

Plant Cell Contact-Dependent Virulence Regulation of *hrp* Genes in *Pseudomonas syringae* pv. *tabaci* 11528

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The *hrp* gene cluster in the plant pathogen *Pseudomonas syringae* is a key determinant of pathogenicity. Recent studies have demonstrated that specific host cell induction of the *Ralstonia solanacearum* *hrp* gene cluster is controlled by the PrhA (plant regulator of *hrp*) receptor. To characterize the role that *P. syringae* PrhA plays in the virulence of plant cells, a *prhA* homolog was isolated from *P. syringae* pv. *tabaci* and a $\Delta prhA$ mutant was constructed by allelic exchange. The $\Delta prhA$ mutant had reduced virulence in the host plant, and co-culture of *P. syringae* pv. *tabaci* and plant cell suspensions induced a much higher level of *hrpA* gene transcription than culture in *hrp*-inducing minimal medium. These results indicate that PrhA of *P. syringae* is a putative pathogen-plant cell contact sensor, therefore, we used a *hrpA-gfp* reporter fusion to monitor the *in situ* expression of PrhA. The results of this study demonstrated that PrhA induces *hrp* gene expression in *P. syringae* pv. *tabaci* in the presence of plant cells.

Key words : Plant regulator of *hrp* (PrhA), plant-pathogen interaction, *Pseudomonas syringae* pv. *Tabaci*, TonB-dependent siderophore receptor

Introduction

Pseudomonas syringae is an important plant pathogenic bacterium commonly used to study plant-microbe interactions. *P. syringae* causes leaf spots and necrosis in host plants and a hypersensitive response (HR) in non-host plants [13]. In host plants, disease symptoms usually develop after several days of bacterial growth in the leaf apoplast. However, on non-host plants, the defense-associated programmed cell death that characterizes the HR occurs within 24 hr of the plant cells coming in contact with the bacteria [25]. More than 50 *P. syringae* pathovars have been identified based on their virulence and host specificity [9]. For example, *P. syringae* pv. *tabaci* causes disease on tobacco plants, but induces a HR on many other non-host plant species. The ability of *P. syringae* to cause diseases on their hosts and to elicit the HR in non-host plants is controlled by the *hrp* (hypersensitive response and pathogenicity) / *hrc* (hypersensitive response and conserved) genes, which reside in a pathogenicity island also known as the Hrp PAI [2,5,18]. The *hrp/hrc* genes (*hrp* gene cluster) are conserved among many gram-negative plant pathogenic bacteria, including *P. syringae*, *Ralstonia solanacearum*, *Xanthomonas campestris*,

Erwinia amylovora, *Pantoea stewartii* sp. *stewartii* and *Erwinia chrysanthemi*, as well as bacteria associated with animals [3,4,6,10,18]. The *hrp/hrc* genes are expressed at a very low level in nutrient-rich media, but are induced in plants or in artificial *hrp*-inducing minimal media that mimics apoplastic conditions [14,24,30]. In addition, recent studies have shown that specific host factors induce expression of the *Ralstonia solanacearum* *hrp* gene cluster, and that this specific host cell induction of the *hrp* gene cluster is controlled by PrhA (plant regulatory of *hrp*), a protein that is homologous to outer-membrane siderophore receptors [1,20].

In this study, we amplified a *prhA* gene of *P. syringae* pv. *tabaci* (ATCC 11528) by PCR and then constructed a $\Delta prhA$ mutant by allelic exchange. We evaluated *prhA* to determine if it regulates the transcription of *hrpA* by comparing the levels of β -galactosidase activity after growth in *hrp*-inducing minimal medium. Despite no differences being found in any of the tested strains, the $\Delta prhA$ mutant had reduced virulence in the host plant. However, co-culture of *P. syringae* and plant cell materials induced a much higher level of *hrpA* gene transcription than culture in *hrp*-inducing minimal medium. Taken together, these results indicate that PrhA of *P. syringae* is a putative pathogen-plant cell contact sensor.

