〔宮崎大学農学部研究報告 56(1−6)(2010)
Bulletin of the Faculty of Agriculture, University of Miyazaki 56(1−6)(2010)〕

Original Articles

Expression of *Pseudomonas syringae* pv. *eriobotryae psvA* Gene, Containing Hrp Box-like Motif, Is Not Regulated by HrpL

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(Accepted on November 26, 2009)

Summary : A conserved promoter sequence, the hrp box, is located upstream of many *hrp* (hypersensitive reaction and pathogenicity) and *avr* (avirulence) genes in *Pseudomonas syringae*. Expression of these genes is positively regulated by *hrpL*, which encodes an alternate sigma factor. An hrp box-like motif was found upstream of the virulence gene *psvA* isolated from a plasmid of *P. s.* pv. *eriobotryae*. To investigate whether the expression of *psvA* is regulated by *hrpL*, *hrpL* mutants were constructed by marker exchange mutagenesis using a suicide vector pUC19LK, which contained *hrpL* interrupted by insertion of the kanamycin resistance gene (Km^r). Marker-exchanged *hrpL* mutants were confirmed by Southern hybridization and PCR analyses. The *hrpL* mutants had lost their virulence, and the expression of *hrpZ* containing the hrp box was not detected by RT-PCR, confirming that the *hrpL* gene was mutated. Western blot and RT-PCR analyses showed that *psvA* was expressed in *hrpL* mutants as well as the wild-type strain. Our data suggest that expression of *psvA* is not regulated by HrpL.

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

Introduction

Pseudomonas syringae pv. *eriobotryae* is the causal agent of stem cankers of loquat trees *(Eriobotrya japonica* Lindl.). All strains cause cankers on the stem, and some strains also cause halo symptoms on the leaf. On the basis of their pigment production and pathogenicity to loquat leaves, the canker bacteria have been classified into three groups as follows : group A, which does not produce pigments and is not pathogenic to leaves ; group B, which does not produce pigments but is pathogenic to leaves ; and group C, which produces pigments and is not pathogenic to leaves (Morita 1978). The virulence gene *psvA* was isolated from a 52 Mdal plasmid in *P. s.* pv. *eriobotryae* NAE6 (group A) and sequenced (Kamiunten 1999). *psvA* consists of 2193 bp encoding a protein of 731 amino-acids. Interestingly, hrp box-like and σ^{70}-like motifs were found upstream of the *psvA* gene. In *P. syringae*, the HrpR-HrpS complex activates expression of the *hrpL* promoter. In turn, the HrpL alternate sigma factor activates *hrp* and *avr* genes by recognizing a consensus sequence motif, the *hrp* box, located upstream of these genes (Ferreira *et al.* 2006 ; Fouts *et al.* 2002 ; Hutcheson *et al.* 2001 ; Thwaites *et al.* 2004 ; Xiao *et al.* 1994a, 1994b).

In this study, we investigated whether expression of *psvA*, which contains an hrp box-like motif in the promoter region, is regulated by HrpL in *P. s.* pv. *eriobotryae*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

P. s. pv. *eriobotryae* NAE6 and its derivatives were grown at 25 °C in YP medium (Kamiunten 1990), King B medium (King *et al*. 1954), or minimal medium (Puri *et al*. 1997). *Escherichia coli* strains were grown at 37 °C in YP medium. For growth on solid medium, 1.5% agar was added. Concentrations of antibiotics were as follows : kanamycin, 50 μg/ml ; nalidixic acid, 50 μg/ml ; rifampicin, 50 μg/ml. For pathogenicity tests, young stems of loquats were pricked with a needle that had been immersed in a

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bacterial suspension containing 10^{8-9} cells/ml. Inoculated plants were placed in a growth chamber (25 °C, 12 h light/12 h dark). Symptoms were assessed at 25 days after inoculation.

