日植病報 65：501-509 (1999)
Ann. Phytopathol. Soc. Jpn. 65：501-509 (1999)

Isolation and Characterization of Virulence Gene *psvA* on a Plasmid of *Pseudomonas syringae* pv. *eriobotryae*

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Abstract

A pLAFR3 cosmid clone, pVIR6, which contains a virulence gene in the 23-kb insert DNA, was previously constructed. This virulence gene originated from the 52 Mdal plasmid of *Pseudomonas syringae* pv. *eriobotryae*. Serial deletion analyses of pVIR6 indicated that *ca.* 7 kb of the insert DNA restored pathogenicity to an avirulent PE0 strain, and the deletion plasmid was designated as pKPN35. A 6961-bp insert DNA of pKPN35 was sequenced, and four possible reading frames (ORF1 480 bp ; ORF2 969 bp ; ORF3 2193 bp ; ORF4 516 bp) were found in tandem. ORF1 and ORF4 had no significant homology to known genes. ORF2 had an amino acid sequence similar to the transposase of IS5 of *E. coli*. A recombinant plasmid pNSF1 containing only the ORF3 region restored pathogenicity to the avirulent PE0 strain. However, an ORF3 mutant of pNSF1, which was constructed by deleting a 580-bp *Bss*HII segment from ORF3, failed to restore virulence to the same strain. Consequently, ORF3 was identified as a virulence gene and was named *psvA*. A HrpL-dependent promoter consensus sequence was found upstream of *psvA*. The *psvA* gene product was 731 amino acids long and had a predicted molecular mass of 83.2 kDa. The deduced protein of the *psvA* gene showed no significant similarity to any protein sequence in the data base, although it had some similarity to the N-terminal region of the *avrA* gene in *Pseudomonas syringae* pv. *glycinea*. Production of the deduced protein of the *psvA* gene was confirmed in *E. coli* by using the expression vector pET-3a. Southern hybridization analysis indicated that the *psvA* gene was conserved in *P. syringae* pathovars *myricae* and *dendropanacis* which are causal agents of woody plant galls in Japan.

(Received March 12, 1999 ; Accepted June 3, 1999)

Key words : virulence gene *psvA*, nucleotide sequence, *Pseudomonas syringae* pv. *eriobotryae*.

INTRODUCTION

Pseudomonas syringae pv. *eriobotryae* causes stem cankers on loquat trees which is a disease of considerable importance in Japan. Loquat stem canker is also reported in Australia and the USA[14,30]. In a previous paper[9], the author reported that several strains of *P. syringae* pv. *eriobotryae* contained 32, 39 and 85 Mdal plasmids. Loss of the 85 Mdal plasmid was correlated with a loss of virulence. Attempts to reintroduce the 85 Mdal plasmid into a cured strain have not been successful. Another strain NAE6 of *P. syringae* pv. *eriobotryae* contained 25, 52 and 60 Mdal plasmids. The 52 Mdal plasmid appeared to be required for virulence[12]. A pLAFR3 cosmid clone containing a 23-kb insert DNA derived from 52 Mdal plasmid, designated as pVIR6, was reintroduced into a cured avirulent PE0 strain by the use of helper plasmid pRK2013. The transconjugants which received pVIR6 regained virulence. Thus, these findings suggest that the 52 Mdal plasmid contains virulence gene(s)[12].

This paper describes the isolation and molecular characterization of a virulence gene coding on the 52 Mdal plasmid of *P. syringae* pv. *eriobotryae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions and chemicals The bacterial strains and plasmids used in this work are listed in Table 1. *P. syringae* pv. *eriobotryae* strains were routinely cultured in YP medium[9] at 25°C. *Escherichia coli* strains were cultured in the same medium at 37°C. For growth on solid media, 1.5% agar was added. The final concentrations of antibiotics were as follows: tetracycline (Tet), 30 μg/ml ; nalidixic acid (Nal), 100 μg/ml ; rifampin (Rif), 50 μg/ml ; kanamycin (Kan), 50 μg/ml ; ampicillin (Amp), 50 μg / ml ; streptomycin (Str), 50 μg / ml. Restriction enzymes, T4 DNA ligase, intestine alkaline phosphatase and polymerase, were obtained from Takara Biochemicals or Nippongene, Japan. All the enzymes were used as recommended by the suppliers.

Plant inoculation Bacteria grown on YP agar medium for 24 hr were inoculated into 1-year-old loquat stems using a needle. The inoculated plants were cov-