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Materials and Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* strains were grown in King's B (*hrp*-repressing medium) or M9 minimal medium supplemented with 5 mM mannitol (*hrp*-inducing medium, pH 5.5) at 30°C. When necessary, antibiotics were added to the media at the following concentrations (µg/ml): ampicillin, 50; kanamycin, 20; tetracycline 10. LB agar containing 10% sucrose was employed for *sacB* gene based counter-selection using the allelic exchange method [11]. Siderophore production was examined by the addition of either FeCl₃ (100 µM) or 2,2'-dipyridyl (200 µM) to chrome azure S (CAS) agar plates to induce iron-depleted or iron-replete conditions, respectively [7,28]. Preparation of cell wall material from *Arabidopsis* cell suspensions was performed using the method described by Aldon *et al* [1].

General DNA manipulations

DNA manipulations were conducted as described by Sambrook *et al.* [27] or using the protocols described by the manufacturers of the reagents. The recombinant suicide plasmid was transferred conjugally into *P. syringae* pv. *tabaci* using *E. coli* S17-1 (*pro*) as a plasmid donor. Standard PCR amplifications were performed with EF-*Taq* DNA polymerase (Solgent, Korea) using GeneAmp 9700 thermal cycler (Applied Biosystems). The oligonucleotides used for cloning are shown in Table 2. Sequencing was performed using a BigDye Terminator v1.1 Ready Reaction cycle sequencing kit on an ABI Prism 377 genetic analyzer (Applied Biosystems). Similarity searches were performed using the BLAST family of programs in National Center for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/>.

Construction of a *prhA* deletion mutant by *in vivo* allelic exchange and complementation

The 2.1-kb fragment of the partial *prhA* gene was ampli-

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics ^a	Source or reference
Strains		
<i>E. coli</i>		
TOP10	Transformation host for cloning vector, F <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80/ <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>araC</i>) <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
S17-1 λ pir	Conjugation donor, Pro ⁻ Res ⁻ Mob ⁺ , <i>recA</i> , integrated plasmid RP4-Tc::Mu-Km::Tn7	[29]
<i>P. syringae</i> pv. <i>tabaci</i>		
ATCC 11528	Wild Type, causal agent of tobacco wild-fire	ATCC
BL11	ATCC 11528 derivative, Δ <i>prhA</i> (1120-bp deletion)	This study
BL18	ATCC 11528 carrying pBL52, Tc ^R	This Study
BL19	BL11 carrying pBL52, Tc ^R	This Study
BL33	ATCC 11528 derivative, Δ <i>fur</i> (299-bp deletion)	[7]
BL31	ATCC 11528 carrying pBL98, Tc ^R	This Study
BL32	BL11 carrying pBL98, Tc ^R	This Study
BL45	BL11 carrying pBL172	This Study
Plasmids		
pGEM-T Easy	Ap ^R , TA Cloning vector for PCR product, ColE1 <i>ori</i>	Promega
pLO1	Suicide vector, Km ^R , <i>sacB</i> , RP4 <i>oriT</i> , ColE1 <i>ori</i>	[17]
pCF1010	Tc ^R , <i>lacZ</i> transcriptional fusion vector, IncQ/IncP4 <i>ori</i>	[16]
pEGFP-C1	Km ^R , It encodes enhanced green fluorescence protein (EGFP)	Clontech
pRK415	Tc ^R ; mobilizable broad-host-range vector, <i>oriV</i> and <i>oriT</i> of RK2	[15]
pRKlac290	Tc ^R , broad-host-range vector, IncP1 <i>ori</i> , Mob ⁺	[12]
pBL39	pLO1 carrying 0.9 kb <i>XbaI</i> - <i>Sad</i> DNA fragment of partial <i>P. syringae</i> pv. <i>tabaci</i> <i>prhA</i> gene	This Study
pBL52	pCF1010 carrying <i>hrpA-lacZ</i> transcriptional fusion	Lab collection
pBL98	pRKlac290 carrying <i>hrpA-gfp</i> transcriptional fusion	This Study
pBL172	pRK415 carrying wild-type <i>prhA</i> gene (complementation)	This Study

^aAp^R, Ampicillin resistance; Km^R, Kanamycin resistance; Tc^R, Tetracycline resistance; *ori* (*oriV*), origin of replication; *oriT*, transfer origin.

