

A LuxR-type Transcriptional Regulator, PsyR, Coordinates Regulation of Pathogenesis-related Genes in *Pseudomonas syringae* pv. *tabaci*

Yeon Hee Choi, Jun Seung Lee, Sora Yun and Hyung Suk Baik*

Department of Microbiology, College of Natural Science, Pusan National University, Busan 609-735, Korea

Received January 20, 2015 / Revised February 4, 2015 / Accepted February 6, 2015

Pseudomonas syringae pathovar *tabaci* is a plant pathogenic bacterium that causes wildfire disease in tobacco plants. In *P. syringae* pv. *tabaci*, PsyI, a LuxI-type protein, acts as an AHL synthase, while primary and secondary sequence analysis of PsyR has revealed that it is a homolog of the LuxR-type transcriptional regulator that responds to AHL molecules. In this study, using phenotypic and genetic analyses in *P. syringae* pv. *tabaci*, we show the effect of PsyR protein as a quorum-sensing (QS) transcriptional regulator. Regulatory effects of PsyR on swarming motility and production of siderophores, tabtoxin, and *N*-acyl homoserine lactones were examined via phenotypic assays, and confirmed by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Further qRT-PCR showed that PsyR regulates expression of these virulence genes in response to environmental signals. However, an upstream region of the gene was not bound with purified MBP-PsyR protein; rather, PsyR was only able to shift the upstream region of *psyI*. These results suggested that PsyR may be indirectly controlled via intermediate-regulatory systems and that auto-regulation by PsyR does not occur.

Key words : Electrophoretic mobility shift assay (EMSA), MBP-*psyR* expression, *Pseudomonas syringae*, Quorum sensing, Real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Introduction

Bacteria undergo a variety of physiological and morphological adaptations in reaction to physical and chemical changes that arise from the environment. Importantly, some of the most active signaling molecules are not only metabolic wastes but also diffusible molecules released from a bacterial population for cell-to-cell communication [5]. This strategy, called quorum sensing (QS), regulates the specific expression of a group of genes according to the bacterial population density. The QS system coordinates physiological reactions such as virulence, production of secondary metabolites, and antibiotic resistance, based on the local density of the bacterial population [12]. When QS signaling molecules reach a threshold, QS bacteria can detect a QS signaling molecule called an autoinducer, resulting in coordinated expression of specific target genes.

In gram-negative bacteria, various QS systems have been found, such as the *Agrobacterium tumefaciens* TraIR system, the *Erwinia carotovora* CarIR system, and the *Pseudomonas aeruginosa* LasIR and RhlIR systems [14, 23, 43, 46, 49]. The QS systems in gram-negative bacteria are similar to the conventional QS circuit of *Vibrio fischeri* that was described in the early 1970s [29, 30]. The QS system consists of two homologues of *V. fischeri* regulatory proteins, LuxI and LuxR. The LuxI family proteins play a role as AHL synthases and produce a specific AHL known as the QS signaling molecule. The LuxR-type proteins regulate QS-related genes. Most of the LuxR-type regulators act as transcriptional activators, but some act as repressors or play a dual role. The amino-terminal domain of LuxR acts as an AHL-binding domain, while its carboxyl-terminal domain acts as a DNA-binding domain. The DNA-binding domain is assumed to form a helix-turn-helix (HTH) motif with a cluster of four helical structures. The HTH motif binds to the major groove of DNA in the presence of AHL [11, 35, 52]. In the absence of AHL, the LuxR-type protein is in the monomeric, inactive conformation. AHL can interact with the amino-terminal domain of LuxR-type transcriptional regulators; this complex then dimerizes or multimerizes [9, 13]. Consequently, the carboxyl-terminal of AHL-bound LuxR binds to a palindromic promoter region, the *lux* box, which affects ex-

*Corresponding author

Tel : +82-51-510-2271, Fax : +82-51-514-1778

E-mail : hsubaik@pusan.ac.kr

This is an Open-Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

pression of QS-controlled target genes.

Pseudomonas is a noteworthy genus, because it includes a human pathogen (*P. aeruginosa*), a phytopathogen (*P. syringae*), and a nonpathogenic bioremediation agent (*P. putida*). Among these strains, *P. syringae* is an important plant pathogen used for studying plant - bacterial interactions. *P. syringae* is a gram-negative bacterium that causes disease in a wide range of plant species. *P. syringae* consists of more than 50 pathovars, based on host-specificities. *P. syringae* pathovar *tabaci* ATCC 11528 causes wildfire disease in tobacco plants and a hypersensitive response (HR) in non-host plants. The AHL molecules produced in *P. syringae* pv. *tabaci* are *N*-(3-oxohexanoyl)-L-homoserine lactone and *N*-(3-oxooctanoyl)-L-homoserine lactone. In *P. syringae* species, the gene *psyI* has a putative *lux* box sequence with dyad symmetry. In *P. syringae* pv. *tabaci*, *PsyI*, a LuxI-type protein, acts as an AHL synthase, while primary and secondary sequence analysis of *PsyR* has revealed that it is a homolog of the LuxR-type transcriptional regulator that responds to AHL molecules [28, 40].

The QS system has been studied intensively in recent years. According to a recent study, the QS system both positively and negatively regulates diverse putative virulence factors. The QS system in *P. syringae* pv. *syringae* was reported to positively regulate production of extracellular polysaccharides (EPSs), as well as tolerance to oxidative stress and maceration of plant tissue, and also to negatively regulate swarming motility [36, 45]. Other phenotypes are determined by QS upon sensing of environmental cues such as nutrient deficiency or iron limitation [42, 50]. Therefore, the QS system is closely associated with other regulons.

The regulation of virulence genes by several regulators has been studied [6, 7, 24, 51]. The iron-uptake regulator *Fur* controls the expression of sigma factors and operon genes associated with iron uptake, acid tolerance, oxidative stress response, chemotaxis, and metabolic pathways [4, 27, 34, 44, 47, 48]. *GacA*, a response regulator of the two-component system, controls a variety of phenotypes, including motility, biofilm formation, and production of secondary metabolites such as antibiotics, siderophores, QS autoinducers, toxins, and extracellular polysaccharides, although the signals that activate the *GacS/GacA* two-component system have not been identified [8, 28, 37].

Another significant virulence factors are the type III effector proteins that are essential for disease progress in host plants and for the HR in non-host plants. These effector pro-

teins translocate into plant cells via the type III secretion system (TTSS) [10, 16]. According to a recent study, the TTSS in plant pathogens is required for the suppression of plant defense during the first step of interaction with their hosts [19]. Several transcriptional factors that mediate the environmental regulation of *P. syringae* *hrp* genes have been identified; however, transcription of the *hrp* genes is mediated by different mechanisms in different species [17, 18]. The TTSS activator *HrpR* is required for the expression of *hrp* regulatory genes. The activation of *hrp* regulatory genes, including *hrpA*, which encodes the key *hrp* pili protein, is not understood. A TTSS-linked regulatory system has been elucidated in *Ralstonia solanacearum*. Specifically, it was found that *PrhI* (an extracytoplasmic function sigma factor), *PrhR* (a periplasmic domain of the inner membrane protein), and *PrhA* (an outer membrane receptor for unknown plant signals [26]) comprise this *hrp* gene regulatory cascade. *PrhA* activates transcription of the *hrp* genes after recognition of tobacco plant cell-contact in *P. syringae* pv. *tabaci* ATCC 11528 [24]. *PrhA* transduces the signal to *PrhR*, which controls *hrp* genes encoding the TTSS, along with *PrhI*. When *PrhR* receives the signals, *PrhI* is released from *PrhR* into the cytoplasm. The regulatory cascade driving the expression of the type III secretion determinants would therefore start with *PrhI* [3, 15].

The purpose of this study was to determine the effect of *PsyR* protein as a transcriptional regulator of various virulence factors in *P. syringae* pv. *tabaci* ATCC 11528. In this study, phenotypes of the *psyR* deletion mutant were compared with those of the wild-type. We showed that *PsyR* has effects on pathogenesis, such as production of siderophores, AHLs, tabtoxin, swarming motility, and pathogenicity to tobacco leaf.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are shown in Tables 1 and 2, respectively. *P. syringae* pv. *tabaci* ATCC 11528 was obtained from the American Type Culture Collection (Manassas, VA, USA). *P. syringae* pv. *tabaci* was cultured in King's B (KB) medium at 28°C with aeration via shaking [22]. *Escherichia coli* Top10 and DH5 α were used for cloning experiments, *E. coli* S17-1 λ _{pir} was used as a donor for conjugation, while *E. coli* BL21 (DE3) was used as host for overexpression of *psyR*; these strains were grown in

Table 1. Bacterial strains used in this study

Strains	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i>		
Top10	Transformation host for cloning vector	Invitrogen, USA
DH5α	Transformation host for cloning vector	Takara, Japan
S17-1 λ _{pir}	S17-1 derivative, RK2 <i>tra</i> regulon, host for <i>pir</i> -dependent plasmids	[6]
BL21(DE3)	Overexpression host for expression vector	Novagen, USA
BL759	<i>E. coli</i> BL21(DE3)/pBL318 [<i>P. syringae</i> pv. <i>tabaci</i> <i>psyR</i> overexpression (pMAL-c5×)]	This study
BL792	BL759/pBL337 [<i>P. syringae</i> pv. <i>tabaci</i> <i>psyR</i> overexpression (pMAL-c5x) with <i>psyl</i> expression vector]	This study
<i>Agrobacterium tumefaciens</i> NT1	<i>A. tumefaciens</i> carrying plasmid pDCI41E33 containing a <i>traG::lacZ</i> fusion and <i>traR</i> cloned into pDSK519, spontaneous Gm ^r	[40]
<i>Pseudomonas syringae</i> pv. <i>tabaci</i>		
ATCC 11528	Plant pathogen, wild-type strain	ATCC, USA
BL37	ATCC 11528 Δ <i>psyR</i> (678-bp deletion)	Lab collection
BL779	BL37 containing pBL336	This study

^aGm^r, gentamycin resistance.

