

**IDENTIFICATION AND CHARACTERIZATION OF *PSEUDOMONAS SYRINGAE*  
MUTANTS ALTERING THE INDUCTION OF TYPE III SECRETION SYSTEM**

by

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B.S., China Agricultural University, Beijing, China, 2001  
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AN ABSTRACT OF A DISSERTATION

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Genetics Inter-department Program  
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## ABSTRACT

*Pseudomonas syringae* bacteria utilize the type III secretion system (T3SS) to deliver effector proteins into host cells. The T3SS and effector genes (together called the T3 genes hereafter) are repressed in nutrient rich medium but are rapidly induced after the bacteria are transferred into minimal medium (MM) or infiltrated into the plant. The induction of the T3 genes is mediated by HrpL, an alternative sigma factor that recognizes the conserved hrp box motif in the T3 gene promoters. The induction of *hrpL* is mediated by HrpR and HrpS, two homologous proteins that bind the *hrpL* promoter.

To identify additional genes involved in regulation of the T3 genes, *P. s. pv. phaseolicola* (*Psph*) NPS3121 transposon insertion mutants were screened for reduced induction of *avrPto-luc* and *hrpL-luc*, reporter genes for promoters of effector gene *avrPto* and *hrpL*, respectively. Determination of the transposon-insertion sites led to the identification of genes with putative functions in signal transduction and transcriptional regulation, protein synthesis, and basic metabolism.

A transcriptional regulator (AefR<sub>NPS3121</sub>) identified in the screen is homologous to AefR, a regulator of the quorum sensing signal and epiphytic (plant-associated) traits that was not known previously to regulate the T3 genes in *P. s. pv. syringae* (*Psy*) B728a. AefR<sub>NPS3121</sub> in *Psph* NPS3121 and AefR in *Psy* B728a are similar in regulating the quorum sensing signal in liquid medium but different in regulating epiphytic traits such as swarming motility, entry into leaves, and survival on the leaf surface.

The two component system RhpRS was identified in *Pseudomonas syringae* as a regulator of the T3 genes (Xiao et al. 2007). In the *rhpS* mutant, the response regulator RhpR

represses the induction of the T3 gene regulatory cascade, but induces its own promoter in a phosphorylation-dependent manner. Deletion and mutagenesis analyses revealed an inverted repeat (IR) element GTATC-N<sub>6</sub>-GATAC in the *rhpR* promoter that confers the RhpR-dependent induction. Computational search of the *P. syringae* genomes for the putative IR elements and Northern blot analysis of the genes with a putative IR element in the promoter region uncovered five genes that were upregulated (PSPTO2036, PSPTO2767, PSPTO3477, PSPTO3574, and PSPTO3660) and two genes that were down-regulated (PSPTO0536 and PSPTO0897) in an RhpR-dependent manner. CHIP assays indicated that RhpR binds the promoters containing a putative IR element but not the *hrpR* and *hrpL* promoters that do not have an IR element, suggesting that RhpR indirectly regulates the transcriptional cascade of *hrpRS*, *hrpL*, and the T3 genes.

To identify additional genes involved in the *rhpRS* pathway, suppressor mutants were screened that restored the induction of the *avrPto-luc* reporter gene in the *rhpS* mutant. Determination of the transposon-insertion sites led to the identification of *rhpR*, an ATP-dependent Lon protease, a sigma 70 family protein (PSPPH1909), and other metabolic genes. A *lon*<sup>-</sup> *rhpS* double mutant exhibited phenotypes typical of a *lon*<sup>-</sup> mutant, suggesting that *rhpS* acts with or through *lon*. The expression of *lon* was elevated in *rhpS* and other T3-deficient mutants, indicating a negative feedback mechanism. Both the *lon*<sup>-</sup> *rhpS* and the *PSPPH1909* *rhpS* double mutant displayed enhanced transcription of *hrpL* in MM than did the *rhpS* mutant.

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## **CHAPTER 1**

### **LITERATURE REVIEW**

#### **Regulation of type three secretion system genes in phytopathogenic bacteria**

## INTRODUCTION

Many Gram-negative pathogenic bacteria rely on the needle-like type three secretion system (T3SS) to secrete a cocktail of effector proteins that help bacteria to infect eukaryotic host organisms (Jin et al. 2003). The expression of the T3SS genes is coordinately regulated by many endogenous regulatory proteins and various external environmental factors (Tang et al. 2006). In phytopathogenic bacteria, the T3SS is encoded by the *hrp* (hypersensitive response and pathogenicity) genes, which are regulated by either an ECF (extracytoplasmic factor) family alternate sigma factor HrpL (Xiao et al. 1994b) or an AraC-like activator (HrpX or HrpB) (Alfano and Collmer 1997). To date, a large number of T3SS-regulating components acting upstream of the *hrp* genes have been identified and characterized in various phytopathogenic bacteria, including two-component systems (TCS), transcription factors, membrane proteins, quorum-sensing genes, plant-derived compounds, and medium components. To better understand the molecular mechanism of bacteria-plant interaction, future studies are needed to elucidate the nature of signals regulating the T3SS genes, the pathways by which bacteria sense the signals, and the connections between the T3SS regulatory genes and the *hrp*/effector genes.

### **The bacterial type three secretion systems.**

The T3SS is a sophisticated molecular machine containing more than 20 different proteins and is essential for bacterial virulence. T3SS-deficient mutants are nonpathogenic (Cunnac et al. 2009).

Although the intact T3SS apparatus has yet to be purified from phytopathogenic bacteria, it has been purified from the mammalian pathogen *Salmonella enterica* (Buttner and He,



2009). The T3SS needle structure measures approximately 80 nm in length and 8 nm in width. It starts at the basal body, which crosses two bacterial membranes, and the needle that protrudes out of the cell. The basal body is composed of two rings that interact with the inner membrane and the outer membrane, respectively. An inner rod connects the basal body to the needle, which is made of 100-150 subunits of one single small protein (Kubori et al. 1998). In phytopathogenic bacteria, the T3SS filament is called *hrp* pilus (Roine et al. 1997).

In *P. syringae*, the basal body of the T3SS is encoded by the so-called *hrc* (hypersensitive response and conserved) genes, which are highly conserved across different bacteria (Collmer et al. 2000). Eight *hrc* genes share high similarity with the flagellar genes, suggesting that the T3SS apparatus is related to a flagellum (He, 1998). HrpA is the major structural protein of the *P. syringae* *hrp* pili, which are much longer than the needle structures of mammalian pathogens. This is probably because the *hrp* pili most span the thick plant cell wall (Jin et al. 2001; Kubori et al. 1998).

The genes encoding the T3SS are located on the chromosome in some bacteria and on a plasmid in others. In the plant pathogen *Pseudomonas syringae*, the T3SS genes are clustered in a chromosomal *hrp* island, which is responsible for pathogenicity on susceptible plants and the hypersensitive response (HR) on resistant and nonhost plants (Collmer et al. 2000). The *hrp* island harbors genes encoding the structure proteins of the T3SS complex and regulatory proteins controlling the expression of the T3SS genes (Buttner and He, 2009).

Effectors are the T3SS-injected virulence proteins that are responsible for bacterial pathogenicity. It has been shown that effector proteins have a signal peptide at the N-terminus of the protein that directs the protein secretion through the T3SS. A conserved motif has not been found in the N-terminal region of effectors, but the amphipathic composition of

the N-terminal amino acid is believed to contain the secretion signal (Arnold et al. 2009; Galan and Wolf-Watz, 2006; Samudrala et al. 2009). A few effector genes are localized in the conserved effector locus (CEL) and an exchangeable effector locus (EEL) flanking the *hrp/hrc* gene cluster. The CEL is crucial for bacterial pathogenicity while the EEL only has a minor role (Alfano et al. 2000; Collmer et al. 2000). A handful of effectors are named Avr proteins because they were initially identified as the proteins that induced the avirulent reaction in the host plants carrying the cognate resistant genes (R genes) (Alfano and Collmer, 2004; Leach and White, 1996). Upon the recognition of the corresponding R genes in resistant plants, the bacterial Avr proteins elicit a hypersensitive response (HR), which is a rapid cell death that inhibits the spread of pathogen from the infection site. In susceptible plants without the corresponding R genes, the Avr effectors function as virulent determinants by interfering with host defense mechanisms and manipulating host cellular activities to the benefit of the pathogen (Alfano and Collmer, 2004). Genome sequencing and bioinformatic analysis have enabled comprehensive identification of effector repertoires in various phytobacteria (Ferreira et al. 2006; Fouts et al. 2002; Zwiesler-Vollick et al. 2002). Many notable studies have characterized the ability of single effectors to suppress the PAMP-mediated defense response (He et al. 2006; Navarro et al. 2006, 2008; Shan et al. 2008), to manipulate hormone-signaling pathways (Chen et al. 2007; de Torres-Zabala et al. 2007; Jelenska et al. 2007; Navarro et al. 2006), and to suppress cell death elicited by other effectors (Wei et al. 2007a).

The secretion of some T3SS effectors needs the corresponding chaperones, which are small cytoplasmic proteins that specifically bind to individual T3SS effectors. It is proposed that T3SS chaperones prevent the cognate effectors from aggregation or degradation in the bacterial cytoplasm and lead effectors to the T3SS machinery (Feldman and Cornelis, 2003;

Losada and Hutcheson, 2005). Most genes encoding T3SS chaperones are linked with the cognate effector genes (Guttman et al., 2002). Most T3SS chaperones are specifically required for the secretion of their corresponding effectors.

### **The gene regulation of the *hrp* genes in phytopathogenic bacteria.**

The expression of the T3SS genes is coordinately regulated by many endogenous regulatory proteins and various external environmental factors (Fass and Groisman, 2009; Rosqvist et al. 1994; Tang et al. 2006; Yahr and Wolfgang, 2006). The *hrp* genes are expressed at a very low level in nutrient rich media, but are activated rapidly in *hrp*-inducing minimal media and in the plants. The *hrp* genes are divided into two groups based on the mechanisms of their regulation. The group I includes the *hrp* genes of *P. syringae*, *Erwinia* spp., and *Pantoea stewartii* that are controlled by an ECF family alternate sigma factor HrpL. The *hrp* genes in group II are activated by an AraC-like transcriptional activator, such as HrpX in *Xanthomonas* spp. and HrpB in *Ralstonia solanacearum* (Alfano and Collmer 1997; Tang et al. 2006).

The group I *hrp* genes are activated by HrpL that recognizes an *hrp* box motif (GGAACC-N<sub>15/16</sub>-CCACNNA) in the *hrp* gene promoters (Nissan et al. 2005; Xiao et al. 1994a). The consensus sequence of the *hrp* box has been used to identify novel candidate T3SS effector genes in the genomes of these bacteria via bioinformatic prediction (Ferreira et al. 2006; Fouts et al. 2002; Zwiesler-Vollick et al. 2002). In addition to the T3SS genes, many non-T3SS genes also contain the *hrp* box in the promoters and are induced by *hrpL*, suggesting coordination between the T3SS induction and the activation of other biological processes (Boch et al. 2002; Lan et al. 2006).

In *P. syringae*, the *hrpL*-based induction of the T3SS genes depends on another alternate sigma factor RpoN ( $\sigma^{54}$ ) and two NtrC-family transcription factors, HrpR and HrpS (Hendrickson et al. 2000; Hutcheson et al. 2001; Xiao et al. 1994b). RpoN controls the transcription of *hrpL* under a  $\sigma^{54}$ -dependent promoter (Hendrickson et al. 2000). The *hrpR* and *hrpS* genes are in the same operon (Grimm et al. 1995; Xiao et al. 1994b). *hrpS* alone induces *hrpL* to a very low level while the full activation of *hrpL* requires both genes (Hutcheson et al. 2001). HrpR and HrpS carry an enhancer-binding motif and a module that associates with the  $\sigma^{54}$ -RNA polymerase. HrpR and HrpS are proposed to form a heterodimer that binds to the *hrpL* promoter and induces the *hrpL* transcription by interacting with the RpoN-RNA polymerase under the T3SS-inducing conditions (Hutcheson et al. 2001). *Erwinia* spp. and *Pantoea stewartii* do not have HrpR, and the induction of *hrpL* is mediated only by *hrpS* (Frederick et al. 2003). Another locus *rsmA/rsmB* in *Erwinia carotovora* has been demonstrated to control the *hrpL* expression (Chatterjee et al. 2002). *rsmA* encodes a small RNA-binding protein and *rsmB* is an RNA (Chatterjee et al. 1995; Liu et al. 1998). The *hrpL* transcription is abolished in an *rsmB*<sup>-</sup> mutant but is elevated in an *rsmA*<sup>-</sup> mutant, suggesting that *rsmA* is a negative regulator and *rsmB* is a positive regulator of the T3SS in *Erwinia carotovora* (Chatterjee et al. 2002).

In *P. syringae*, HrpS activity is repressed by HrpV, a T3SS negative regulator that physically interacts with HrpS (Preston et al. 1998; Wei et al. 2005). In the inducing medium, an *hrpV* mutant displays a higher level of the T3SS gene expression, whereas the strain overexpressing *hrpV* compromises the T3SS gene induction. HrpV-mediated repression can be cleared by HrpG, a chaperone-like protein that interacts with HrpV and liberates HrpS from HrpV-mediated repression without changing the transcription of *hrpV* (Wei et al. 2005). In *Erwinia* spp. and *Pantoea stewartii*, the *hrpS-hrpL-hrp* cascade is positively regulated by a

two-component system *hrpXY* (Merighi et al. 2003; Wei et al. 2000b). The phosphorylation of the response regulator HrpY, likely by the cognate histidine kinase HrpX, is required for the activation of the *hrpS-hrpL-hrp* cascade (Nizan-Koren et al. 2003). However, how *hrpXY* activates the *hrpS* expression is still unknown.

The *P. syringae* HrpR protein is degraded by an ATP-dependent protease Lon, which degrades unstable or misfolded proteins involved in a variety of biological processes in bacteria (Bretz et al. 2002). HrpR is unstable in KB but is stabilized in a *lon*<sup>-</sup> mutant, leading to an elevated expression of the T3SS genes in the nutrient rich medium (Bretz et al. 2002; Lan et al. 2007). In addition, the *lon*<sup>-</sup> mutant hypersecretes a few T3SS effectors, suggesting a Lon-associated degradation of these effectors. The effectors have been shown to be protected from the Lon degradation by their cognate chaperones prior to secretion (Losada and Hutcheson, 2005). Conversely, the expression of *hrpL* in the *lon*<sup>-</sup> mutant exhibits a dynamic change in the T3SS-inducing minimal medium. *hrpL* is transcribed at a higher level in the *lon*<sup>-</sup> mutant than in the wild-type strain shortly after the induction in the minimal medium, but it is more abundant in the wild-type strain at later time points (Lan et al. 2007).