Table 2. Primers used in this study

No	Name	Oligonucleotide sequence ^a (5'-3')
p134	prhA-m-F(<i>Xba</i> I)	AGTCTAGATACTTGGCCGTC AACGCATAGC
p136	prhA-m-R(<i>Sac</i> I)	TGGAGCTCGTTTTCTTTTACCCCTGCCCTG
p140	long-prhA-F	GCGCAGGATGAGTTCCTTGTT
p141	long-prhA-R	GCTTGGAGCCCAGATAGCC
p159	hrpA-p-F(<i>Xba</i> I)	TATCTAGATCTGTCCGCTGCTCAGC
p160	hrpA-p-R(<i>Ban</i> HI)	TAGGATCCGCCGTCTCTTCGTTCCG
p161	egfp-F(<i>Ban</i> HI)	TAGGATCCATGGTGAGCAAGGGCGAG
p163	egfp-R(<i>Hind</i> III)	TAAAGCTTCTGTACAGCTCGTCCATG
p196	prhA-cp-F(<i>Eco</i> RI)	TAGAATTCGCATTATCAGGTTGCAGACG
p198	prhA-c-R(<i>Ban</i> HI)	TAGGATCCTCAGAAGCTGTA CTGGCCG

^aUnderlines are the restriction enzyme sites for the enzyme indicated in the primer names

fied by PCR from *P. syringae* pv. *tabaci* 11528 genomic DNA using the primer pairs p134 and p136. The PCR product was then subcloned into pGEM-T Easy vector and digested with *Hind*III/*Sma*I enzymes. Next, the resultant 3.9-kb DNA fragment was blunted with Mung Bean nuclease and self-ligated, which gave pBL26. A 0.9-kb *Xba*I-*Sac*I fragment of pBL26 was then cloned into pLO1 vector to generate the recombinant suicide plasmid, pBL39. Next, the pBL39 plasmid was transferred from *E. coli* S17-1 to *P. syringae* pv. *tabaci* 11528 by a spot mating technique [29] and then introduced into *P. syringae* pv. *tabaci* 11528 by allelic exchange [17] using the suicide plasmid pBL39, yielding *P. syringae* pv. *tabaci* BL11. The presence of the 1.2-kb deletion was confirmed by Southern blotting (data not shown).

To complement the Δ *prhA* mutant (BL11), the complete *prhA* gene was PCR-amplified from genomic DNA using primer pairs p196 and 198, and then cloned into pRK415 vector. The construct was then introduced into the strain BL11 by mating, yielding *P. syringae* pv. *tabaci* BL45. Complementation tests were then conducted as phenotypic experiments.

Construction of a *hrpA::gfp* transcriptional fusion plasmid

In order to determine the *hrpA* expression at the transcriptional levels, an *hrpA::gfp* fusion plasmid was constructed in the pRKlac290 vector. A 0.6-kb *hrpA* promoter region encompassing the putative *hrpA* promoter was then synthesized by PCR amplification using the *P. syringae* pv. *tabaci* 11528 genomic DNA as a template and primers p159 and p160. Next, a 0.7-kb *egfp* (enhanced green fluorescent protein) gene was amplified from the pEGFP-C1 plasmid using primers p161 and p163. The resulting PCR products were then restricted using the *Ban*HI enzyme and ligated together

within the pGEM-T Easy vector to give pBL96. Next, the 1.3-kb *Xba*I-*Hind*III fragment of pBL96 was cloned into the pRKlac290 vector to generate pBL98. The resultant plasmid, pBL98, was then confirmed by PCR and subsequent DNA sequencing using primers p159 and p163. The pBL98 plasmid was then transferred from *E. coli* S17-1 to *P. syringae* pv. *tabaci* by a spot mating technique [29].

