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Two-Component Sensor RhpS Promotes Induction of *Pseudomonas syringae* Type III Secretion System by Repressing Negative Regulator RhpR

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The *Pseudomonas syringae* type III secretion system (T3SS) is induced during interaction with the plant or culture in minimal medium (MM). How the bacterium senses these environments to activate the T3SS is poorly understood. Here, we report the identification of a novel two-component system (TCS), RhpRS, that regulates the induction of *P. syringae* T3SS genes. The *rhpR* and *rhpS* genes are organized in an operon with *rhpR* encoding a putative TCS response regulator and *rhpS* encoding a putative biphasic sensor kinase. Transposon insertion in *rhpS* severely reduced the induction of *P. syringae* T3SS genes in the plant as well as in MM and significantly compromised the pathogenicity on host plants and hypersensitive response-inducing activity on nonhost plants. However, deletion of the *rhpRS* locus allowed the induction of T3SS genes to the same level as in the wild-type strain and the recovery of pathogenicity upon infiltration into plants. Overexpression of RhpR in the *ΔrhpRS* deletion strain abolished the induction of T3SS genes. However, overexpression of RhpR in the wild-type strain or overexpression of RhpR(D70A), a mutant of the predicted phosphorylation site of RhpR, in the *ΔrhpRS* deletion strain only slightly reduced the induction of T3SS genes. Based on these results, we propose that the phosphorylated RhpR represses the induction of T3SS genes and that RhpS reverses phosphorylation of RhpR under the T3SS-inducing conditions. Epistasis analysis indicated that *rhpS* and *rhpR* act upstream of *hrpR* to regulate T3SS genes.

Pseudomonas syringae consists of a large group of phytopathogens that are divided into numerous pathovars, each specialized on one or several plant species (Hirano and Upper 2000). The ability of *P. syringae* to infect their host plants depends on a cluster of genes, termed hypersensitive response and pathogenicity (*hrp*) genes, that are also essential for induction of a hypersensitive response (HR) on nonhost plants (Collmer et al. 2000). *hrp* genes encode the type III secretion system (T3SS) that is conserved in numerous gram-negative bacterial pathogens of plants and animals (Galan and Collmer 1999). The T3SS is a syringe needle–like structure consisting of inner and outer membrane rings and a protruding filament called a pilus (Kubori et al. 1998). The type III pilus functions as a conduit to deliver an array of virulence proteins, termed type III effectors, into host cells (Jin and He 2001). Type III effectors promote parasitism largely by suppressing host defenses. In resistant plants, the recognition of the type III effectors by the corresponding disease resistance proteins triggers HR and disease resistance (Alfano and Collmer 2004).

The expression of T3SS structural and effector genes (collectively called T3SS genes hereafter) in *P. syringae* is coordinately regulated by various environmental and host factors (Huynh et al. 1989; Rahme et al. 1992; Xiao et al. 1992). When grown in nutrient-rich medium such as KB (King et al. 1954), the expression of T3SS genes is suppressed. However, these genes are rapidly induced after the bacteria are infiltrated into plants or cultured in minimal medium (MM) (Huynh et al. 1989; Rahme et al. 1992; Xiao et al. 1992). Studies in *Ralstonia solanacearum* suggest that the induction of T3SS genes in plants involves a contact-dependent signal (Aldon et al. 2000; Marenda et al. 1998). The perception of this signal is mediated by PrhA, a membrane protein with significant similarities to siderophore receptors (Aldon et al. 2000). However, it is not known if the same contact-dependent signal also activates the *P. syringae* T3SS genes in the plant. The MM is believed to resemble the condition of plant intercellular spaces in which bacteria proliferate (Huynh et al. 1989). It is possible that some inducing signals in MM also exist in plant apoplast and contribute to the regulation of T3SS genes in planta. However, the biochemical nature of the signal, the pathway for signal perception and transduction, and the significance of the signal to bacterial pathogenicity are largely unknown.

A few signal transduction components regulating *P. syringae* T3SS genes have been identified. HrpL, an alternate σ factor, recognizes the hrp box motif conserved in the promoter of many T3SS genes and activates their transcription (Fouts et al. 2002; Xiao and Hutcheson 1994). Transcription of *hrpL* is under the control of a σ^{54}-dependent promoter in an alternate σ factor RpoN-dependent manner (Chatterjee et al. 2003; Hendrickson et al. 2000). Activation of *hrpL* also requires HrpR and HrpS, two homologous DNA binding proteins encoded by the *hrpRS* operon (Xiao et al. 1994). Small differences were reported in different *P. syringae* strains on the transcription of *hrpRS* operon. In pathovars *syringae* and *tomato*, promoter upstream of *hrpR* was reported to be the main promoter driving the expression of this operon (Hutcheson et al. 2001), whereas in pathovar *phaseolicola*, a promoter upstream of *hrpS* was believed to drive the expression of *hrpS* (Grimm et al. 1995). The HrpR and HrpS proteins form a heterodimer that binds the *hrpL* promoter to

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activate *hrpL* transcription (Hutcheson et al. 2001). The stability of the HrpR protein is negatively controlled by LonB, an ATP-dependent protease (Bretz et al. 2002). Additional T3SS regulatory proteins in *P. syringae* include HrpA, HrpV, and HrpG. HrpA is a pilus structural protein, but the *hrpA* mutation down-regulates the *hrpRS* operon, resulting in reduced T3SS gene expression in MM (Wei et al. 2000). HrpV negatively regulates T3SS genes (Preston et al. 1998) and physically interacts with HrpS and HrpG (Wei et al. 2005). HrpG derepresses T3SS gene expression in the presence of HrpV, probably by freeing HrpS from the suppression by HrpV (Wei et al. 2005).

Expression of T3SS genes in *P. syringae* is also influenced by the two-component system (TCS) GacS/GacA (Chatterjee et al. 2003). The GacS/GacA system exists in many gram-negative bacteria, regulating a variety of traits (Heeb and Haas 2001). This system controls expression of T3SS genes through the *hrpRS-hrpL* regulatory cascade in *P. syringae* pv. *tomato* DC3000. GacA-deficiency attenuates significantly the transcription levels of *hrpRS*, *rpoN*, and *hrpL* (Chatterjee et al. 2003), suggesting that GacA positively regulates the transcript levels of *hrpL*, most likely due to its effects on *hrpRS* and *rpoN*, and consequently, T3SS genes. The nature of signals perceived by GacS and the mechanism by which GacA regulates the expression of *hrpRS* and *rpoN* are unclear.

To study how *P. syringae* senses the plant apoplast environment to activate T3SS genes, we screened for *P. syringae* pv. *phaseolicola* mutants displaying diminished induction of the type III effector gene *avrPto* in *Arabidopsis*. Two mutants carrying transposon insertions in a putative TCS sensor kinase gene designated as *rhpS* were identified that poorly induced *avrPto* in plants as well as in MM. *rhpS* is located immediately downstream of a putative TCS response-regulator gene termed *rhpR,* and the two genes are organized in an operon. The deletion mutant of the whole *rhpRS* locus (*ΔrhpRS*) and the wild-type strain showed similar induction of *avrPto*. Further analysis suggested that RhpR represses T3SS genes in a phosphorylation-dependent manner and that the repressing activity of RhpR is inhibited by RhpS under the T3SS-inducing conditions.

RESULTS

Isolation of *rhpS*$_{Pph}^{-}$ mutants.

avrPto has a typical hrp box motif in its promoter and is strongly induced in plants (Xiao et al. 2004). To understand the signal transduction events involved in activation of hrp box promoters, the *avrPto* promoter was fused to the firefly luciferase gene *luc*, the resulting *avrPto-luc* reporter gene was introduced into *P. syringae* pv. *phaseolicola* NPS3121, and the strain was subject to transposon insertion mutagenesis. Mutants defective in *avrPto-luc* induction were screened in *Arabidopsis att1*plants. *att1* plants carry a mutation in the fatty acid ω-hydroxylase gene and super-induce the *P. syringae* T3SS genes (Xiao et al. 2004). Two plant insensitive (*pin*) mutants, *pin9* and *pin19*, were identified carrying transposon inserted in a putative TCS sensor kinase gene (Fig. 1A). Both mutants displayed poor induction of *avrPto-luc* in the nonhost *Arabidopsis* plants (Fig. 2A), the host bean plants (Fig. 2B), and MM (Fig. 2C). We named this gene *rhpS* (regulator of hrp genes, sensor) to reflect the requirement of this gene for the induction of hrp box promoters. Immediately upstream of *rhpS* is a putative TCS response regulator gene that is designated as *rhpR* (regulator of hrp genes, response regulator). Mutation of *rhpS* did not affect significantly the activity of the control promoter of a replicase gene (Supplementary Fig. 1A). No growth defect was observed for *pin19* and *pin9* mutants on KB agar plate or in KB liquid culture (data not shown).

Mutation of *rhpS* compromises the bacterial pathogenicity and elicitation of HR.

T3SS genes are required for *P. syringae* virulence on host plants and HR induction on nonhost plants. To test how the *rhpS*$_{Pph}^{-}$ mutation affects the bacterial pathogenicity and HR-inducing activity, the *pin19* mutant was inoculated into host Red Kidney bean and nonhost tobacco W38 plants, respectively. Unlike the wild-type strain that caused severe diseases, the *pin19* mutant did not cause visible symptoms on bean plants (Fig. 3A). The bacterial population of *pin19* was consistently 20- to 50-fold smaller than that of the wild-type strain six days after inoculation but much larger than that of the *hrpR*$^{-}$ mutant obtained in the *pin* mutant screen (Fig. 3B). The wild-type strain triggered HR on tobacco plants in 12 h (Fig. 3C), but *pin19* and *hrpR*$^{-}$ mutants did not cause HR in two days. *pin9* showed the same phenotype as did *pin19* (data not shown).