Table 2. Plasmids used in this study

Plasmids	Relevant properties ^a	Reference or source
pGEM-T easy	Cloning vector, Ap ^r	Promega, USA
T-blunt	Cloning vector, Ap ^r , Km ^r	Solgent, Korea
pDMS197	Suicide vector, Tc ^r	[6]
pRK415	Broad host range cloning vector, Tc ^r	[20]
pMAL-c5x	Expression vector carrying an N-terminal maltose binding protein sequence and <i>lacI</i> , Ap ^r	NEB, UK
pBL108	1.7-kb DNA containing 5' -flanking and 3' -flanking regions of <i>psyR</i> in pGEM-T easy vector, Ap ^r	This study
pBL111	Derivative of pDMS197 containing the insert DNA of pBL108, DAP required, Km ^r	This study
pBL122	Derivative of pGEM-T Easy containing the 1.34-kb wild type <i>psyR</i> with promoter region, Ap ^r	This study
pBL186	Derivative of pGEM-T Easy containing the 946-bp wild type <i>psyl</i> , Ap ^r	This study
pBL278	749-bp <i>psyR</i> -coding region in T-blunt vector, Ap ^r , Km ^r	This study
pBL318	Derivative of pMAL-c5x containing the 749-bp <i>psyR</i> , Ap ^r	This study
pBL336	Derivative of pRK415 containing the 1.34-kb wild-type <i>psyR</i> , Tc ^r	This study
pBL337	Derivative of pRK415 containing the 946-bp wild-type <i>psyl</i> , Tc ^r	This study

^aAp^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; DAP; diaminopimelic acid.

Luria-Bertani (LB) medium at 37°C [2, 25]. The *Agrobacterium tumefaciens* NT1 (pDCI41E33) indicator strain was maintained in AB minimal medium at 28°C [33].

Media were solidified using 1.5% (w/v) agar. When required, antibiotics were added to media at the following concentrations: 100 µg/ml ampicillin (Ap), 20 µg/ml kanamycin (Km), 20 µg/ml tetracycline (Tc), and 15 µg/ml gentamicin (Gm).

DNA manipulation

Plasmid DNA was prepared with a Plasmid Mini Prep kit (Solgent; Daejeon, Korea) and DNA fragments were ex-

tracted from agarose gels using a QIAquick Gel Extraction kit (Qiagen; Hilden, Germany). Transformation of *E. coli* or *P. syringae* strains was done by either RbCl-CaCl₂ heat shock or electroporation (Bio-Rad; Hercules, CA, USA) at 2,500 volts, 200 ohms, and 25 µF. Transfer of the recombinant suicide plasmid to *Pseudomonas* was accomplished by conjugation with *E. coli* S17-1 λ_{pir} as the plasmid donor.

Oligonucleotide primers used in this study are described in Table 3. Polymerase chain reaction (PCR) amplification was performed with *Taq* or *Pfu* polymerase from Solgent, under the following reaction conditions: denaturation at 95°C for 20 s, primer annealing according to melting temper-

Table 3. Primers used in this study

Primers	Forward primer (5' - 3')	Reverse primer (5' - 3')
Oligonucleotides used for construction of gene deletion mutations and complementation assays		
<i>psyR</i> -L	TATCTAGACATCMGCAGGGTCRTAAG	TAGGATCCCGGTGGRCAGTCAAGTTG
<i>psyR</i> -R	TAGGATCCCATCAASATGGGCATGGTCT	TAGAGCTCAGAACGCKRAGCAGATTT
<i>psyR</i> -C	TAGAATTCCGCTGCTGATCATGTAGATCAG	TCAGACCATGCCCATSTTG
Oligonucleotides for quantitative real-time RT-PCR		
qRT-16S rRNA	CACTCTAAGGAGACTG	TACTAGCGATTCCGAC
qRT- <i>fur</i>	CACTCTAAGGAGACTG	CACCAGATTGTGGTCC
qRT- <i>gacA</i>	AGTGCAAGTTGTCCAC	TGCATGCACAGCGCTG
qRT- <i>psyl</i>	ATCGATCATCAGGTG	AGGTCATCCGTGACAG
qRT- <i>prhA</i>	CATGTACAACGACATC	AACGCATAGCTGCGTG
qRT- <i>prhI</i>	GATTCTGGAAGACCTC	TGTCGGCTCACCGTAG
qRT- <i>hrpR</i>	TGCTCAGCCATGACTG	ACGTCTAGGCAGTTCG
qRT- <i>hrpA</i>	TGACAGTATCGACGCT	CTGGACGACCGAGTTC
Oligonucleotides for plasmid construction		
<i>psyR</i> -P	GGAATTCCATATGATGGAGGTCGTACCGTGA	CGGGATCCTCAGACCATGCCCATGTT
<i>psyl</i> -P	TCGATACCGGCATATAGACTG	ATCAACATGGGCATGGTCT
Oligonucleotides for electrophoretic mobility shift assays		
EM- <i>fur</i>	TGCATCGGTGGGCGAC	AGGGTTACCTTTAGTC
EM- <i>gacA</i>	CAAGTTGTCGAGAGTC	GTCATCGACAAGTAGC
EM- <i>psyR</i>	GCACCTTAATGGAACG	TGGCTGTACTCATTATC
EM- <i>psyl</i>	CGCCGAAGCAGATTTC	CCAGTGTGAAGCATCG
EM- <i>prhA</i>	GCTCATCTGATGTACG	CGTCCATGGCTGGATG
EM- <i>prhI</i>	GCATTTACACTGGAG	GATCTAACTAAAGAGAATC
EM- <i>hrpR</i>	ACAGTCTGGCTCTATG	GATCACTCTCACTGTG
EM- <i>hrpA</i>	GCTCTATCGACGGATC	GAAGTCATGATGCTC
EM- <i>omsR</i>	GGTGGACGTGCAGATG	TAGAGGGAAACTCTCG

ature for 40 s, polymerization at 72°C for 1-3 min depending on the length of DNA fragment; these steps were repeated for 25 cycles, and were followed by a final extension at 72°C for 5 min.

Construction of the *psyR* mutant strain and complementation strain

The *psyR* deletion mutant of *P. syringae* pv. *tabaci* ATCC 11528 was constructed by the allelic-exchange method [38]. The 5' -flanking region of *psyR* was amplified by PCR using *psyR*-L forward and reverse primers designed from the *P. syringae* pv. *tabaci* sequence (GenBank Accession No. AF110468). In addition, the 3' -flanking region of *psyR* was amplified using *psyR*-R forward and reverse primers. Amplified DNA fragments were cloned into the pGEM-T easy vector, yielding pBL108. A 1.7-kb DNA fragment from pBL108 was digested with *Xba*I and *Sac*I and cloned into the suicide vector pDMS197, creating pBL111. Then, the recombinant suicide plasmid was introduced into *E. coli* S17-1 λ_{pir} carrying the plasmid as the plasmid donor, via

electroporation. Cloning was verified by analyses of the DNA fragment profile after restriction enzyme digestion. pBL111 was introduced into *P. syringae* pv. *tabaci* strain by mating. Colonies carrying the chromosomal insertion of the recombinant suicide plasmid were selected on plates supplemented with kanamycin. Loss of the suicide plasmid after the second recombination between two homologous regions was selected using the *sacB*-based sucrose sensitivity counter selection system. Finally, the *psyR* deletion mutant strain was confirmed via PCR amplification using *psyR*-L forward and *psyR*-R reverse primers.

To generate a construct for complementation of the *psyR* deletion, the DNA fragments containing the intact *psyR* gene were amplified from the chromosomal DNA using the *psyR*-C primer set (Table 3). The purified PCR product was cloned into the pGEM-T easy vector, resulting in pBL122. Then, the 1.34-kb DNA fragment from pBL122 was digested with *Eco*RI and cloned into the broad-host-range cloning vector pRK415, creating pBL336. pBL336 was transferred into *P. syringae* pv. *tabaci* strain from *E. coli* S17-1 λ_{pir} by means

of conjugation [20]. Finally, the complement strain, BL779, was constructed using the above procedures.