β -Galactosidase assays

β -galactosidase activity in bacterial cells was estimated using the procedures described by Miller [21]. Briefly, *P. syringae* cells grown under various conditions were permeabilized with chloroform and 1% SDS in Z buffer. The enzyme activity was then determined by measuring the absorbance at 420 nm to determine the rate at which the chromogenic substrate, ONPG (o-nitrophenyl- β -D-galactopyranoside), was cleaved. The activity was expressed in Miller units.

Phenotypic and virulence tests

Phenotypic (growth curve, CAS universal siderophore detection assay, swarming motility) [7] and virulence tests [26] were conducted as described previously. For these tests, bacteria were grown overnight at 30°C, after which, the cultures were adjusted to approximately 2×10^8 CFU/ml in distilled water. Swarming motility was observed on King's B agar (0.4% agar) plates using the method described by Quinones *et al* [23]. The inoculated plates were incubated for 48 hr at 30°C and photographs were taken with an Olympus C3020 zoom digital camera. The image contrast and brightness were adjusted using Photoshop CS2 (Adobe, San Jose, CA). All assays were repeated in three separate experiments.

Confocal laser scanning microscopy

Fluorescence images were recorded on a Zeiss LSM510 confocal laser scanning microscope (Zeiss, Germany). An argon laser was then used to generate an excitation source at 488 nm, and enhanced GFP fluorescence was recorded using a BP 505-515 nm filter set. A HeNe laser was used to generate an excitation source at 543 nm, and the chlorophyll fluorescence was recorded using an LP 550 nm filter set.

Statistics

Data are reported as the means±S.D., with the overall statistical significance of differences within the groups being determined using a student's t-test. All analyses were performed using SPSS for Windows (version 12.0K, SPSS Inc., Chicago, IL). For all statistical analyses, a p value <0.05 was considered significant.

Nucleotide sequence accession number

The sequence of the 2,169-nucleotide, which encodes the *prhA* gene from *P. syringae* pv. *tabaci* 11528, has been deposited in the GenBank database under accession no. DQ672633.

Results and Discussion

Nucleotide sequence and characterization of the *prhA* gene in *Pseudomonas syringae* pv. *tabaci* 11528

In our attempt to identify a *Ralstonia solanacearum* *prhA* homolog [20] in *P. syringae* pv. *tabaci* 11528, degenerate primers (Table 2) were used to PCR-amplify a 17-kb DNA fragment. These primers were designed using the sequences of *P. syringae* pv. *phaseolicola* 1448A (GenBank database under accession no. NC_005773), *P. syringae* pv. *syringae* B728a (GenBank database under accession no. CP_000075), and *P. syringae* pv. *tomato* DC3000 (GenBank database under accession no. NC_004578). Sequence analysis of the *prhA* gene was performed using the p196 and p198 primers (Table 2). The putative protein encoded by the *prhA* gene (2,169 bp) shares homology with outer membrane siderophore receptor proteins and encodes a protein of 722 amino acids, with a calculated molecular mass of 79.5 kDa. PrhA showed significant similarities to numerous TonB-dependent siderophore receptor proteins (data not shown) that are believed to bind specific iron-siderophore complexes to form a chan-

nel that allows the transport of these complexes into the periplasm [20].

Characterization of a $\Delta prhA$ mutant strain of *P. syringae* pv. *tabaci* 11528

Allelic exchange mutagenesis was used to construct a *prhA* deletion mutant strain that harbored a 1,172-bp deletion of the internal fragment of the *prhA* gene. When compared to the parent strain, growth of the $\Delta prhA$ mutant strain was slower, however, the stationary phase was reached at the same cell density as of the parent strain when grown in King's B media (Fig. 1a). Conversely, the growth rate of the $\Delta prhA$ mutant strain was equal to that of the wild-type strain, however, the stationary phase of the mutant strain was reached at a lower cell density than that of the wild-type strain when grown in *hrp*-inducing media (Fig. 1b). These results indicate that the phenotypic differences between the mutant and wild-type strains were most likely not due to a serious defect in the growth of the mutant. Swarming motility in the plant-pathogen interaction is critical as a virulence factor, most likely because it facilitates entry of the pathogen into plant tissues [7]. As shown in Fig. 1c, the swarming motility of the wild-type strain and the mutant were equal, which indicates that PrhA does not regulate swarming motility. In addition, the $\Delta prhA$ mutant produced slightly more siderophores than the wild-type strain under iron-depleted and iron-replete conditions (Fig. 1d), which indicates that *prhA* is not a key determinant in iron homeostasis.