The *hrpRS* transcription of *P. syringae* displays a two to four-fold induction in both the minimal medium and the host plant (Lan et al. 2006; Rahme et al. 1992; Thwaites et al. 2004). The expression of *hrpRS* is regulated by at least two two-component systems (TCS), GacAS and RhpRS (Chatterjee et al. 2003; Lebeau et al. 2008; Xiao et al. 2007). The GacAS system plays crucial roles in regulating multiple biological processes in various bacteria, such as motility, virulence, quorum-sensing and production of toxin, antibiotics, exopolysaccharides and biofilm (Heeb and Haas 2001). In *P. syringae*, a mutation in the response regulator gene *gacA* severely reduces the expression of *hrpRS*, *rpoN*, and *hrpL*, suggesting that *gacA* is an

important T3SS regulator that is located at the top of the regulatory cascade (Chatterjee et al. 2003). Recent research with *Erwinia chrysanthemi* 3937 has also demonstrated that GacA is required for the expression of the T3SS genes (Lebeau et al. 2008). The signal perceived by GacS and the connection between GacA and *hrpRS* still remain to be elucidated.

Another TCS mutant of *P. syringae* has also been shown to display diminished induction of the T3SS genes in the minimal medium and the host plants (Deng et al. 2009; Xiao et al. 2007). The mutant carries a transposon insertion in the *rhpS* gene encoding a putative sensor kinase. *rhpS* is located immediately downstream of a putative response regulator gene *rhpR*, and the two genes are organized in an operon. The *rhpS*<sup>-</sup> mutant shows reduced transcriptional induction of *hrpR*, *hrpL*, and *avrPto* in both the minimal medium and the plant. In addition, the *rhpS*<sup>-</sup> mutant is severely reduced in pathogenicity, suggesting that *rhpS* is a key sensor for the activation of the T3SS genes. Interestingly, the deletion mutant of the whole *rhpRS* locus,  $\Delta$ *rhpRS*, and the wild-type strain show similar induction of *avrPto* and pathogenicity in the host plants, suggesting that RhpR is a negative regulator of the T3SS. Overexpression of RhpR in  $\Delta$ *rhpRS* suppresses the induction of the T3SS genes in a phosphorylation-dependent manner (Xiao et al. 2007). Based on these observations, RhpR is proposed to be phosphorylated by an unknown factor in the *rhpS*<sup>-</sup> mutant and the phosphorylated RhpR represses the T3SS genes. In wild-type bacteria, RhpS acts as a phosphatase and retains RhpR in a dephosphorylated state when bacteria are grown in the T3SS-inducing conditions.

In addition to GacAS and RhpRS, *hrpA* and *corR* also regulate *hrpRS* transcription in *P. syringae* (Sreedharan et al. 2006; Wei et al. 2000a). A mutation in *hrpA* that encodes the major *hrp* pilus component severely compromises the transcription of *hrpRS* and *hrpL*, which

can be restored by the overexpression of *hrpRS*. However, the mechanism by which HrpA controls *hrpRS* is unknown (Wei et al. 2000a). Similarly, a mutation in *corR*, which encodes a response regulator controlling the expression of the phytotoxin coronatine in *Pseudomonas syringae* pv. *tomato*, shows a reduction in the expression of *hrpL*, compared with the wild-type strain. A putative CorR-binding site is located upstream of *hrpL*, and the gel shift assay confirms the binding of CorR to this DNA motif (Sreedharan et al. 2006).

The group II *hrp* genes in *Xanthomonas* spp. and *Ralstonia solanacearum* are regulated by the AraC-type transcriptional activator HrpX and HrpB, respectively (Genin et al. 1992; Wengelnik and Bonas, 1996). The protein sequences of HrpX and HrpB are highly conserved. In *Xanthomonas* spp., HrpX specifically binds to a conserved motif named PIP (plant inducible promoter)-box (TTCGC-N<sub>15</sub>-TTCGC), which is present in the promoter regions of most HrpX-regulated genes (Coebnik et al. 2006). Similarly, many HrpB-regulated genes in *R. solanacearum* contain an hrpII-box (TTCG-N<sub>16</sub>-TTCG) in the promoters (Cunnac et al. 2004). Although computational searches for the PIP/hrpII motifs have been successful to identify the T3SS effector genes (Occhialini et al. 2005), some HrpX/HrpB-regulated T3SS genes lack the PIP/hrpII-box, such as *avrBs1* and *avrBs3* family genes in *Xanthomonas* spp. (Thieme et al. 2005).

*hrpX* and *hrpB* are activated by another key regulator HrpG, an OmpR-type two-component response regulator containing a DNA-binding domain (Brito et al. 1999; Wengelnik et al. 1996). HrpG of *Xanthomonas axonopodis* pv. *citri* physically interacts with an two-component system histidine kinase, suggesting that HrpG may be phosphorylated by this protein (Alegria et al. 2004). In *X. campestris* pv. *vesicatoria*, three point mutations of HrpG are constitutively active in the T3SS-repressing medium, suggesting that HrpG may

need the conformational change to activate the T3SS gene expression (Wengenilk et al. 1999). In addition, five other loci have been recently reported to regulate the T3SS in *Xanthomonas* spp. First, like *Erwinia* spp., an *rsmA*-like gene in *X. campestris* pv. *campestris* plays a negative role in regulating the T3SS genes. The *rsmA*<sup>-</sup> mutant displayed an enhanced induction of the T3SS genes and bacterial virulence (Chao et al. 2008). Second, in *X. campestris* pv. *campestris*, *hpaR*, a putative *marR* family transcriptional regulator, is required for the induction of the T3SS genes. Mutation of *hpaR* renders the bacterium nonpathogenic to the host cabbage plants. *hpaR* is regulated by *hrpG/hrpX* and is repressed in the nutrient rich medium but induced in the T3SS-inducing medium (Wei et al. 2007b). Third, Zur, the key regulator for zinc homeostasis in *X. campestris*, positively regulates the *hrp* genes through *hrpX*, but not through *hrpG* (Huang et al. 2009). Fourth, using mutational analysis, a two-component system *colRS* has been identified as another novel regulator of the T3SS of *X. campestris* (Zhang et al. 2008). Finally, *X. oryzae* pv. *oryzae* two-component system PhoPQ positively controls the *hrpG* expression and the virulence (Lee et al. 2008).

In *R. solanacearum*, *hrpG* is constitutively expressed in both the rich medium and the minimal medium but induced in the plant (Brito et al. 1999). It is proposed that upon sensing the plant signal, the expression of *hrpG* is activated by five upstream signal transduction components (*prhA*, *prhJ*, *prhI*, *prhR*, and *phcA*), which are discussed in a following section.

### **The bacterial two-component transduction systems.**

Bacteria primarily utilize two-component systems (TCS) to couple environmental signals to adaptive responses (Hoch. 2000). TCSs play important roles in regulating multiple biological processes, such as metabolism, growth, motility, quorum-sensing, and



pathogenicity (Gao and Stock. 2009). TCSs generally include two components, a sensor histidine kinase (HK) and a cognate response regulator (RR). Upon sensing specific signals, the HK autophosphorylates the conserved histidine (His) residue of the kinase domain, and the high-energy phosphoryl group is subsequently transferred to the aspartate (Asp) residue of the cognate RR. The phosphorylation of RR induces its conformational change that activates the downstream responses (Stock et al. 2000).

HKs and RRs are modular proteins with variable domains, suggesting that they are versatile in sensing various environmental signals. The typical HKs have a N-terminal signal input domain, a transmembrane domain, and a cytoplasmic kinase core. The N-terminal signal input domain is diverse in sequence and enables the HKs to perceive a wide variety of stimuli, such as ions, metabolic molecules, light, osmolarity, humidity, cell envelope stress, reactive oxygen species, and electrochemical gradients (Gao and Stock, 2009). Although great advancements have been achieved in understanding the signal-sensing mechanisms in a few HKs in recent years, the exact signal for most HKs still remains unknown (Mascher et al. 2006; Szurmant et al. 2007).

The kinase core, where the HKs usually autophosphorylate spontaneously, contains a dimerization and histidine phosphotransfer (DHp) domain in the N-terminus and a catalytic and ATP binding (CA) domain in the C-terminus. The CA domain has the kinase activity that phosphorylates the conserved His residue in the DHp domain using ATP (Stock et al. 2000). In many cases, HKs are bifunctional and have both kinase and phosphatase activities, which control the level of RR phosphorylation and response afterwards (Laub and Goulian, 2007). The DHp domain has the phosphatase activity, which is also affected by the CA domain. The conserved His residue is responsible for the phosphatase activity of *Escherichia coli*

osmosensor HK EnvZ, suggesting a reverse phosphorylation from the Asp residue of the RR to the His residue of the HK (Dutta et al. 1996; Zhu et al. 2000). However, some HK mutants that change the conserved His to other residues still retain the phosphatase activity, indicating that the phosphatase activity of the HKs involves other mechanisms (Chamnongpol et al. 2003). The level of RR phosphorylation and output response are largely controlled by either the HK kinase activity (Fleischer et al. 2007), the HK phosphatase activity (Brandon et al. 2000), or both (Chamnongpol et al. 2003), suggesting a big diversity of mechanisms in HK signal transduction. HKs always function as dimers that are controlled by a *trans*-phosphorylation mechanism. The CA domain of one dimer subunit phosphorylates the His residue in the DHp domain of the other dimer subunit (Stock et al. 2000).

The typical RR carries an N-terminal REC domain that receives the phosphoryl group from HK and a C-terminal variable effector domain that is regulated by the REC domain. The REC domain is a phosphorylation-activated switch that controls the conformation of RRs. An unphosphorylated REC domain exists in the inactive conformation, whereas the phosphorylation at the conserved Asp residue switches it to the active conformation (Gardino et al. 2007). The REC domain has both the phosphoryl transfer and the autodephosphorylation activities, which determine the level of RR phosphorylation that controls the activity of the effector domain (Stock et al. 2000).

A wide variety of output responses are generated by different effector domains, which can be categorized into at least four groups (Gao and Stock, 2009). First, the effector domains of 63% of all RRs carry a DNA-binding domain that can be further grouped into four major subfamilies, including OmpR (30% of all RRs) (Martinez-Hackert et al. 1997), NarL (17%) (Milani et al. 2005), NtrC (10%) (Batchelor et al. 2008), and LytTR (3%) (Sidote et al. 2008).

The DNA-binding RRs regulate expression of target genes by modulating their own phosphorylation status, inducing the dimerization or higher-order oligomerization, thereby controlling its affinity to DNA motifs in the promoter region of downstream genes (Martinez-Hackert et al. 1997). The second group carries enzymatic domains that are found in ~13% of all RRs. About half of these enzymatic RRs play a role in the regulation of cyclic diguanylate, a secondary messenger of the bacterial cells (Romling et al. 2005). The third group is represented in 3% of all RRs of which the effector domains interact with other proteins or ligands (Gao and Stock, 2009). The fourth group includes only 1% of all RRs containing an RNA-binding domain that function as anti-termination factors (O'Hara et al. 1999). Unlike the prototypical RR structures, nearly 17% of all RRs have only REC domains. Most of these RRs regulate the bacterial motility by interacting with motor proteins or phosphorylating intermediates in phosphorelay pathways (Varughese et al. 2005). A RR can regulate its downstream gene(s) as activator, repressor, or both (Gao and Stock, 2009).

Most sequenced bacterial genomes encode dozens of TCS proteins, which makes it possible for the cross-phosphorylation between similar DHP and REC domains, resulting in complicated one-to-many, many-to-one, and many-to-many networks between HKs and RRs (Gao and Stock, 2009). Approximately 25% of all HKs have a REC domain that can be phosphorylated by the kinase domain of HK (Ogino et al. 1998). The phosphorylated REC domain can transfer the phosphoryl group to a His-containing HPt domain and then phosphorylate an RR, which forms a sophisticated His-Asp-His-Asp phosphorelay. This HPt domain can be a part of an HK, a single protein, or a part of another membrane protein (Stock et al. 2000).

In addition to regulating the downstream genes, many RRs are capable of autoregulation (Bijlsma and Groisman, 2003). Autoregulation is mediated by direct binding of an RR to its own promoter (Bijlsma and Groisman, 2003). Many RRs autoactivate their own expression (Bang et al. 2002; Clarke and Sperandio, 2005; Gonzalo-Asensio et al. 2008; Soncini et al. 1995), but a few RRs such as CovR and TorR are capable of autoinhibition (Ansaldi et al. 2000; Gusa and Scott, 2005). Direct autoregulation enables bacteria to respond more rapidly and efficiently to environmental changes (Hoffer et al. 2001; Shin et al. 2006).

Many TCSs play critical roles in bacterial pathogenicity. As discussed previously, a group of phyto bacterial TCSs act as important regulators in controlling the expression of the *hrp* genes, such as GacAS (Chatterjee et al. 2003; Lebeau et al. 2008), and RhpRS (Xiao et al. 2007) in *P. syringae*; ColRS (Zhang et al. 2008) and PhoPQ (Lee et al. 2008) in *Xanthomonas* spp.; and HrpXY in *Pantoea stewartii* (Merighi et al. 2003; Wei et al. 2005).

### **Host and environmental signals regulating the phyto bacterial T3SS genes.**

Host sensing is essential for the activation of the bacterial T3SS genes, which is responsible for disease development (Brencic and Winans, 2005). Even though little is known about host signals for phytopathogenic bacteria, the elegant work carried out in *Ralstonia solanacearum* indicated the presence of host specific signal to regulate the T3SS genes. Like many other Gram-negative phytopathogenic pathogens, the *R. solanacearum* T3SS genes are induced upon the bacteria-plant cell contact (Aldon et al. 2000). A mutation in *prhA*, a gene encoding an outer membrane protein that is homologous to siderophore receptors, disrupts the induction of the T3SS genes by the plant, but not by the T3SS-inducing medium (Marenda et al. 1998). PrhA might sense an unidentified plant-specific signal, likely a non-

diffusible component in the plant wall (Aldon et al. 2000).

Two genes acting downstream of *prhA* are *prhI* and *prhR*, which are organized in the same operon in the *hrp* gene cluster and encode a transmembrane protein and an ECF sigma factor, respectively (Brito et al. 2002). A *prhIR*<sup>-</sup> mutant compromises the pathogenicity and the HR elicitation. PrhIR are required for the activation of the T3SS gene expression in the plant, but not in the minimal medium. It is proposed that a plant signal sensed by PrhA is transferred to PrhR and passed through the membrane. In the cytoplasm, PrhI is activated by PrhR, and then sequentially activates a signal transduction cascade consisting of three transcription factors, PrhJ, HrpG, and HrpB (Brito et al. 1999; 2002). In addition, a LysR family transcriptional regulator PhcA negatively regulates the protein level but not the transcription level of HrpG in the rich medium (Genin et al. 2005). It is recently reported that PhcA binds to the *prhIR* promoter and represses the transcription of *prhIR* (Yoshimochi et al. 2009).