Phenotypic tests and plant pathogenicity assays

We performed chrome azurol S (CAS) universal siderophore detection assays, as described by Schwyn and Neiland [39]. Swarming motility was determined using KB agar (0.4% agar) plates [7]. Tabtoxins were detected using standard bioassays with *A. tumefaciens* NT1 as the indicator bacteria. To detect AHLs production in a liquid medium, AHLs extracts were prepared as described by Shaw *et al.* [41]. The concentrated extracts were characterized by thin-layer chromatography (TLC) using *A. tumefaciens* NT1 as the AHL-sensor strain. Plant pathogenicity assays were performed using tobacco plants (*Nicotiana tabacum* L. cv. Samsun) as described previously [24]. Each treatment was repeated three times. All assays were repeated in three separate experiments.

RNA isolation and quantitative real-time reverse transcription (qRT)-PCR analysis

Cells were grown to the early (OD_{600} 0.5) and late (OD_{600} 3.5) exponential phases. Total RNA isolation and qRT-PCR were conducted as described previously [6]. Briefly, total RNA was extracted from bacteria using an RNeasy Mini RNA Isolation kit (GE Healthcare; Pittsburgh, PA, USA) according to the manufacturer's instructions. The total RNA concentration was quantified using a Nanodrop 2000 spectrometer (Thermo Fisher Scientific; Waltham, MA, USA). cDNAs were synthesized using a Reverse Transcription Premix kit (ElpisBiotech; Daejeon, Korea) according to the manufacturer's protocols, using target gene-specific primers, and were stored at -20°C until required for use.

Real-time RT-PCR was carried out using SYBR Primix Ex TaqTM (TaKaRa; Osaka, Japan) with a StepOne Real-Time PCR System (Applied Biosystems; Foster City, CA, USA), as previously described [6]. Specific primer sets used to amplify the genes of interest were designed based on sequences in the GenBank database. The PCR conditions were as follows: 94°C for 5 min, followed by 40 cycles each consisting of 10 s at 94°C , 10 s at 55°C , and 15 s at 72°C , followed by a 7-min incubation at 72°C . To normalize the fold-induction of mRNA, the expression level of 16S rRNA (endogenous control) was quantified and then normalized to the threshold value acquired for the wild-type strain [1]. The relative expression ratios were calculated using a mathe-

matical model, which contained an efficiency correction for the real-time RT-PCR efficiency of each transcript [32]. The data are shown as the average of triplicate samples.

Overproduction and purification of the PsyR protein

The *psyR*-coding region was amplified from the genomic DNA of *P. syringae* pv. *tabaci* by PCR, using the *psyR*-P forward and reverse primers. The 749-bp DNA fragment was cloned into the T-blunt vector, creating pBL278; this was then digested with *Nde*I and *Bam*HI and the digested fragment was cloned into the pMAL-c5x vector, resulting in pBL318. To support the production of soluble PsyR protein, a co-expression vector carrying the AHL-encoding *psyl* was constructed. The *psyl* fragment, including its promoter region, was amplified by PCR using *psyl*-P forward and reverse primers. Amplified DNA fragments were cloned into the pGEM-T Easy vector, resulting in pBL186. A 946-bp DNA fragment from pBL186 was digested with *Eco*RI and cloned into the broad host range cloning vector pRK415, creating pBL337. The integrity of the *psyR* and *psyl* sequences was confirmed by DNA sequencing.

For PsyR overexpression, pBL318 expressing the C-terminally maltose-binding protein (MBP)-tagged PsyR proteins, was introduced into *E. coli* BL21 (DE3) by electroporation, yielding strain BL759. To enhance the production of soluble PsyR protein, pBL337 expressing AHL synthase from *psyl* was transformed into the BL759 strain. In this way, the PsyR overproduction strain, harboring the pBL318 and pBL337 plasmids, was constructed. The PsyR overproduction strain, BL792, was cultured overnight in 5 ml LB broth containing tetracycline (20 $\mu\text{g}/\text{ml}$), ampicillin (50 $\mu\text{g}/\text{ml}$), and 0.2% glucose. The overnight culture was diluted 100-fold with 50 ml LB medium containing ampicillin (50 $\mu\text{g}/\text{ml}$) and incubated at 37°C . At the exponential phase (OD_{600} 0.4-0.6), the cell cultures were induced with 0.1 mM IPTG and incubated at 15°C for 12 hr. After incubation, cells were harvested by centrifugation and the cell pellet was suspended in 5 ml column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4). The suspended cells were lysed with 1 mg/ml lysozyme and using an Ultrasonic generator US-300 (Nissei; Osaka, Japan). Cell debris was removed by centrifugation at $20,000\times g$ for 20 min at 4°C . The 1.5×10 -cm column, filled with amylose resin (New England Biolabs; Ipswich, MA, USA), was washed with five column volumes of column buffer and the cleared cell lysate was loaded. The column was washed with 12 column volumes of column buf-

fer and subsequently eluted with five column volumes of elution buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 10 mM maltose). The protein-containing fractions were concentrated to about 1 mg/ml in an Amicon Ultra-15 (Millipore; Billerica, MA, USA).

Electrophoretic mobility shift assay

To investigate the interaction of PsyR protein with the virulence gene upstream region, an electrophoretic mobility shift assay (EMSA) was performed using an EMSA kit (Invitrogen; Waltham, MA, USA) [19]. DNA fragments were generated by PCR amplification of the regulatory regions of *omsf*, *psyR*, *psyL*, *gacA*, *fur*, *prhA*, *prhI*, *hrpR*, and *hrpA* using the primer pairs listed in Table 3. As a positive control for the DNA fragment, we used the promoter region of *psyL*, encoding *N*-acyl homoserine lactone synthase, whose expression is known to be regulated by PsyR. The EMSA reaction mixture (final volume of 10 μ l) contained 0.16 pM DNA, 0~2.4 nM protein, and 5 \times binding buffer (50 mM Tris-HCl [pH 7.4], 750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA), and was incubated at room temperature for 20 min. After the addition of 2 μ l of 6 \times EMSA gel-loading solution, the reaction mixtures were analyzed by electrophoresis in 0.5 \times TBE buffer (1.1 M Tris-HCl [pH 8.3], 9 M boric acid, 25 mM EDTA). All reactions were loaded onto native 5% polyacrylamide gels (1:30 bis-acrylamide to acrylamide ratio) at 110 V (15 V/cm) for 55 min at 4°C. The gel was stained in SYBR Green EMSA staining solution at room temperature for 20 min, and then washed twice with sterile distilled water to remove excess stain. The stained DNA gel was visualized using a Lumi-imager F1 (Roche; Basel, Switzerland).

Results

Phenotypes of the *psyR* deletion mutant

CAS agar plates were used to detect production of siderophores in *P. syringae* pv. *tabaci* ATCC 11528. As shown in Fig. 1A, both wild-type (WT) and Δ *psyR* mutant (BL37) strains did not produce siderophores under high-iron condition, but produced siderophores under low-iron condition. In the Δ *psyR* mutant strain, the orange halo was slightly smaller than in the WT strain. These results suggest that the production of siderophores may be co-regulated by PsyR in *P. syringae* pv. *tabaci* ATCC 11528.

Swarming motility is the rapid and coordinated move-

ment of a bacterial population across a semi-solid medium, which is driven by flagella. In comparison with the WT strain, the Δ *psyR* mutant strain demonstrated enhanced swarming motility (Fig. 1B). These results indicated that PsyR represses the swarming motility of *P. syringae* pv. *tabaci* ATCC 11528, and functions as a negative regulator of this activity.

To determine whether the Δ *psyR* mutation affects tabtoxin synthesis, tabtoxin production was measured using the *A. tumefaciens* NT1 growth inhibition assay. The produced tabtoxin formed zones of inhibition in the case of the WT strain of *P. syringae* pv. *tabaci* ATCC 11528 (Fig. 1C). The zones of inhibition of the Δ *psyR* mutant strain were slightly greater than that of its parent strain. Therefore, PsyR may be a negative regulator of tabtoxin synthesis in *P. syringae* pv. *tabaci* ATCC 11528.

The *A. tumefaciens* NT1 indicator strain carrying a *lacZ-traG* fused plasmid was used for the detection of AHL. Upon TLC analysis, two spots were observed on the TLC plates with a culture of the *A. tumefaciens* NT1 strain (Fig. 1D). The top spot represented *N*-(3-oxohexanoyl)-L-homoserine lactone and the bottom spot *N*-(3-oxooctanoyl)-L-homoserine lactone. In the Δ *psyR* mutant strain, production of AHL was considerably reduced as compared to the wild-type strain. These results indicated that synthesis of AHL in *P. syringae* pv. *tabaci* ATCC 11528 is regulated by PsyR.

The WT and Δ *psyR* mutant strains were observed at 1, 3, 5, and 7 days after inoculation onto tobacco leaves (Fig. 2A). Disease symptoms, such as brown necrotic lesions surrounded by chlorosis, were on the tobacco leaves inoculated with the WT strain. However, inoculation with the Δ *psyR* mutant strain resulted in a slight delay in disease symptom development. Consequently, symptom development of wild-fire disease appears to be regulated by PsyR.