The $\Delta prhA$ mutant had reduced virulence in the host plant

The ability of the $\Delta prhA$ mutant strain to cause wild-fire disease was assessed in tobacco leaves (host plant). *P. syringae* pv. *tabaci* 11528, BL11, and BL45 overnight cultures were adjusted to an OD₆₀₀ of 1.0 (approximately 2.0×10^8 CFU/ml) and then diluted serially to a concentration of 2×10^5 CFU/ml. After 3 days, the wild-type strain appeared to elicit disease symptoms at all concentrations, however, the $\Delta prhA$ mutant strain only appeared to elicit weak disease symptoms at a concentration of 2×10^8 CFU/ml (4×10^7 CFU/20 μ l inoculum). In addition, the wild-type strain elicited small brown necrotic lesions that were surrounded by chlorosis after 24 hr of incubation, but no such symptoms were observed when the plants were infected with less than 2×10^7 CFU/ml (4×10^6 CFU/20 μ l) of strain BL11. As shown

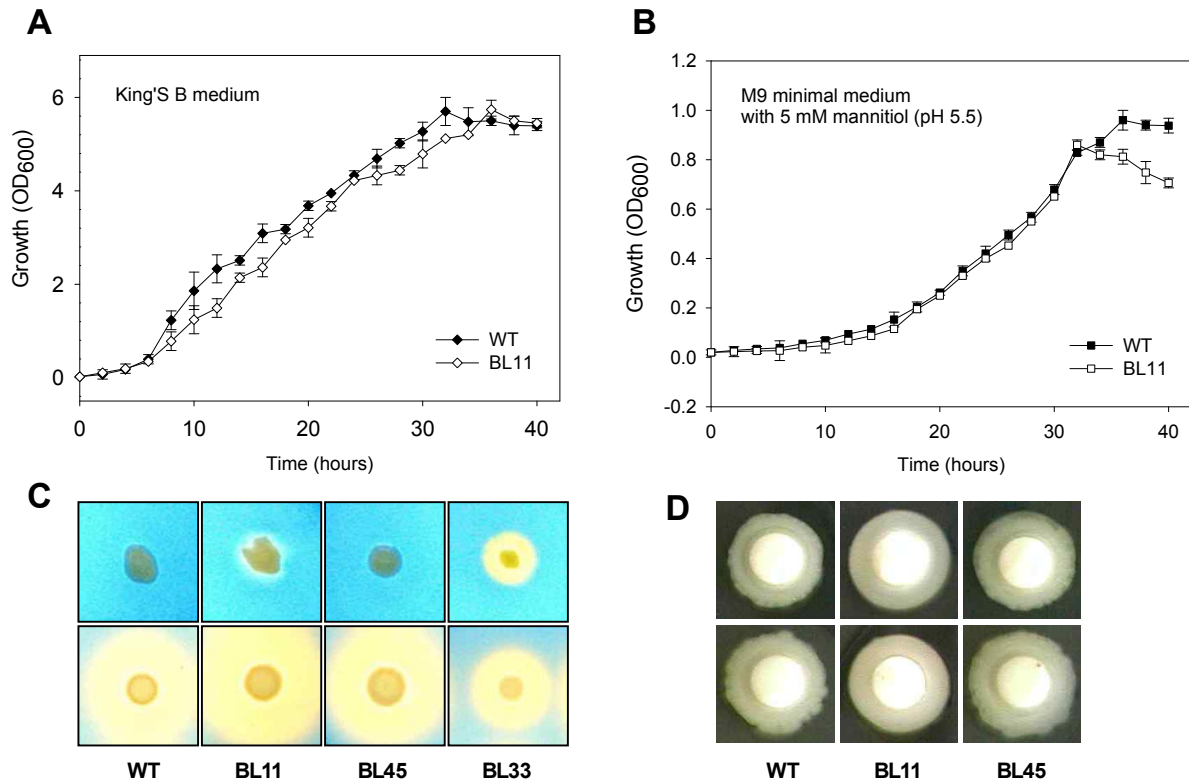


Fig. 1. Phenotypic characterization of the $\Delta prhA$ mutant. Growth curves of the wild-type (WT) and $\Delta prhA$ mutant (BL11) strains of *P. syringae* pv. *tabaci* 11528 cultured in *hrp*-repressing (A) and *hrp*-inducing medium (B), respectively. Values represent independent means \pm standard deviations of OD₆₀₀ readings obtained for triplicate cultures. Siderophore biosynthesis was compared using a CAS universal siderophore detection assay under iron-depleted or iron-replete conditions (C). BL33 (Δfur mutant) was used as a CAS assay control strain. BL45 is BL11 carrying pBL172 (*prhA* complementation). Swarming motility test in 0.4% King's B agar plate (D).

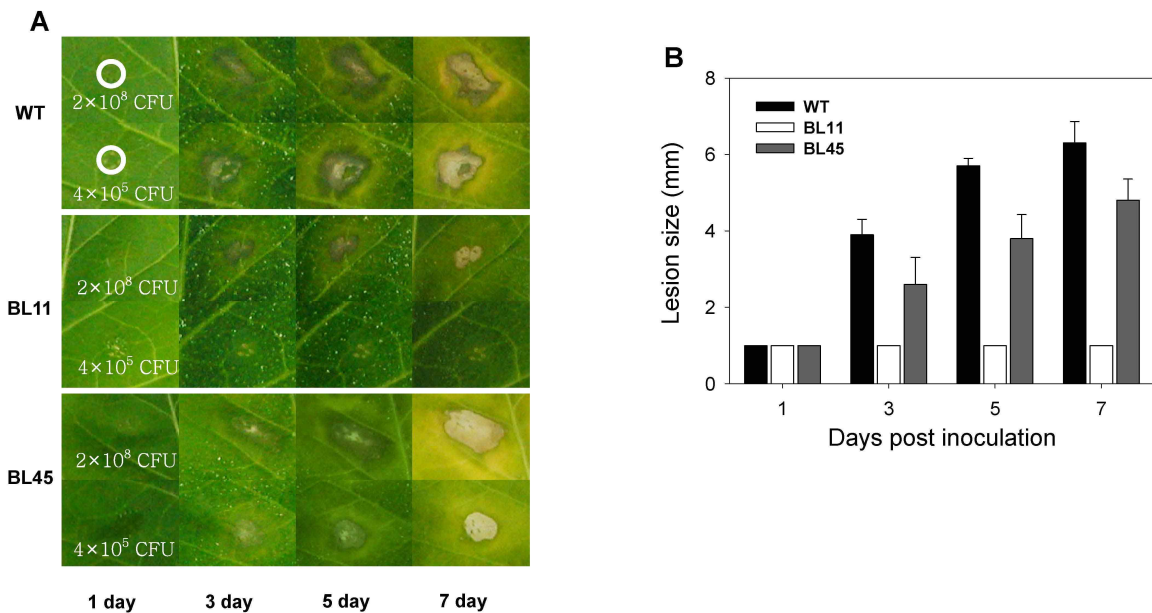


Fig. 2. Pathogenicity test of the $\Delta prhA$ mutant on tobacco leaves. (A) Disease symptoms caused by the *P. syringae* pv. *tabaci* wild-type (11528; WT), $\Delta prhA$ mutant (BL11), and its complementation strain (BL45). (B) Size of lesions on tobacco leaves produced by infection with the wild-type, BL11, and BL45 strains at a concentration of 4×10^5 CFU (20 μ l). The lesion diameter was measured in millimeters. The vertical lines above each bar indicate the standard errors of the mean of three individual tests.

in Fig. 2a, the ability of the $\Delta prhA$ mutant strain BL11 to produce symptoms in tobacco leaves was significantly weaker than that of the wild-type. In addition, strain BL11 is less virulent than the wild-type, therefore, PrhA may play an important role in the interaction between tobacco leaves and *P. syringae* pv. *tabaci* 11528 (Fig. 2a).