Recombinant DNA techniques

The *hrpL* gene was amplified from genomic DNA of NAE6 by PCR with forward primer HrpL-f (5'-CGGATCCTTGGCTGGCACGGTTATC, *Bam*HI site underlined) and reverse primer HrpL-r (5'-CGGATCCGACAGATATCCACTCAGG, *Bam*HI site underlined), which were outside the ORF. The 639-bp PCR product was cloned into the *Bam*HI site of pUC19 generating pUC19L. The kanamycin-resistance (Km^r) gene was amplified from R388(ts) :: Tn5 (Sasakawa & Yoshikawa 1987) using forward primer Km-f (5'-TCCCCCGGGCTTGCAGTGGGCTTACAT, *Sma*I site underlined) and reverse primer Km-r (5'-TCCCCCGGGTAGAAGGCGGCGGTGGAATCGAAA, *Sma*I site underlined), which were outside the ORF. The amplified 1,086-bp fragment was digested with *Sma*I and inserted into the internal *Nru*I site of the *hrpL* gene in pUC19L, generating pUC19LK containing the *hrpL*::Km^r gene. To generate an *hrpL* mutant of NAE6, the plasmid pUC19LK was introduced into NAE6 competent cells by electroporation for marker-exchange mutagenesis.

Competent cell preparation and electroporation

P. s. pv. *eriobotryae* NAE6 was grown in King B medium (2% [w/v] protcose peptone No. 3, 1% [w/v] glycerol, 6 mM $MgSO_4 \cdot 7H_2O$, 8 mM K_2HPO_4) at 25 °C until OD_{600} reached 0.4 - 0.5. After incubating cultures on ice for 20 min, cells were harvested by centrifugation at 6,000 × *g* for 15 min. Cell pellets were washed twice with 10% glycerol and resuspended in 10% glycerol at 1/10th of the original volume. Competent cells were used immediately. Electroporation was carried out using gene transfer equipment (Shimadzu GTE-10) and consisted of two pulses at 3 μF, 10 kV/cm and 9-10 ms. Transformants were selected on plates containing kanamycin.

RT-PCR

Total RNAs were isolated from *P. s.* pv. *eriobotryae* strains grown in *hrp*-inducing minimal medium (13 mM potassium phosphate buffer, pH 5.5, 17 mM NaCl, 30 mM $(NH_4)_2SO_4$, 2.8 mM $MgSO_4$, 10 mM sucrose) using an SV total RNA isolation system (Promega). Polyadenylated RNA was isolated using an Oligotex-dT (Super) mRNA purification kit (TaKaRa). The mRNAs were then reverse transcribed using Random 9-mers (IIigh Fidelity RNA PCR Kit, TaKaRa). The following primers were used for RT-PCR analyses : for PsvA, forward primer PsvA-f (5'-ATGTGGAATTTTAAT) and reverse primer PsvA-r (5'-CTACAACTTCCACCA) ; for HrpZ, forward primer HrpZ-f (5'-ATGCAGAGTCTCAGT) and reverse primer HrpZ-r (5'-TCAGGCAGCAGCCTG).

Southern blot analysis

Southern blot analysis was performed with an ECL direct nucleic acid labelling and detection system (GE Healthcare). Genomic DNAs of wild-type and *hrpL*-mutant strains digested with *Eco*RI were separated by gel electrophoresis and then transferred onto a nitorocellulose membrane. The Km^r gene probe generated by PCR was separated by electrophoresis on a 1% agarose gel and purified with a QIAEX II gel extraction kit (Qiagen). Labeling of probe DNA, hybridization, and signal detection were carried out according to the manufacturer's instructions.