A handful of reports suggest that perception of plant signals is important for the activation of the T3SS genes in *P. syringae* and other bacteria. For example, *hrpL* of *P. syringae* is induced much greater in the plant than in the minimal medium, suggesting the presence of a plant-specific signal for the T3SS in *P. syringae* (Rahme et al. 1992). It is also recently reported that the induction of the *P. syringae* *hrpA* promoter is enhanced by cell-free exudates from the plant cell suspension cultures. Further analysis suggests that some water-soluble plant-cell-derived compounds are the signals that are sensed by bacteria (Haapalainen et al. 2009). Furthermore, a study in *Dickeya dadantii* (*Erwinia chrysanthemi*) 3739 has found two plant phenolic acids that induce the T3SS gene expression, which are the first identified specific T3SS-inducers in phytobacteria (Yang et al. 2009).

On the other hand, some plant signals may act as T3SS repressors that inhibit *in planta* T3SS gene induction. In a study to identify the host signals for the induction of the T3SS genes, an *Arabidopsis* (a host of *P. syringae* pv. *tomato* DC3000) mutant *attI<sup>-</sup>* (aberrant induction of type three genes) has been isolated. The *attI<sup>-</sup>* mutant significantly enhances *in planta* expression of the bacterial T3SS genes, suggesting a negative role of ATT1 in regulating the T3SS gene expression. ATT1 encodes CYP86A2, a cytochrome P450 monooxygenase that catalyzes fatty acid oxidation, which regulates cutin formation (Xiao et al. 2004). Certain lipids may reduce the T3SS gene expression from the intercellular spaces. These lipids might be either cutin monomers or cutin-related fatty acids that CYP86A2 synthesizes. In support of this hypothesis, a variety of commercial cutin-related fatty acids were found to be capable of repressing the *hrp* promoter activity (Xiao et al. 2004). The negative cutin-related signals may inhibit the T3SS genes expression during bacterial growth on leaf surface. However, how the cutin-related signals are perceived by *P. syringae* is not clear (Xiao et al. 2004). In addition, several examples of plant components acting as negative signals for the T3SS genes have been reported in other phyto bacteria. For example, in *Dickeya dadantii* (*Erwinia chrysanthemi*), a plant-derived *p*-coumaric acid represses the T3SS genes expression, suggesting a plant defense mechanism against bacterial pathogens (Li et al. 2009). Similarly, a low molecular weight (<10kDa) plant extract also inhibits the *hrp* genes expression in *Xanthomonas campestris* pv. *campestris* (Watt et al. 2009).

The bacterial quorum-sensing system has also been recently demonstrated to regulate the T3SS in *Pseudomonas syringae* and *Pantoea agglomerans* (Chalupowicz et al. 2008; 2009; Chatterjee et al. 2007; Deng et al. 2009). *P. syringae* produces *N*-acyl homoserine lactones (AHLs) as the signal of the quorum-sensing system that coordinates multiple bacterial genes expression adaptive to local population density (Fuqua et al. 1994). A transposon insertion

mutation in *psrA*, a *Pseudomonas* sigma regulator, results in enhanced expression of the AHL synthase gene *psyI* and reduced pathogenicity in the host tomato, implicating a regulatory interaction between the quorum-sensing and the T3SS (Chatterjee et al. 2007). In support of this observation, AefR, a TetR-type transcriptional regulator that controls the AHL production in *P. syringae* pv. *syringae* (Quinones et al. 2004; 2005), has been recently discovered to positively control the expression of *hrpRS* and *hrpL* in *P. syringae* pv. *phaseolicola* (Deng et al. 2009). In gall-forming *Pantoea agglomerans*, *pagI* and *pagR* are responsible for production and perception of the quorum-sensing signals *N*-l-homoserine lactones (HSL) (Chalupowicz et al. 2008). The *hrpL* expression in a *pagI*<sup>-</sup> mutant or a *pagR*<sup>-</sup> mutant is significantly repressed compared with the wild-type, suggesting that the T3SS regulation is also subject to the quorum-sensing system in *P. agglomerans* pv. *gypsophilae* (Chalupowicz et al. 2008, 2009).

Interestingly, an *iaaH* mutant lacking the auxin biosynthesis and an *etz*<sup>-</sup> mutant disrupting the cytokinin pathway substantially compromise the transcription of *hrpS* and *hrpL* in the plant, indicating the involvement of auxin and cytokinin in regulating the T3SS in *P. agglomerans* pv. *gypsophilae* (Chalupowicz et al. 2009).

In many phyto bacteria, the T3SS genes are suppressed by nutrient rich media but rapidly induced after being transferred into minimal media (Tang et al. 2006). Even though chemically defined minimal media have been widely used to induce the phyto bacterial *hrp* genes, it is hard to identify the specific component that is responsible for the induction (Kim et al. 2009). Multiple environmental factors, such as temperature, medium components, osmolaric strength, pH, and nutritional conditions, affect the T3SS gene expression in the liquid media (Huynh et al. 1989; Schulte et al. 1992; van Dijk et al. 1999). The best

temperature for the induction of the T3SS genes in *P. syringae* is between 20 and 30°C (van Dijk et al. 1999). In *P. syringae*, iron has been recently found to induce the transcription of *hrpL* and an effector gene *hopAAI-1* while it repress the bacterial growth in the minimal medium (Kim et al. 2009). Complex nutrient sources, high pH, and high osmolarity are responsible for the T3SS gene repression in rich media. On the other hand, the physiological and chemical environment in the plant is thought to be mimicked by the T3SS-inducing media that are low in pH and nutritionally poor (Huynh et al. 1989). The T3SS-inducing medium composition varies between different pathogens, which may suggest that the apoplastic conditions of different host plants vary. For example, fructose and sucrose induce the T3SS genes better than other carbon sources tested in *P. syringae*, while the induction of the T3SS genes in *Xanthomonas* spp. needs sucrose and multiple sulfur-containing amino acids (Huynh et al. 1989; Schulte et al. 1992).

## **Perspectives**

Tremendous progress has been made in better understanding the phyto bacterial T3SS, especially for novel functions of the T3SS effectors and new components controlling the *hrp* genes. Despite studies in the previous years that have identified a large number of T3SS-regulating genes in various phytopathogenic bacteria, how these genes regulate the downstream T3SS pathways is largely unknown. Even though a handful of two-component systems have been demonstrated to regulate the T3SS genes, the nature of the T3SS signals and mechanism by which bacteria perceive and transduce the signals remain to be elucidated.

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## CHAPTER 2

*Pseudomonas syringae* pv. *phaseolicola* mutants compromising the induction of the type  
III secretion system

## ABSTRACT

*Pseudomonas syringae* bacteria utilize the type III secretion system (TTSS) to deliver effector proteins into host cells. The TTSS and effector genes (together called the type III genes hereafter) are repressed in nutrient rich medium but rapidly induced after the bacteria are transferred into minimal medium (MM) or infiltrated into the plant. The induction of the type III genes is mediated by HrpL, an alternative sigma factor that recognizes the conserved hrp box motif in the type III gene promoters. The induction of *hrpL* is mediated by HrpR and HrpS, two homologous proteins that bind the *hrpL* promoter. To identify additional genes involved in regulation of the type III genes, the *P. s. pv. phaseolicola* (*Psph*) NPS3121 transposon insertion mutants were screened for reduced induction of *avrPto-luc* and *hrpL-luc*, reporter genes for promoters of effector gene *avrPto* and *hrpL*, respectively. Determination of the transposon-insertion sites led to the identification of genes with putative functions in signal transduction and transcriptional regulation, protein synthesis, and basic metabolism. A transcriptional regulator (AefR<sub>NPS3121</sub>) identified in our screen is homologous to AefR, a regulator of the quorum sensing signal and epiphytic traits that was not known previously to regulate the type III genes in *P. s. pv. syringae* (*Psy*) B728a. AefR<sub>NPS3121</sub> in *Psph* NPS3121 and AefR in *Psy* B728a are similar in regulating the quorum sensing signal in liquid medium but different in regulating epiphytic traits such as swarming motility, entry into leaves, and survival on the leaf surface.

## INTRODUCTION

Like many Gram-negative bacterial pathogens, *Pseudomonas syringae* bacteria rely on the type III secretion system (TTSS) for parasitism (Jin et al. 2003). The TTSS is encoded by the hypersensitive response and pathogenicity (*hrp/hrc*) genes that are essential for disease

development on host plants and hypersensitive response (HR) on resistant plants and non-host plants (Collmer et al. 2000). Through the TTSS, bacteria inject an array of virulence proteins termed type III effectors into the host cells (Galan and Collmer 1999). A number of type III effectors have been shown with a function to suppress the host basal defense systems (Zhou and Cai 2008). In resistant plants, however, the effectors are recognized by the plant disease resistance proteins, which trigger the HR and disease resistance (Alfano and Collmer 2004).

The type III genes of *Pseudomonas syringae* bacteria are repressed in nutrient rich medium such as KB (King et al. 1954), but are induced in minimal medium (MM) and in the plant (Tang et al. 2006). The induction of the type III genes is mediated by HrpL, an alternate sigma factor that recognizes the conserved *hrp* box motif in the promoter of type III genes (Xiao and Hutcheson 1994; Fouts et al. 2002; Ferreira et al. 2006; Lan et al. 2006). The *hrpL* gene itself is induced under the same conditions (Thwaites et al. 2004). *hrpL* induction is mediated by HrpR and HrpS, two DNA binding proteins that form a heterodimer on the *hrpL* promoter (Xiao et al. 1994; Hutcheson et al. 2001). The HrpR/HrpS heterodimer activates *hrpL* transcription by interacting with the alternate sigma factor RpoN (Hendrickson et al. 2000; Chatterjee et al. 2003). Deletion of either *hrpR* or *hrpS* abolishes *hrpL* induction and bacterial pathogenicity (Xiao et al. 1994). Additional regulators required for maximal *hrpL* induction include CorR, which regulates coronatine synthesis, and PsaA, which regulates production of the quorum sensing signal *N*-acyl homoserine lactones (AHL). CorR regulates *hrpL* by binding directly to the *hrpL* promoter (Sreedharan et al. 2006). PsaA directly binds the *rpoS* promoter and *aeiR* operator to positively regulate *rpoS* and negatively regulate *aeiR*, respectively (Chatterjee et al. 2007). The regulation of PsaA on *hrpL* gene is likely indirect

(Chatterjee et al. 2007). Mutation of either *corR* or *psrA* reduces bacterial pathogenicity (Sreedharan et al. 2006; Chatterjee et al. 2007).

HrpR and HrpS are encoded by the *hrpRS* operon that is moderately expressed in KB medium and further induced 2-4 fold in MM and in the plant (Rahme et al. 1992; Grimm et al. 1995; Hutcheson et al. 2001; Thwaites et al. 2004). The expression of the *hrpRS* operon is regulated by two loci encoding the two-component systems GacS/GacA and RhpS/RhpR (Chatterjee et al. 2003; Xiao et al. 2007). GacS is a sensor histidine kinase, while GacA is the cognate response regulator of GacS. In *Pst* DC3000 strain, *gacA*<sup>-</sup> mutation significantly attenuates the transcription of *hrpRS*, *rpoN*, and *hrpL* (Chatterjee et al. 2003). It is believed that GacS is activated by a yet unidentified signal, which in turn phosphorylates GacA, and the phosphorylated GacA further induces, either directly or indirectly, the *hrpRS* operon (Chatterjee et al. 2003). The role of RhpS/RhpR in regulating the *hrpRS* operon is indicated by mutation of the sensor kinase gene *rhpS*, which abolishes *hrpRS* induction in the plant and in MM (Xiao et al. 2007). However, disruption of the corresponding response regulator gene *rhpR* in the *rhpS*<sup>-</sup> mutant or deletion of the *rhpRS* locus completely restores the *hrpRS* induction, suggesting that RhpR is a negative regulator of *hrpRS*. In addition to GacS/GacA and RhpS/RhpR, the *hrpA* gene encoding type III pilin was also found to affect *hrpRS* induction through an unknown mechanism. In a *hrpA*<sup>-</sup> mutant, transcription of the *hrpRS* operon, *hrpL*, and the type III genes is severely reduced (Wei et al. 2000).

The stability of the HrpR protein is regulated by Lon, an ATP-dependent protease (Bretz et al. 2002). HrpR is stabilized in a *lon*<sup>-</sup> mutant, which leads to elevated expression of *hrpL* and type III genes in KB medium (Bretz et al. 2002; Lan et al. 2007). In MM, however, the impact of *lon*<sup>-</sup> mutation on *hrpL* expression exhibits a dynamic change. *hrpL* is expressed at a



higher level in a *lon*<sup>-</sup> mutant than in the wild type (WT) strain shortly after induction in MM, but the expression pattern is reversed at later time points, e.g., *hrpL* mRNA is more abundant in the WT strain than in *lon*<sup>-</sup> mutant (Lan et al. 2007). HrpS protein stability does not appear to be affected by the *lon*<sup>-</sup> mutation. However, the activity of HrpS protein is repressed by HrpV, a negative regulator of the type III genes, which physically interacts with HrpS (Preston et al. 1998; Wei et al. 2005). This repression can be cleared by HrpG, a protein that interacts with HrpV and liberates HrpS from the HrpV repression (Wei et al. 2005).

The strong induction of the type III genes in MM provides an assay system for genetic identification of regulators for the type III genes. Hendrickson et al (2000) screened for *P. s. pv. maculicola* (*Psm*) ES4326 mutants that compromised the induction of *hrpZ-uidA*, the reporter gene for the *hrpZ* promoter. From a total 14,000 colonies, 297 mutants were identified. Complementation of the mutants with a cosmid harboring the *hrpRS* locus restored the induction of the reporter gene in all mutant strains. None of these mutant genes has been cloned.

To identify additional regulators of the type III genes, we screened for *Psph* NPS3121 transposon-insertion mutants that poorly induce the expression of *avrPto-luc* (done by Drs. Tang and Xiao) and *hrpL-luc* reporter genes in MM. *avrPto* is a type III effector gene of *Pst* DC3000 that carries a typical *hrp* box motif in its promoter (Salmeron and Staskawicz 1993). *avrPto-luc* in *Psph* NPS3121 is induced in MM and in the plant in a *hrpL*- and *hrpRS*-dependent manner (Xiao et al. 2004; Xiao et al. 2007). In this chapter, the mutant genes identified in our screening are reported.

## RESULTS

### **Isolation of *Psph* mutants using *avrPto-luc* reporter (done by Drs. Tang and Xiao).**

*Psph* NPS3121 carrying the *avrPto-luc* reporter gene displayed low luciferase (LUC) activity in KB medium but high LUC activity in MM. This strain was subjected to EZ::TN<Kan-2> transposon insertion mutagenesis. A total of 11,872 mutant clones were screened for reduced LUC activity 6 hr after induction in MM. Clones that displayed a LUC activity less than 20% of the parental strain were selected and named MM insensitive (*min*) mutants. 84 mutants were recovered from the screening (Table II-1; Fig. II-S1).