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Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Source or reference
E. coli		
HB101	*hsdS20* ($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	Nippongene
JM110	*rpsL* (Str^r) *thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F^- *traD36 proAB* $lacI^q$ ZΔM15]	Stratagene
DH5	*recA1 endA1 gryA96 thi-1 hsdR17 supE44 relA1*	Nippongene
BL21 (DE3)	F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal* (λ*CI857 ind1 sam7 nin5 lac*UV5-T7gene*1*) *dcm* (DE3)	Stratagene
P. syringae		
pv. *eriobotryae*		
NAE 6	Parent strain (harbored a virulence plasmid and 2 cryptic plasmids)	12
PE0	Nal^r Rif^r; avirulent NAE6 derivative cured of 3 plasmids	12
pv. *myricae*		
(302457)	A causal agent of gall of Yamamomo (*Myrica rubra* S. et Z.)	MAFF[a)]
pv. *dendropanacis*		
(SUPP453)	A causal agent of gall of Kakuremino (*Dendropanax trifidus* Mak.)	Y. Takikawa
Plasmids		
pLAFR 3	Tet^r; cosmid vector	26
pCPP46	Tet^r; broad host range vector	D. Bauer
pUC118	Amp^r; intergenic region of M13 in pUC18	29
pRK2013	Kan^r; helper plasmid	7
pPL6	Tet^r; pLAFR3 containing the *hrp* cluster of *P. s.* pv. *phaseolicola*	17
pTET40	Tet^r; pRK414 containing *iaaM* and *iaaH*	T. Yamada
pET-3a	Amp^r; pT7 protein expression vector	Stratagene
pVIR6	Tet^r; 23-kb *Bam*HI fragment from 52 Mdal plasmid in pLAFR3	12
pKPN30	Tet^r; 14-kb *Kpn*I fragment form pVIR6 in pLAFR3	This study
pKPN35	Tet^r; 7-kb *Bam*HI fragment from pKPN30 in pLAFR3	This study
pECO1	Tet^r; 3.6-kb *Eco*RI fragment from pKPN35 in pLAFR3	This study
pECO3	Tet^r; 2.7-kb *Eco*RI fragment from pKPN35 in pLAFR3	This study
pHIN1	Tet^r; 4.0-kb *Hin*dIII fragment from pKPN35 in pLAFR3	This study
pHIN3	Tet^r; 2.5-kb *Hin*dIII fragment from pKPN35 in pLAFR3	This study
pXXA1	Tet^r; 3.2-kb *Xho*I-*Xba*I fragment deletion from pKPN35	This study
pCLA1	Tet^r; 2.4-kb *Cla*I fragment deletion from pKPN35	This study
pCLA3	Tet^r; 2.4-kb *Cla*I fragment from pKPN35 in pCPP46	This study
pKUT1	Amp^r; 6.9-kb *Bam*HI fragment from pKPN35 in pUC118	This study
pNS1-KUT	Amp^r; 2.7-kb *Nru*I-*Stu*I fragment deletion from pKUT1	This study
pNSF1-KUT	Amp^r; 1.3-kb *Fba*I fragment deletion from pNS1-KUT	This study
pNSF1	Tet^r; 3-kb *Bam*HI fragment from pNSF1-KUT in pLAFR3	This study
pNSF15	Tet^r; 0.58-kb *Bss*HII fragment deletion from *psvA* in pNSF1	This study

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ered with a polyethylene bag for 12 hr to keep the humidity high. The inoculated plants were kept in a growth chamber at 25°C with a 12-hr photoperiod and observed for development of symptoms for up to 90 days after inoculation.

General DNA manipulations and DNA sequencing Plasmid extraction, restriction enzyme digestion, DNA ligation, and agarose gel electrophoresis were performed using standard procedures[22]. Plasmids were introduced into *E. coli* by transformation and into *P. syringae* pv. *eriobotryae* by triparental matings with pRK2013 as a conjugative plasmid.

Nucleotide sequence analysis was performed for both DNA strands by shotgun sequencing and custom primer walking at the Gene Analysis Center of Takara Biochemicals. The nucleotide sequence data were analyzed with DNASIS and GENETYX computer softwares. Homology searches were carried out with BLAST and FASTA programs.

Expression of ORF3 (virulence gene psvA) in E. coli Tailed primers incorporating *Bam*HI restriction site (underlined) at the 5′(5′-CGGGATCCATGTGG AATTTTAAT) and 3′ ends of ORF3 (5′-CGGGATCCC TACAACTTCCACCA) were used to amplify ORF3 from a pUC118 derivative, pNSF1-KUT. PCR was performed with a MiniCycler (MJ Research) in a final volume of 100 μl containing the template DNA (50 ng), deoxynucleotide triphosphates (200 μM each), primers (0.5 μM), 2.5 U of LA Taq polymerase (Takara Biochemicals) and the buffer supplied with enzyme. Am-

plification involved 3 cycles of 94°C for 45 sec, 37°C for 45 sec, and 72°C for 90 sec ; 28 cycles of 94°C for 45 sec, 54°C for 45 sec, 72°C for 90 sec ; a final incubation at 72°C for 5 min.

The PCR products were precipitated with ethanol, digested with *Bam*HI, electrophoresed on an agarose gel and extracted from the gel with SpinBind (FMC). The purified PCR product of ORF3 was ligated into the *Bam*HI cloning site of pET-3a and transformed into *E. coli* DH5. For expression of ORF3, the recombinant plasmid was isolated from *E. coli* DH5 with a Plasmid Midi Kit (Quiagen) and transformed into *E. coli* BL21 (DE3). Expression of ORF3 was induced by the addition of IPTG. The proteins expressed in pET-3a are known to be translationally fused to the first 11 amino acids of the phage gene 10 protein. Total cell proteins were electrophoresed on 10% acrylamide gels which were then visualized with Coomassie brilliant blue.

Hybridization experiments Isolation of total genomic DNA was performed as described previously[11]. The cosmid vector pLAFR3 was digested with *Bam*HI to convert to a single linear fragment and electorophoresed. The linearized pLAFR3 was then isolated from the agarose gel with an Easytrap Kit (Takara Biochemicals) and used as probe DNA to detect the integrated pKPN35 into chromosomal DNA. To investigate whether the virulence gene *psvA* (ORF3) was conserved in other bacteria, the purified PCR product of ORF3 was used as a hybridization probe. The labeling of probe DNA with horseradish peroxidase, hybridization and detection by exposure on autoradiography film were done using an ECL direct nucleic acid labeling and detection kit (Amersham), according to the manufacturer's instructions.

Nucleotide sequence accession number The nucleotide sequence of the 6.961 kb *Bam*HI fragment of pKPN35 has been deposited in DDBJ under accession no. AB018553.

RESULTS

Deletion analyses of pVIR6

In preliminary experiments, it became clear that *Eco*RI, *Kpn*I, *Pst*I, *Sac*I and *Xba*I had three to five cleavage sites in a 23-kb insert DNA of pVIR6. Only *Eco*RI had a cleavage site in the multi-cloning site of pLAFR3. Therefore, pVIR6 was digested with each restriction endonuclease and religated for deletion analysis. The religated molecules were transformed into *E. coli* HB101 or DH5, selecting for Tet^r cells. A total of thirty transconjugants containing plasmids smaller than pVIR6 was obtained. These plasmids were introduced into an avirulent strain of *P. syringae* pv. *eriobotryae* PE0 by triparental matings. Loquat stems were then inoculated with the PE0 transconjugants. Only one transconjugant, carrying a *Kpn*I deletion plasmid which was designated pKPN30, regained virulence. Plasmid pKPN30 was found to contain a 14-kb insert, which was cleaved with *Bam*HI to yield two 7-kb fragments. pKPN30 was cleaved with *Bam*HI and religated to yield pKPN25 and pKPN35, each containing a 7-kb fragment. Mobilization of pKPN25 and pKPN35 from *E. coli* into the avirulent PE0 strain was accomplished by triparental matings, and Tet^r transconjugants were selected. The avirulent recipient PE0 regained virulence on receipt of pKPN35. However, pKPN25 failed to restore virulence to this same strain.