The wild-type and Δ *psyR* mutant strains were grown in LB medium. As shown in Fig. 2B, the Δ *psyR* mutant strain grew slightly faster than did the WT strain.

Regulation of gene expression by PsyR

To choose regulatory factors related with the regulation of virulence-associated gene expression, qRT-PCR was performed using total RNA from the Δ *psyR* mutant and WT strains, which were incubated until the early and late exponential phases. The relative levels of mRNA were determined from the threshold values that were normalized to 16S rRNA gene expression. The genes chosen for this anal-

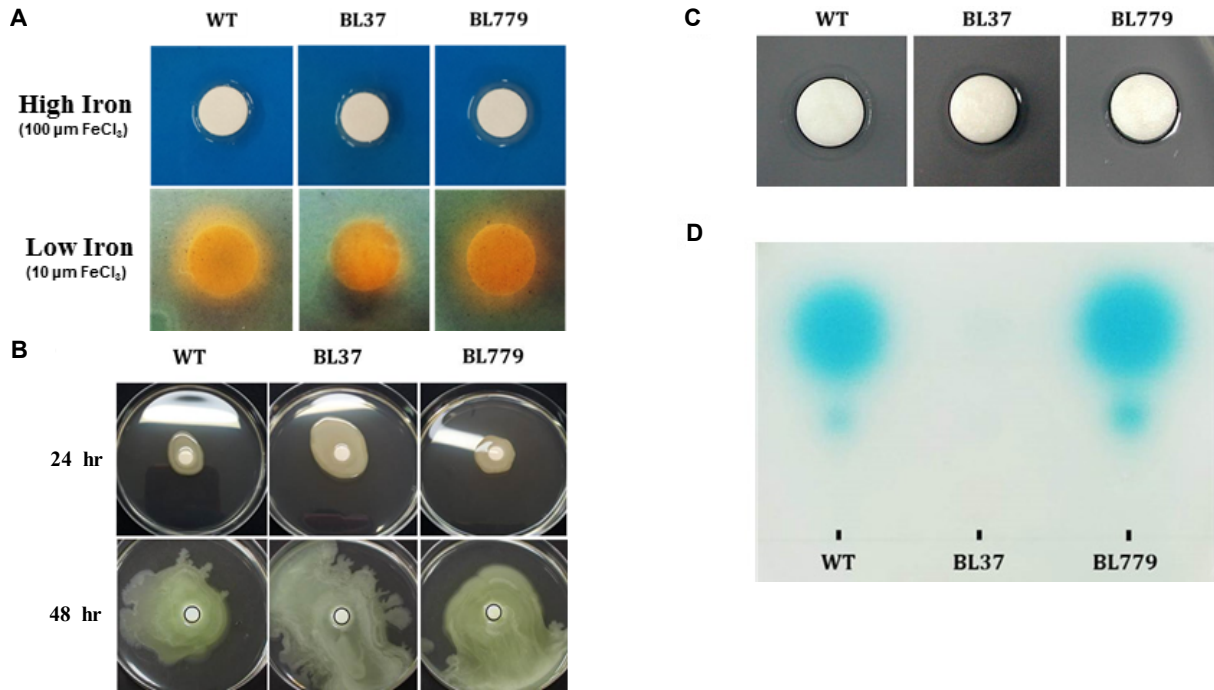


Fig. 1. (A) Siderophore assay with wild-type (WT), *ΔpsyR* mutant (BL37), and *ΔpsyR* mutant harboring pBL336 (BL779) strain of *Pseudomonas syringae* pv. *tabaci* ATCC 11528. *P. syringae* pv. *tabaci* strains were grown overnight and diluted to 2×10^8 CFU/ml in sterile water. Aliquots of 20 μl were spotted on chrome azure S (CAS) agar plates containing high or low iron concentrations. The inoculated plates were incubated for 48 hr at 30°C. (B) Comparison of swarming motility in a wild-type (WT) strain of *P. syringae* pv. *tabaci*, *ΔpsyR* mutant (BL37), and a *ΔpsyR* mutant-carrying pBL336 (BL779) strain. Overnight cultures of bacteria were inoculated onto 6-mm sterile filter discs in KB medium containing 0.4% agar. The plates were incubated at 30°C and observed at 24 hr and 48 hr post-inoculation. (C) Detection of tabtoxin-productive capacity with WT, *ΔpsyR* mutant (BL37), and *ΔpsyR* mutant-harboring pBL336 (BL779) strain of *P. syringae* pv. *tabaci* ATCC 11528. Overnight cultures of bacteria were inoculated onto 6-mm sterile filter discs in KB agar plates containing *Agrobacterium tumefaciens* NT1 indicator bacteria. The plates were incubated at 30°C and the zones of inhibition were observed at 48 hr. (D) Thin layer chromatography analysis of N-acetyl homoserine lactone (AHL) production. *A. tumefaciens* NT1 was used for the detection of AHL produced in *P. syringae* pv. *tabaci* ATCC 11528 (WT); *ΔpsyR* mutant (BL37) and the *ΔpsyR* mutant-harboring pBL336 (BL779) strain. A, N-(3-oxohexanoyl)-l-homoserine lactone; B, N-(3-oxooctanoyl)-l-homoserine lactone.

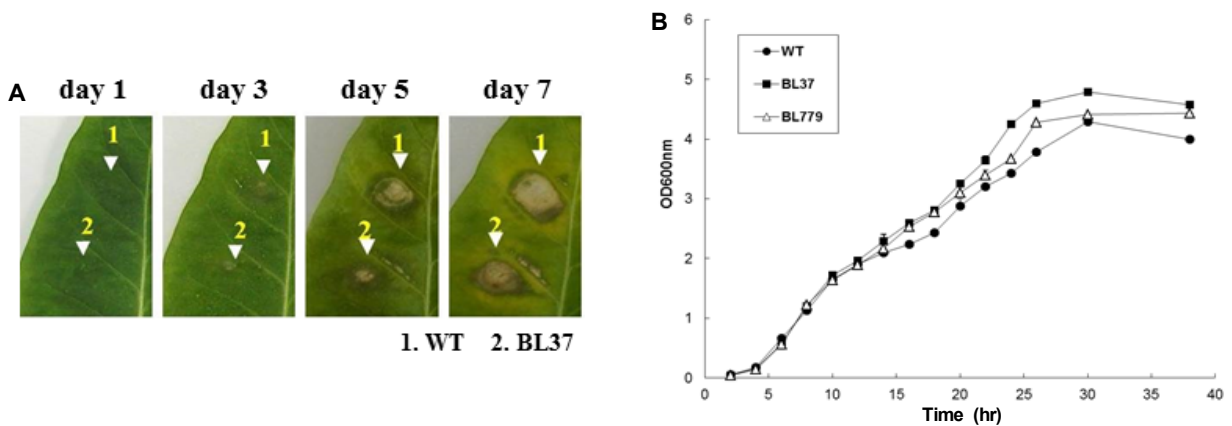


Fig. 2. (A) Test for disease symptoms on tobacco leaves. Disease symptoms generated by the wild-type (WT), *ΔpsyR* mutant- (BL37), and *ΔpsyR* mutant-harboring pBL336 (BL779) strain of *Pseudomonas syringae* pv. *tabaci* ATCC 11528. The tobacco leaves were photographed 1, 3, 5, and 7 days post-inoculation. 1, WT; 2, BL37. (B) Growth characteristics of the WT, BL37, and BL779 *P. syringae* pv. *tabaci* ATCC 11528 strains.

ysis were those encoding a global regulator (*gacA*), a QS signal synthase (*psyl*), a ferric uptake regulator (*fur*), a TTSS regulator, structural components (*hrpR*, *hrpA*), and a TTSS-linked regulatory system (*prhI*, *prhA*).

The WT and Δ psyR mutant strains showed a higher transcript level of *gacA* at the late-exponential phase in comparison to levels at the early exponential phase (Fig. 3A). At the early exponential phase, the mRNA levels of *gacA* in the Δ psyR mutant strain were increased approximately 3.9-fold. At the late exponential phase, the expression levels of *gacA* in the Δ psyR mutant strain was decreased approximately 0.8-fold compared to that in the WT strain. These

results showed that expression of *gacA* was not significantly different between WT and Δ psyR mutant strains.

The expression level of *psyl* in Δ psyR mutant strain was decreased approximately 160-fold at the late-exponential phase (Fig. 3B). Therefore, expression of *psyl* at a high cell-density is positively regulated by PsyR.

As shown in Fig. 4A, in the wild-type strain, the *fur* mRNA levels at the late exponential phase was higher than that at the early exponential phase, indicating that bacterial cell density affects the mRNA expression of *fur*. At the early exponential phase, the transcript levels of *fur* were slightly higher in the Δ psyR mutant strain than in the WT strain.