Transposon insertion sites in these mutants were determined by two-stage semi-degenerated PCR (Jacobs et al. 2003), and the flanking sequences were searched against the *Psph* 1448A genomic sequence (Joardar et al. 2005). These mutants are distributed among 45 loci, which can be divided into four groups based on the putative functions of the gene products (Table II-1). Genes in Group A encode proteins with a role in signal perception and transduction, including *hrpS*, *rhpS*, and *PSPPH\_3244<sub>NPS3121</sub>*. *hrpS* and *rhpS* are known regulators of the *P. syringae* type III genes (Xiao et al. 1994; Xiao et al. 2007). *PSPPH\_3244<sub>NPS3121</sub>* encodes a transcriptional regulator that is 87% identical to the AefR protein that regulates the production of quorum sensing signal AHL and epiphytic fitness in *Psy* B728a strain (Quinones et al. 2004; 2005). Mutants of *hrpL*, *hrpR*, and other known regulatory genes were not identified in the screening. Group B has two genes encoding putative membrane proteins. *PSPPH\_4907<sub>NPS3121</sub>* encodes a porin protein in the OprD family that functions in trafficking small molecules across the outer membrane (Hancock and Brinkman 2002). *PSPPH\_5137<sub>NPS3121</sub>* encodes a putative integral membrane protein of the YeeE/YedE family with an unknown function (Joardar et al. 2005). Group C has only one gene *trmE* that encodes the tRNA modification GTPase (Joardar et al. 2005). Mutation of

*trmE* increases the rate of misincorporation of amino acids and frame-shifting during the translation process (Cabedo et al. 1999). Mutation of this gene in *Shigella flexneri* reduces bacterial virulence (Durand et al. 1997). Most of the mutant genes belong to Group D encoding functions in basic metabolism. Twelve genes are involved in the porphyrin metabolism (Table II-1). Nineteen genes are involved in the biosynthesis of amino acids arginine, glutamine, glutamic acid, histidine, valine, isoleucine, leucine, methionine, serine, and tryptophan (Table II-1). The induction of LUC activity in the amino acid biosynthesis mutants was restored by adding corresponding amino acids to 0.1 mM in the MM. Three genes are in an operon required for fructose uptake and metabolism (Joardar et al. 2005). Two genes encode enzymes that function in purine synthesis. Four additional metabolic genes are *eno-1* encoding enolase, *PSPPH\_2878<sub>NPS3121</sub>* encoding a glycosyl hydrolase, *sypA* encoding a putative peptide/siderophore synthase, and *PSPPH\_0569<sub>NPS3121</sub>* encoding a putative ATP phosphoribosyltransferase subunit.

All the mutants were examined for growth in MM. Mutants in groups A, B, and C displayed 7-9 fold multiplication 36 hr after culture in MM, similar to the WT strain. However, mutants in group D did not exhibit obvious growth in MM.

### **Mutant screening using the *hrpL-luc* reporter.**

The use of *avrPto-luc* as reporter led to the identification of a large number of metabolic mutants and only three genes with regulatory functions. Many genes with a known function in regulating the type III genes were not identified in the screening. Because the screening was ~2x coverage of the *Psph* genome, I decided to screen more mutants using *hrpL-luc* as reporter. To avoid metabolic mutants, mutants with low *hrpL-luc* reporter activity were assayed for growth in MM, and those with growth defect were discarded.

Eight mutants were obtained from a total of 16,000 clones. Five mutations were in the *rhpS* gene, one in *PSPPH\_3244<sub>NPS3121</sub>*, one in *trmE*, and one in *miaA*. Mutants of *rhpS*, *PSPPH\_3244<sub>NPS3121</sub>*, and *trmE* genes were obtained in the screening with *avrPto-luc* reporter. *miaA* encodes tRNA isopentenyltransferase, and like *trmE*, also has a function in protein translation (Leung et al. 1997). Except *rhpS* and *PSPPH\_3244<sub>NPS3121</sub>*, no gene with a regulatory function was identified in this screening.

### **Evaluation of the reporter genes.**

It was puzzling that a large number of metabolic mutants and only a few regulatory mutants were recovered from the mutant screening. One possibility was that the metabolic mutants are lethal in MM, as indicated by the lack of bacterial growth in MM. I therefore decided to evaluate the reporter genes with the mutant strains to determine if the reporter gene activities were consistent with the *hrpL* RNA expression in the mutants.

I first analyzed the levels of *hrpL* RNA in representative mutants of each functional group that were isolated based on the *avrPto-luc* reporter activity (Fig. II-1A). All mutants except *min93* exhibited good correlation between the *avrPto-luc* reporter activity (Fig. II-6) and the level of *hrpL* RNA (Fig. II-1A). The *min93* mutant displayed normal induction of *hrpL* RNA, but the LUC activity derived from *avrPto-luc* was almost undetectable in this mutant (Fig. II-6).

I then tested if the *hrpL-luc* reporter activity was consistent with *hrpL* RNA abundance. *min93* and six other mutants (*min24*, 4, 32, 42, 49, and 62) were cured of the *avrPto-luc* reporter plasmid, the *hrpL-luc* reporter plasmid was introduced into each, and LUC activity derived from *hrpL-luc* was measured (Fig. II-1B). The *hrpL-luc* reporter activity was almost

undetectable in *min93* mutant, but the *hrpL-luc* reporter activities in other mutants generally agreed with the levels of *hrpL* RNA.

I further conducted a western blot analysis of the LUC protein derived from *hrpL-luc* in *min93* and other mutants (Fig. II-1C). A high level of LUC protein was detected in the *min93* mutant, suggesting that this mutation impaired the detection of LUC enzymatic activity. The levels of LUC protein in other mutants were generally consistent with the levels of *hrpL-luc* reporter activities and the levels of *hrpL* RNA. These results indicated that the activities of *avrPto-luc* and *hrpL-luc* reporter genes generally reflected the level of *hrpL* RNA expression in the mutant bacteria.

The mutant gene in *min93* (*PSPPH\_4907<sub>NPS3121</sub>*) encodes an outer membrane protein of the porin D family that is involved in uptake and excretion of small molecules (Hancock and Brinkman 2002). Both mutant alleles of *PSPPH\_4907<sub>NPS3121</sub>* (*min79* and *min93*) showed normal levels of LUC protein but very poor LUC activity. In an experiment to test how tetracycline treatment of bacteria affected the induction of type III genes in MM, I observed that both *min79* and *min93* mutants were insensitive to tetracycline-mediated inhibition of *hrpA* gene expression (data not shown). These results suggested that the low LUC activities exhibited by *min79* and *min93* mutants probably resulted from defect in uptake of luciferin, the substrate for LUC enzymatic assay. The *min79* and *min93* mutants displayed only a slight reduction in pathogenicity (Table II-1).

### **Pathogenicity and HR assays of *min* mutants.**

Although metabolic mutations severely compromised induction of the type III genes in MM, many of the mutants were not reported in previous studies to isolate *P. syringae*

mutants that abolished the pathogenicity or HR-inducing activity. To determine if there is a correlation between the type III gene expression in MM and pathogenicity in host plants, all the mutant strains at  $2 \times 10^4$  CFU/mL were infiltrated into the primary leaves of bean plants. The degree of disease symptoms was evaluated according to the disease indices shown in Fig. II-7. The WT strain caused the most concentrated specks and was assigned with index 3. The *hrpS* mutants were symptom-free and assigned with index 0. Mutants with the disease symptoms in between were assigned with index 1 or 2. A number of metabolic mutants, including *min24/35/36* for histidine synthesis, *min9/14/21/39/61/60* for leucine synthesis, *min32/86* for valine and isoleucine synthesis, *min62/71* for tryptophan synthesis, and *min27* for purine synthesis, failed to elicit visible disease symptoms. Mutants with defects in histidine, leucine, valine, isoleucine, tryptophan, and purine biosynthesis were also identified by Brooks et al (2004) in a screening of *Pst* DC3000 mutants that compromised the pathogenicity in Arabidopsis plants.

Representative mutants that did not elicit visible symptoms were assayed for bacterial growth 6 days after inoculation into bean plants (Fig. II-2). All these mutants displayed a significant reduction of bacterial growth in the host plants, lower than that of the *rhpS* mutant (*min12*). With the exception of *min24(hisF)*, the growth of the remaining metabolic mutants was even lower than that of the *hrpS* mutant (*min8*). Although *min32(ilvD)* displayed a significant level of *hrpL* RNA in MM (Fig. II-1A), the growth of this mutant in host bean plants was even lower than that of the *hrpS* mutant (Fig. II-2). In contrast, many metabolic mutants with completely abolished *hrpL* expression in MM were only slightly reduced in pathogenicity compared with the WT strain (Fig. II-1A; Table II-1).

Representative mutants of each functional group were also assayed for the HR-inducing activity on tobacco W38 plants (Table II-2). A number of mutants that elicited significant disease symptoms on bean plants such as *min4(gltB<sup>-</sup>)*, *min18(fruK<sup>-</sup>)*, *min42(serA<sup>-</sup>)*, *min49(metF<sup>-</sup>)*, *min83(cibD<sup>-</sup>)*, *min85(Psph\_2818<sub>NPS3121</sub><sup>-</sup>)*, *min93(Psph\_4907<sub>NPS3121</sub><sup>-</sup>)*, and *min47(trmE<sup>-</sup>)* also elicited normal HR. On the other hand, mutants such as *min12(rhpS<sup>-</sup>)*, *min24(hisF<sup>-</sup>)*, *min27(purK<sup>-</sup>)*, *min32(ilvD<sup>-</sup>)*, *min62(trpA<sup>-</sup>)*, and *min71(trpD<sup>-</sup>)* that were significantly reduced in pathogenicity, elicited a delayed HR or failed to induce a HR. However, *min14(leuA<sup>-</sup>)* and *min21(leuB<sup>-</sup>)* mutants, although were significantly reduced in pathogenicity on the host bean plants, elicited normal HR as did the WT strain on tobacco W38 plants.

**PSPPH\_3244<sub>NPS3121</sub> is functionally similar to AefR in regulating the *ahlI* gene promoter and its own promoter (done by Dr. Lan).**

PSPPH\_3244<sub>NPS3121</sub> is 87% identical to AefR that is known to regulate the synthesis of quorum sensing signal AHL in *Psy* B728a (Quinones et al. 2004). AefR positively regulates the *ahlI* gene encoding AHL synthase and auto-inhibits its own promoter (Quinones et al. 2004). To determine if PSPPH\_3244<sub>NPS3121</sub> is functionally similar to AefR of *Psy* B728a, Dr. Lan generated a deletion mutant of *PSPPH\_3244<sub>NPS3121</sub>* by marker exchange and examined how PSPPH\_3244<sub>NPS3121</sub> regulates the *ahlI* gene promoter and its own promoter. As observed in *Psy* B728a, *ahlI-luc* exhibited ~20% expression in the  $\Delta$ *PSPPH\_3244<sub>NPS3121</sub>* mutant relative to the WT strain (Fig. II-3A), and overexpression of *PSPPH\_3244<sub>NPS3121</sub>* using the pNm promoter in pML122 plasmid (Labes et al. 1990) in the deletion mutant severely inhibits the *PSPPH\_3244<sub>NPS3121</sub>* promoter, as indicated by the low LUC activity (Fig. II-3B).

These results suggested that PSPPH\_3244<sub>NPS3121</sub> is functionally similar to AefR of *Psy* B728a in regulating the AHL signal and is hereafter named AefR<sub>NPS3121</sub>.

### **Regulation of the type III genes by AefR<sub>NPS3121</sub>.**

Dr. Lan and I used the  $\Delta aefR_{NPS3121}$  mutant to investigate if AefR<sub>NPS3121</sub> regulates the type III genes. Consistent with the reduced *avrPto-luc* and *hrpL-luc* activities in insertion mutants, *hrpL* RNA was reduced to 30% of the WT level in the  $\Delta aefR_{NPS3121}$  mutant, and this was largely complemented by expressing the WT *aefR\_{NPS3121}* gene (done by Dr. Lan, Fig. II-4A). The *hrpR* promoter activity (as indicated by the *hrpR-luc* reporter) in the  $\Delta aefR_{NPS3121}$  mutant was 20-25% of that in the WT strain (Fig. II-4B). Several regulatory genes acting upstream of *hrpRS*, including *lonB*, *rpoS*, *gacA*, and *psrA*, did not show an altered expression pattern in the  $\Delta aefR_{NPS3121}$  mutant (done by Dr. Lan, Fig. II-4A). The induction of *avrPto-luc* and *hrpL-luc* in the  $\Delta aefR_{NPS3121}$  mutant was also monitored in the bean plants. Both *avrPto-luc* and *hrpL-luc* displayed lower activities in the  $\Delta aefR_{NPS3121}$  mutant than in the WT strain 4 hr after the bacteria were infiltrated into the bean plants (Fig. II-4, C and D). Consistently, the  $\Delta aefR_{NPS3121}$  mutant elicited weaker disease symptoms than did the WT strain after infiltration into the bean plants (Fig. II-8). Both insertion and deletion mutants of *aefR\_{NPS3121}* showed ~3 fold reduction in bacterial growth compared with the WT strain 4 days after infiltration inoculation (Fig. II-4E); this difference was observed consistently in four repeat experiments and was statistically significant according to F test ( $P < 0.05$ ). When infiltrated into the non-host tobacco W38 plants, the *aefR\_{NPS3121}*<sup>-</sup> mutants did not show a visible difference from the WT strain in the induction of HR (data not shown). I also investigated if the *aefR\_{NPS3121}* RNA expression is altered in mutants corresponding to the *hrpR*, *hrpS*, *rhpS*, *rhpRS*, and *lon* genes that are known to regulate the type III genes (Xiao et al. 2007; Lan et



al. 2007). The expression of *aefR*<sub>NPS3121</sub> RNA was not altered by any of these mutations in KB medium, but was moderately elevated in MM when the *rhpRS* locus was deleted (Fig. II-4F).

### **Regulation of swarming motility and epiphytic traits by AefR<sub>NPS3121</sub>.**

AefR regulates epiphytic behaviors in *Psy* B728a. Mutation of *aefR* enhances swarming motility on semisolid agar and invasion into leaves but reduces bacterial survival on the leaf surface (Quinones et al. 2004; 2005). These traits were examined in the  $\Delta aefR$ <sub>NPS3121</sub> mutant.

Unlike *Psy* B728a and *Pst* DC3000 that displayed clear swarming motility on semisolid agar plate, *Psph* NPS3121 strain did not show swarming motility (Fig. II-5A). Mutation of *aefR*<sub>NPS3121</sub> did not enhance swarming motility on semisolid agar plate (Fig. II-5A).

To determine if AefR<sub>NPS3121</sub> regulates bacterial invasion into leaves, two-week old bean plants were dip-inoculated with a bacterial suspension (10<sup>6</sup> CFU/mL), each plant was covered with a plastic bag, and the numbers of bacteria on the leaf surface and inside the leaves were measured. Unlike mutation of *aefR* in *Psy* B728a that increased the bacterial invasion into leaves by ~1000 folds 4-7 hr after inoculation (Quinones et al. 2005), mutation of *aefR*<sub>NPS3121</sub> in *Psph* NPS3121 did not alter the number of bacteria inside the leaves within 28 hr after inoculation (Fig. II-5B).