Characterization of deletion plasmids

When pKPN35 was introduced into the avirulent strain PE0, the transconjugants became tetracycline resistant and regained virulence. However, the plasmid could not be detected by agarose gel electrophoresis of total genomic DNA isolated from these transconjugants [PE0(pKPN35)]. Because it seemed probable that pKPN35 was integrated into the chromosomal DNA of PE0, hybridization analysis was carried out. The labeled pLAFR3 hybridized to the chromosomal DNA of PE0 (pKPN35), not to that of the avirulent PE0 (Fig. 1). Furthermore, there were no hybridizations with the chromosomal DNA isolated from the spontaneous mutants of PE0 (pKPN35), which became tetracycline sensitive and avirulent by subculturing (data not shown).

To estimate the segregational stability of pKPN35 and pKPN30 in the PE0-colonized loquat tissue, populations of bacteria at 30 days after inoculation were measured using selective media (Table 2). The results are the average of three measurements. In the case of PE0 (pKPN35), the population of Tet^r bacteria was only 1.9×10^5 cfu. per lesion, although that of total bacteria reached 1.8×10^8 cfu. per lesion. The Tet^s colonies selected at random had lost virulence, and the chromosomal DNA of these colonies did not hybridize to the pLAFR3 probe (data not shown). In the case of PE0 (pKPN30), Tet^r bacteria were only 10^5 cfu. per lesion, whereas the total bacteria reached 1.8×10^8 cfu. per lesion. The Tet^s colonies selected at random had lost the

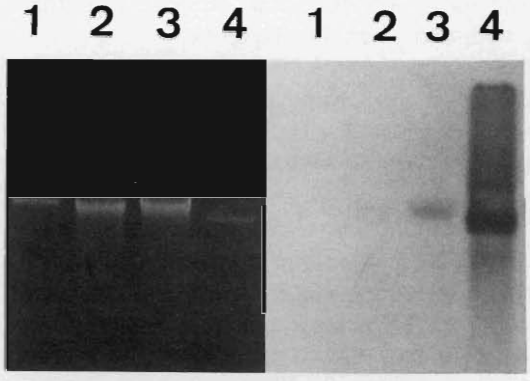

Fig. 1. Southern hybridization of total genomic DNA of *P. syringae* pv. *eriobotryae* strains with the probe pLAFR3. Lanes: 1, 60, 52 and 25 Mdal plasmids of NAE6 ; 2, total genomic DNA of PE0 ; 3, total genomic DNA of PE0 received pKPN35 by triparental mating ; 4, pKPN35 isolated from *E. coli*.

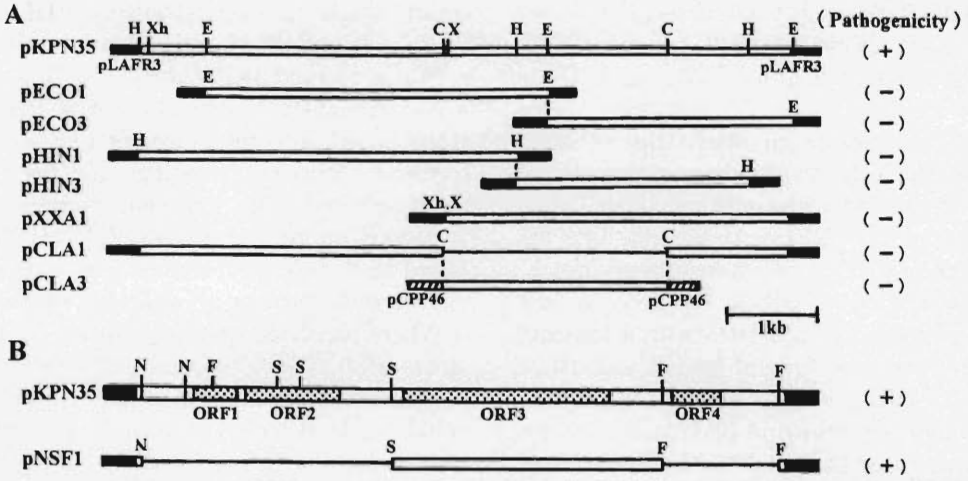

Fig. 2. Physical maps of the insert DNA of pKPN35 and scheme of virulence gene mapping. Restriction sites are abbreviated as follows: H, *Hin*dIII; Xh, *Xho*I; E, *Eco*RI; C, *Cla*I; X, *Xba*I; N, *Nru*I; S, *Stu*I; F, *Fba*I. The resulting plasmids were transferred to the avirulent PE0 by triparental matings and tested for their pathogenicity on loquat stems. The transconjugants produced (+) or did not produce (−) symptoms on loquat stems 30 days after inoculation. (A) Restriction map and various subcloned regions, (B) Position of predicted ORFs and subcloned region which restored virulence to the avirulent PE0.

Table 2. The number of bacteria in loquat stems 30 days after inoculation

Strains	Symptom	Selective antibiotics[a)]	CFU/lesion
PE0 (pKPN35)	+	Nal, Rif, Tet	1.9×10^5
		Nal, Rif	1.8×10^8
PE0 (pKPN30)	+	Nal, Rif, Tet	1.0×10^5
		Nal, Rif	1.8×10^8
PE0	−	Nal, Rif	1.0×10^4
NAE6	+	Str	1.4×10^8

a) Nal, nalidixic acid; Rif, rifampin; Tet, tetracycline; Str, streptomycin.

plasmids and had become avirulent. pKPN35 and pKPN30 may have been lost from PE0 in the loquat tissue with considerable frequency.