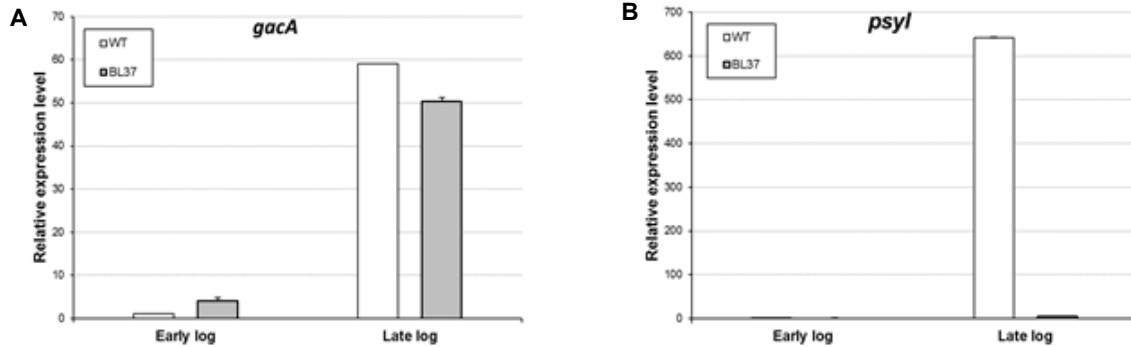


Fig. 3. The relative mRNA expression levels of *gacA* and *psyl* in wild-type (WT) and the Δ psyR mutant *Pseudomonas syringae* pv. *tabaci* ATCC 11528 strains. The relative mRNA levels of each gene were measured by quantitative real-time RT-PCR and normalized to 16S rRNA gene expression as the endogenous control. Bacterial cells were incubated until the early (OD₆₀₀ of 0.5) (A) and late (OD₆₀₀ of 3.5) exponential phases (B). The data are expressed as the average of three replicates \pm the standard deviation.

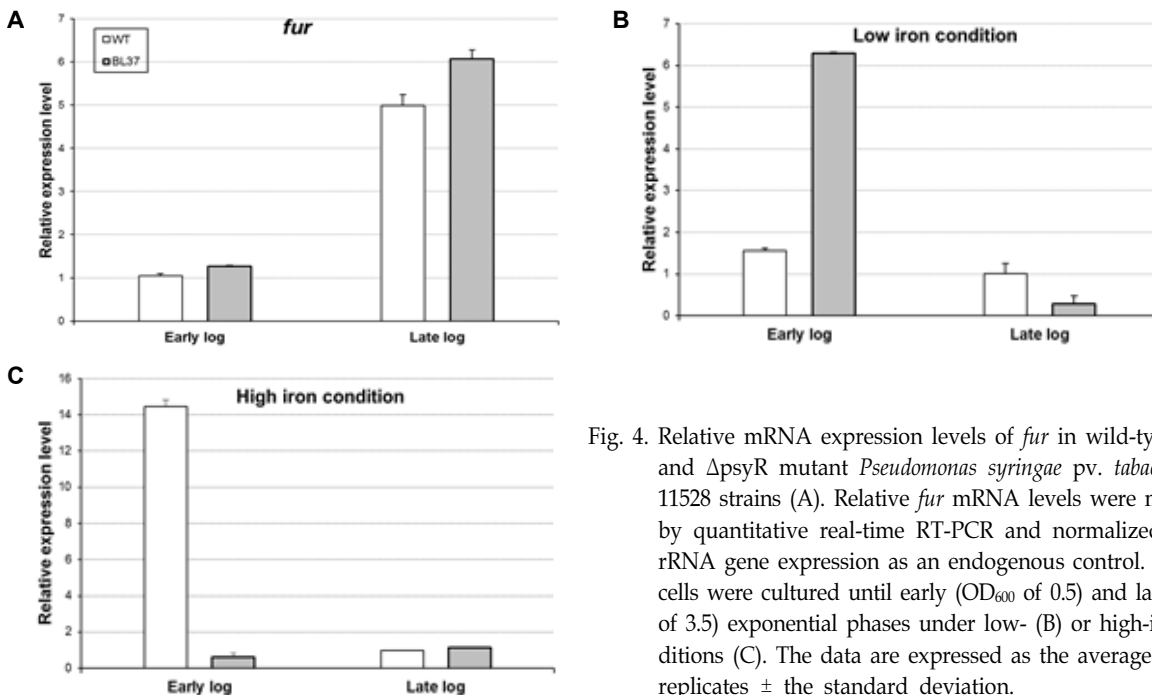


Fig. 4. Relative mRNA expression levels of *fur* in wild-type (WT) and Δ psyR mutant *Pseudomonas syringae* pv. *tabaci* ATCC 11528 strains (A). Relative *fur* mRNA levels were measured by quantitative real-time RT-PCR and normalized to 16S rRNA gene expression as an endogenous control. Bacterial cells were cultured until early (OD₆₀₀ of 0.5) and late (OD₆₀₀ of 3.5) exponential phases under low- (B) or high-iron conditions (C). The data are expressed as the average of three replicates \pm the standard deviation.

Furthermore, the *fur* mRNA levels of the Δ *psyR* mutant strain at the late exponential phase was slightly higher than that of the WT strain.

In addition, the *fur* mRNA levels were also assayed in *P. syringae* pv. *tabaci* strains grown under high- or low-iron conditions (Fig. 4B and 4C). Under low-iron conditions, the mRNA levels of *fur* in the Δ *psyR* mutant strain at the early exponential phase were higher than that in the WT strain. However, these mRNA expression levels were reversed at the late exponential phase. A lower amount of *fur* mRNA was observed in the Δ *psyR* mutant strain grown under high-iron conditions in comparison with the WT strain, but this was again reversed at the late exponential phase.

In order to investigate the correlation between TTSS-associated regulatory factors and the QS regulator PsyR, qRT-PCR was conducted as described previously [6]. *P. syringae* pv. *tabaci* PrhI, corresponding to *R. solanacearum* PrhI, exhibited sequence similarity to other sigma factors.

The expression levels of *prhA* in the WT strain at the early exponential phase were greater than that at the late exponential phase (Fig. 5A). In comparison with the WT strain, the *prhA* mRNA levels in Δ *psyR* mutant strain were increased approximately 0.8-fold at the early exponential phase; slightly greater levels of *prhA* mRNA were also observed at the late exponential phase. These results showed

that the mRNA expression levels of *prhA* are not significantly different between WT and Δ *psyR* mutant strains.

For *prhI*, the mRNA levels in the WT strain were increased at the late exponential phase (Fig. 5B). As compared with the WT strain, the Δ *psyR* mutant strain exhibited higher levels of this transcript at the early exponential phase. The mRNA levels of *prhI* in the Δ *psyR* mutant strain were decreased at the late exponential phase. Therefore, expression of *prhI* is regulated optimally by PsyR in a cell density-dependent manner.

Along with PrhA and PrhI, TTSS regulatory factors influencing expression of HrpR (an activator of TTSS) and HrpA (a major protein of TTSS pili) were investigated in the WT and Δ *psyR* mutant strains (Fig. 5C). In the WT strain, the mRNA levels of *hrpR* decreased at the late exponential phase. In general, lower *hrpR* mRNA levels were observed in the Δ *psyR* mutant strain as compared with the WT strain. However, the mRNA expression levels of *hrpR* were not significantly different between WT and Δ *psyR* mutant strains.

The WT strain showed a higher *hrpA* mRNA levels at the late exponential phase (Fig. 5D). In the Δ *psyR* mutant strain, the mRNA levels of *hrpA* at the late exponential phase was greater than that of the WT strain. These observations suggested that PsyR may repress *hrpA* expression at a high cell density. Therefore, *hrpA* expression at the late exponential

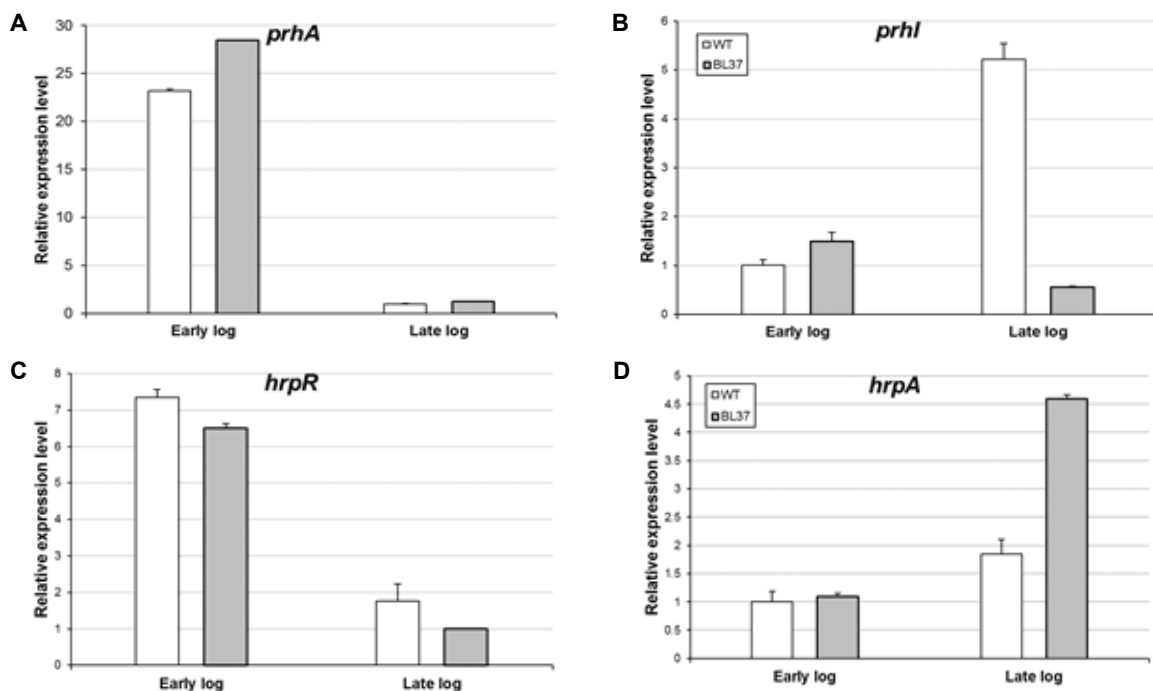


Fig. 5. Relative mRNA expression levels of *prhA*, *prhI*, *hrpR*, and *hrpA* in wild-type (WT), and Δ *psyR* mutant in *Pseudomonas syringae* pv. *tabaci* ATCC 11528 strains. (A) *prhA*, (B) *prhI*, (C) *hrpR*, (D) *hrpA*. Other details are as described in the legend to Fig. 4.

phase was negatively regulated by PsyR.