*Psy* B728a and *aefR*<sup>-</sup> mutant grew ~50 folds epiphytically after the surface-inoculated plants were incubated in moist conditions for 48 hr (Quinones et al. 2004). After transferred into a dry environment, epiphytic bacteria of both strains decreased ~20 folds in 2 hr, and the WT strain stayed stable, but the *aefR*<sup>-</sup> mutant decreased another ~5-7 folds in 50 hr (Quinones et al. 2004). Unlike *Psy* B728a and the *aefR*<sup>-</sup> mutant, *Psph* NPS3121 and the  $\Delta aefR$ <sub>NPS3121</sub>

mutant did not exhibit significant epiphytic growth after incubation inside the plastic bags for 48 h (Fig. II-5C). After the plants were placed in the greenhouse, both strains displayed ~2 fold reduction of the epiphytic population in the first 6 hr, and then the epiphytic populations of *Psph* NPS3121 and  $\Delta aefR_{NPS3121}$  mutant increased 4 and 7 folds, respectively, in 48 hr (Fig. II-5C). These results indicated that  $AefR_{NPS3121}$  in *Psph* NPS3121 and *AefR* in *Psy* B728a function differently under the dry environments.

In contrast with what was observed in infiltration inoculation (Fig. II-4E), the  $\Delta aefR_{NPS3121}$  mutant showed ~2-4 fold higher bacterial numbers inside the leaves than did the WT strain 48 hr after dip-inoculation, and the fold-difference remained till the end of experiment (Fig. II-5C). The better growth of  $\Delta aefR_{NPS3121}$  mutant on the bean leaf surface and inside the bean plants was observed consistently in four repeat experiments, including one experiment with an inoculum of  $10^8$  CFU/mL. Although the inoculated leaves did not show water soaked lesions, they did senesce earlier than the uninoculated leaves. Leaves dip-inoculated with the  $\Delta aefR_{NPS3121}$  mutant usually senesced 1-2 days earlier than leaves dip-inoculated with the WT strain (data not shown).

## DISCUSSION

We screened a total of ~28,000 transposon-insertion mutants of *Psph* NPS3121 based on the compromised induction of *avrPto-luc* and *hrpL-luc* reporter genes in MM, and we isolated 46 mutant genes. The screening was ~5x coverage of the ~6 Mb *Psph* genome (Joardar et al. 2005), assuming that average bacterial genes are 1 kb. Some of the mutants may be polar, because the mutant gene is organized in an operon with other genes. Most of the 46 mutant genes encode metabolic enzymes, only three genes encode regulatory functions. Characterization of the reporter gene activities and *hrpL* RNA expression in

various mutant strains indicated that the reporter gene activities generally reflected the type III gene expression, indicating that the reporting systems are valid.

Many genes that were reported to regulate the TTSS genes in other *P. syringae* strains were not identified in our screen. One explanation is that some of these regulatory genes do not have a regulatory function in *Psph* NPS3121. Another explanation is that the regulatory genes small in size were missed by the transposon insertion. But it is more likely that the mutant screening protocol has intrinsic defects. We used the protocol for analysis of type III gene induction in MM for mutant screening, e.g., an individual mutant colony was picked from a KB plate and grown to saturation in KB liquid medium in 96-well plates, the culture was then washed twice with MM, and the reporter genes were subsequently induced with MM. Because it was impossible to pick equal quantities of bacterial cells from each mutant colony, and the growth rates of the mutants differed in KB medium, prolonged growth in KB medium was allowed to achieve saturation before induction in MM in order to minimize the variations in bacterial numbers. However, we later found that an extended stationary phase in KB medium reduced the induction of *avrPto-luc* and *hrpL-luc* reporter genes in MM (Deng and Tang, unpublished result). This practice, albeit enabling a uniform number of cells to be compared, narrowed the difference between strains showing wild type levels of induction of the reporter genes and mutants showing partially reduced induction of the reporter genes, rendering the identification of the latter difficult. Washing to remove KB medium from the culture before induction of the reporter genes is another step that may affect identification of partial mutants. We noticed that contamination of MM with a small amount of KB medium significantly reduced the induction of the reporter genes in *Psph* NPS3121. However, it was technically difficult to completely remove KB medium from all the microtiter wells, and the residual amount of KB medium in the microtiter wells may inhibit the induction of the

reporter genes. We also noticed bacterial loss when supernatant was discarded from the plate after centrifugation. Because both residual KB medium and bacterial loss caused variations among strains in the plate, we set criteria to select mutants with a LUC activity 20% or less of the majority of other strains in the same plate. It is apparent that these intrinsic problems of the screening protocol discriminated against the selection of mutants with partial induction of the reporter genes. As a result, most of the mutants isolated for further analysis had extremely low reporter activities. In fact, we even missed a *hrpR* mutant known to be in the mutant library; this mutant was later isolated in a screen based on the *avrPto-luc* induction in Arabidopsis plants (Xiao et al., 2007). Therefore, the mutant screening procedures need to be improved for isolation of mutants with partially reduced induction of the type III genes.

Mutant screening experiments conducted by us and by Hendrickson et al (2000) indicated that as much as 4-5% of *P. syringae* mutants showed decreased induction of the type III genes in MM. To identify the regulatory genes from the large pool of candidate mutants was a major obstacle. We deployed the two-stage semi-degenerated PCR method (Jacobs et al. 2003) to determine the transposon insertion sites in 84 mutants that were identified by *avrPto-luc* reporter. Most of these mutant genes encode metabolic enzymes. Although these genes provide little insight into the regulatory mechanisms, they do reveal the nature of *P. syringae* mutations that compromise the induction of the type III genes in MM. These results led us to test if bacterial growth assays in MM can differentiate metabolic mutants from regulatory mutants. We found that all metabolic mutants showed no or poor growth, whereas all regulatory mutants grew normally in MM. We therefore added this procedure to the mutant screening with the *hrpL-luc* reporter. This procedure eliminated all metabolic mutants from the pool of candidate mutants and significantly enhanced the

efficiency of isolation of regulatory genes. However, regulatory genes which are also required for bacterial growth in MM would be excluded by this procedure.

Pathogenicity assays indicated that the level of type III gene expression in many metabolic mutants in MM was not correlated with their pathogenicity in host plants. In fact, a large number of metabolic mutants were almost as pathogenic as the WT strain, although they displayed no induction of the type III genes in MM. The pathogenicity of most metabolic mutants was positively correlated with the abundance of the corresponding metabolites in the apoplastic fluid (Ritte et al. 1999; Solomon and Oliver 2002; Tanaka and Tanaka 2007; Rico and Preston 2008), indicating that nutrient auxotroph plays a critical role in the type III gene induction in the plant and bacterial pathogenesis. The pathogenicity was well correlated with the HR-inducing activity in most metabolic mutants; this is consistent with the fact that apoplastic fluids from different plant species are similar in nutrient compositions (Solomon and Oliver 2002; Rico and Preston 2008).

The only novel regulatory gene identified in our mutant screening was *aeFR*<sub>NPS3121</sub>. AefR in *Psy* B728a regulates the AHL signal by up-regulating the *ahlI* promoter, and it auto-inhibits its own promoter (Quinones et al. 2004). Both functions were observed with AefR<sub>NPS3121</sub>, suggesting that AefR<sub>NPS3121</sub> is orthologous to AefR in *Psy* B728a. The role of AefR in regulating the type III genes was not reported, although the *aeFR*<sup>-</sup> mutant was found to cause smaller disease lesions than did *Psy* B728a when injected into bean pods (Quinones et al. 2005). Here we found that the *aeFR*<sub>NPS3121</sub><sup>-</sup> mutants displayed reduced induction of the type III genes in MM and in the plant. The *aeFR*<sub>NPS3121</sub><sup>-</sup> mutants also exhibited reduced pathogenicity after injection into bean leaves. Because induction of the type III genes is essential for bacterial pathogenesis, the reduced type III gene induction may be accountable

for the reduced pathogenicity. The finding that AefR<sub>NPS3121</sub> regulates both the quorum sensing signal and type III gene expression is exciting and suggests that *Psph* NPS3121 may regulate the bacterial virulence in response to cell density. Quorum sensing signals are known to regulate the production of exoenzymes and exopolysaccharides, two major virulence factors, in numerous plant pathogenic bacteria (Von Bodman et al. 2003); but its role in regulating the type III genes has not been well documented in *P. syringae*. Our studies showed that AefR<sub>NPS3121</sub> regulates the type III genes possibly by modulating the *hrpR* promoter activity. It remains to be determined if AefR<sub>NPS3121</sub> regulates the *hrpR* promoter directly by binding to the promoter or indirectly via the AHL-mediated signaling pathway.

Although AefR of *Psy* B728a and AefR<sub>NPS3121</sub> of *Psph* NPS3121 have similar functions in regulating AHL synthesis, the respective mutants exhibit significant difference in several epiphytic traits that are proposed to be regulated by AHL in *Psy* B728a (Quinones et al. 2004; 2005). Compared with *Psy* B728a, the *aefR*<sup>-</sup> mutant is hypermotile on semisolid agar and invades leaves more rapidly (Quinones et al. 2005). The hypermotility on semisolid agar was assumed to be related to the more rapid invasion of leaves (Quinones et al. 2005). Unlike the WT *Psy* B728a and *Pst* DC3000 strains, the WT *Psph* NPS3121 strain did not show visible swarming motility on semisolid agar, and mutation of *aefR*<sub>NPS3121</sub> did not enhance the swarming motility. Nonetheless, both *Psph* NPS3121 and the *aefR*<sub>NPS3121</sub><sup>-</sup> mutant were capable of invading bean leaves following dipping-inoculation. However, the two strains displayed similar rates of invasion, as indicated by the similar numbers of bacteria inside the leaves 4-28 hrs after dipping-inoculation (Fig. II-5, B and C). This result indicated that AefR<sub>NPS3121</sub> did not play a major role in regulating *Psph* NPS3121 invasion into the bean leaves.

*Psy* B728a and *aefR*<sup>-</sup> mutant grew ~50 folds on bean leaf surfaces within 2 days under the moist conditions, and a short exposure to a dry environment reduced the epiphytic population by 20-50 folds. Extended incubation in the dry conditions further reduced the epiphytic population, and mutation of *aefR* rendered the bacterial cells more susceptible to the stress (Quinones et al. 2004). Different from *Psy* B728a and the *aefR*<sup>-</sup> mutant, *Psph* NPS3121 and the *aefR*<sub>NPS3121</sub><sup>-</sup> mutant did not grow significantly on bean leaf surface under the moist conditions, and a short exposure to the dry conditions reduced the epiphytic population only by ~2 folds. Interestingly, both strains grew slowly on leaf surface after the small reduction of the epiphytic population in the dry environment (Fig. II-C). These results suggested that AefR<sub>NPS3121</sub>, like AefR, does not affect bacterial growth under the moist environments. However, under the dry conditions, AefR positively regulates the epiphytic survival of *Psy* B728a, while AefR<sub>NPS3121</sub> negatively regulates the epiphytic growth of *Psph* NPS3121. The remarkable difference of AefR and AefR<sub>NPS3121</sub> in modulating the epiphytic behaviors of their respective bacteria also suggested that the epiphytic associations of *Psph* NPS3121 and *Psy* B728a with their host plants involve different mechanisms.

Although the *aefR*<sub>NPS3121</sub><sup>-</sup> mutant is less pathogenic than the WT strain after infiltration into bean plants, it is more pathogenic than the WT strain following dipping inoculation, as indicated by the larger *aefR*<sub>NPS3121</sub><sup>-</sup> mutant population inside the leaves at the end of experiment (Fig. II-5C) and the earlier senescence of the inoculated leaves. Similar results were also reported for the *aefR*<sup>-</sup> mutant that elicited more disease lesions on bean leaves if the spay-inoculated plants were incubated in moist conditions for more than 48 hr before placed in a dry environment (Quinones et al. 2005). It was proposed that more *aefR*<sup>-</sup> mutant cells entered the leaves at the end of moist incubation, which was responsible for the more severe symptoms (Quinones et al. 2005). Indeed, I detected a larger internal population of the

*aefR<sub>NPS3121</sub>*<sup>-</sup> mutant 48 hr after incubation in the moist conditions (Fig. II-5C). Two possible routes may lead to the larger internal population of the *aefR<sub>NPS3121</sub>*<sup>-</sup> mutant. 1. Although the *aefR<sub>NPS3121</sub>*<sup>-</sup> mutant was not found to enter the leaves more rapidly during the first 24 hr of incubation, the mutant cells might enter the leaves more rapidly during the later hours under the moist conditions. 2. Although the internal *PspH* NPS3121 grew better than did the *aefR<sub>NPS3121</sub>*<sup>-</sup> mutant in the greenhouse conditions, the *aefR<sub>NPS3121</sub>*<sup>-</sup> mutant might grow better in the wet conditions. In addition to the bacteria internalized under the wet conditions, the epiphytic bacteria could also contribute to the disease development because these cells could enter the leaves during the period in the greenhouse. In this regard, I noticed that the *aefR<sub>NPS3121</sub>*<sup>-</sup> mutant produced a larger epiphytic population in the greenhouse (Fig. II-5C), which would allow more bacterial cells to enter the leaves during the prolonged interaction with the plants. Other than these scenarios, it should be pointed out that infiltration-inoculation and surface-inoculation involve different biological processes, and that *AefR<sub>NPS3121</sub>* may have a different role in regulating the bacterial pathogenicity in these different processes.

## **MATERIALS AND METHODS**

### **Plant materials and culture media.**

Bean (*Phaseolus vulgaris* cv. Red Kidney) plants and tobacco W38 plants were used for pathogenicity and HR assays, respectively. Plant materials were grown in a greenhouse as described previously (Xiao et al. 2007). *E. coli* strains were cultured in LB at 37°C. *P. syringae* bacteria were cultured in KB medium (King et al. 1954) at room temperature. Induction of TTSS was performed at room temperature in MM (50 mM KH<sub>2</sub>PO<sub>4</sub>, 7.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.7 mM MgCl<sub>2</sub>, 1.7 mM NaCl, 10 mM fructose, pH 5.7; Huynh et al. 1989).



Bacteria were plated on TSA plates (Xiao et al., 2007) for counting of colony forming units (CFUs). Antibiotics (in mg/L) for selection of *P. syringae* strains are: rifampicin, 25; kanamycin, 10; spectinomycin, 50; tetracycline, 10; and gentamycin, 10. Antibiotics (in mg/L) for selection of *E. coli* are: ampicillin, 100; kanamycin, 50; spectinomycin, 100; and gentamycin, 20.