Subcloning and deletion analyses of pKPN35

For further characterization of pKPN35, a physical map was constructed with five restriction enzymes (Fig. 2A). pKPN35 was digested with *Eco*RI or *Hin*dIII, and the resulting fragments were subcloned into pLAFR3. The recombinant plasmids were designated pECO1, pECO3, pHIN1 and pHIN3 (Fig. 2A). A plasmid pXXA1 was constructed from pKPN35 by digesting with *Xba*I and *Xho*I, making bluntends using T4 DNA polymerase, and religated (Fig. 2A). The plasmid pKPN35 was also digested with *Cla*I and religated to yield pCLA1. pCLA3 was constructed by cloning the 2.4-kb *Cla*I fragment of pKPN35 into pCPP46 (Fig. 2A). All of the constructed plasmids failed to restore virulence to the avirulent PE0.

DNA sequencing analysis

The location of virulence gene(s) on the 7-kb of insert DNA of pKPN35 could not be ascertained by further deletion analysis or subcloning. Since the expression of virulence probably needs relatively large gene(s) or many clustered genes, a 7-kb insert of pKPN35 was sequenced. Contrary to expectation, analysis of sequence data revealed that four possible ORFs were arranged in tandem (Fig. 2B and Accession No. AB018553).

ORF1 was predicted to encode a polypeptide of 160 residues with a predicted molecular weight of 18.7 kDa and an isoelectric point (pI) of 9.57 (sequence data not shown). This coding region had a G+C content of 51%. The predicted polypeptide sequence had no significant similarity to any polypeptide sequence in the data base.

ORF2 encoded a protein with 323 amino acids and had a calculated molecular weight of 36.4 kDa. The predicted ORF2 protein had a high pI (9.8) (sequence data not shown). The G+C content of ORF2 was 57%. A GenBank data base search found significant nucleotide sequence similarities between ORF2 and *tnpA* of plasmid pPGH1 of *Pseudomonas putida* (Accession No. AFO52751). The predicted amino acid sequence of ORF2 was compared with sequences in the data base and was about 55% identical to a hypothetical protein of the *E. coli* transposable element IS5 (Accession No. A64964).

The 2790-bp nucleotide sequence that contains ORF3 is presented with the deduced polypeptide sequence in Fig. 3. This coding region had a G+C content of 50%. A putative ribosome-binding site was located five bases upstream of the deduced initiation codon. A HrpL-dependent promoter consensus sequence[31)] and potential σ^{70} promoter regions were identified upstream of ORF3. ORF3 was comprised of 2193-bp and encoded a protein of 731 amino acids with a deduced molecular weight of 83.2-kDa and a pI of 5.8. No signal peptide secretion sequence was present in ORF3. A search of the data base failed to locate known polypeptides with significant

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GTTCCTTGCCCTCCTGCAACCTCTTTTTTTCGGTTTTTTTGATCCAAGTCAATAGCGAGAAAAACTGGCCCACTGCTGGTGGAACCTAAT      90