PrhA does not regulate transcription of the *hrpA* gene in *hrp*-inducing or nutrient rich media

The *hrp* gene cluster can be classified into three categories according to their functions, a regulatory system, a type III secretion system (TTSS), and the substrates of TTSS (harpin, Hop and pilus proteins, etc) [8,19]. Three intracellular regulatory proteins, HrpR, HrpS, and HrpL, are known to activate the *hrp* gene cluster expression. In addition, HrpL is an alternative sigma factor in the ECF (extracytoplasmic factor) family [30], and HrpR, HrpS, and HrpL appear to function in a regulatory cascade in which HrpS and HrpR synergistically activate the expression of *hrpL* in response to sig-

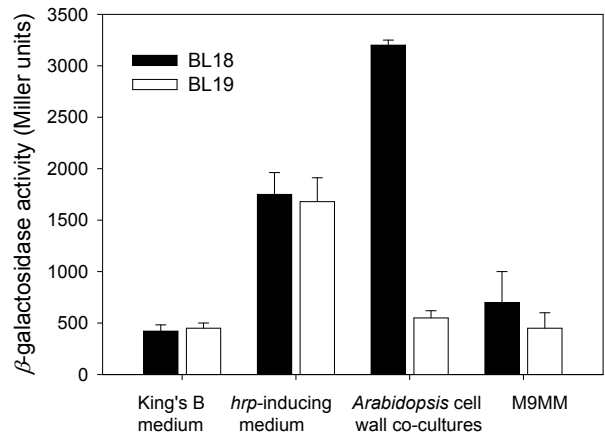


Fig. 3. Effect of a *prhA* mutation on expression of the *hrpA* gene during co-culture of *P. syringae* with *A. thaliana* cell wall material. β -galactosidase activity was measured in BL18 (11528 carrying pBL52) and BL19 (BL11 carrying pBL52) grown in *hrp*-repressing medium and *hrp*-inducing medium, respectively. The co-culture medium used was M9 minimal medium (M9MM).