### **Plasmids.**

Plasmids and primers are listed in tables II-3 and II-4, respectively. To construct the reporter gene for the *aeFR<sub>NSP3121</sub>* promoter, the *luc* gene was released from pHM2::*avrPto-luc* by *Bam*HI and *Xba*I digestion and ligated into pBluescript-SK(+), resulting pBluescript-SK-*luc*. The promoter of *aeFR<sub>NSP3121</sub>* was PCR amplified using pspph3244-P-F (carrying *Eco*RI) and pspph3244-P-R (carrying *Bam*HI) as primers and *PspH* NPS3121 genomic DNA as template. The PCR product was digested with *Eco*RI and *Bam*HI and cloned into pBluescript-SK-*luc*, resulting in pBS-*aeFR<sub>NSP3121</sub>-luc*. After sequencing, the *Eco*RI and *Xba*I fragment in pBS-*aeFR<sub>NSP3121</sub>-luc* was released and then cloned into pHM2, resulting in pHM2::*aeFR<sub>NSP3121</sub>-luc* for the *aeFR<sub>NSP3121</sub>* promoter assay. The same strategy was used to generate the reporter gene for the *ahII* (PSPPH\_1614) promoter. The *ahII* promoter was PCR-amplified using pspph1614-P-F and pspph1614-P-R as primers.

To construct the *hrpR-luc* reporter gene, a 1kb fragment of the *hrpR* promoter was amplified using primers pspH-hrpR-PF and pspH-hrpR-PR. The PCR product was digested with *Eco*RI and *Bam*HI, cloned into pBluescript-SK(+) plasmid, and sequence confirmed. The promoter DNA was then released from the plasmid with *Eco*RI/*Bam*HI digestion and cloned upstream of the *luc* gene in the pPTE6::*luc* plasmid (Xiao et al. 2004) to generate

pPTE6::*hrpR-luc*. To generate pLT::*hrpR-luc*, the *kanR* gene in pPTE6::*hrpR-luc* plasmid was knocked out by EZ-Tn5<TET-1> transposon insertion.

To construct pML122::*aefR<sub>NSP3121</sub>*, the coding region of *aefR<sub>NSP3121</sub>* was PCR-amplified using primers pspph3244-O-F (carrying *XhoI*) and pspph3244-O-R (carrying *ClaI*). The PCR fragment was cloned into pGEM-T and confirmed by sequencing. The fragment was then released by *XhoI* and *ClaI* digestion and cloned into pML122, resulting in pML122::*aefR<sub>NSP3121</sub>* for *aefR<sub>NSP3121</sub><sup>-</sup>* mutant complementation and *aefR<sub>NSP3121</sub>* overexpression.

### **Mutant screen.**

The transposon insertion mutant library was constructed in *PspH* NPS3121 strain carrying the pHM2::*avrPto-luc* and pHM2::*hrpL-luc* reporter plasmids as described previously (Xiao et al. 2007). Mutant colonies grown on KB plates containing rifampicin, kanamycin, and spectinomycin were picked with sterile toothpicks into 100  $\mu$ L of liquid KB medium containing the same antibiotics in 96-well plates and cultured for 36 hr till complete saturation. The 96-well plates were centrifuged, and the bacteria were washed twice with MM and resuspended in 500  $\mu$ L MM. After induction in MM for 6 hr, 100  $\mu$ L of cell suspension was transferred from each sample to a new 96-well plate and mixed with 10  $\mu$ L 0.1mM luciferin. LUC activity was measured using a cooled charge-couple device (CCD, Roper Scientific, Trenton, NJ). Mutants with ~20% LUC activity relative to the other clones in the same plate were selected as putative mutants. These mutants were confirmed for the induction of reporter genes in MM as described previously (Xiao et al. 2007).

### **Measurement of reporter gene activities in MM and in the plant.**

The WT *PspH* NPS3121 and mutant colonies were grown in liquid KB medium containing rifampicin and spectinomycin to  $OD_{600}=2.0-2.5$ . To induce the reporter genes in MM, bacteria were washed twice with MM, resuspended in MM to  $OD_{600}=0.1$ , and incubated for 4 hr for the induction of *hrpL-luc* and 6 hr for the induction of *avrPto-luc*. 100  $\mu$ L of cell suspension was mixed with 10  $\mu$ L of 0.1mM luciferin, and the LUC activity was measured using a cooled CCD (Roper Scientific, Trenton, NJ). After LUC measurement, the bacteria were diluted and plated on TSA plates for counting of colony forming units. The relative LUC activity was normalized to the numbers of bacteria in MM. To induce the reporter genes in the plant, bacteria were washed twice with sterile water, resuspended in sterile water to  $OD_{600}=0.5$ , and infiltrated into the primary leaves of 2-week-old bean plants. The inoculated leaves were excised 4 and 6 hr, respectively, after inoculation for measurement of *hrpL-luc* and *avrPto-luc* reporter activities. The excised leaves were sprayed with 1 mM luciferin dissolved in 0.01% Tween-20, and the LUC activity was determined using a cooled CCD (Roper Scientific, Trenton, NJ). After LUC measurement, the numbers of bacteria inside the inoculated leaves was measured as described previously (Xiao et al. 2007), and the relative LUC activity was normalized to the bacterial number inside the leaves. Each data point represents an average of 3–4 replicates. Each experiment was repeated 3-5 times with similar results.

### **Mapping of transposon insertion sites.**

The transposon insertion sites were determined by a two stage semi-degenerated PCR according to Jacobs and associates (2003) using two transposon-specific primers (Kan2-SP1 and Kan2-SP2) and four degenerated primers (CEKG 2A, CEEKG 2B, CEEKG 2C, and

CEKG). The PCR product was sequenced using the third transposon-specific primer Kan2-SP3. Sequences flanking the transposon DNA were searched against the *Psph* 1448A genome sequence using Blastn. The mutant genes of *Psph* NSP3121 were distinguished from the *Psph* 1448A genes by adding a subscript “*NSP3121*”.

#### **Assay of bacterial growth in MM.**

Mutant colonies were grown in KB medium to  $OD_{600}=2.5$ . Bacterial cells were collected by centrifugation, washed twice with MM, and resuspended in MM. The bacteria were diluted in MM to  $OD_{600}=0.1$  and grown at room temperature for 36 hr with constant shaking at 250 rpm. The bacterial density was determined using a spectrophotometer.

#### **Infiltration inoculation and HR assay.**

Preparation of bacterial inoculum for plant inoculation was as described previously (Shan et al. 2000). Bacteria at  $2 \times 10^4$  CFU/mL were hand-injected into the primary leaves of two week-old bean plants for pathogenicity assays. Disease symptoms on bean leaves were documented 5 days after inoculation. For bacterial growth assays, leaf discs ( $1 \text{ cm}^2$ ) were removed at 0, 4 or 6 days after inoculation and ground in sterile water. Bacteria were diluted to proper concentration and plated on TSA plate containing 30 mg/L rifampicin (Xiao et al., 2007) for counting of CFUs. For HR assay, bacteria at  $10^8$  CFU/mL were injected into the fully expanded tobacco W38 leaves. Eight hr after injection, death of the inoculated area was visually examined hourly.

### **Motility Assay.**

Swarming motility was assessed on semisolid KB plates containing 0.4% agar, as described by Quinones et al (2005). Cells were grown in KB liquid overnight and resuspended in KB to  $OD_{600}=1$ . Filter discs (6 mm in diameter) were soaked in bacterial suspensions and placed in the center of the plate. Plates were then incubated at 28°C for 24 hr before photography.

The procedures described by Quinones et al (2005) were used to determine the ability of bacteria to enter the interior of leaves. Two-week-old bean plants were dipped into a bacterial suspension of  $1 \times 10^6$  CFU/mL in 10 mM potassium phosphate buffer pH 7.0 (PB) plus 50  $\mu$ L/L silwet for 1 min. The plants were covered with plastic bags immediately after dipping inoculation to maintain the humidity. Primary leaves were excised at various times and surface sterilized with 15% hydrogen peroxide for 10 min, followed by a treatment with catalase at 7.5  $\mu$ g/mL for 10 min. Leaves were then macerated in sterile PB, and the released bacteria were plated on a TSA plate containing 30 mg/L rifampicin for counting of CFUs. The number of bacteria was normalized according to the surface area of leaves. Each experiment was repeated at least 3 times with similar results.

### **Epiphytic fitness assay.**

Two-week-old bean plants were dipped into a bacterial suspension ( $1 \times 10^6$  CFU/mL in PB plus 50  $\mu$ L/L silwet) for 1 min. Plants were covered with plastic bags immediately after dipping inoculation, removed from the plastic bag 48 hr after inoculation, and placed in the greenhouse. Primary leaves were excised at various times after inoculation and immersed individually into 50 mL washing buffer (100 mM potassium phosphate buffer containing

0.1% Bacto-peptone) in a plastic tube. The plastic tubes were sonicated in an ultrasonic bath for 7 min and vortexed briefly to wash off the surface bacteria. Bacteria in washing solution were plated on a TSA plate containing 30 mg/L rifampicin for bacterial count. To measure the bacteria inside the leaves, the leaves were further treated with 15% hydrogen peroxide for 10 min, followed by a treatment with catalase at 7.5 µg/mL for 10 min. Leaves were then macerated in sterile PB, and the released bacteria were plated on a TSA plate containing 30 mg/L rifampicin for bacterial count. The numbers of bacteria were normalized according to the surface area of leaves. Each experiment was repeated at least 3 times with similar results.

#### **RNA extraction and Northern blotting.**

Bacterial RNA was extracted using a modified hot phenol method (Aiba et al., 1981; Lan et al., 2006). DNase I (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 *hrpL*, *rpoS*, *psrA*, *lonB*, *gacA*, and *aefR<sub>NPS3121</sub>* coding regions were PCR amplified using primers listed in Table II-4 and radio-labeled with <sup>32</sup>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.

#### **Construction of $\Delta aefR_{NPS3121}$ mutant.**

A 1.8-kb DNA fragment upstream of *aefR<sub>NPS3121</sub>* was PCR-amplified using primers P3244LF and P3244LR (*Xba*I and *Bam*HI sites are underlined). A 1.8-kb DNA fragment downstream of *aefR<sub>NPS3121</sub>* was PCR-amplified using primers P3244RF and P3244RR (*Bam*HI and *Sac*I sites are underlined). The PCR products were digested with *Xba*I and *Bam*HI and *Bam*HI and *Sac*I, respectively, and cloned into the *Xba*I and *Sac*I sites of pGEM-

7Z, resulting in p7Z-3244FR. A DNA fragment containing the kanamycin resistance gene was PCR-amplified from EZ::Tn< KAN-2> (Epicentre, Wisconsin, MD), using primers Kan-BF and Kan-BR (*Bam*HI sites underlined), digested with *Bam*HI, and cloned into the *Bam*HI site of p7Z-3244FR, resulting in p7Z-3244FkanR. The *Xba*I and *Sac*I fragment in p7Z-3244FkanR was cloned into pHM1, and the resulting pHM1::3244FkanR plasmid was introduced into *P. s. pv. phaseolicola* NPS3121 strain for marker exchange. Colonies sensitive to spectinomycin but resistant to kanamycin were further verified by PCR and Southern blotting using DNA probes derived from the *ae*fR<sub>NPS3121</sub> coding region.

### **Western blot analysis.**

Bacteria grown in KB and MM media were adjusted with corresponding medium to OD<sub>600</sub>=1. Thirty microliter of bacteria was boiled in 1x SDS sample buffer and loaded to a SDS PAGE gel. Western blot was performed as described (Shan et al. 2000) with the anti-LUC antibodies (NeoMarkers, Fremont, CA).

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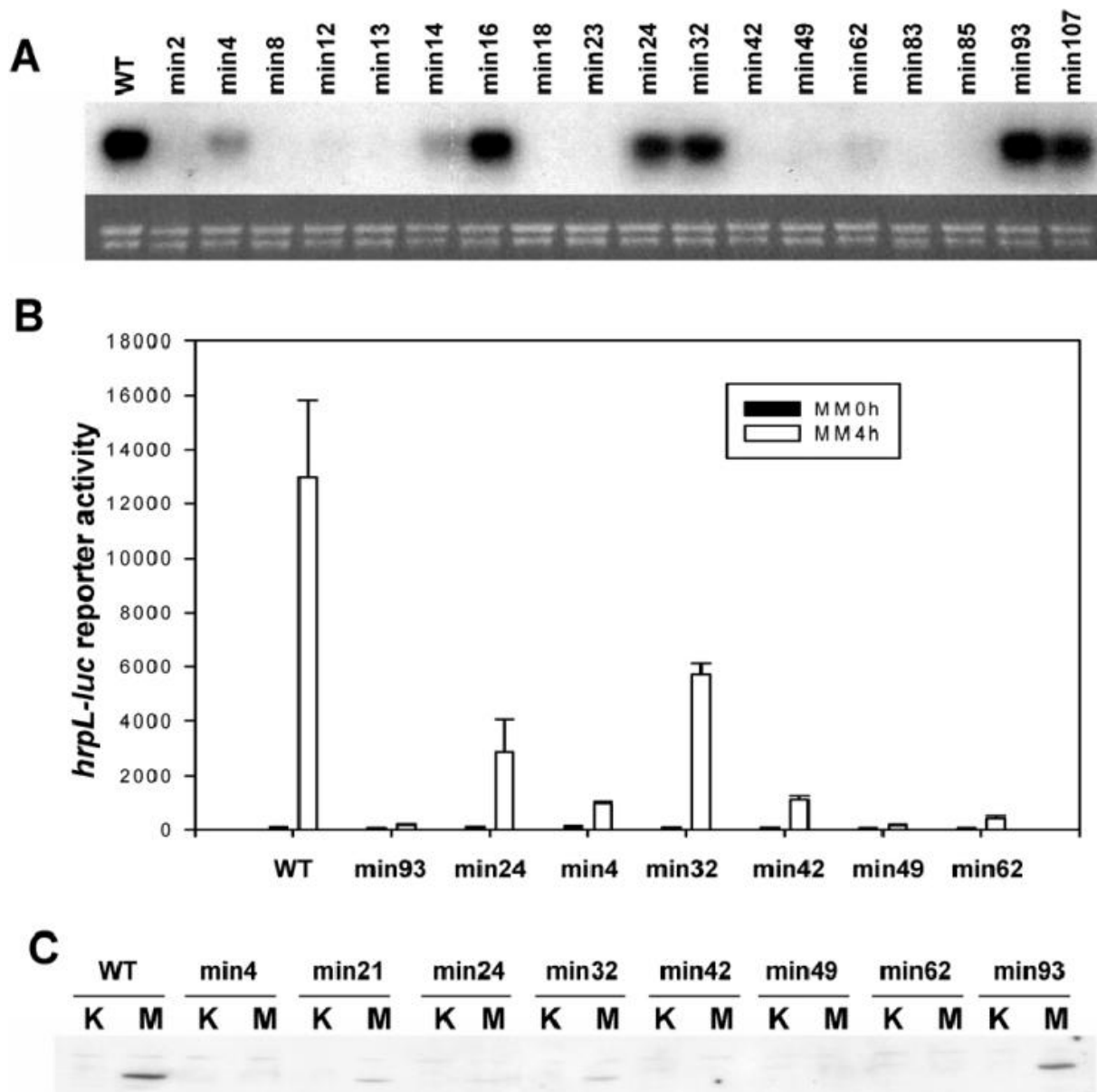
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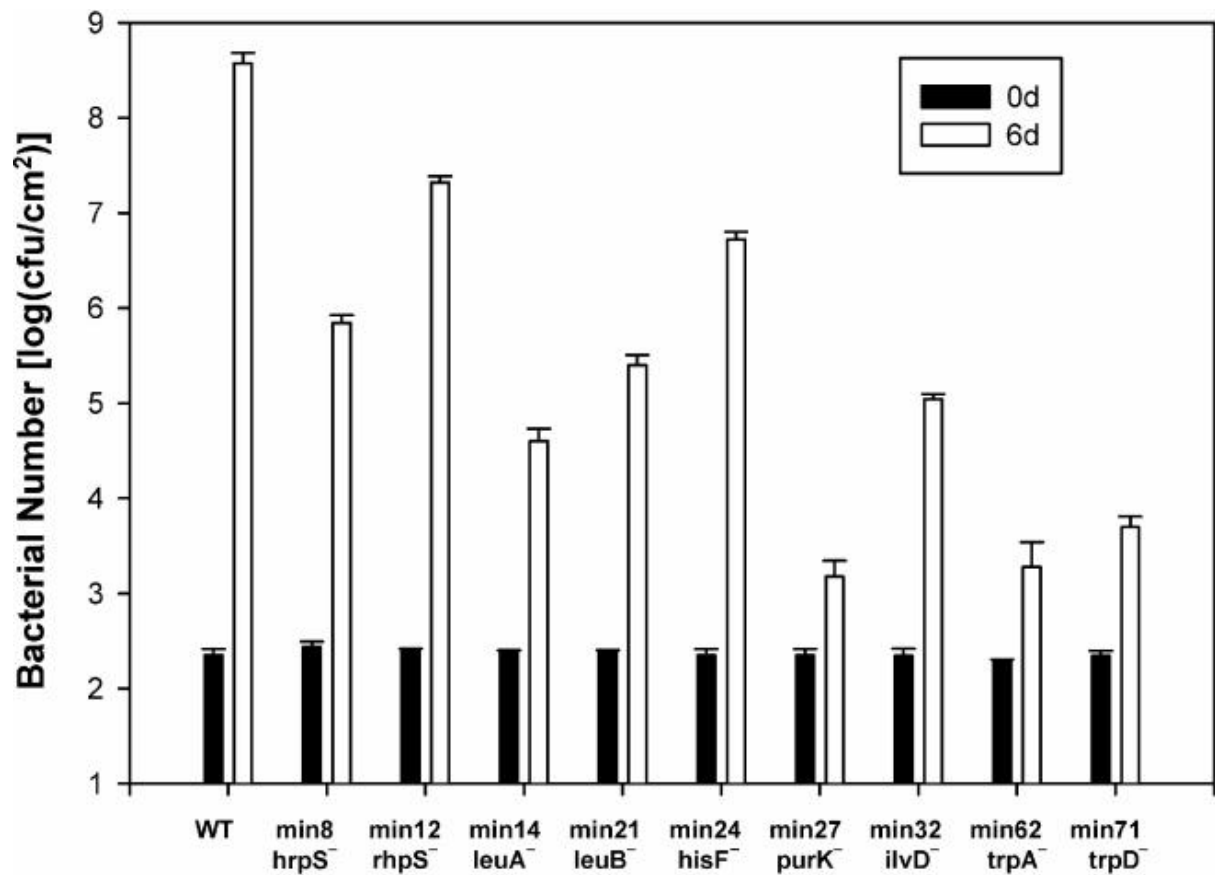