CGCTGGAGAGGCCTACTAATGATTGTCTTGCTTAAGAGATGCGAATATTAAAATTCAAATCAACCTGTTGATTATTATTTATGGCTTTCT     180

AATGGCAAACCAGTATCGACGCTAAAATTACGGAAAACCCCAATACAGAAAAGTATATGGGTATCACAGCAAGAATAATACAAAATTATT     270

TTAACATCTCAGAGAGATTTTCTGACAGAATTGCCATCAACTAGGAGGAATATATGTGGAATTTTAATAACTGGAGCAAAGGCTTGGACG     360
                                                          M  W  N  F  N  N  W  S  K  G  L  D
CTTATCAGAGACTCCAGGAAACGCAAAGTAAACTTCATGAGTTTCTTTCTTCTGATACAACATCCTCAGTCCAACCCGATGGGGGGGGCG     450
A  Y  Q  R  L  Q  E  T  Q  S  K  L  H  E  F  L  S  S  D  T  T  S  S  V  Q  P  D  G  G  G
CGCACGACCTGCCGCAAAGACAGCGATATTCTATTCAGCAAGCTGAGTCTCAGGGCAGGCGGCTCGTAGATCAAGCAGAACTACAAACGC     540
A  H  D  L  P  Q  R  Q  R  Y  S  I  Q  Q  A  E  S  Q  G  R  R  L  V  D  Q  A  E  L  Q  T
AGGTTGAGCGCCGCTTTTCAAAAAAGTCTGAGACAAGATATGTAACGGAGGTTCAATTTGTTCCAGATCACCTTGAGGATACTGAGTATC     630
Q  V  E  R  R  F  S  K  K  S  E  T  R  Y  V  T  E  V  Q  F  V  P  D  H  L  E  D  T  E  Y
GATACAGCTCTAGAAAAGTCCCCTATTGTGAGGATATAGCAACTCATTGAAAGATTTTGCGAGGGTGCTCTGCTAGGTGGTAGCAACTCTA     720
R  Y  S  S  R  K  V  P  Y  C  E  D  I  A  L  I  E  R  F  C  E  G  A  L  L  G  G  S  N  S
ACACCGTGGACTTTTATAAAAATCAGTTGATCTCTATTAGTGACTATCTTCAGCGACAGCACATGCCAGCGATTAACGCACGTTTATTCT     810
N  T  V  D  F  Y  K  N  Q  L  I  S  I  S  D  Y  L  Q  R  Q  H  M  P  A  I  N  A  R  L  F
CTGATAGTCTTGAAAGCGATTTAAAACAGTATGCGTTTCAAAACAATCGCAGTGATACGCTGGCAATTATAGGTCACTTACGACGTATTG     900
S  D  S  L  E  S  D  L  K  Q  Y  A  F  Q  N  N  R  S  D  T  L  A  I  I  G  H  L  R  R  I
AATCTAACAAACACGGGGTCAGTGCTATTCTGCCTTTCAAAACAAAAAGTTCAGATTTAGACGAATGGTTAATCGATCAAGTGTTTTCCG     990
E  S  N  K  H  G  V  S  A  I  L  P  F  K  T  K  S  S  D  L  D  E  W  L  I  D  Q  V  F  S
ACGAAAACCAGACTACTTCCTCATATAGATCAACTTTGCGCGCGCTCAGTCATTGGCTCGCAGCACAAGAAAAGCCGGGGGCTGTGTGATC    1080
D  E  N  Q  T  T  S  S  Y  R  S  T  L  R  A  L  S  H  W  L  A  A  Q  E  K  P  G  L  C  D
CGGACTATCTCCATTCCCATGAATTGACGGAAGATGTGCTGAAGTTCAGCTGTTTGCCAGGCCGTCACCAATGTAGCGCGGCGTTGCAAC    1170
P  D  Y  L  H  S  H  E  L  T  E  D  V  L  K  F  S  C  L  P  G  R  H  Q  C  S  A  A  L  Q
ACATGCGAAACTATGACCTTGGTAGTAAAGTTCGCCTAAAGAAGCAACGTGATACCCGTAATATCCCAGACGAGGACCAAACGTTAATCT    1260
H  M  R  N  Y  D  L  G  S  K  V  R  L  K  K  Q  R  D  T  R  N  I  P  D  E  D  Q  T  L  I
CGCACTACCAAAAAATTGCTAACGATGCCTTGGTAATAAAAAATAGTAAAGCCGGAAAAAAAAACGAATCGTGATCCTCATGGAAGAACGA    1350
S  H  Y  Q  K  I  A  N  D  A  L  V  I  K  N  S  K  A  G  K  K  T  N  R  D  P  H  G  R  T
GCGTTGACAAGTACGCGTCTGTACTACGCTCATTCAGCGCGTGGCTTAAGGAGGAGGGAAAAGGAAGCTTGTCAACTCTTCTTCACGATC    1440
S  V  D  K  Y  A  S  V  L  R  S  F  S  A  W  L  K  E  E  G  K  G  S  L  S  T  L  L  H  D
CAGAGTTGGATACGTATAGGGATTTATGGACGCACAACAAAAGCTCTTCTAATGCCAAAACGGTTGTAACCCTGCTAATAAAATTGCGTG    1530
P  E  L  D  T  Y  R  D  L  W  T  H  N  K  S  S  S  N  A  K  T  V  V  T  L  L  I  K  L  R
AAATATTTCCACCCTTCTCAGTAGAAGCGGTACAAGAACCTAGTCACTCCTCTTTTACGTTGCCAAATTCAGAGTGGTCAGGTTGGGGCT    1620
E  I  F  P  P  F  S  V  E  A  V  Q  E  P  S  H  S  S  F  T  L  P  N  S  E  W  S  G  W  G
GGAATCCAGATACGCCGCAATACCCCCCTCAAAGTCCAGCTTCGACCTTCAACGGACTCTCCTCCCTGAGTGATTACGGCCGCGAATTCG    1710
W  N  P  D  T  P  Q  Y  P  P  Q  S  P  A  S  T  F  N  G  L  S  S  L  S  D  Y  G  R  E  F
ACCTCAATACGCCCCAGCAAGAGCAGCCGTGGAGCACCTATGGGGACTATGGCACTCAGGCTACAATGGAGCACTCGGCCCTGCCCCCCA    1800
D  L  N  T  P  Q  Q  E  Q  P  W  S  T  Y  G  D  Y  G  T  Q  A  T  M  E  H  S  A  L  P  P
TGAGTCCCGAGAGGATCGATGTGGACAATCTGCCGTTTCCCCAGGACGTCGAAGACCCCGAGCTGCCTCAAGTGACTGAGACTTCGTGGC    1890
M  S  P  E  R  I  D  V  D  N  L  P  F  P  Q  D  V  E  D  P  E  L  P  Q  V  T  E  T  S  W
TGCTAGACGGACATTTGCACGCCTACACCAACGACCTAGCTCGCCGATTGCAAGAGGAGTCCAATGCCCATTTACTCCACTTTGCCGACT    1980
L  L  D  G  H  L  H  A  Y  T  N  D  L  A  R  R  L  Q  E  E  S  N  A  H  L  L  H  F  A  D
CGCAAATAGTCACTATGCTGAACTCCGAGGATGAAGCACAGAGAAACGTTGCGTTGCGCCGTCTAGTCGGAGACGCAGTCAACCCTGCCC    2070
S  Q  I  V  T  M  L  N  S  E  D  E  A  Q  R  N  V  A  L  R  R  L  V  G  D  A  V  N  P  A
CACCCATCGCGTTCATGCCCATCAACCGAGATAACGTCCATTGGTCGCTTCTCGTCGTCGACCGTCGAGATAACCACTCGCCTGCAGCCT    2160
P  P  I  A  F  M  P  I  N  R  D  N  V  H  W  S  L  L  V  V  D  R  R  D  N  H  S  P  A  A
ACCATTACGATTCCATGGGAACTCCGCATCCACATCAGCACTGGCATGCCCAAATGGCAGCCTGGCGCCTGGGCCTTGATGCTTCGCAAG    2250
Y  H  Y  D  S  M  G  T  P  H  P  H  Q  H  W  H  A  Q  M  A  A  W  R  L  G  L  D  A  S  Q
TCTATAAAATGCCCACCGCCATACAGCCGGACGGTTATTCTTGCGGCGATCATGTGCTGACCGGTATAGAGGTGTTGGCTCATAGGGTGA    2340
V  Y  K  M  P  T  A  I  Q  P  D  G  Y  S  C  G  D  H  V  L  T  G  I  E  V  L  A  H  R  V
TCGACGGCATGTTCGATTACGCGGGCGGCAAGGACCTGAGCGATATCAAGCCAGACCGCGACTTCATCAGGGATCGTCTTGCCCCAGCGG    2430
I  D  G  M  F  D  Y  A  G  G  K  D  L  S  D  I  K  P  D  R  D  F  I  R  D  R  L  A  P  A
ATCAAGCGCCAGCAGAAAGCAGCGTCAGGTCAGTTCCCGAGCCGCCCGTCGAACAGAAGAAAAAGAAAAGCAAGTGGTGGAAGTTGTAGC    2520
D  Q  A  P  A  E  S  S  V  R  S  V  P  E  P  P  V  E  Q  K  K  K  K  S  K  W  W  K  L  *
GGCTTGATGTATAGGAATTTCAAAGTTTCTATACAGGGCAATCGTCCCCAAAAGTCACCTCTGCGTGATGCCGATGTGGCGTTGAGGCTG    2610
GTGAAACGCTTTTCTCACGCCACATCGGCGTCTAAAAACGGGGCCATTGCGACGTTCTGTGTAGTGGTCAACTGATCCCGGACACGACGT    2700
TAAGGTTGAGGGGGCGCCAGTCCACCTTTGAACTGGTGTGGTCGAATCCAGTTGTAACGATGCATCAGATAATGACTGATATCCCGCTGA    2790
```

Fig. 3. Nucleotide and deduced amino acid sequence of ORF3. Stop codon is indicated by asterisk. Potential ribosome binding site is underlined. HrpL-dependent promotor consensus sequence is double underlined, and potential σ^{70} promoter regions is overlined.