Characteristics of *rhpS* and *rhpR*.

rhpR and *rhpS* are organized in an operon (Fig. 1A). Northern blot analyses with *rhpS* and *rhpRS* DNA as probes both detected RNA of approximately 1.8 kb, indicating that the two genes are transcribed into a polycistronic RNA (data not

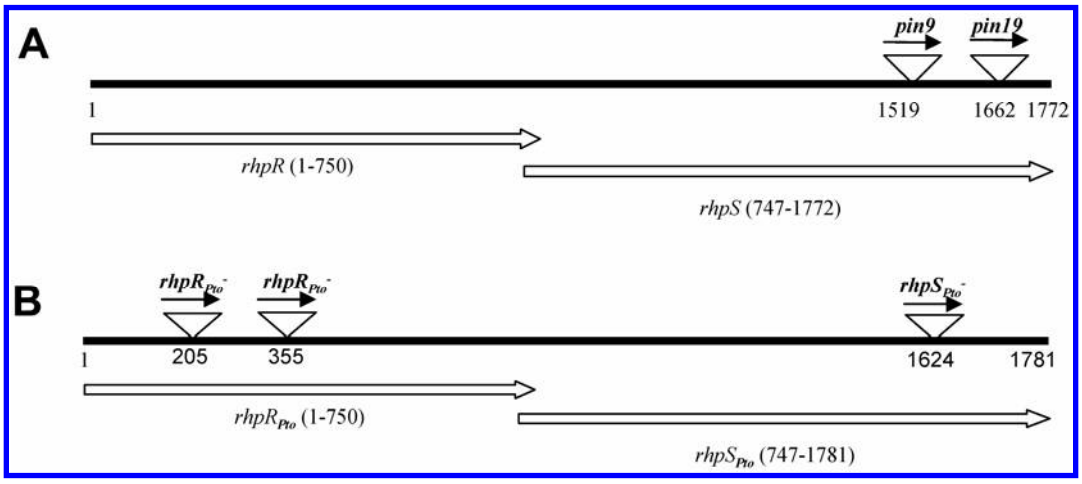

Fig. 1. Gene organization and transposon insertions in the *rhpRS* loci of *Pseudomonas syringae* pv. *phaseolicola* NPS3121 and *P. syringae* pv. *tomato* DC3000. **A,** Transposon insertions in the NPS3121 *rhpRS* locus. **B,** Transposon insertions in the DC3000 *rhpRS* locus. Solid lines illustrate nucleotide sequences containing the open reading frames. Transposon insertions are indicated by triangles. Solid arrows above the transposons indicate the direction of antibiotic resistance genes. Open arrows denote open reading frames. Numbers indicate the open reading frames and transposon insertion sites of *rhpRS* DNA.

shown). The corresponding loci in three sequenced *P. syringae* genomes consist of PSPPH2004-2003 in *P. syringae* pv. *phaseolicola* 1448A (Joardar et al. 2005), PSPTO2223-2222 in *P. syringae* pv. *tomato* DC3000 (Buell et al. 2003), and Psyr_2032-2031 in *P. syringae* pv. *syringae* B728a (Feil et al. 2005). According to annotation of these genomes, the coding regions of *rhpR* and *rhpS* overlap by 4 bp, and the coding region of *rhpS* starts at the fourth nucleotide before the end of *rhpR*.

Predicted by TopPred II (Claros and von Heijne 1994), RhpS proteins from all three *P. syringae* strains possess two transmembrane domains flanking a short periplasmic domain and a long C-terminal cytoplasmic domain. The transmembrane domains and cytoplasmic region are highly conserved, but the periplasmic domain is variable (Supplementary Fig. 2A). RhpS proteins display high similarities to the biphasic TCS sensor kinase EnvZ (Park et al. 1998). Biphasic sensor kinases have both the kinase activity to phosphorylate the cognate response regulator and the phosphatase activity to dephosphorylate the phosphorylated response regulator (Hsing et al. 1998; Zhu et al. 2000). His147 of $RhpS_{Pph}$ is predicted to be the autophosphorylation site required for both the kinase and phosphatase activities (Hsing and Silhavy 1997; Skarphol et al. 1997).

The RhpR proteins in the three *P. syringae* pathovars are nearly identical and highly homologous to OmpR (Supplementary Fig. 2B) (Martinez-Hackert and Stock 1997). Pfam analysis indicated that RhpR proteins possess two conserved domains, the N-terminal CheY-like signal receiver domain and C-terminal winged-helix DNA-binding effector domain (Volz 1993). The Asp70 residue is predicted to be the phosphorylation site of the receiver domain. Phosphorylation of the receiver domain alters the DNA-binding affinity or specificity, which in turn alters the target gene expression (Stock et al. 2000).

Complementation of *pin19* mutant.

Complementation experiments were performed to verify the role of RhpS in controlling the *pin19* mutant phenotype. Three versions of the $rhpS_{Pph}$ gene differing in the predicted start codon were used in the test: $rhpS_{pph}(TTG)$ (identical to the genomic sequence in the NPS3121 strain with TTG as the predicted start codon), $rhpS_{Pph}(ATG)$ (TTG substituted by ATG), and $rhpS_{Pph}(TTC)$ (TTG substituted by TTC). TTC was never reported to act as a start codon in any organism. These genes were cloned under a strong, constitutive promoter in plasmid pML123 (Labes et al. 1990) and were introduced into *pin19* mutant. Compared with the wild-type strain, the *pin19* mutant expressing $rhpS_{Pph}(ATG)$ displayed the same induction of *avrPto-luc* in MM and in plants (Fig. 2A, B, and C), full virulence on Red Kidney plants (Fig. 3A and B), and normal HR on tobacco plants (Fig. 3C). However, $rhpS_{Pph}(TTG)$ and $rhpS_{Pph}(TTC)$ did not complement the *avrPto-luc* induction (Table 1) and other mutant phenotypes, suggesting that TTG as the start codon could not initiate effective translation of RhpS if it is not in the polycistronic context. Complementation of *pin19* mutant by $rhpS_{Pph}(ATG)$ indicated that $rhpS_{Pph}$ is responsible for the observed phenotype.

We further tested if the putative phosphorylation site His147 is important for RhpS function by changing this residue to alanine in $rhpS_{Pph}(ATG)$. A FLAG-tag was added to the C-terminus of $Rhp_{Pph}S$ and $RhpS_{Pph}$(H147A) to monitor the protein. FLAG-tagging did not affect the regulatory activity of $Rhp_{Pph}S$. Unlike $rhpS_{Pph}(ATG)$, $rhpS_{Pph}(H147A)$ did not complement the *avrPto-luc* induction in *pin19* mutant (Table 1). Western blot analysis showed that the H147A mutant protein accumulated to the same level as did the wild-type protein, indicating that the inability of this mutant to complement the *pin19* mutant

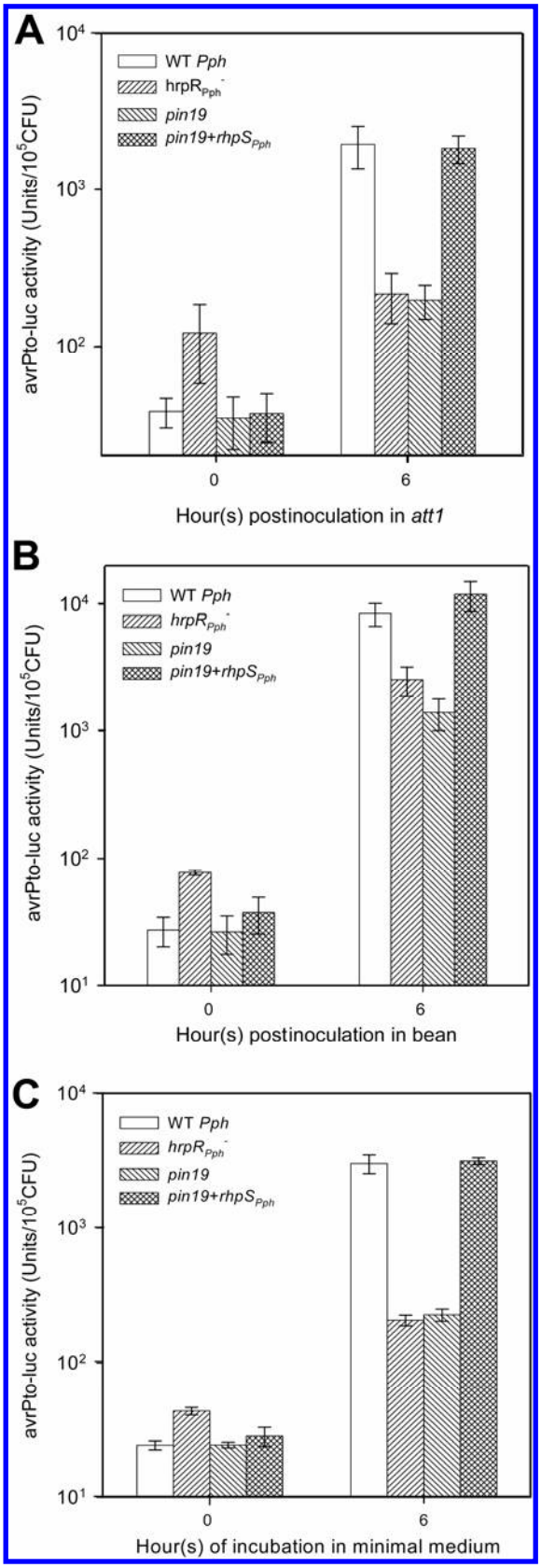

Fig. 2. Mutation of *rhpS* compromises the *avrPto-luc* induction. The wild-type NPS3121, *pin19*, *hrpR*$^-$, and the $rhpS(ATG)_{Pph}$-complemented *pin19* strains were tested for *avrPto-luc* induction in **A,** *Arabidopsis att1* plants, **B,** bean plants, and **C,** minimal medium. The luciferase activity was measured at 0 and 6 h after induction, using a cooled charge-couple device, and was normalized by the bacterial number. Error bars indicate standard error. The experiments were repeated at least five times with similar results.