**Fig. II- 1 Assessment of *hrpL* RNA, *hrpL-luc* reporter activity, and HrpL protein abundance in representative *min* mutants.**

**A**, *hrpL* RNA. The WT *Psph* NPS3121 strain and *min* mutants were grown in KB medium and induced in MM for 4 hr. Ten microgram of total RNA from each sample was electrophoresed in a denaturing agarose gel. The blot was hybridized with DNA probes

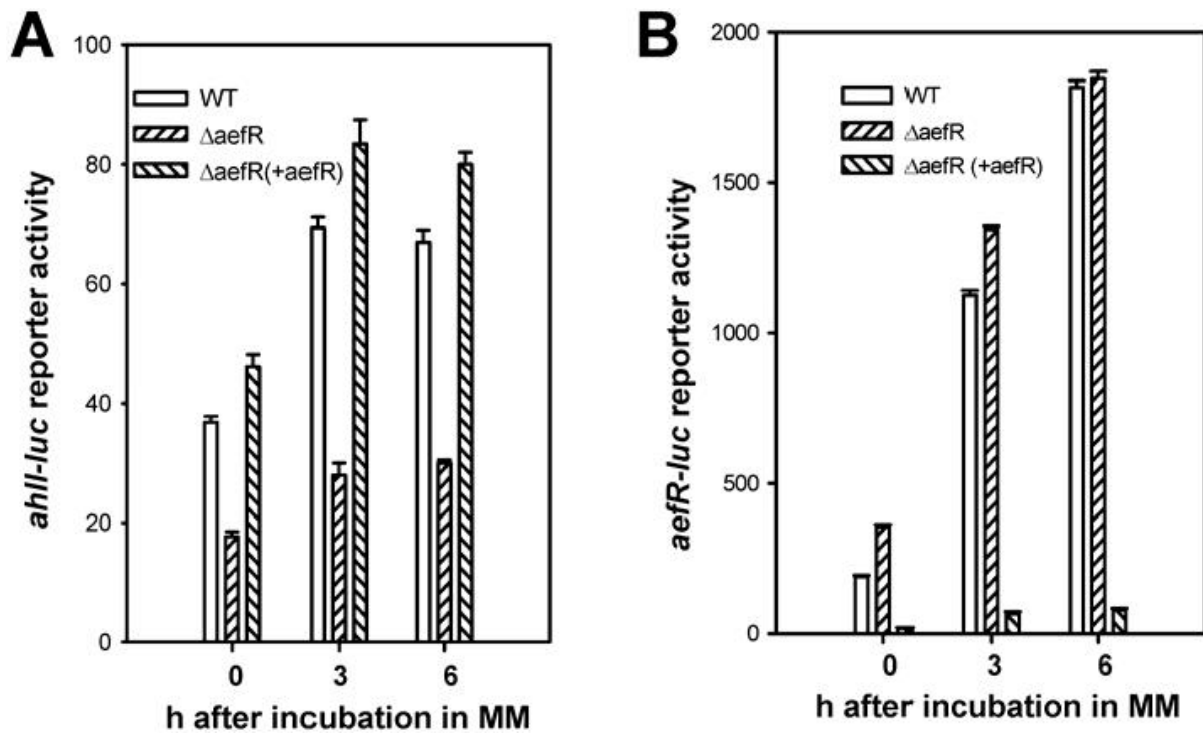
derived from the *hrpL* coding region. Loading of RNA samples is indicated by rRNA. **B**, LUC activity derived from *hrpL-luc* reporter. Bacteria were grown in KB medium and induced in MM for 4 hr. 100  $\mu$ l of bacterial culture was mixed with 10  $\mu$ l of 0.1 mM luciferin. LUC activity was measured with a cooled CCD. Each data point represents three replicates. Error bars indicate standard error. **C**, LUC protein derived from *hrpL-luc* reporter. The WT and *min* mutant strains carrying *hrpL-luc* reporter were grown in KB and induced in MM for 4 hr. Bacteria were harvested by centrifugation and resuspended in MM to OD<sub>600</sub>=1. Thirty  $\mu$ l of bacteria was boiled in 1x SDS sample buffer and loaded to a SDS PAGE gel. Western blot was performed using anti-LUC antibodies.





**Fig. II- 2 *In planta* growth of *min* mutants that did not elicit disease symptoms.**

The WT strain and *min* mutants that did not elicit visible disease symptoms were inoculated into primary bean leaves at  $2 \times 10^4$  CFU/mL. For each data point, three leaf discs (1 cm<sup>2</sup>) were removed at 0 and 6 days after inoculation and ground separately in sterile water. Bacteria were plated on TSA plates for bacterial counting. Error bars represent standard error. The experiment was repeated two times with similar results.



**Fig. II- 3 Regulation of *ahII* and *aefR* promoters by  $AefR_{NPS3121}$ (done by Dr. Lan).**

The *ahII-luc* and *aefR-luc* reporter genes were introduced into the WT *Psph* NSP3121,  $\Delta aefR_{NPS3121}$  mutant, and  $\Delta aefR_{NPS3121}$  mutant carrying the pML122::*aefR\_{NPS3121}* plasmid (for *aefR\_{NPS3121}* overexpression). The bacteria were grown in KB medium and induced in MM for 0, 3, and 6 hr before measurement of LUC activities. **A**, mutation of *aefR\_{NPS3121}* reduces *ahII* promoter activity in KB (indicated by 0 hr) and MM. **B**, Overexpression of *aefR\_{NPS3121}* inhibits its own promoter. Error bars indicate standard error.

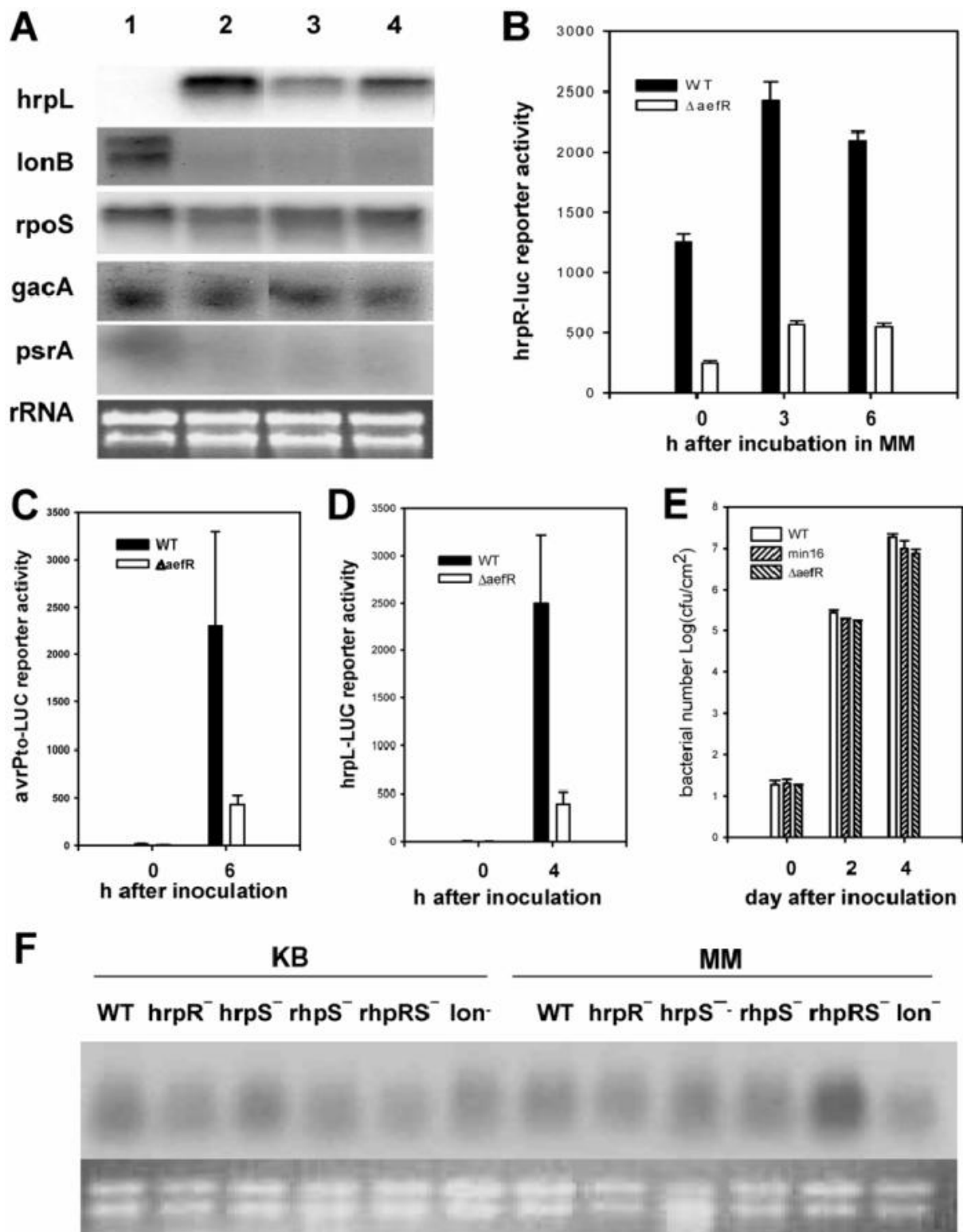
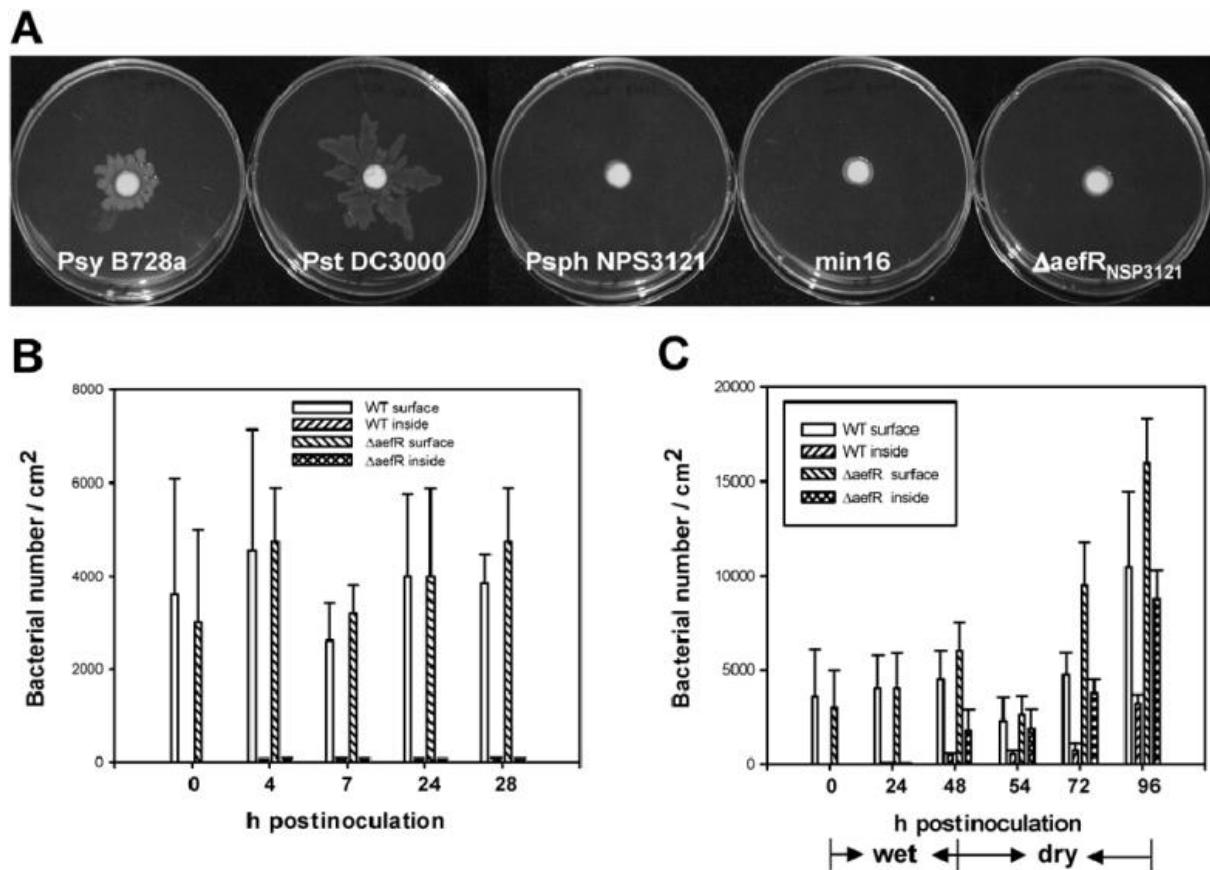


Fig. II- 4 Regulation of the TTSS and pathogenicity by AefR<sub>NPS3121</sub>.