Putative promoter region related to virulence genes of *P. syringae* pv. *tabaci* ATCC 11528

A specific palindromic sequence, called a *lux* box, has been reported in the promoter regions of many QS-regulated genes [31]. In this study, we searched for a putative *lux* box sequence in the promoter regions of eight genes of interest (Fig. 6). Interestingly, this regulatory box lies between the -35 and -10 position, and overlaps the putative -10 sequence. Thus, all these genes have a putative *lux* box with homologous dyad symmetry.

DNA-binding activity of PsyR

PsyR was purified from BL792 containing the plasmids pBL318 and pBL337, as described in the Materials and Methods. Protein samples from each phase of purification, as well as the eluted protein fractions, were analyzed by SDS-PAGE (Fig. 7). The molecular weight of the purified MBP-PsyR was determined as being about 70 kDa, which matched the predicted molecular weights of this protein.

The ability of *P. syringae* pv. *tabaci* 11528 PsyR to bind the *lux* box in vitro was investigated using EMSA (Fig. 8). The purified MBP-PsyR caused a band mobility shift of the PCR fragment on a 5% polyacrylamide gel. The DNA fragment was amplified from the upstream region of virulence-related genes in assays using as little as 160 fM of the purified DNA fragment. PsyR derived from the BL792 harboring pBL318 and pBL337 was not able to shift DNA frag-

```

psyI  ACCTGTTGTTAGGTACAG-T
Fur   ACCTGCATTAA-GAGAAGCT
gacA  ACC-GTTGCG-CG---AGGT
psyR  ACCT-TA-ATGGAAC-GAA
prhA  ACCTGT-GC-TCG-CAG-T
prhI  CACTGGAGCATCG-GCACGA
hrpR  ACGTGTATTATAGTCCGCTT
hrpA  ATCTGTACCAA-G-CAATT

      ACCTGTTCTTAAGTACAGTA
          <----->
    
```

Fig. 6. Alignment of the upstream regions of virulence-related genes in *Pseudomonas syringae* pv. *tabaci* ATCC 11528 with *lux* box sequences; alignments were created using CLUSTAL X. Arrows above the sequences indicate dyad areas.

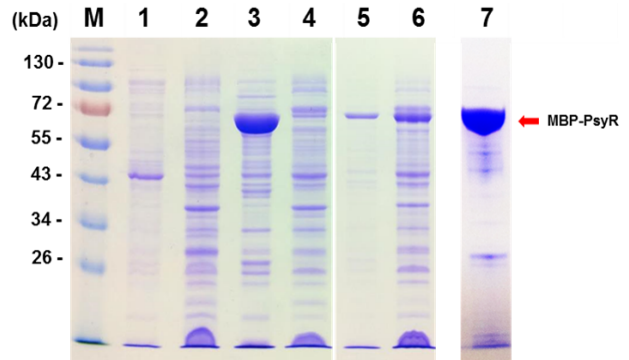


Fig. 7. SDS-PAGE of the PsyR-tagged maltose-binding protein (MBP-PsyR) of *Pseudomonas syringae* pv. *tabaci* ATCC 11528. Lane M, prestained protein ladder (Fermentas); lane 1, insoluble proteins in cell lysates (BL792) without IPTG; lane 2, soluble proteins in cell lysates (BL792) without IPTG; lane 3, insoluble proteins in cell lysates (BL759) with 0.1 mM IPTG; lane 4, soluble proteins in cell lysates (BL759) with 0.1 mM IPTG; lane 5, insoluble proteins in cell lysates (BL792) with 0.1 mM IPTG; lane 6, soluble proteins in cell lysates (BL792) with 0.1 mM IPTG; lane 7, MBP-PsyR purified from BL792.

ments containing the *lux* box of any of the genes, except for the upstream region of *psyl*. These results confirmed that PsyR binds specifically to the *lux* box region in *psyl*, but does not directly regulate virulence-related genes.

Discussion

Pseudomonas syringae pv. *tabaci* is a plant pathogenic bacterium that causes wildfire disease in tobacco plants. Studies on the genetic basis of the pathogenesis of *P. syringae* pathovars has been reported, and have revealed the role of TTSS-related genes, global regulatory genes, and motility and virulence-associated factors such as various phytotoxins, EPSs, and siderophores [3]. *P. syringae* pv. *tabaci* exhibits QS that is based on the production of diffusible signal molecules; however, the role of QS in this organism has not yet been clearly defined. In this study, the effect of PsyR protein as a QS transcriptional regulator was demonstrated using phenotypic and genetic analyses in *P. syringae* pv. *tabaci* ATCC 11528. We investigated the regulation of virulence-related genes by PsyR and observed differences in the patterns of regulation of *psyl*, *fur*, *prhI*, and *hrpA* in the Δ *psyR* mutant strain as compared to that in the WT strain.

We also investigated the regulatory effects of PsyR on swarming motility and production of siderophores, tabtoxin, and AHLs, as well as symptoms of wildfire disease in tobac-

co leaves by comparing the mutant and WT strains. Previous studies of tabtoxin reported that tabtoxin causes chlorosis around necrotic lesions and assists the development of

lesions. Our results demonstrated that PsyR coordinated the production of tabtoxin. In addition, swarming motility was increased in the Δ psyR mutant strain, indicating that PsyR