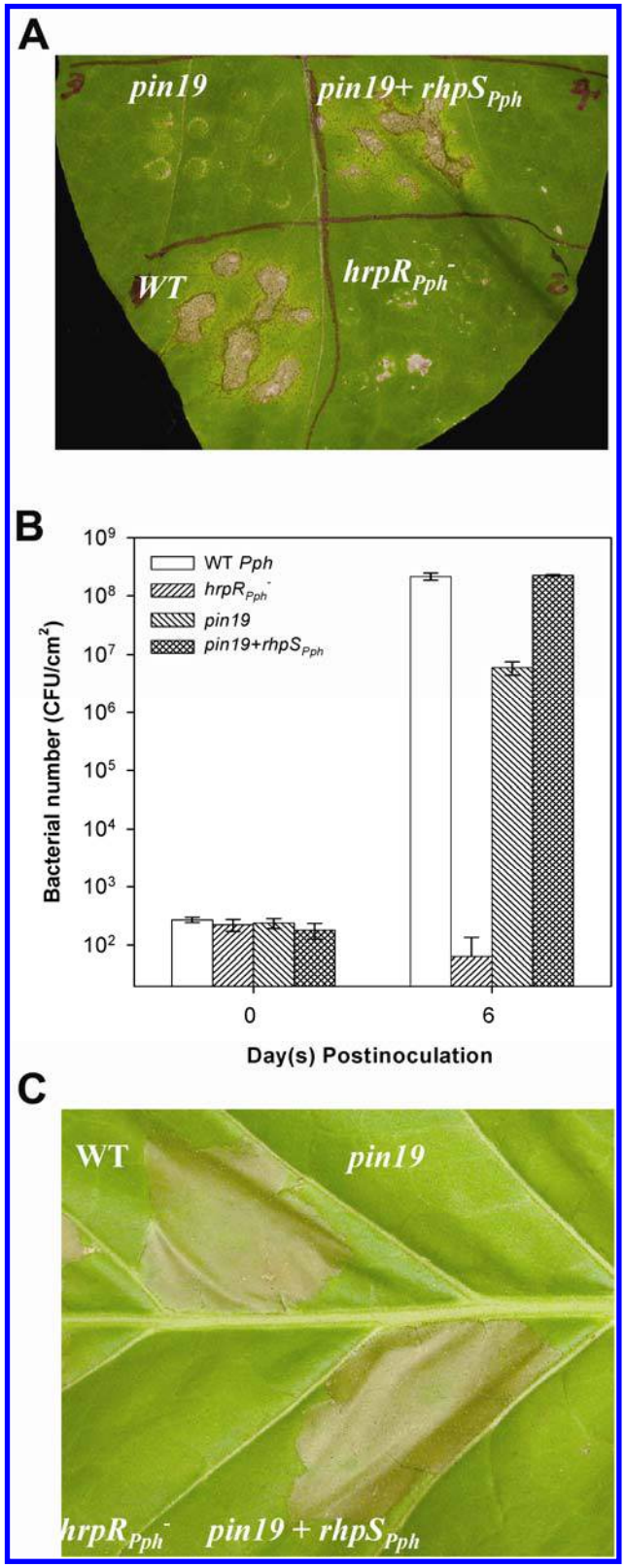

Fig. 3. Mutation of *rhpS* compromises pathogenicity and hypersensitive response (HR)-inducing activity. Primary leaves of Red Kidney bean plants were inoculated with 10^4 CFU of bacteria per milliliter. **A,** Disease symptoms were photographed 7 days after inoculation. **B,** Bacterial numbers were measured at 0 and 6 days after inoculation. Numbers represent the average of bacteria in four leaf disks. Error bars indicate standard error. W38 plants were inoculated with 10^8 CFU of bacteria per milliliter. **C,** The nonhost HR was photographed at 16 h after inoculation. Bacteria tested are the wild-type NPS3121, *pin19*, *hrpR*⁻, and the *rhpS(ATG)*$_{Pph}$-complemented *pin19* strains. The experiments were repeated at least three times with similar results.

was not caused by the stability of the protein (Supplementary Fig. 3). Expression of *rhpS*$_{Pph}$*(ATG)* and *rhpS*$_{Pph}$*(H147A)* in the wild-type strain did not alter the *avrPto-luc* induction (Table 1).

RhpS is functionally conserved in *P. syringae* pv. *tomato* DC3000.

Because the orthologs of *rhpS* and *rhpR* from three *P. syringae* pathovars are highly similar, we tested if the RhpS proteins are functionally conserved among the pathovars. We thus screened a *P. syringae* pv. *tomato* DC3000 transposon insertion library by polymerase chain reaction (PCR) and identified a mutant with a transposon inserted in the *rhpS*$_{Pto}$ gene (Fig. 1B). This mutant was tested for the *avrPto-luc* induction, pathogenicity, and HR-inducing ability. As expected, the *rhpS*$_{Pto}$⁻ mutation reduced the induction of *avrPto-luc* in MM and tomato plants, pathogenicity on tomato, and the HR-inducing activity on tobacco plants (Supplementary Fig. 4). *rhpS*$_{Pto}$ was able to restore the full induction of *avrPto-luc* in MM when expressed in *P. syringae* pv. *phaseolicola pin19* mutant (Table 1). These results indicated the functional conservation of *rhpS* genes in various *P. syringae* pathovars.

RhpR represses the induction of *avrPto*.

As our screening failed to identify a mutant of the *rhpR*$_{Pph}$ gene, we speculated that *rhpR* is either essential for bacterial growth or that the mutant screen was not saturated. To test this, we screened the *P. syringae* pv. *tomato* DC3000 transposon insertion library by PCR for mutants carrying insertions in *rhpR*. Two DC3000 mutants with transposon inserted in the *rhpR* gene were identified (Fig. 1B). The induction of *avrPto* was tested in these mutant strains. Surprisingly, the expression of *avrPto-luc* in these mutants was almost the same as that in the wild-type DC3000 strain (data not shown).

To further investigate the role of RhpR in T3SS gene regulation, we generated the *rhpRS* deletion (Δ*rhpRS*) mutant in DC3000 by marker exchange (Fig. 4A). The deletion strain was used for further experiments because transposon insertion in *rhpR* may affect the function of *rhpS* gene and complicate data interpretation. The Δ*rhpRS* mutant supported almost the same of *avrPto* induction as did the wild-type strain in MM (Fig. 4B), indicating that RhpS is not essential for T3SS gene induction when RhpR is absent. However, overexpressing *rhpR* from a plasmid in the Δ*rhpRS* mutant strain severely repressed the *avrPto* induction in MM (Fig. 4B), indicating that RhpR is a negative regulator of the *hrp* promoter and that the RhpR repressor activity is suppressed in the presence of RhpS. Consistently, upon infiltration into tomato plants, the Δ*rhpRS* mutant and

Table 1. Induction of *avrPto-luc* in NPS3121, *pin19*, and their derivatives in minimal medium (MM)[a]

Bacterial strain	LUC induction
WT NPS3121	171.2 ± 15.7
pin19	11.0 ± 1.2
pin19 + *rhpS(ATG)*$_{Pph}$	167.1 ± 24.8
pin19 + *rhpS(TTC)*$_{Pph}$	12.5 ± 3.6
pin19 + *rhpS(TTG)*$_{Pph}$	10.3 ± 1.6
pin19 + *rhpS(H147A)*$_{Pph}$	10.5 ± 1.6
pin19 + *rhpS*$_{Pto}$	174.6 ± 1 6.8
WT NPS3121 + *rhpS(ATG)*$_{Pph}$	170.5 ± 10.7
WT NPS3121 + *rhpS(H147A)*$_{Pph}$	169.4 ± 18.7

[a] The wild-type (WT) NPS3121 and *pin19* mutant strains were transformed with pML123 plasmid carrying *rhpS(ATG)*$_{Pph}$, *rhpS(TTC)*$_{Pph}$, *rhpS(TTG)*$_{Pph}$, *rhpS*$_{Pto}$, and *rhpS(H147A)*$_{Pph}$ genes. Luciferase activity was measured at 0 and 6 h after induction in MM. The tested strains displayed similar LUC activities at 0 h. The numbers represent fold induction of LUC activity and standard error. Each number represents an average of four replicates. The experiments were repeated four times with similar results.

wild-type strains displayed almost the same levels of bacterial growth and disease symptoms, but overexpressing *rhpR* in the Δ*rhpRS* background severely reduced the bacterial pathogenicity (Fig. 4D and E). Deletion of the *rhpRS* locus and overexpression of *rhpR* in the Δ*rhpRS* mutant did not alter the bacterial growth in KB medium (Supplementary Fig. 5) and the activity of the control replicase promoter (Supplementary Fig. 1B).

