニンに置換するとPsvACの酵素活性が失われた．これらの実験結果はPsvACがSUMOシステインプロテアーゼとして機能することを示していた．また，PsvANはエフェクター蛋白質と部分的に相同生があり，アデニル酸シクラーゼ（Cya）レポーターシステムを用いてPsvAが植物細胞内に分泌されることを明らかにした．PsvAが細菌の外膜に関連していることを以前に報告したが，そのことを確かめるため，*psvAN*遺伝子とChloramphenicol acetyltransferase（CAT）遺伝子を融合させ，*Pse*に導入し，CAT 抗血清を用いてウエスターンブロッティングで調べた結果，PsvAN-CAT融合蛋白質は外膜と細胞質画分に検出された．なお，CATのみの場合は細胞質画分にのみ検出された．更にN末端側が欠落したPsvAN-CATは外膜には検出されなかった．これらの実験結果はPsvANが外膜局在性の役割を果たしていることを示唆していた．