```
                10        20        30        40        50        60
A: MWNFNNWSKGLDAYQRLQETQSKLHEFLSSDTTSSVQPDGGGAHDLPQRQRYSIQQAESQ
   ::: .. :..: ::.   :.:.  ...   :. :..::.: . ::  : ..: :::::..
B: MWNVSKSSNNLGAYKLPLEAQTPPEKISPFDAMSAAQPEGKAPHDQLQNDQYPIQQAEDR

                70        80        90      97
A: GRRLVDQAELQTQVERRFSKKSETRYVTEVQFVPDHL
   ::.::.:::.:..:..  ::  :   .:..: : ..:
B: GRHLVEQAEIQAHVQHCHSKAPEIGDATKTQSVSEKL
```

Fig. 4. Comparison of amino acid sequences of the N-terminal regions (1 to 97) of ORF3 (A) and the *avrA* gene of *P. syringae* pv. *glycinea* (B). Colons and periods indicate identical and similar amino acids, respectively.

homology with the putative protein encoded by ORF3. However, the amino acid sequences of the N-terminal region (1-97) of ORF3 and the *avr*A gene of *P. syringae* pv. *glycinea*[18] resembled each other (Fig. 4). The identity is 40%.

ORF4 was 516-bp long and encoded a potential

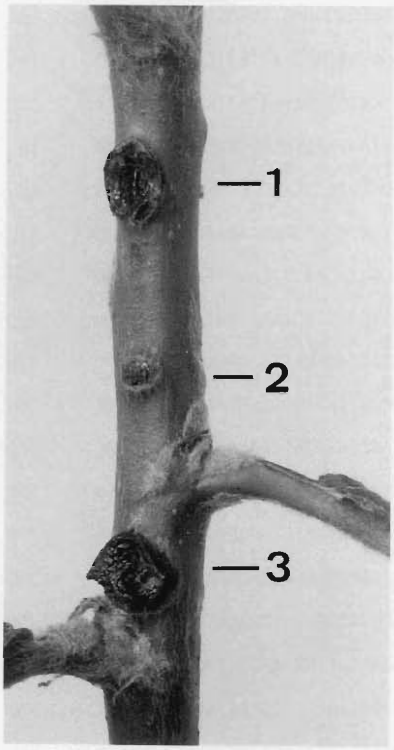

Fig. 5. Symptoms induced on a loquat stem by the derivative strains of *P. syringae* pv. *eriobotryae*. The stem was photographed 30 days after inoculation. (1) PE0 containing pNSF1, (2) avirulent PE0 strain derived from NAE6, (3) parent strain NAE6.

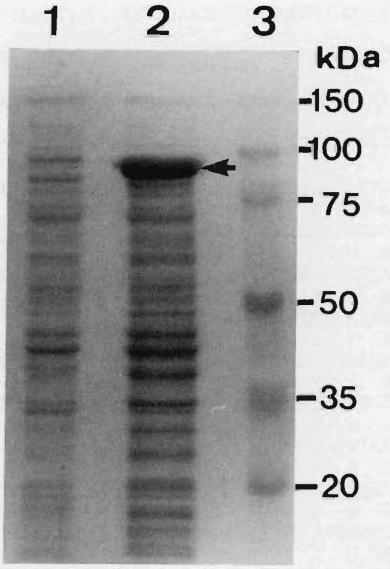

Fig. 6. Expression of *psvA* (ORF3). Proteins from crude cell lysate of *E. coli* BL21 (DE3) containing pET-3a with the *psvA* insert were separated on a 10% SDS polyacrylamide gel and stained with Coomassie blue. Lanes: 1, protein pattern of cells which have grown without IPTG; 2, protein pattern of cells which had been induced with IPTG; 3, molecular weight standards. The arrow indicates the synthesis of an 83-kDa protein.

18.9-kDa protein with 172 amino acids (pI 7.0) (sequence data not shown). This coding region had a 43% G+C content. The predicted peptide sequence of ORF4 had no significant similarity to known protein sequences.

Construction of plasmid carrying ORF3 (psvA)

From the results of the subcloning and sequencing analyses of pKPN35, ORF3 might be a virulence gene. Therefore, construction of the recombinant plasmid containing only the ORF3 region was attempted in two steps on the basis of the physical map of pKPN35 (Fig. 2B). First, a pUC118 derivative, pKUT1, which contained a 6.9-kb insert fragment from pKPN35, was digested with *Nru*I and *Stu*I to remove the ORF1 and ORF2 regions. End-blunt ligation was performed. A resulting deletion plasmid pNS1-KUT contained ORF3 and ORF4. Second, pNS1-KUT, which had been replicated in *E. coli* JM110 deficient in Dam methylase, was digested with Dam-sensitive *Fba*I to remove the ORF4 region, then religated. The resulting plasmid pNSF1-KUT contained only the ORF3 region. The 3-kb *Bam*HI insert fragment of pNSF1-KUT was subcloned into pLAFR3, and the recombinant plasmid was designated as pNSF1 (Fig. 2B). A 0.58-kb *Bss*HII fragment was deleted from ORF3 in pNSF1 to construct a deletion mutant pNSF15. The plasmid pNSF1 was able to confer wild-type pathogenicity to the avirulent PE0 (Fig. 5). The pNSF15, however, falied to restore virulence to the same strain. These results indicated that ORF3 is a virulence gene of *P. syringae* pv. *eriobotryae* and was designated as *psvA* (*Pseudomonas syringae* virulence).

Expression of the virulence gene psvA in E. coli

A single colony of *E. coli* BL21 (DE3) containing pET-3a with the *psvA* insert was grown in YP medium until the OD_{600} reached 0.6. IPTG was then added to the culture to a final concentration of 0.4 mM, and the incubation was continued for 3 hr. The cells were harvested by centrifugation and resuspended in a 15% culture volume of 125 mM Tris-HCl (pH 6.8), 40% SDS, 20% glycerol, 0.01% BPB and 10% β-mercaptoethanol. After boiling for 5 min, the mixture was centrifuged. The supernatant was analyzed by SDS polyacrylamide-gel electrophoresis. Lysate of IPTG-induced cells contained a *ca.* 83-kDa protein (Fig. 6). The molecular weight of the newly synthesized protein was in close agreement with the size predicted from *psvA* of 83.2 kDa. The 83-kDa, newly synthesized protein was not detected in the IPTG-induced cell lysate of *E. coli* BL21 (DE3) which contained only pET-3a.

Homology of psvA with DNA from other plant pathogenic bacteria