The phosphorylation site of RhpR is required for T3SS gene repression.

Phosphorylation of response regulators often plays a crucial role in their activity in gene regulation. We therefore tested if the predicted phosphorylation site, Asp70, is required for T3SS gene repression. The *rhpR(D70A)* mutant with Asp70 substituted by alaline was expressed from a plasmid in the Δ*rhpRS* mutant strain. Western blot indicated that RhpR(D70A) and RhpR proteins were expressed at similar levels in the Δ*rhpRS* mutant strains (Fig. 4C). However, overexpression of *rhpR(D70A)* only reduced approximately twofold of the *avrPto* expression in MM (Fig. 4B), suggesting that the nonphosphorylated RhpR is a poor repressor. Δ*rhpRS* overexpressing *rhpR(D70A)* displayed almost the same levels of bacterial growth and disease symptoms as did the wild-type strain (Fig. 4D and E), suggesting that the small reduction of T3SS gene

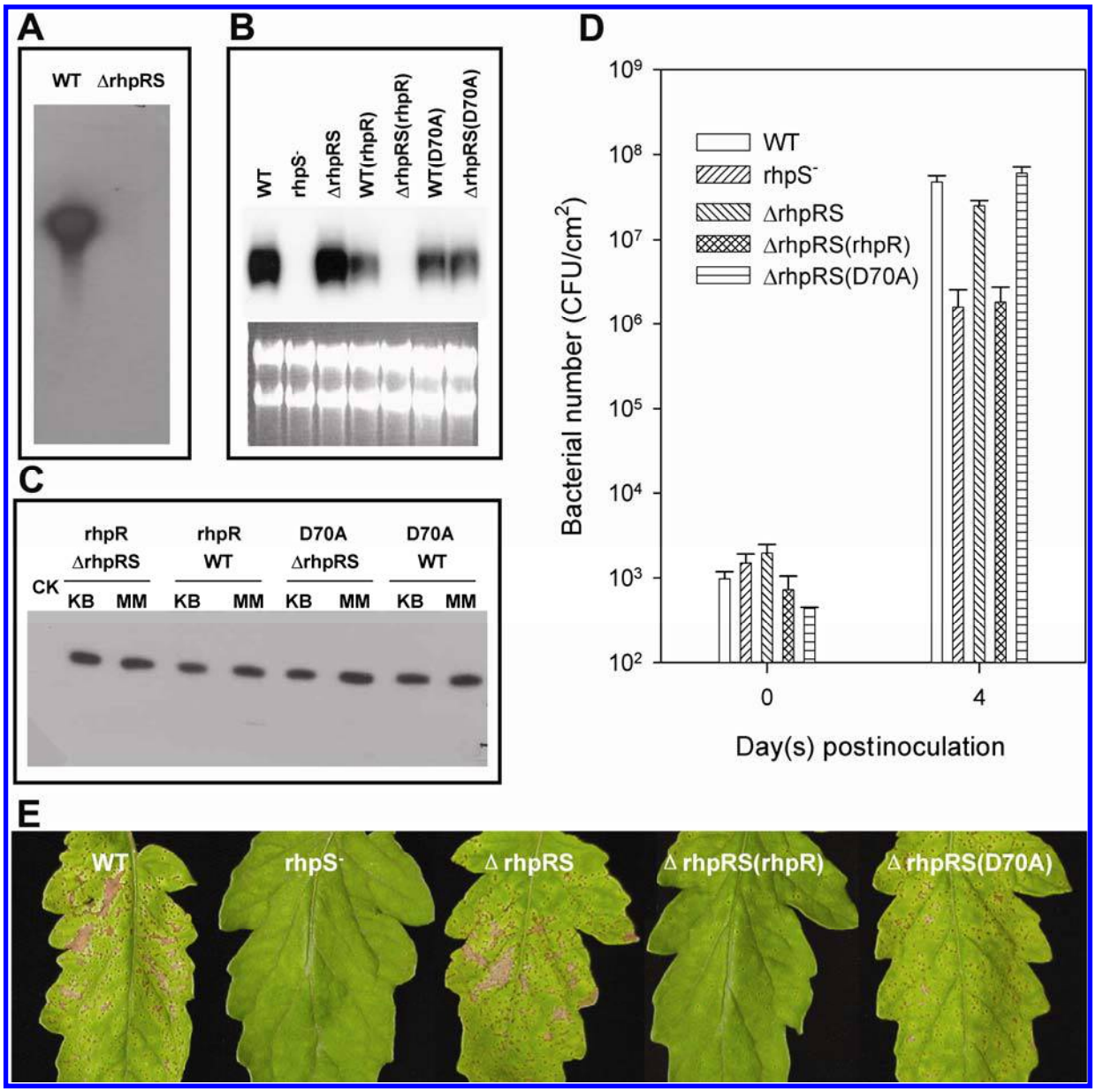

Fig. 4. Characterization of the DC3000 *rhpS* and *rhpR* genes in regulating *avrPto* gene expression and pathogenicity. **A,** Southern blot analysis of the *rhpRS* locus in the wild-type (WT) and *ΔrhpRS* strains. Genomic DNA from the two strains was digested with *Eco*RI and *Apa*I and was probed with radio-labeled probes derived from DNA encompassing the *rhpRS* locus. No hybridization signal was detected in the *ΔrhpRS* strain. **B,** Northern blot analysis of *avrPto* expression in DC3000 and its derivative strains. The HA-tagged *rhpR* and *rhpR*(*D70A*) genes were cloned in the pML122 plasmid and were expressed in the wild-type DC3000 and *ΔrhpRS* strains. Bacteria were induced in minimal medium (MM) for 6 h before being harvested for RNA extraction. RNA blot was hybridized to radio-labeled *avrPto* probes. The ethidium bromide-stained RNA gel indicates the loading of RNA samples. **C,** Western blot analysis of the RhpR-HA and RhpR(D70A)-HA proteins expressed in bacteria. The bacteria were grown in KB medium (King et al. 1954) and were then induced in minimal medium (MM). Bacteria cells (3×10^7 CFU) were boiled in sodium dodecyl sulfate (SDS) sample buffer and analyzed using SDS-polyacrylamide gel electrophoresis. The membrane was hybridized with the anti-HA antibodies. CK indicates the nontransformed DC3000 wild-type strain as control. **D,** Growth of bacterial strains in tomato plants. DC3000 and its derivative bacteria at 2×10^4 CFU/ml were vacuum-infiltrated into tomato plants. Bacterial numbers were measured at 0 and 4 days after inoculation. Error bars indicate standard error. **E,** Disease symptoms caused by the bacteria. Leaves were photographed 5 days after inoculation.

expression did not have a significant impact on the bacterial pathogenicity. Asp70 was also replaced by Glu, a mutation that renders several response regulators constitutively active (Gupte et al. 1997; Klose et al. 1993; Lan and Igo 1998) but disrupts the activity of others (Bourret et al. 1990; Hagen and Meeks 1999). RhpR(D70E) and RhpR(D70A) displayed similar activity in regulating bacterial pathogenicity (Supplementary Fig. 6) and *avrPto* expression when overexpressed in the Δ*rhpRS* mutant. Western blot analysis indicated that the D70E mutation did not affect protein stability.

rhpR and *rhpR(D70A)* were also overexpressed in the wild-type DC3000 strain. The two transformed strains displayed approximately twofold lower *avrPto* expression than did the wild-type DC3000 strain (Fig. 4B). Interestingly, the levels of *avrPto* mRNA in these two strains were similar to that in the Δ*rhpRS* mutant overexpressing *rhpR(D70A)* (Fig. 4B), suggesting a role of RhpS in preventing phosphorylation of RhpR in the wild-type strain. Expressing $rhpS_{Pph}(ATG)$ from a plasmid in the wild-type DC3000 and NPS3121 strains did not alter the expression of *avrPto-luc* (data not shown).

The $rhpS_{Pph}^-$ mutant displayed reduced expression of *hrpL* and *hrpR*.

In *P. syringae*, *hrpR*, *hrpS*, and *hrpL* are required for the activation of the *hrp* promoters. *hrpR* and *hrpS* are organized in an operon controlled by the *hrpR* promoter. To determine if RhpS regulates the T3SS genes by altering the expression of these activators, we examined the expression of *hrpR* and *hrpL* in the *pin19* mutant. The expression of the *hrpL-luc* reporter was strongly induced in MM and in planta in the wild-type strain, but this induction was attenuated in the *pin19* mutant (Fig. 5A), indicating that the induction of *hrpL* requires RhpS. Overexpression of *hrpL* in the *pin19* mutant led to constitutive activation of *avrPto-luc* (Fig. 5B), further indicating that *rhpS* acts upstream of *hrpL*. The *hrpR* RNA in the wild-type strain was expressed at a low level when grown in KB medium, but was significantly induced in MM (Fig. 5C). This induction is consistent with the induction of *hrpR* promoter (Rahme et al. 1992). In the *pin19* mutant, however, *hrpR* RNA was not detectable in either KB or MM (Fig. 5C), indicating an RhpS-mediated induction of *hrpR*. Overexpression of *hrpR* in both the wild-type strain and *pin19* mutant elevated the expression of *avrPto-luc* in KB, and *avrPto-luc* expression was further induced in the MM (Fig. 5D). Complementation of *avrPto-luc* expression in the *pin19* mutant by *hrpR* overexpression indicates that *rhpS* acts upstream of *hrpR*. Mutation of *rhpS* did not affect the expression of *gacA* and *rpoN* genes in MM, suggesting that *rhpS* regulates the expression *hrpR* and *hrpL* independent of *gacA* and *rpoN* (data not shown).