Western blot analysis

Cultures of *P. s.* pv. *eriobotryae* strains were grown in *hrp*-inducing minimal medium. To obtain whole-cell proteins, cells from 1.5 ml overnight culture were harvested by centrifugation at 6,500 × *g* for 5 min and resuspended in 100 μl sample buffer (0.063 mM Tris-HCl [pH 6.8], 1 mM EDTA, 3% SDS, 2.5% 2-mercaptoethanol, 10% glycerol, 0.013% bromophenol blue). After incubation in a boiling water bath for 5 min, samples were separated by electrophoresis on 10% SDS gels and electroblotted onto Hybond nitrocellulose membranes. The blotted membranes were blocked with 5% non-fat milk and then incubated for 1 h with antiserum against PsvA. The membranes were washed three times with TBS-T (150 mM NaCl, 20 mM Tris, and 0.1% Tween, pH 7.5) and then incubated for 1 h with peroxidase-labeled anti-rabbit antibody. After three washes with TBS-T, immunoactive proteins were visualized using ECL Western Blotting Detection Reagents (Amersham).

Results and Discussion

Construction of *hrpL* mutant of *P. s.* pv. *eriobotryae* NAE6

The nucleotide sequence of a 6,961-bp DNA fragment of the 52 Mdal plasmid containing the *psvA* gene has been deposited in the DDBJ database under Accession No. AB018553. Analysis of the upstream region of *psvA* revealed a putative hrp box motif (KGGARCY [N15-16] CCACNNA) (Table 1). The *hrpL* gene is necessary for transcription of *hrp*-box-

Table 1. Hrp box-like motif upstream of *psvA*

Gene	hrp box			Position[c]
	Motif 1	Spacing	Motif 2	
	KGGARCY[a]	[N15-16]	CCACNNA[b]	
P.s. pv. *eriobotryae*				
psvA	TGGAACC	15	CCTACTA	- 216
P.s. pv. *syringae* (Xiao *et al.* 1994b)				
hrmA (hopPsyA)	TGGAACC	16	CCACCCA	- 523
hrpK	TGGAACC	15	CCACACA	- 32
hrpJ	GGGAACC	16	CCACTCA	- 30
hrcU	TGGAACT	15	CCACTTA	- 296
hrpH	TGGAACC	16	CCACTCA	- 33
hrpZ	TGGAACC	16	CCACCTA	- 47

a) K : T or G. R : A or G. Y : C or T.
b) N : A, C, G or T.
c) Numbers indicate nucleotide positions relative to start codon of each ORF.

containing genes. Thus, we examined the possibility that HrpL could regulate expression of the *psvA* gene.

To determine whether *psvA* expression was HrpL-dependent, we disrupted *hrpL* in *P. s.* pv. *eriobotryae* NAE6 by marker-exchange to produce mutants 6MEa and 6MEb. The marker exchange was confirmed by Southern blot and PCR analyses. Southern blot analyses showed that the Km^r gene probe hybridized with a fragment of *Eco*RI-digested genomic DNAs from 6MEa and 6MEb strains, but not with DNA from the NAE6 strain (Fig. 1). Furthermore, when genomic DNAs extracted from 6MEa and 6MEb were amplified using primers HrpL-f and HrpL-r, the band observed was the expected length (ca.1.7 kb). However, a band that was the same size as the *hrpL* gene (ca. 0.6 kb) was observed in NAE6 (Fig. 2). These observations indicate that homologous recombination occurred between the *hrpL* region of the host genome and the *hrpL::Km* region in the plasmid pUC19LK.

The marker-exchanged mutant strains 6MEa and 6MEb had lost their pathogenicity, because *hrpL* is a transcription unit necessary for pathogenicity (Fig. 3). It is known that *hrpZ*, which encodes an extracellular hypersensitive response (HR) elicitor, is regulated by *hrpL* in *P. syringae* (He *et al.* 1993 ; Xiao *et al.* 1994b). Therefore, expression of *hrpZ* was examined using RT-PCR. The PCR analyses showed that *hrpZ* was expressed in the wild-type strain NAE6 but not in mutant strains 6MEa and 6MEb (Fig. 4). The results of pathogenicity and *hrpZ* expression tests confirmed that the *hrpL* gene had been mutated.