Southern hybridization was carried out to determine whether *psvA* could have sequences homologous to genes carried in other pathovars, or other plant pathogenic bacteria. The total genomic DNA digested with *Bam*HI was electrophoresed, and Southern transfers were hybridized with the *psvA* probe. Hybridization

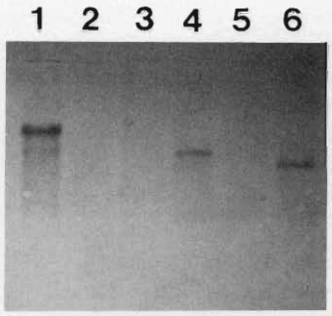

Fig. 7. Southern hybridization analysis of total genomic DNA from *P. syringae* pathovars using the virulence gene *psvA*. Lanes: 1, *P. syringae* pv. *eriobotryae* NAE6; 2, *P. syringae* pv. *syringae* 301432; 3, *P. syringae* pv. *actinidiae* 302146; 4, *P. syringae* pv. *dendropanacis* SUPP453; 5, *P. syringae* pv. *daphniphylli* SUPP451; 6, *P. syringae* pv. *myricae* 302457.

occurred with a *Bam*HI-fragment of *P. syringae* pv. *myricae* 302457[19], pv. *dendropanacis* SUPP453[20] and pv. *eriobotryae* NAE6 (Fig. 7). No hybridization occurred with the digested DNA of *Pantoea agglomerans* pv. *millettiae* Wist801, *Erwinia carotovora* subsp. *carotovora* N7133, *Agrobacterium tumefaciens* Ku7411 and *Xanthomonas oryzae* pv. *oryzae* T7174 (data not shown).

DISCUSSION

The virulence of *Agrobacterium tumefaciens* and *P. savastanoi* pv. *savastanoi* is due to the presence of the Ti[27,28] and pIAA[6] plasmids, respectively. The functions of these plasmids are the synthesis of cytokinines and of auxins. Virulence plasmids of other phytopathogenic bacteria have also been reported. The chlorosis-inducing phytotoxin, coronatine, is plasmid-borne in several pathovars of *P. syringae*[1,2,25]. In *Ralstonia* (*Pseudomonas*) *solanacearum*, the *hrp* gene cluster is on a megaplasmid[4,5]. In preliminary hybridization experiments, pTET40 containing *iaaM* and *iaaH*[33] and pPL6[16] containing the *hrp* gene of *P. syringae* pv. *phaseolicola* did not hybridize with the 52 Mdal plasmid. Besides, *P. syringae* pv. *eriobotryae* is not known to produce coronatine. Therefore, the function of the 52 Mdal plasmid of *P. syringae* pv. *eriobotryae* might be different from that of the already known virulence plasmids of phytopathogenic bacteria.

The deletion plasmids pKPN30 and pKPN35 contained 14 kb and 7 kb of insert DNA, respectively. When the deletion plasmids were introduced into the avirulent strains of *P. syringae* pv. *eriobotryae* PE0 from *E. coli* by triparental mating, the pKPN30 existed as an extrachromosomal DNA and restored pathogenicity to the recipient. The pKPN35, however, could not be detected as an extrachromosomal DNA in the recipient in spite of the restoration of pathogenicity. It was suggested from the hybridization analysis that pKPN35 was integrated into the chromosomal DNA of the PE0. The total bacterial cells of transconjugants in the inoculated tissue reached levels of 10^8 cells after 30 days of inoculation. However, the PE0 strain maintaining pKPN30 or pKPN35 reached levels of 10^5 cells. The extremely slow development of symptoms after 1 or 2 months after inoculation with PE0 (pKPN30) or PE0 (pKPN35) might be due to the loss of the plasmids.

It became clear that the virulence gene(s) was encoded in the 7 kb of insert DNA of pKPN35. However, seven kinds of derivative plasmids constructed from pKPN35 by deletion or subcloning could not restore pathogenicity to the avirulent PE0 (Fig. 2A). At that time, getting a further subcloned plasmid containing the virulence gene(s) seemed to be difficult. Therefore, sequence analysis of the 7-kb insert DNA was carried out. The result indicated that insert DNA carried four possible open reading frames.

It became evident after the sequence determination that seven kinds of derivative plasmids constructed from pKPN35 contained ORF1, ORF2, ORF4 or a part of ORF3. However, these plasmids failed to restore virulence to the avirulent PE0. None of the plasmids contained the entire ORF3. Therefore, ORF3 might be closely related to pathogenicity. In fact, the plasmid pNSF1 containing only the ORF3 region restored the wild-type virulence to the avirulent PE0. Furthermore, deletion of 0.58-kb *Bss*HII segment from ORF3 in pNSF1 resulted in avirulence. Thus, it became evident that ORF3 is a virulence gene of *P. syringae* pv. *eriobotryae*. The author then designated this gene as *psvA*.