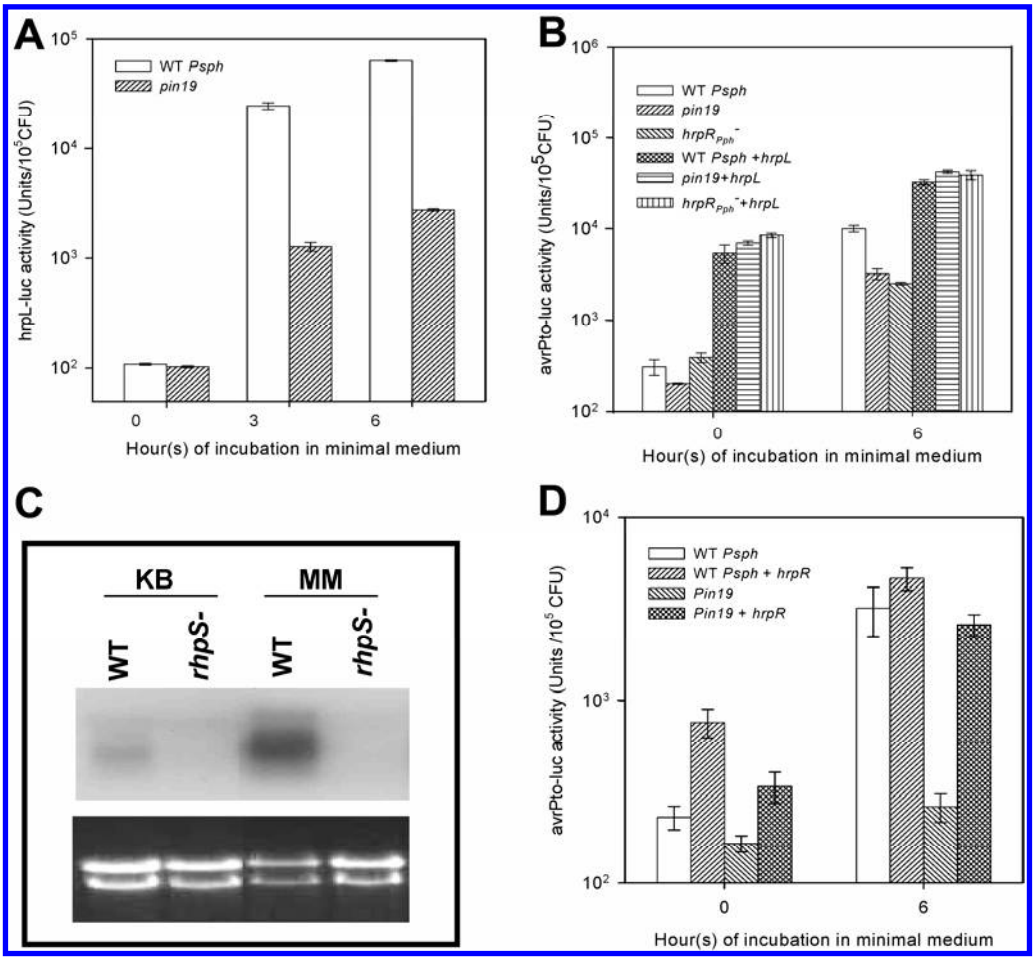

Fig. 5. Mutation of *rhpS* compromises the induction of *hrpL* and *hrpR*. **A,** The wild-type NPS3121 and *pin19* mutant carrying the *hrpL-luc* reporter gene were induced in minimal medium (MM). The luciferase activity was measured at 0 and 6 h after induction. **B,** The *hrpL* gene from the NPS3121 strain was constitutively expressed in the wild-type strain NPS3121 (WT), *pin19*, and $hrpR^-$ mutants carrying the reporter gene *avrPto-luc*. LUC activity was measured at 0 and 6 h after induction in MM. **C,** Wild-type NPS3121 and *pin19* mutant were grown in KB medium (King et al. 1954) and were subsequently induced in MM for 6 h before RNA extraction. Total RNA (10 μg per sample) was analyzed by RNA blotting with radio-labeled *hrpR* probes. The RNA gel picture (bottom) indicates the loading of RNA samples. **D,** The *hrpR* gene from NPS3121 was constitutively expressed in wild-type NPS3121 and the *pin19* mutant carrying the *avrPto-luc* reporter. LUC activity was measured at 0 and 6 h after induction in MM. Error bars indicate standard error.

RhpR represses *hrpR* expression.

Characterization of RhpS and RhpR in T3SS gene regulation suggests that RhpS regulates the T3SS genes by modulating the RhpR activity. This implies that RhpR also acts upstream of *hrpR*. We tested this possibility by determining if overexpression of *rhpR* represses the *hrpR* expression in the *ΔrhpRS* strain. Figure 6 shows that constitutive expression of *rhpR* in the Δ*rhpRS* mutant strain severely repressed the accumulation of *hrpR* transcripts in MM. Overexpression of RhpR(D70A) in the *ΔrhpRS* strain only slightly reduced the *hrpR* expression (Fig. 6). The levels of *hrpR* and *avrPto* transcripts are well correlated in the tested strains (Figs. 4B and 6).

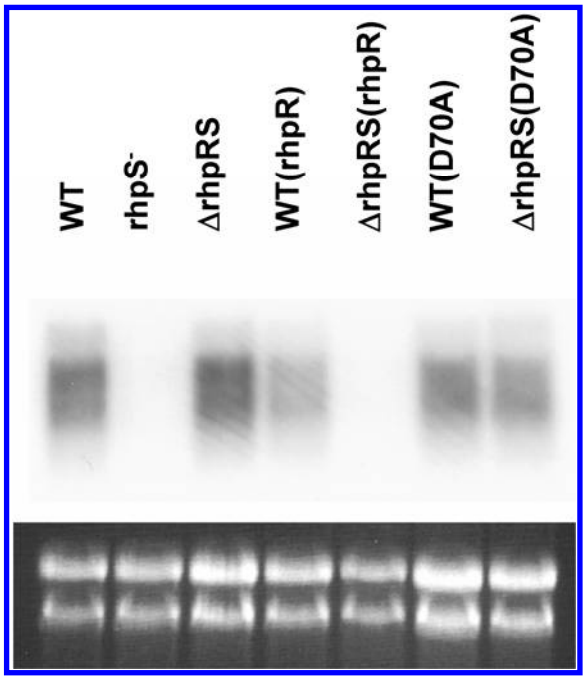

Fig. 6. Inhibition of *hrpR* expression by RhpR. *rhpR* and *rhpR*(*D70A*) were expressed in the wild-type DC3000 (WT) and *ΔrhpRS* strains from a plasmid. The bacterial strains were grown in KB medium (King et al. 1954) and were subsequently induced in minimal medium for 6 h before RNA extraction. Total RNA was analyzed by RNA blotting with radiolabeled *hrpR* probes. The ethidium bromide-stained RNA gel indicates the loading of RNA samples.

DISCUSSION

We report here the identification and characterization of the putative TCS RhpRS in regulating the *P. syringae* T3SS genes. Our results suggested that phosphorylated RhpR acts as a repressor of the *hrpR*S-*hrpL*-T3SS regulatory pathway and that RhpS reverses phosphorylation of RhpR under the T3SS-inducing conditions.

Bacteria rely primarily on TCS to sense and respond to environmental changes. A TCS usually consists of a sensor kinase and a response regulator. In general, the sensor kinase autophosphorylates at a highly conserved histidine residue in the transmitter domain and subsequently transfers the phosphoryl group to an aspartate residue in the receiver domain of its cognate response regulator. Phosphorylation of the response regulator, in turn, either stimulates or represses the transcription of its target genes (Stock et al. 2000). Under this circumstance, the sensor kinase serves as the positive regulator of the response regulator by stimulating its regulatory activity. However, many TCS sensor kinases also possess the phosphatase activity that can dephosphorylate the cognate response regulators (Russo and Silhavy 1993). The phosphatase activity of various sensor proteins can be constitutive or regulated by environmental conditions. Under conditions that repress the kinase activity or stimulate the phosphatase activity, the biphasic sensor protein serves as a negative regulator of the corresponding response regulator by dephosphorylating the protein. The negative regulation of the response regulator by the cognate sensor was reported for TCS of CovRS of group A *Streptococcus pyogenes* and VanRS of *Streptomyces coelicolor*. A *Streptococcus pyogenes* strain with mutation of the sensor kinase CovS does not