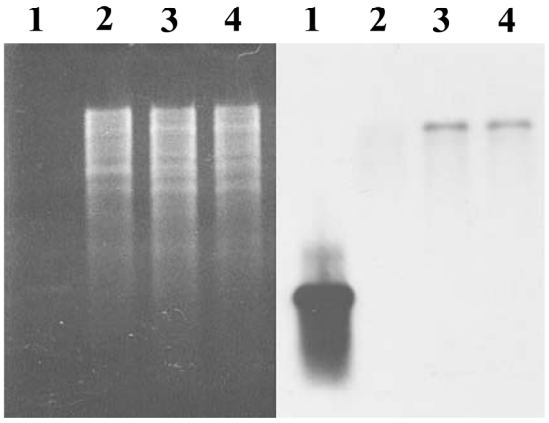

Fig. 1. Southern blot analysis of genomic DNA from marker exchange mutants. Genomic DNAs were digested with *Eco*RI and probed with kanamycin-resistance gene (Km^r). Lane 1, Km^r gene ; lane 2, *P. s.* pv. *eriobotryae* NAE6 ; lane 3, *P. s.* pv. *eriobotryae* 6MEa ; lane 4, *P. s.* pv. *eriobotryae* 6MEb.

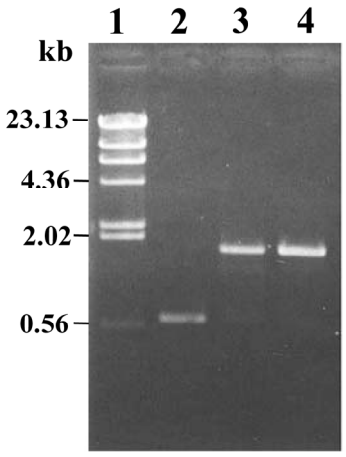

Fig. 2. PCR analysis of marker-exchanged *hrpL* mutants using *hrpL*-specific primers. Lane 1, λ/*Hin*dIII ; lane 2, *P. s.* pv. *eriobotryae* NAE6 ; lane 3, *P. s.* pv. *eriobotryae* 6MEa ; lane 4, *P. s.* pv. *eriobotryae* 6MEb.

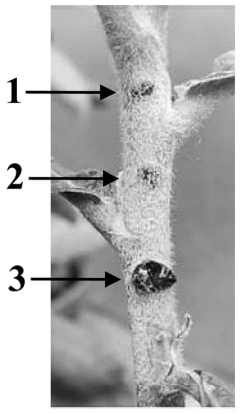

Fig. 3. Pathogenicity of marker exchange mutants. 1, *P. s.* pv. *eriobotryae* 6MEa ; 2, *P. s.* pv. *eriobotryae* 6MEb ; 3, *P. s.* pv. *eriobotryae* NAE6.

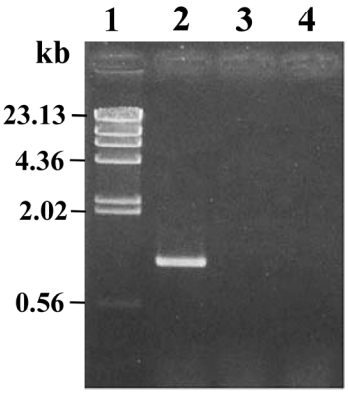

Fig. 4. RT-PCR analysis of *hrpZ* expression. Lane 1, λ/*Hin*dIII ; lane 2, *P. s.* pv. *eriobotryae* NAE6 ; lane 3, *P. s.* pv. *eriobotryae* 6MEa ; lane 4, *P. s.* pv. *eriobotryae* 6MEb.

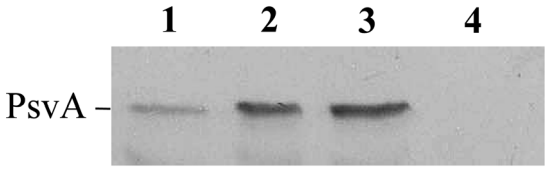

Fig. 5. Western blot analysis of PsvA expression. Lane 1, *P. s.* pv. *eriobotryae* NAE6 ; lane 2, *P. s.* pv. *eriobotryae* 6MEa ; lane 3, *P. s.* pv. *eriobotryae* 6MEb ; lane 4, *P. s.* pv. *eriobotryae* PE0.