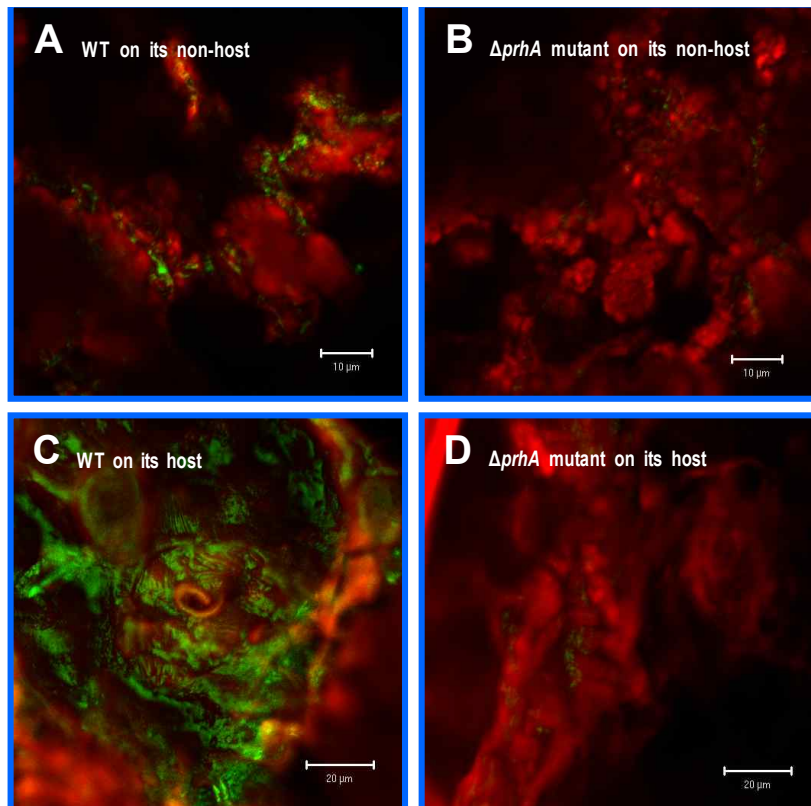


Fig. 4. The plant signal recognized by PrhA, which induces *hrpA* gene expression, is present in plant leaves. Observations by confocal laser scanning microscopy after 4 hr of co-cultivation of *Arabidopsis thaliana* (non-host) (A, B) and *Nicotiana tabacum* (host) (C, D) cell wall materials with the *P. syringae* pv. *tabaci* BL31 (11528 carrying pBL98) (A, C) or BL32 (BL11 carrying pBL98) (B, D). The bar represents 10 or 20 μ m. The red color is plant cell wall material and the green color is *P. syringae* carrying pBL98 (*hrpA-gfp* reporter fusion).

nals from plants or in *hrp*-inducing minimal medium. HrpL is also presumed to induce expression of the *hrp* and *avr* genes by recognizing a consensus sequence motif (*hrp* box) in the upstream regions [31]. Finally, the HrpL-dependent *hrpA* gene is a member of TTSS, therefore expression of the *hrpA* gene represents an *hrp* gene cluster expression. The transcription of the *P. syringae* pv. *tabaci* *hrp* gene cluster, which was identified by measuring the expression level of the *hrpA-lacZ* promoter fusion (pBL52), is induced in *hrp*-inducing medium (including M9 minimal medium with 5 mM mannitol, pH 5.5) and repressed in rich medium (including King's B medium) [20,22]. As shown in Fig. 3, PrhA does not regulate transcription of the *hrpA* gene in either *hrp*-inducing or nutrient rich media. Taken together, these results indicate that PrhA may regulate expression of the *hrp* gene cluster in response to an unknown plant cell factor.

Transcription of the *hrpA* gene is induced upon co-culture with its host or non-host plant cells, and this induction is dependent on the presence of PrhA

Co-culture of *P. syringae* pv. *tabaci* 11528 and *A. thaliana* cell wall material induced a much higher level of *hrp* gene transcription than culture in *hrp*-inducing medium (Fig. 3). In addition, an enhanced green fluorescent protein (eGFP) reporter gene system was used to monitor the *in situ* expression of the *hrpA* gene upon cultivation of *P. syringae* pv. *tabaci* 11528 with plant cell material. This *hrpA-gfp* reporter fusion was also strongly induced upon contact of the bacteria with plant (both host and non-host) cell wall material, but was not as strongly expressed in an $\Delta prhA$ mutant strain (Fig. 4). Based on these observations, we conclude that *P. syringae* pv. *tabaci* PrhA is required for full virulence in plant.

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초록 : *Pseudomonas syringae* pv. *tabaci*에서 식물세포접촉에 의한 병원성 유전자의 조절

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Pseudomonas syringae pv. *tabaci*는 숙주인 담배에 감염하여 들불병(wild fire)을 일으키는 식물 병원성 세균이다. 이 세균의 pathogenicity island (PAI)는 Type III secretion system 및 병원성 유전자들을 암호화하고 있으며, 병원성 조절에 있어 핵심적인 역할을 한다. 최근 식물 병원성 세균인 *Ralstonia solanacearum*에서 식물 세포 접촉을 매개로 하여 *hrp* gene cluster를 양성조절하는 PrhA (plant regulator of *hrp*) receptor가 발견되었다. 본 연구에서는 *P. syringae*에서 식물세포에 의해 *hrp* 유전자가 유도되는지 확인하기 위해, *prhA* 유사체를 동정하고 PrhA 결실돌연변이주(BL11)를 구축하였다. BL11은 숙주 감염 실험에서 병원성이 현저히 감소하였고, 식물 세포현탁액에서 *hrpA* 유전자의 발현수준이 *hrp* 유도배지에서 보다 3배 더 높게 나타났다. 이러한 결과들을 근거로 PrhA가 식물세포접촉에 의한 조절에 중요한 역할을 한다는 것을 확인하였으며, *hrpA-gfp* reporter fusion을 사용하여 이를 다시 검증하였다.