Table 2. Plasmids

Plasmids	Description	Reference
pBluescript-SK(+)	Cloning and sequencing	Stratagene, La Jolla, CA, U.S.A.
pGem7Z	Cloning and sequencing	Promega, Madison, WI, U.S.A.
pBluescript-HA	Modified from pBluescript-SK(+), for HA-tagging and sequencing	This study
pBluescript-FLAG	Modified from pBluescript-SK(+), for FLAG-tagging	This study
pHM1	Broad-host plasmid	Zhu et al. 1999
pHM2	Modified from pHM1, for *avrPto-luc* reporter expression	This study
pML123	Broad-host plasmid	Labes et al. 1990
pML122	Broad-host plasmid	Labes et al. 1990
pPTE6::*avrPto-luc*	*avrPto-luc* reporter in pPTE6	Xiao et al. 2004
pPTE6::*hrpL-luc*	*hrpL-luc* reporter in pPTE6	Xiao et al. 2004
pLT::*hrpL-luc*	Derived from pPTE6::*hrpL-luc* by EZ-Tn*5*<TET-1> transposon insertion mutagenesis	This study
pHM2::*avrPto-luc*	*avrPto-luc* reporter in pHM2	This study
pHM2::*rep-luc*	*Rep-luc* reporter in pHM2	This study
pML123::*rhpS(TTG)*$_{Pph}$	*rhpS(TTG)*$_{Pph}$ in pML123 plasmid, under pNm promoter	This study
pML123::*rhpS(ATG)*$_{Pph}$	*rhpS(ATG)*$_{Pph}$ in pML123 plasmid, under pNm promoter	This study
pML123::*rhpS(TTC)*$_{Pph}$	*rhpS(TTC)*$_{Pph}$ in pML123 plasmid, under pNm promoter	This study
pML123::*rhpS*$_{Pto}$	*rhpS*$_{Pto}$ in pML123 plasmid, under pNm promoter	This study
pHM2::*rhpRS-Flag*	*rhpRS* operon under native promoter with *rhpS* FLAG-tagged at C-terminus	This study
pHM2::*rhpRS(H147)-Flag*	Derived from pHM2::*rhpRS-Flag*, His147 replaced by alanine	This study
pML123:: *rhpS(H147A)*$_{Pph}$	Derived from pML123::*rhpS*(ATG)$_{Pph}$, His147 replaced by alanine	This study
pML122::*rhpR-HA*	*rhpR*$_{Pph}$ in pML122 plasmid, under pNm promoter	This study
pML122::*rhpR(D70A)-HA*	Derived from pML122::*rhpR-HA*, with Asp70 replaced by Ala	This study
pML122::*rhpR(D70E)-HA*	Derived from pML122::*rhpR-HA*, with Asp70 replaced by Glu	This study
p7Z::FR	Intermediate construct for marker exchange	This study
p7Z::FKanR	Intermediate construct for marker exchange	This study
pHM1-FKanR	For maker exchange	This study
pML122::*hrpR*$_{Pph}$	*hrpR*$_{Pph}$ in pML122 plasmid, under pNm promoter	This study
pML122::*hrpL*$_{Pph}$	*hrpL*$_{Pph}$ in pML122 plasmid, under pNm promoter	This study

grow under stress conditions, and second-site mutations that inactivate the response regulator CovR restore the wild-type phenotype (Dalton and Scott 2004). Similarly, the *Streptomyces coelicolor* wild-type strain is sensitive to vancomycin but the mutant of VanS sensor kinase confers resistance to vancomycin. Deletion of VanR, the response regulator corresponding to VanS, individually or together with VanS, renders the bacterium sensitive to vancomycin (Hutchings et al. 2006). Both CovR and VanR require the putative phosphorylation site for their regulatory activities. It was proposed that CovS and VanS exert their negative regulation on CovR and VanR by dephosphorylating the phosphorylated response regulators under the tested conditions (Dalton and Scott 2004; Hutchings et al. 2006). The negative regulation was also reported for the *Eschirichia coli* osmosensor EnvZ towards the response regulator OmpR in low medium osmolarity. EnvZ displays a higher phosphatase activity over the kinase activity at low medium osmolarity. As a result, the level of the phosphorylated OmpR is low and unable to activate the *ompC* gene expression (Yoshida et al. 2006). In the *ΔenvZ* deletion strain, overexpression of OmpR elevates the *ompC* gene expression in a phosphorylation-dependent manner (Matsubara and Mizuno 1999). Our studies suggest that RhpRS represents another TCS in which the sensor RhpS negatively regulates the response regulator RhpR under T3SS-inducing conditions.

Three observations indicated that RhpR alone in the *P. syringae* cells acts as a repressor of T3SS genes. First, the induction of *avrPto* was abolished in the *rhpS*$^-$ mutants. Transposon insertion in *rhpS* did not appear to cause a polar effect on *rhpR* because i) *rhpS* is downstream of *rhpR*, ii) the kanamycin-resistant gene carried by transposon is inserted in the same direction as the *rhpRS* operon, and iii) *rhpS* alone in a plasmid completely complemented the *avrPto* induction in *pin19* mutant. Second, mutants with transposon inserted in the *rhpR* gene and with the *rhpRS* locus deleted showed normal induction of the *avrPto* gene compared with the wild-type strain, and the expression of *avrPto* in the *ΔrhpRS* deletion strain was not affected by the plasmid-borne *rhpS*. Thus, removal of RhpR from bacterial cells leads to derepression of T3SS genes. Third, overexpression of the wild-type *rhpR* gene alone in the *ΔrhpRS* mutant strain abolished the *avrPto* induction, unequivocally demonstrating that RhpR can repress T3SS genes.

Sequence alignment predicts that Asp70 is the phosphorylation site of RhpR. Although phosphorylation of this site remains to be demonstrated, mutagenesis of Asp70 to Ala in RhpR reduced severely the T3SS-repressing activity of RhpR in the *ΔrhpRS* deletion strain, suggesting that phosphorylation of RhpR is required for its T3SS-repressing activity. The low activity displayed by RhpR(D70A) in the *ΔrhpRS* mutant is not uncommon among response regulators. A number of response regulators of similar mutations were reported to have residual activities, especially when the mutant proteins are overexpressed (Abdel-Fattah et al. 2005; Comolli et al. 2002; Powell and Kado 1990). Replacement of Asp by Glu was proposed to mimic phosphorylation of Asp in changing the conformation of response-regulator proteins. Indeed, a few response regulators, including OmpR, NtrC, and RcsB, with such mutations are constitutively active (Gupte et al. 1997; Klose et al. 1993; Lan and Igo 1998). However, this proposal does not apply to all response regulators. It was reported that replacement of Asp by Glu in CheY and DevR disrupts the response-regulator activity (Bourret et al. 1990; Hagen and Meeks 1999). BlastP analysis indicated that RhpR has a CheY-like receiver domain. Northern blot analysis of *avrPto* expression and pathogenicity analysis of the *ΔrhpRS* strain overexpressing D70E indicated that D70E, like D70A, severely reduced repressor activity. Although the D70E mutation did not turn RhpR into a constitutively active form, it did indicate that Asp70 is a site critical for RhpR activity.

In the absence of RhpS, what is the mechanism phosphorylating RhpR? It was reported that response regulators can be phosphorylated by unrelated sensor kinases or by small phosphate donor molecules such as acetyl-phosphate in the absence of the cognate sensor kinase (McCleary et al. 1993; Soncini and Groisman 1996). The *P. syringae* genomes carry a large number of TCS sensor kinases (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). The DC3000 genome also carries phosphate acetyl-transferase (PSPTO1169) and acetate kinase (PSPTO1669) involved in acetyl-phosphate production, and the *P. syringae* pv. *phaseolicola* 1448A genome only carries phosphate acetyl-transferase. While phosphorylation of RhpR by acetyl-phosphate has yet to be demonstrated, phosphorylation by acetyl-phosphate was shown to be responsible for the activation of a number of response regulators, including VanR and OmpR (Bouché et al. 1998; Hutchings et al. 2006; Matsubara and Mizuno 1999).

Table 3. Primers[a]

Primer name	Sequence
Adaptor-F	TCCGAATTCAAGCTTCTCGAGGGTACCTCTAGA
Adaptor-R	TCTCTAGAGGTACCCTCGAGAAGCTTGAATTCG
rhpS-F	TTGAATTCCAGGGAGCTGGGTTGA
rhpS-R	TTTCTAGAGTCAAAGGCGCGGCAG
rhpS(ATG)-F	TTGGATCCCAGGGAGCTGGGATGATCCGCCGGTTCGACAC
rhpS(TTC)-F	GGAGCTGGGTTCATCCGCCGGTTCGAC
rhpS(TTC)-R	GTCGAACCGGCGGATGAACCCAGCTCC
rhpS(H147A)-F	CTGGCTGCGGTATCCGCTGACCTGCGCACG
rhpS(H147R)-R	CGTGCGCAGGTCAGCGGATACCGCAGCCAG
Kan2-SP1	GATAGATTGTCGCACCTGATTG
Kan2-SP2	AAGACGTTTCCCGTTGAATATG
Kan2-SP3	GCAATGTAACATCAGAGATTTTGAG
CEKG 2A	GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG
CEKG 2B	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
CEKG 2C	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
CEKG 4	GGCCACGCGTCGACTAGTAC
Kan	CTACCTTTGCCATGTTTCAG
pin19-3'in	GAGAATGCGCAGGACAATGG
TN-Tet-L1	TACCGGCATAACCAAGCCTATGCCTACAG
TN-Tet-L2	AGGATGACGATGAGCGCATTGTTAGATTTC
TN-Tet-R1	CACATGGAACGGGTTGGCATGGATTGTAG
TN-Tet-R2	ACTCCAAGAATTGGAGCCAATCAATTCTTG
DCrhpS-R1	GCATCGGTACGATAGTCACACACGTCTAA
DCrhpS-R2	ATGGACAATCGCTTTCGTGAGCACGCTCA
rhpR-F	TTAAGCTTCAGGGAGCTGGGTTGA
rhpR-R	CTAGCTAGCACCCAGCTCCCTGGCATC
rhpR(D70A)-F	GACGTGGTGGTGCTC**GCC**CTGATGCTGCCCGGT
rhpR(D70A)-R	ACCGGGCAGCATCAG**GGC**GAGCACCACCACGTC
rhpR(D70E)-F	GACGTGGTGGTGCTC**GAA**CTGATGCTGCCCGGT
rhpR(D70E)-R	ACCGGGCAGCATCA**GTT**CGAGCACCACCACGTC
rhpRS-FLAG-F	TTGGGCCCAAACGTCCTTGTTCAACG
rhpRS-FLAG-R	AATTCGAAAAGGCGCGGCAGATTCAG
FME-LF	AGGGTACCGTTGCACCGGGCGACT
FME-LR	ATCCCGGGTGTTCTCGGGAAGT
FME-RF2	TCCCCGGGAGTGAGCGTGCTCA
FME-RR2	CTGAGCTCGGTCACCTATCAGTTA
Kan-F	CACCCGGGATCGATGAATTGTGTCT
Kan-R	GTCCCGGGTGGACCAGTTGGTGAT
hrpRS-F	ATGCTCTAGACGGGTGTTGCTC
hrpR-R	GAGACATAAGCTTTTTGGACTCC
hrpL-F	ATCGCTCTAGAGCTTGCACACC
hrpL-R	AATCAAGCTTCCAGACAGATATCCACTCAGG