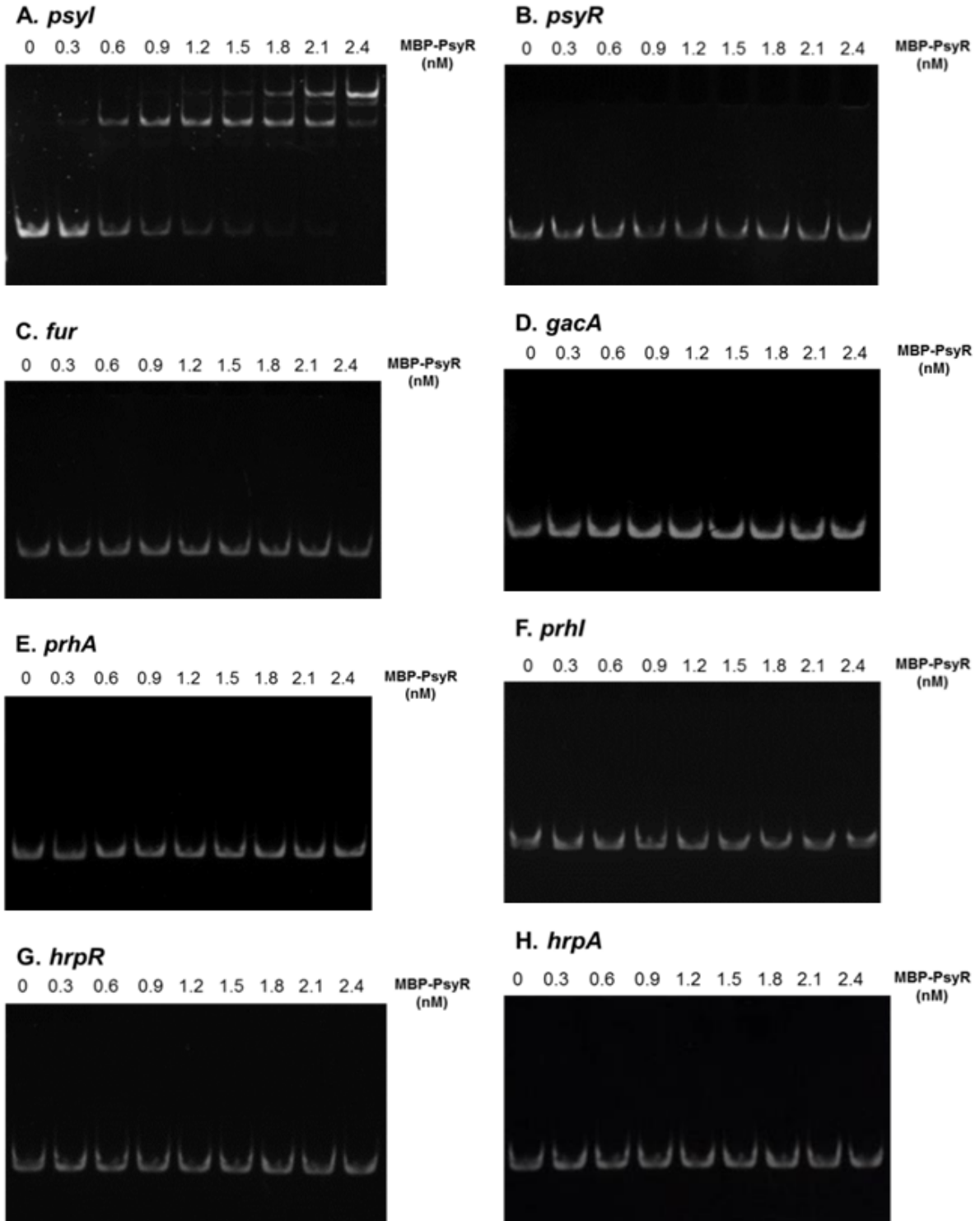


Fig. 8. Electrophoretic mobility shift assays (EMSA) of binding of MBP-PsyR to the putative promoter-containing regions of target genes: (A) *psyl*, (B) *psyR*, (C) *fur*, (D) *gacA*, (E) *prhA*, (F) *prhI*, (G) *hrpR*, (H) *hrpA*. All lanes contained 160 fM of DNA and the indicated concentration of MBP-PsyR. The DNA - protein reaction mixtures were loaded onto 5% polyacrylamide gels.

was involved in the regulation of swarming motility. The $\Delta psyR$ mutant strain caused a delay in the development of disease symptoms compared to the WT strain. However, PsyR did not affect the growth rate of *P. syringae* pv. *tabaci* 11528. Thus, the phenotypic characteristics of the $\Delta psyR$ mutant may not be due to differences in growth rates.

Furthermore, the $\Delta psyR$ mutant strain produced less siderophores than the WT strain under low-iron conditions and did not produce siderophores under high-iron conditions. This was confirmed by the results from real-time RT-PCR. Under low-iron conditions, the *fur* mRNA level in the $\Delta psyR$ mutant strain at the early exponential phase was higher than that in the WT strain, suggesting that the expression of *fur* repressed production of siderophores and may be positively regulated by PsyR. Moreover, the *fur* mRNA level in the $\Delta psyR$ mutant strain was lower under high-iron conditions than in the WT strain. This may suggest that expression of *fur* in the presence of high iron concentrations is controlled by PsyR, using another mechanism.

Production of AHL was regulated by PsyR in response to cell density; this finding was supported by the real-time RT-PCR results. The *psyl* mRNA levels in the $\Delta psyR$ mutant strain were significantly reduced at the late exponential phase. These results suggested that expression of *psyl* at a high density of bacteria is positively controlled by PsyR. Thus, *psyl* expression is tightly controlled by PsyR in response to bacterial cell density. There was no marked difference in the *gacA* mRNA levels between WT and $\Delta psyR$ mutant strains. Previous studies have reported that GacA positively controls QS signaling and PsyR [6, 8, 31]. More specifically, GacA regulates expression of *psyR*, according to a recent study, but PsyR does not regulate expression of *gacA*. Expression levels of *prhI* in the $\Delta psyR$ mutant strain were higher at the late exponential phase. In addition, the mRNA levels of *hrpA* at the late exponential phase were greater than those in the WT strain. These results showed that *prhI* and *hrpA* are expressed via regulation by PsyR in response to bacterial cell density; thus, QS influences expression of TTSS genes. These results suggest that PsyR has a dual role as an activator or repressor.

To determine whether PsyR binds upstream of pathogenesis-related genes in order to regulate their expression, EMSA was performed. A PsyR-overproduction strain was constructed specifically for use in EMSA in this study. A recent study reported that cognate AHLs are necessary for protein stability and solubility during the purification of sev-

eral LuxR-type regulators [21]. *psyl*, encoding an AHL synthase, was co-expressed in the PsyR-overproduction strain to enhance the production of soluble protein. Soluble PsyR proteins were successfully overproduced when the overproduction strain harbored both *psyl* and *psyR*. The identification of *cis*-regulatory sequences and the binding of PsyR to the upstream region of each virulence gene provided evidence of their involvement in the regulation of these genes. No binding of a purified MBP-PsyR protein to the upstream region of these genes, except *psyl*, was detected. Surprisingly, PsyR did not bind to the upstream region of *psyR*. These results suggested that PsyR may be indirectly controlled via intermediate-regulatory systems and that auto-regulation by PsyR does not occur.

In conclusion, this study shows that PsyR controls production of the effectors by modulating transcript levels. These findings indicate that PsyR-mediated regulation plays important roles in infection of plants by *P. syringae* pv. *tabaci*. Integration of the regulatory network including PsyR is required in order to obtain a better understanding of the pathogenetic mechanism common to all *P. syringae* pathovars and to identify the major components of each regulatory system. Elucidating pathogenetic mechanisms in phytopathogens will offer valuable insight into diseases caused by plant pathogens and will lay the basis for studies into antimicrobial therapies, as well as in the medical, food, and agriculture industries.

Acknowledgement

This work was supported by a 2-year Research Grant of Pusan National University.

References

1. Allen, S. S. and McMurray, D. N. 2003. Coordinate cytokine gene expression *in vivo* following induction of tuberculous pleurisy in guinea pigs. *Infect. Immun.* **71**, 4271-4277.
2. Bertani, G. 1951. A method for detection of mutations, using streptomycin dependence in *Escherichia coli*. *Genetics* **36**, 598-611.
3. Brito, B., Aldon, D., Barberis, P., Boucher, C. and Genin, S. 2002. A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum* *hrp* genes. *Mol. Plant Microbe Interact.* **15**, 109-119.
4. Calderwood, S. B. and Mekalanos, J. J. 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. *J. Bacteriol.* **169**, 4759-4764.