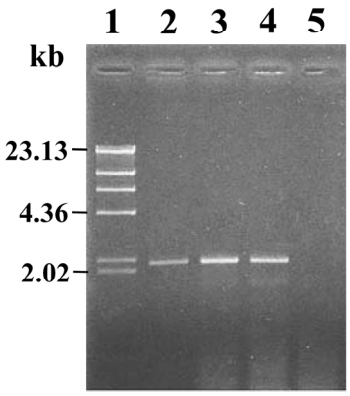

Fig. 6. RT-PCR analysis of *psvA* gene expression. Lane 1, λ/*Hin*dIII ; lane 2, *P. s.* pv. *eriobotryae* NAE6 ; lane 3, *P. s.* pv. *eriobotryae* 6MEa ; lane 4, *P. s.* pv. *eriobotryae* 6MEb ; lane 5, *P. s.* pv. *eriobotryae* PE0.

Expression of *psvA* is not regulated by HrpL

First, we investigated the expression of *psvA* gene in the wild-type and *hrpL* mutant strains using Western blotting analysis. The PsvA protein was detected as a single band with a molecular mass of ca. 83 kDa in the wild-type strain (NAE6) and in the *hrpL* mutant strains (6MEa and 6MEb), but was not detected in the PE0 strain lacking the *psvA* gene (Fig. 5). Next, we analyzed *psvA* expression in each strain by RT-PCR. The expected 2.1 kb DNA fragment comprising the *psvA* gene was detected in the wild-type strain and *hrpL* mutant strains but not in the PE0 strain (Fig. 6). Together, these data indicate that HrpL does not regulate *psvA* expression. Therefore, we speculate that the expression of *psvA* gene may be controlled by σ^{70}-like promoter, but its precise location remains unknown.

Expressions of *hrp* genes and many effector genes that contain an hrp box sequence in their promoters are regulated by the HrpL alternative sigma factor (reviewed by Collmer *et al.* 2002). However, our results show that expression of *psvA* containing the hrp box-like motif was independent of the *hrp* regulatory system. Our study suggests that the presence of a putative hrp box consensus sequence in the promoter region of a gene may not accurately predict whether that gene is regulated by HrpL.

The PsvA protein was predominantly detected in the outer membrane fraction (Kamiunten & Yoshinaga 2004). However, its functions remain largely unknown. The fact that *psvA* is not regulated by HrpL may become important when we try to uncover the origin and functions of *psvA*.

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ビワがんしゅ病細菌の病原性遺伝子*psvA*はhrp box様モチーフを有しているがその発現はHrpLの制御を受けない

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要　約

hrp boxプロモーターは*Pseudomonas syringae*の*hrp*遺伝子や*avr*遺伝子の上流にあり，これらの遺伝子の発現は*hrpL*遺伝子にコードされているシグマ因子により制御されている．hrp box様モチーフが*P. s.*pv. *eriobotryae*のプラスミドから分離された病原性遺伝子*psvA*の上流に存在することが明らかになった．hrp box様モチーフを有する*psvA*の発現が*hrpL*によって制御されるかどうかを調べるために，*hrpL*にカナマイシン耐性遺伝子を挿入した自殺ベクターpUC19LKを用いてマーカーエクスチェンジによる*hrpL*変異株を構築し，サザンハイブリダイゼーションおよびPCRで確認した．これら*hrpL*変異株は病原性を失い，hrp boxを有する*hrpZ*の発現もRT-PCRで認められず，*hrpL*に変異が起きていることが裏付けられた．得られた*hrpL*変異株における*psvA*の発現をウエスタンブロッティングおよびRT-PCRで調べた結果，野生株と同じように発現しており，我々の実験結果は*psvA*の発現はHrpLに制御されていないことを示唆していた．

キーワード：ビワがんしゅ病細菌，病原性遺伝子*psvA*，HrpL