[a] Primers were designed based on the genomic sequences of *Pseudomonas syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola* 1448A strains. Added cloning sites are underlined.

Based on the results that RhpR requires the putative phosphorylation site for the repression of T3SS genes and that RhpS derepresses T3SS genes, we propose that RhpS acts as a phosphatase preventing the formation of phospho-RhpR under T3SS-inducing conditions. This hypothesis explains several observations made in our study. First, expression of *rhpS* from a plasmid in the *rhpS*$^-$ *(pin19)* mutant elevated the *avrPto-luc* induction to the wild-type level but had no effect on *avrPto* induction in the Δ*rhpRS* mutant. Thus *rhpS* positively regulates *avrPto* expression only when *rphR* is present, which is consistent with the notion that RhpS derepresses T3SS gene expression by regulating the RhpR activity. Second, expression of the RhpS(H147A) mutant protein did not restore the *avrPto-luc* induction in the *pin19* mutant. His147 is the predicted autophosphorylation site in RhpS required for both the kinase and phosphatase activities. The lack of phosphatase activity likely accounts for the inability of the H147A mutant to restore the RhpS function. Further test of this possibility will require the identification of RhpS mutations that specifically abolish the phosphatase activity. Nonetheless, the requirement of His147 for RhpS function further supports the involvement of RhpR phosphorylation in regulating T3SS genes. Third, the wild-type DC3000 strains overexpressing RhpR and RhpR(D70A) displayed the same levels of *avrPto* expression as did the *ΔrhpRS* mutant strain overexpressing RhpR(D70A), suggesting that RhpR exists in the unphosphorylated state in the wild-type strain, possibly due to the phosphatase activity of RhpS. The moderate reduction of *avrPto* in these strains was probably due to the overexpression of RhpR protein, because *rhpR* and *rhpR(D70A)* genes in the plasmid are controlled by a strong constitutive promoter.

rhpRS likely acts upstream of *hrpRS* to repress the transcriptional cascade of *hrpRS-hrpL*-T3SS genes. This statement is supported by the reduced expression of *hrpL-luc* reporter and *hrpR* RNA in the *rhpS*$^-$ mutant and the *ΔrhpRS* strain overexpressing RhpR. In addition, overexpression of *hrpR* or *hrpL* in the *rhpS*$^-$ mutant strongly increased the *avrPto-luc* expression even in the KB medium, indicating that *rhpRS* regulates T3SS genes through these intermediate regulators. We do not yet know if the reduced *hrpR* RNA is completely responsible for the repression of T3SS genes and whether RhpR represses *hrpR* expression by direct binding and inhibiting the *hrpR* promoter. Nonetheless, it is clear that repression of T3SS genes by RhpR is not caused by nonspecific repression of overall cellular activities, because the control promoter of a replicase gene was expressed normally in the *rhpS*$^-$ mutant and *ΔrhpRS* mutant overexpressing RhpR.

It is interesting to note that *avrPto-luc* reporter is similarly reduced in the *rhpS*$^-$ and *hrpR*$^-$ mutants, but *hrpR*$^-$ mutant is nonpathogenic while *rhpS*$^-$ mutants only lost partially the pathogenicity on host plants. One explanation to this result is that *hrpR* integrates both RhpS-dependent and -independent pathogenicity mechanisms, thereby mutation of *rhpS* only compromises partially the pathogenicity. Another explanation is that *rhpS* mutation reduces the cellular HrpR, and the low level of HrpR is able to active only partially the virulence. This explanation is supported by Northern blot analysis that showed a significant reduction of *hrpR* RNA in *rhpS*$^-$ mutants in MM. Similar reduction of *hrpR* RNA in *rhpS*$^-$ mutants may occur in the plant, because the downstream *hrpL* gene is significantly reduced in the plant. The reduced HrpR is probably insufficient to activate virulence mechanisms represented by the *avrPto-luc* reporter.

In conclusion, we propose that RhpS acts as a phosphatase negatively regulating RhpR, a repressor of T3SS genes, under the T3SS-inducing conditions. A possible extension of this hypothesis is that *P. syringae* employs this TCS to regulate the T3SS in response to different environmental conditions. Conditions that either repress the RhpS kinase activity, stimulate the phosphatase activity, or both would induce T3SS genes, while conditions stimulating the RhpS kinase activity would suppress T3SS genes. The two different conditions may differ in the presence or absence of the signal perceived by RhpS.

MATERIALS AND METHODS

Plant materials.

Arabidopsis att1 mutant plants (Xiao et al. 2004) were used for the screening of *pin* mutants because they provide a sensitive assay for the *avrPto-luc* reporter. Bean (*Phaseolus valgaris* cv. Red Kidney) (Lindgren et al. 1986), tomato (*Lycopersicon esculentum* cv. Rio-Grande PtoS), and tobacco (*Nicotiana tabacum* cv. W38) plants (Shan et al. 2000) were used for pathogenicity and nonhost HR assays. Growth of plant materials was described previously (Shan et al. 2000).

Construction of plasmids.

Plasmids and oligo primers are listed in Tables 2 and 3, respectively. pBluescript-HA plasmid was modified from pBluescript-SK(+) by replacing the sequence between the *Hin*dIII and *Bam*HI sites with 5′-AAGCTT**CTGCAGCTAGC***TACCC ATACGACGTCCCAGACTACGCT*TAGGATCC-3′ (the *Hin*dIII and *Bam*HI sites are underlined; the HA-tagged sequences are italicized; *Pst*I and *Nhe*I are in bold face) for HA-tagging of a gene at the 3′ end. pBluescript-FLAG plasmid was modified from pBluescript-SK(+) by replacing the DNA between *Xho*I and *Spe*I sites with 5′-CTCGAGCCAGGGCCCTGGTTCGAA**GACTACAAAGACCATGATGGAGACTATAAGGATCAC GACATCGATTACAAGGACGATGACGATAAG**TGAACT AGT-3′ containing *Xho*I, *Apa*I, *BstX*I, *Csp45*I, *Cla*I, and *Spe*I sites (underlined) and the 3xFLAG epitope sequence (bold). pHM2 was modified from pHM1 plasmid (Zhu et al. 1999) through the following steps to facilitate the expression of the *avrPto-luc* reporter gene. First, the pHM1 plasmid was digested with *Bam*HI to remove the *lacZ* promoter and multiple cloning sites. The vector was then partially filled in with Klenow and dATP/dGTP and was subsequently ligated with the adaptor DNA formed by association of the oligo nucleotides Adaptor-F and Adaptor-R. The resulting pHM2 plasmid has *Bam*HI, *Eco*RI, *Hin*dIII, *Xho*I, *Kpn*I, and *Xba*I sites.

pPTE6::*avrPto-luc* and pPTE6::*hrpL-luc* reporter plasmids were constructed previously (Xiao et al. 2004). To clone the *avrPto-luc* reporter gene into pHM2, pPTE6::*avrPto-luc* was digested with *Eco*RI and *Xba*I, and the released *avrPto-luc* expression cassette was subsequently cloned into the pHM2 plasmid predigested with the same enzymes, resulting in pHM2::*avrPto-luc*. To generate the pLT::*hrpL-luc* reporter construct, the kanamycin-resistant gene in pPTE6::*hrpL-luc* plasmid was disrupted by EZ-Tn*5*<TET-1> transposon. The resulting plasmid confers tetracycline resistance.