5. Camilli, A. and Bassler, B. L. 2006. Bacterial small-molecule signaling pathways. *Science* **311**, 1113-1116.
6. Cha, J. Y., Lee, D. G., Lee, J. S., Oh, J. I. and Baik, H. S. 2012. GacA directly regulates expression of several virulence genes in *Pseudomonas syringae* pv. *tabaci* 11528. *Biochem. Biophys. Res. Commun.* **417**, 665-672.
7. Cha, J. Y., Lee, J. S., Oh, J. I., Choi, J. W. and Baik, H. S. 2008. Functional analysis of the role of Fur in the virulence of *Pseudomonas syringae* pv. *tabaci* 11528: Fur controls expression of genes involved in quorum-sensing. *Biochem. Biophys. Res. Commun.* **366**, 281-287.
8. Chatterjee, A., Cui, Y., Yang, H., Collmer, A., Alfano, J. R. and Chatterjee, A. K. 2003. GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. *tomato* DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol. Plant Microbe Interact.* **16**, 1106-1117.
9. Choi, S. H. and Greenberg, E. P. 1992. Genetic dissection of DNA binding and luminescence gene activation by the *Vibrio fischeri* LuxR protein. *J. Bacteriol.* **174**, 4064-4069.
10. Deng, W. L., Rehm, A. H., Charkowski, A. O., Rojas, C. M. and Collmer, A. 2003. *Pseudomonas syringae* exchangeable effector loci: sequence diversity in representative pathovars and virulence function in *P. syringae* pv. *syringae* B728a. *J. Bacteriol.* **185**, 2592-2602.
11. Ducros, V. M., Lewis, R. J., Verma, C. S., Dodson, E. J., Leonard, G., Turkenburg, J. P., Murshudov, G. N., Wilkinson, A. J. and Brannigan, J. A. 2001. Crystal structure of GerE, the ultimate transcriptional regulator of spore formation in *Bacillus subtilis*. *J. Mol. Biol.* **306**, 759-771.
12. Dulla, G., Marco, M., Quinones, B. and Lindow, S. 2005. A Closer Look at *Pseudomonas syringae* as a Leaf Colonist - The pathogen *P. syringae* thrives on healthy plants by employing quorum sensing, virulence factors, and other traits. *Asm News* **71**, 469-475.
13. Fuqua, C. and Greenberg, E. P. 2002. Listening in on bacteria: Acyl-homoserine lactone signalling. *Nat. Rev. Mol. Cell Biol.* **3**, 685-695.
14. Fuqua, C., Parsek, M. R. and Greenberg, E. P. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **35**, 439-468.
15. Genin, S., Brito, B., Denny, T. P. and Boucher, C. 2005. Control of the *Ralstonia solanacearum* Type III secretion system (Hrp) genes by the global virulence regulator PhcA. *FEBS Lett.* **579**, 2077-2081.
16. Hauck, P., Thilmoney, R. and He, S. Y. 2003. A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc. Natl. Acad. Sci. USA* **100**, 8577-8582.
17. Hendrickson, E. L., Guevera, P., Penaloza-Vazquez, A., Shao, J., Bender, C. and Ausubel, F. M. 2000. Virulence of the phytopathogen *Pseudomonas syringae* pv. *maculicola* is rpoN dependent. *J. Bacteriol.* **182**, 3498-3507.
18. Jin, Q., Thilmoney, R., Zwiesler-Vollick, J. and He, S. Y. 2003. Type III protein secretion in *Pseudomonas syringae*. *Microbes Infect.* **5**, 301-310.
19. Jing, D., Agnew, J., Patton, W. F., Hendrickson, J. and Beechem, J. M. 2003. A sensitive two-color electrophoretic mobility shift assay for detecting both nucleic acids and protein in gels. *Proteomics* **3**, 1172-1180.
20. Keen, N. T., Tamaki, S., Kobayashi, D. and Trollinger, D. 1988. Improved Broad-Host-Range Plasmids for DNA Cloning in Gram-Negative Bacteria. *Gene* **70**, 191-197.
21. Kim, J. and Park, W. 2013. Identification and characterization of genes regulated by AqsR, a LuxR-type regulator in *Acinetobacter oleivorans* DR1. *Appl. Microbiol. Biotechnol.* **97**, 6967-6978.
22. King, E. O., Ward, M. K. and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* **44**, 301-307.
23. Lazdunski, A. M., Ventre, I. and Sturgis, J. N. 2004. Regulatory circuits and communication in Gram-negative bacteria. *Nat. Rev. Microbiol.* **2**, 581-592.
24. Lee, J. S., Cha, J. Y. and Baik, H. S. 2011. Plant cell contact-dependent virulence regulation of *hrp* genes in *Pseudomonas syringae* pv. *tabaci* 11528. *J. Life Sci.* **21**, 227-234.
25. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190-206.
26. Marendra, M., Brito, B., Callard, D., Genin, S., Barberis, P., Boucher, C. and Arlat, M. 1998. PrhA controls a novel regulatory pathway required for the specific induction of *Ralstonia solanacearum* *hrp* genes in the presence of plant cells. *Mol. Microbiol.* **27**, 437-453.
27. Mey, A. R., Wyckoff, E. E., Kanukurthy, V., Fisher, C. R. and Payne, S. M. 2005. Iron and fur regulation in *Vibrio cholerae* and the role of fur in virulence. *Infect. Immun.* **73**, 8167-8178.
28. Miller, M. B. and Bassler, B. L. 2001. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**, 165-199.
29. Nealson, K. H. and Hastings, J. W. 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiol. Rev.* **43**, 496-518.
30. Nealson, K. H., Platt, T. and Hastings, J. W. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* **104**, 313-322.
31. Parsek, M. R. and Greenberg, E. P. 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc. Natl. Acad. Sci. USA* **97**, 8789-8793.
32. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
33. Piper, K. R., Beck von Bodman, S. and Farrand, S. K. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**, 448-450.
34. Prince, R. W., Storey, D. G., Vasil, A. I. and Vasil, M. L. 1991. Regulation of *toxA* and *regA* by the *Escherichia coli* fur gene and identification of a Fur homologue in *Pseudomonas aeruginosa* PA103 and PA01. *Mol. Microbiol.* **5**, 2823-2831.
35. Pristovsek, P., Sengupta, K., Lohr, F., Schafer, B., von Trebra, M. W., Ruterjans, H. and Bernhard, F. 2003. Structural analy-

- sis of the DNA-binding domain of the *Erwinia amylovora* RcsB protein and its interaction with the RcsAB box. *J. Biol. Chem.* **278**, 17752-17759.
36. Quinones, B., Dulla, G. and Lindow, S. E. 2005. Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Mol. Plant Microbe Interact.* **18**, 682-693.
 37. Schwartz, T., Walter, S., Marten, S. M., Kirschhofer, F., Nusser, M. and Obst, U. 2007. Use of quantitative real-time RT-PCR to analyse the expression of some quorum-sensing regulated genes in *Pseudomonas aeruginosa*. *Anal. Bioanal. Chem.* **387**, 513-521.
 38. Schweizer, H. P. 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. *Mol. Microbiol.* **6**, 1195-1204.
 39. Schwyn, B. and Neilands, J. B. 1987. Universal chemical-assay for the detection and determination of siderophores. *Anal. Biochem.* **160**, 47-56.
 40. Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Jr., Rinehart, K. L. and Farrand, S. K. 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci. USA* **94**, 6036-6041.
 41. Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Rinehart, K. L. and Farrand, S. K. 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci. USA* **94**, 6036-6041.
 42. Sircili, M. P., Walters, M., Trabulsi, L. R. and Sperandio, V. 2004. Modulation of enteropathogenic *Escherichia coli* virulence by quorum sensing. *Infect. Immun.* **72**, 2329-2337.
 43. Smith, R. S. and Iglewski, B. H. 2003. *Pseudomonas aeruginosa* quorum sensing as a potential antimicrobial target. *J. Clin. Invest.* **112**, 1460-1465.
 44. Thompson, D. K., Beliaev, A. S., Giometti, C. S., Tollaksen, S. L., Khare, T., Lies, D. P., Nealson, K. H., Lim, H., Yates, J., 3rd, Brandt, C. C., Tiedje, J. M. and Zhou, J. 2002. Transcriptional and proteomic analysis of a ferric uptake regulator (*fur*) mutant of *Shewanella oneidensis*: possible involvement of *fur* in energy metabolism, transcriptional regulation, and oxidative stress. *Appl. Environ. Microbiol.* **68**, 881-892.
 45. Ulrich, R. L., Deshazer, D., Brueggemann, E. E., Hines, H. B., Oyston, P. C. and Jeddleloh, J. A. 2004. Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. *J. Med. Microbiol.* **53**, 1053-1064.
 46. Von Bodman, S. B., Bauer, W. D. and Coplin, D. L. 2003. Quorum sensing in plant-pathogenic bacteria. *Annu. Rev. Phytopathol.* **41**, 455-482.
 47. Watnick, P. I., Eto, T., Takahashi, H. and Calderwood, S. B. 1997. Purification of *Vibrio cholerae fur* and estimation of its intracellular abundance by antibody sandwich enzyme-linked immunosorbent assay. *J. Bacteriol.* **179**, 243-247.
 48. Wertheimer, A. M., Tolmashy, M. E., Actis, L. A. and Crosa, J. H. 1994. Structural and functional analyses of mutant Fur proteins with impaired regulatory function. *J. Bacteriol.* **176**, 5116-5122.
 49. Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J. and Salmond, G. P. 2001. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol. Rev.* **25**, 365-404.
 50. Withers, H., Swift, S. and Williams, P. 2001. Quorum sensing as an integral component of gene regulatory networks in Gram-negative bacteria. *Curr. Opin. Microbiol.* **4**, 186-193.
 51. Yang, H. J., Lee, J. S., Cha, J. Y. and Baik, H. S. 2011. Negative regulation of pathogenesis in *Pseudomonas syringae* pv. *tabaci* 11528 by ATP-dependent Lon protease. *Mol. Cells* **32**, 317-323.
 52. Zhang, R. G., Pappas, K. M., Brace, J. L., Miller, P. C., Oulmassov, T., Molyneaux, J. M., Anderson, J. C., Bashkin, J. K., Winans, S. C. and Joachimiak, A. 2002. Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* **417**, 971-974.

초록 : *Pseudomonas syringae* pv. *tabaci*에서 LuxR-type 전사조절자인 PsyR에 의한 병원성 유전자들의 조절

최연희 · 이준승 · 윤소라 · 백형석*
(부산대학교 미생물학과)

Pseudomonas syringae pv. *tabaci* 11528은 담배를 숙주로 하여 wildfire disease를 일으키는 식물 병원성 세균이다. *P. syringae* pv. *tabaci* *psyR* deletion mutant를 이용하여 swarming motility, tabtoxin 생산능, siderophore 생산능, AHL 생산능 등의 phenotypic test를 수행하였다. *psyR* deletion mutant는 wild-type 균주보다 swarming motility가 증가하였고, tabtoxin 생산 또한 증가하였다. 하지만 siderophore와 AHL 생산능은 감소하였고 virulence 또한 지연되었다. 이러한 결과로 PsyR이 QS regulator로 작용한다는 사실과 더불어 병원성 유전자의 조절에도 관여한다는 것을 확인하였다. PsyR이 각각의 병원성 유전자의 발현을 조절하는 regulator들에게 미치는 영향을 전사단계에서 확인하기 위해 *fur*, *gacA*, *psyI*, *prhI*, *prhA*, *hrpR*, *hrpA* 유전자들을 정량적 real-time PCR (qRT-PCR) 방법으로 확인하였다. 또한 PsyR에 의한 병원성 유전자 조절이 DNA상에 직접적으로 결합하여 일어나는 것인지 아니면 다른 경로를 통해 간접적으로 일어나는 것인지를 확인할 필요가 있어 정제한 PsyR 단백질과 병원성 관련 유전자들의 upstream region 서열을 이용하여 electrophoretic mobility shift assay (EMSA)를 수행한 결과 본 연구에서 선정한 병원성 관련 유전자들이 PsyR에 의해 직접적으로 조절되지는 않는다는 사실을 밝혔다.