P. syringae and other phytopathogenic bacteria possess *hrp* genes needed for basic pathogenicity. *hrp* mutants are unable to multiply in susceptible plant tissue and fail to elicit the hypersensitive reaction (HR) in resistant plants[3,8]. In Southern analysis, a region homologous to the *hrp* gene of pPL6[16] was observed in chromosomal DNA of the avirulent PE0 (data not shown). The avirulent PE0 could elicit HR in tobacco leaves as well as in the parent strain NAE6. The results suggested that *psvA* is one of the virulence genes of *P. syringae* pv. *eriobotryae*.

The putative *avr* and *hrp* promoter consensus sequence of *P. syringae*[31] and the *E. coli* consensus promoter sequence were found upstream of *psvA*. It is unknown whether these sequences function as a transcripional initiation signal. However, the presence of an HrpL-dependent promoter consensus sequence would suggest that the product of *psvA* is dependent upon the type III protein translocation system encoded by the *hrp* gene cluster in the strain's genome. It will be of special interest to investigate the phenotypic interdependence of *psvA* and *hrp* genes.

Using the research tools (SOSUI and PSORT) in GenomeNet, the deduced amino acid sequence of *psvA* was predicted to code for a soluble protein. Its localization site is cytoplasm. Comparison of the overall amino acid sequence encoded by *psvA* did not show significant

homology to those of other known proteins. However, the putative peptide encoded by *psvA* showed partial similarity to the *avrA* gene from *P. syringae* pv. *glycinea*[18]. It is known that the *avr* genes play a role in virulence on a susceptible host[13,17,24]. These avirulence genes appear to be essential for the growth of the pathogen in the host plants. The derivative strain cured of the 85 Mdal plasmid could not multiply in the loquat tissue, and the bacterial cells were distorted[10]. Structural homology existed between the 85 Mdal plasmid and the *psvA* gene[12]. The PE0 cured of 52-Mdal plasmid containing the *psvA* also could not multiply in the loquat tissue. The *psvA* gene might then affect the multiplication of *P. syringae* pv. *eriobotryae* in the loquat tissue. However, whether the failure to propagate in the host tissue is a cause or a result of the loss of pathogenicity is unclear. It will be interesting to study the functional relationship between *psvA* and the avirulence gene.

The G+C content of *P. syringae* is in the range of 59-61%[21]. The G+C content of *psvA* (50%) was relatively low by comparison. It could be implied that an IS5-like element (ORF2) located adjacent to the *psvA* assumes one end of responsibility of horizontal transfer of *psvA*, as suggested in the transfer of IAA biosynthesis genes[15,32]. Southern hybridization analysis indicated that the *psvA* gene was conserved in two pathovars of *P. syringae* which induced galls on woody plants in Japan. Recently, an IS5-like element was also found in the coronatine biosynthetic gene cluster of a plasmid in *P. syringae* pv. *glycinea*[23]. It is interesting to speculate about the potential role of IS5-like elements in the transfer of virulence genes.

The roles of ORF1 and ORF4 in the expression of pathogenicity were not obvious from the present study. The functions of *psvA* gene are currently under investigation.

The author thanks Y. Takikawa, and T. Yamada for the providing the plasmids pCPP46, pPL6, and pTET40, respectively.

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和 文 摘 要

上運天博 : *Pseudomonas syringae* pv. *eriobotryae* プラスミド上の病原性遺伝子 *psvA* の分離と解析

ビワがんしゅ病菌 (*Pseudomonas syringae* pv. *eriobotryae*) の 52 Mdal プラスミド由来の病原性遺伝子を含む DNA 断片 (23 kb) を pLAFR 3 に挿入して得られた pVIR 6 から病原性遺伝子を単離するためサブクローニングや欠失を試みた。その結果，挿入断片が約 7 kb で，病原性を有する pKPN 35 が得られた。この挿入断片の塩基配列を調べた結果，全長が 6961 bp で，4 つのオープンリーディングフレーム (OPF) の存在が示唆された。ORF 1 (480 bp) と OPF 4 (516 bp) は既知の遺伝子と相同性はなかった。ORF 2 (969 bp) の塩基配列から想定されるアミノ酸配列は大腸菌 IS 5 のトランスポザーゼと相同性が認められた。ORF 3 (2193 bp) 領域のみを含む pNSF 1 は 52 Mdal プラスミドが欠落し，病原性を失ったビワがんしゅ病菌 PE 0 に病原性を回復させた。しかし，pNSF 1 の ORF 3 から 580 bp の *Bss*HII 断片を欠失させたプラスミドは病原性を回復させることはできなかった。以上の結果は ORF 3 が病原性遺伝子であることを示しており，*psvA* と命名した。*psvA* の上流には HrpL-dependent promoter の共通配列が認められた。*psvA* の塩基配列から想定されるタンパク質 psvA は 731 のアミノ酸からなり，その分子量は 83.2 kDa であった。psvA は既知のタンパク質と相同性は認められなかったが，*Pseudomonas syringae* pv. *glycinea* の *avrA* 遺伝子の N 末端領域との相同性が認められた。*psvA* を発現用ベクター pET-3 a に組み込み，大腸菌でのタンパク質発現を調べた結果，psvA の想定分子量とほぼ同じ分子量を有するタンパク質の発現が認められた。サザンハイブリダイゼーション分析により，*psvA* がヤマモモこぶ病菌 (*P. syringae* pv. *myricae*) と カクレミノこぶ病菌 (*P. syringae* pv. *dendropanacis*) にも保持されていることが明らかになった。