The pML123 and pML122 plasmids (Labes et al. 1990) were used to express the $rhpS_{Pph}$ and $rhpS_{Pto}$ genes in *P. syringae* strains. $rhpS(TTG)_{Pph}$ and $rhpS(ATG)_{Pph}$ were amplified by PCR from the NPS3121 strain using the primer pairs rhpS-F and rhpS-R and rhpS(ATG)-F and rhpS-R, respectively, and were cloned into pBluescript-SK plasmid. Confirmed by sequencing, the $rhpS_{Pph}$ insert was released by *Bam*HI/*Xba*I digestion and was cloned into the pML123 plasmid predigested with the same enzymes. QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) and the primers rhpS(TTC)-F and rhpS(TTC)-R were used to change the start codon of $rhpS_{Pph}$ in pBluescript-SK from TTG to TTC. The same kit and the primers rhpS(H147A)-F and rhpS(H147R)-R were used to generate the H147A mutant in $rhpS_{Pph}$. The mu-

tant *rhpS*$_{Pph}$ genes were verified by sequencing and were cloned into the pML123 plasmid. Similarly, the *rhpS*$_{Pto}$ gene was amplified by PCR from the DC3000 strain, using the rhpS-F and rhpS-R primers, and the sequence was confirmed and cloned into the *Bam*HI and *Xba*I sites of pML123.

To construct the pML122::*rhpR-HA* plasmid, the *rhpR* gene from the NPS3121 strain was amplified using the rhpR-F and rhpR-R primers, was cloned into the pBluescript-HA plasmid between the *Hin*dIII and *Nhe*I sites, and sequence was confirmed. The resulting pBluescript::*rhpR-HA* plasmid was digested by *Hin*dIII and *Bam*HI, and the insert was subsequently cloned into pML122 predigested with the same enzymes, resulting in pML122::*rhpR-HA*. QuickChange site-directed mutagenesis kit (Stratagene) and primer pairs rhpR(D70A)-F and rhpR(D70A)-R and rhpR(D70E)-F and rhpR(D70E)-R were used to change the phosphorylation site of *rhpR* gene in pBluescript::*rhpR-HA* from GAC to GCC and GAA, respectively. After verification by sequencing, the inserts were released by *Bam*HI and *Hin*dIII digestion and were cloned into plasmid pML122, resulting in pML122::*rhpR(D70A)-HA* and pML122::*rhpR(D70E)-HA*, respectively. For the construction of pHM2::*rhpRS*-FLAG, the *rhpRS* locus containing the native promoter was PCR-amplified with rhpRS-FLAG-F and rhpRS-FLAG-R primers and NPS3121 genomic DNA as template. The PCR product was digested using *Apa*I and *Csp45*I and was cloned into pBluescript-FLAG. After sequence verification, the clone was digested with *Kpn*I and *Xba*I, and the insert was cloned into pHM2.

The coding regions of *hrpL* and *hrpR* were amplified from NPS3121 using primer pairs hrpL-F and hrpL-R and hrpR-F and hrpR-R, respectively. The PCR products were digested with *Xba*I and *Hind*III and were cloned into pBluescript-SK plasmid. After sequencing verification, the inserts were released from pBluescript-SK with *Xba*I and *Hin*dIII digestion and were cloned into pML122, resulting in pML122::*hrpL* and pML122::*hrpR* plasmids.

Construction of transposon-insertion libraries.

The *P. syringae* pv. *phaseolicola* NPS3121 strain carrying the pHM2::*avrPto-luc* reporter plasmid and the *P. syringae* pv. *tomato* DC3000 strain were used for construction of the EZ-Tn*5*<KAN-2> and EZ-Tn*5*<TET-1> (Epicentre, Madison, WI, U.S.A.) transposon insertion libraries, respectively. Briefly, electro-competent cells were mixed with transposon and transposase as instructed by the manufacturer. Following electroperation, the NPS3121 mutant library was plated on KB medium containing 10 mg of kanamycin per liter (selection for EZ::TN<KAN-2> transposon) and 10 mg of spectinomycin per liter (selection for pHM2::*avrPto-luc* plasmid). The DC3000 mutant library was plated on KB plate containing 10 mg of tetracycline per liter (selection for EZ-Tn5<TET-1>).

Screen of *pin* mutants and mapping of transposon insertion sites.

The NPS3121 mutant colonies were grown in liquid KB medium containing spectinomycin and kanamycin, were washed twice with sterile water, and were resuspended in sterile water. For *pin* mutant screen, bacteria at optimal density at 600 nm (OD_{600}) = 0.5 were injected into *Arabidopsis att1* plants. After 6 h, the inoculated leaves were excised and sprayed with 1 mM luciferin dissolved in 0.01% Tween-20, and the luciferase activity was determined using a cooled charge-couple device (Roper Scientific, Trenton, NJ, U.S.A.). Putative mutants with <30% of the wild-type LUC activity were selected and confirmed. A total of 6,000 colonies were screened.

The transposon insertion sites were determined by a two-stage semidegenerated PCR according to Jacobs and associates (2003), using two transposon-specific primers (Kan2-SP1 and Kan2-SP2) and four degenerated primers (CEKG 2A, CEKG 2B, CEKG 2C, and CEKG). The PCR product was sequenced using the third transposon-specific primer Kan2-SP3. Blastn was used to search for the homologous sequence in the National Center for Biotechnology Information database. To confirm the transposon insertion sites in *pin19* and *pin9* mutants, gene-specific primer rhpS-R and transposon–specific primer Kan2-SP3 were used to PCR amplify the transposon-flanking DNA. The PCR products were sequenced to determine the transposon insertion sites.

Isolation of mutants from the DC3000 transposon insertion library.

rhpS$_{Pto}^{-}$ and *rhpS*$_{Pto}^{-}$ mutants were isolated from the DC3000 transposon insertion library via DNA pooling and nested PCR strategy. Nested primers DCrhpR1 and DCrhpR2 downstream of the DC3000 *rhpS* gene in combination with the nested transposon-specific primer pairs TN-Tet-L1 and TN-Tet-L2 and TN-Tet-R1 and TN-Tet-R2 were used in PCR. A total of 19,200 colonies from the DC3000 mutant library were grown in 200 96-well plates. Bacteria grown in each plate were pooled for DNA isolation. A total of 10 pools of DNA representing 960 colonies were combined to form a superpool, and the superpool DNA was used for screening of *rhpS*$_{Pto}^{-}$ and *rhpR*$_{Pto}^{-}$ mutants using nested PCR. Once the superpool carrying the desired mutant was identified, the 10 corresponding subpools were PCR-screened to identify the plate carrying the mutant. The plate was then subjected to two-dimensional pooling, forming eight horizontal pools (each pool represents 12 colonies) and 12 vertical pools (each pool represents eight colonies). These pools were PCR-screened to identify the well of mutant. Bacteria from the well were streaked onto a KB plate, and individual colonies were reconfirmed by PCR and sequencing.

Construction of the *ΔrhpRS* deletion mutant in DC3000.

A 1.8-kb DNA fragment upstream of *rhpR* was PCR-amplified using primers FME-LF and FME-LR (*Kpn*I and *Sma*I sites are underlined). A 1.8-kb DNA fragment downstream of *rhpS* was PCR-amplified using primers FME-RF2 and FME-RR2 (*Sac*I and *Sma*I sites are underlined). The PCR products were digested with *Kpn*I and *Sma*I and *Sma*I and *Sac*I, respectively, and were ligated and cloned into the *Kpn*I and *Sac*I sites of pGEM-7Z, resulting in p7Z-FR. A DNA fragment containing the kanamycin resistance gene was PCR-amplified from EZ::Tn< KAN-2> (Epicentre), using primers Kan-F and Kan-R (*Sma*I sites underlined) digested with *Sma*I, and cloned into the *Sma*I site of 7Z-FR, resulting in p7Z-FkanR. The *Kpn*I and *Sac*I fragment in 7Z-FkanR was cloned into pHM1, and the resulting pHM1::FkanR plasmid was introduced into DC3000 for marker exchange. Colonies sensitive to spectinomycin but resistant to kanamycin were further verified by PCR and Southern blotting using DNA probes synthesized from DNA encompassing *rhpRS*.

Bacterial growth, disease symptoms, and HR assays.

Preparation of bacterial inoculum and bacterial growth assay were as described previously (Shan et al. 2000). NPS3121 at 2×10^4 CFU/ml was hand-injected into the primary leaves of 10-day-old bean plants. DC3000 at 2×10^4 CFU/ml was vacuum-infiltrated into 4-week-old tomato plants. Disease symptoms on bean and tomato leaves were photographed 7 and 4 days after inoculation, respectively. For the nonhost HR assay, bacteria at 1×10^8 CFU/ml was injected into the fully expanded tobacco W38 leaves. The lesions were visually examined every 2 h after inoculation.

RNA extraction and Northern blotting.

Bacterial RNA was extracted using a modified hot phenol method (Aiba et al. 1981). RQ1 DNase (Promega, Madison, WI, U.S.A.) treatment was used to remove the contaminating DNA in RNA samples. Total RNA (10 μg) was used for Northern blotting. The *hrpR* and *avrPto* coding regions were PCR-amplified and radio-labeled with ^{32}P dCTP using the Random primed DNA labeling kit (Ambion, Austin, TX, U.S.A.) as probes. Procedures described by Tang and associates (1999) were followed for hybridization and washing.

Western blot analysis.

Bacteria grown in KB and MM media were harvested by centrifugation and were resuspended in water to OD_{600} = 1. Bacteria (30 μl) was boiled in 1× sodium dodecyl sulfate (SDS) sample buffer and was loaded to a SDS-polyacrylamide electrophoresis gel. Western blot was performed as described (Shan et al. 2000) with the anti-HA and anti-FLAG antibodies (Sigma, St. Louis).

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