**A**, Mutation of *aefR*<sub>NPS3121</sub> reduces the expression of *hrpL* in MM. WT *Psph* NPS3121,  $\Delta$ *aefR*<sub>NPS3121</sub> mutant, and  $\Delta$ *aefR*<sub>NPS3121</sub> mutant carrying the pML122::*aefR*<sub>NPS3121</sub> plasmid were grown in KB medium and induced in MM for 4 hr before being harvested for RNA extraction. Ten microgram of total RNA from each sample was analyzed by Northern blotting using DNA probes derived from the coding regions of *hrpL*, *lonB*, *rpoS*, *gacA*, and *psrA*. Loading of RNA samples is indicated by rRNA. 1, WT in KB; 2, WT in MM; 3,  $\Delta$ *aefR*<sub>NPS3121</sub> mutant in MM; 4, the complemented  $\Delta$ *aefR*<sub>NPS3121</sub> mutant in MM. **B**, Mutation of *aefR*<sub>NPS3121</sub> reduces *hrpR* promoter activity in MM. *hrpR-luc* reporter gene was introduced into the WT strain and  $\Delta$ *aefR*<sub>NPS3121</sub> mutant. Bacteria were grown in KB and induced in MM. LUC activity was measured at 0, 3, 6 hr after induction. Error bars indicated standard error. **C and D**, Mutation of *aefR*<sub>NPS3121</sub> reduces the *avrPto* and *hrpL* promoter activities in plant. The WT strain and  $\Delta$ *aefR*<sub>NPS3121</sub> mutant carrying the *avrPto-luc* (C) and *hrpL-luc* (D) reporter genes were resuspended in water to OD<sub>600</sub>=0.5. The bacteria were infiltrated into the primary leaves of two-week-old bean plants. Leaves were removed and sprayed with 1 mM luciferin dissolved in 0.01% Tween-20, and the LUC activity was determined using a cooled CCD. The experiments were repeated three times with similar results. **E**, Mutation of *aefR*<sub>NPS3121</sub> reduces the bacterial growth inside the host plant. Bacteria at  $2 \times 10^4$  CFU/mL were injected into the primary bean leaves. For each data point, three leaf discs (1 cm<sup>2</sup>) were removed at 0, 2, and 4 days after inoculation and ground separately in sterile water for counting of bacterial numbers. Error bars represent standard error. The experiment was repeated four times with similar results. F test indicated that differences between the WT and mutant strains at 2 and 4 day are significant (P<0.05). **F**, Expression of *aefR*<sub>NPS3121</sub> RNA in *hrpR*, *hrpS*, *rhpS*, *rhpRS*, and *lon* mutants. The WT *Psph* NPS3121 and mutant strains were grown in KB medium and induced in MM for 6 hr before being harvested for RNA extraction. Ten ug RNA was

analyzed by RNA blotting using probes derived from the coding region of aefRNPS3121.

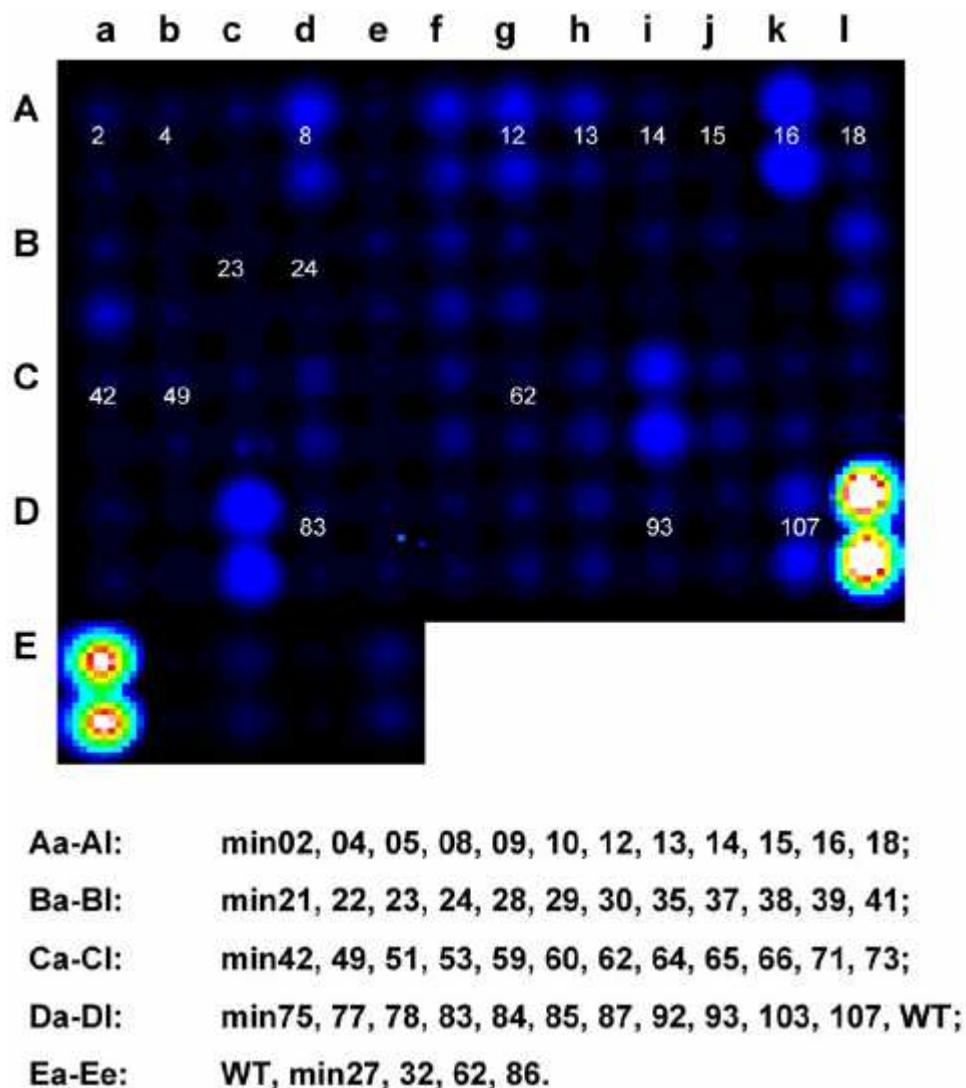
Loading of RNA samples is indicated by rRNA.



**Fig. II- 5 Effect of *aefR*<sub>NPS3121</sub> mutation on bacterial motility and epiphytic fitness.**

**A**, Swarming motility on semisolid agar. Bacteria were grown in KB liquid overnight and resuspended in KB to  $OD_{600}=1$ . Filter discs (6 mm in diameter) were soaked in bacterial suspensions and placed in the center on semisolid KB plates. Plates were photographed after incubation at 28°C for 24 hr. **B**, The ability of bacteria to enter the interior of leaves. Two-week-old bean plants were dipped into bacterial suspension ( $1 \times 10^6$  CFU/mL in 10 mM PB plus 50  $\mu$ l/L silwet) for 1 min and covered with plastic bags immediately after inoculation. Primary leaves were removed at the indicated times and surface sterilized with 15% hydrogen peroxide, followed by treatment with catalase. Leaves were then macerated in sterile PB for bacterial counts. Population sizes of WT and *aefR*<sub>NPS3121</sub><sup>-</sup> mutant are not significantly different ( $P=0.7 > 0.05$ ) according to F test. **C**, Epiphytic fitness assay. Two-week-old bean

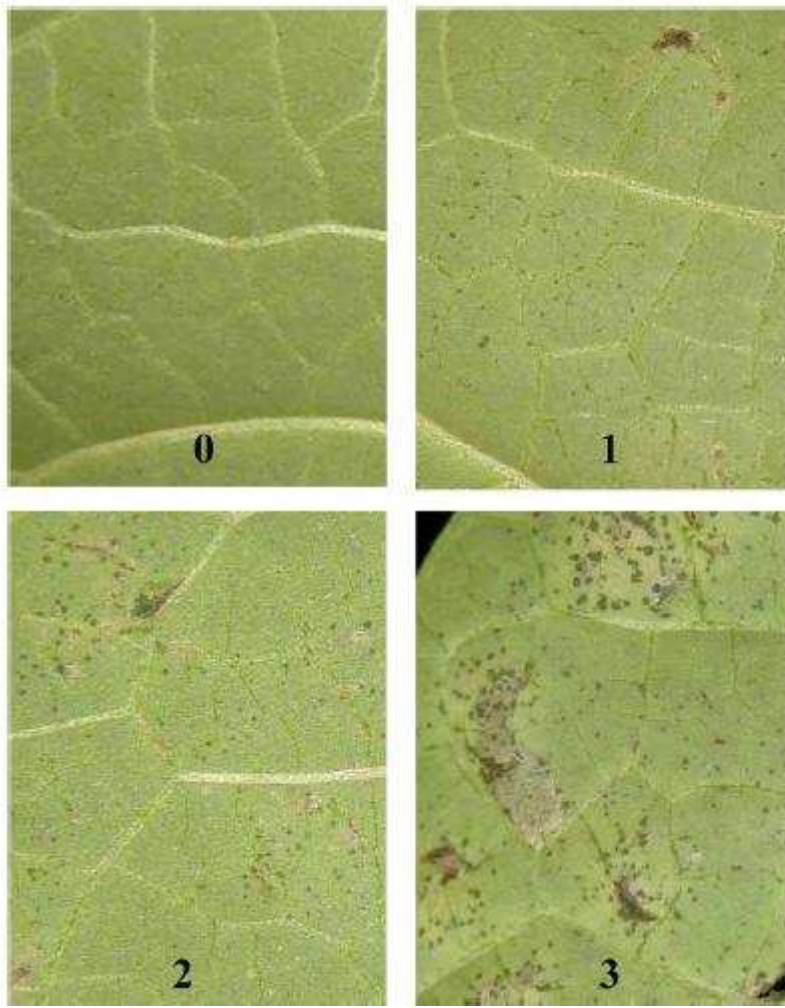
plants were dip-inoculated and covered with plastic bags immediately after inoculation. The plants were removed from the plastic bags 48 hr after inoculation and placed in the greenhouse. Surface bacteria were determined by washing the primary leaves with washing buffer in an ultrasonic bath, and the bacteria in washing solution were determined by plating on TSA plates. Bacteria inside the leaves were determined by treating the washed leaves with 15% hydrogen peroxide, followed by a treatment with catalase. Leaves were then macerated in sterile PB to release the bacteria for counting. Epiphytic population sizes of the WT and mutant strains are not significantly different at 0, 24, 48 and 54 hpi, but are significantly different after 72 hpi according to F test. The numbers of internal bacteria of the WT and mutant strains are significantly different after 48 hpi. Error bars indicate standard error. Experiments of B and C were repeated 4 times with similar results.



**Fig. II- 6 Induction of *avrPto-luc* reporter gene in *min* mutants in MM.**

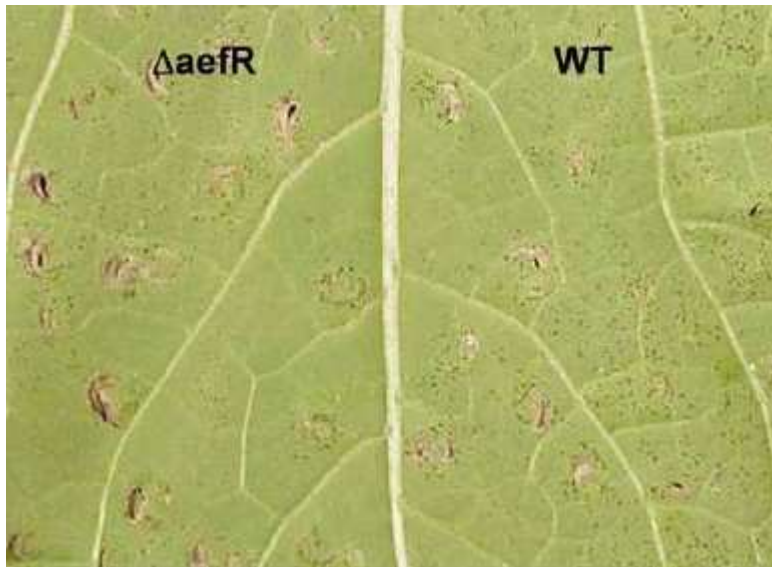
WT strain and *min* mutants were grown in KB medium containing proper antibiotics, washed twice with MM, resuspended in MM to  $OD_{600}=0.1$ , and incubated for 6 hr. 100  $\mu$ l of bacterial culture was mixed with 10  $\mu$ l of 0.1 mM luciferin in a 96-well plate, and the image of LUC activity was captured using a cooled CCD. The LUC activity is reflected by light intensity in the wells. The bacterial strains in the wells are indicated by the text under the CCD image. Two alleles were tested for some of the mutants (see Table II-1). Clones indicated by the numbers on the CCD image were tested for *hrpL* RNA expression in Fig. II-1A.





**Fig. II- 7 Disease Indices.**

Pathogenicity assays were performed in Red Kidney bean plants. Bacteria were infiltrated into the primary leaves of two-week-old plants at  $2 \times 10^4$  CFU/mL. Shown are four indices to score disease symptoms 5 days after inoculation.



**Fig. II- 8 Disease symptoms elicited by aefRNPS3121 mutant.**

WT *Psph* NSP3121 and aefRNPS3121 mutant at  $2 \times 10^4$  CFU/mL were infiltrated into the primary leaves of two-week-old bean plants. Disease symptoms were photographed 5 days after inoculation.

Table II- 1 *min* mutants.

Genes	Mutants	Gene products	Pathways of biological processes	Disease index
<b>Group A: signal sensor or transcriptional regulator</b>				
<i>rhpS</i>	<i>min12*</i> , 80, <i>L1</i> , <i>L2</i> , <i>L3</i> , <i>L5</i> , <i>L7</i>	Sensor histidine kinase	Signal transduction	0
<i>hrpS</i>	<i>min08*</i> , 94, 96	Type III transcriptional regulator	Signal transduction	0
<i>aefR</i>	<i>min16*</i> , <i>L4</i>	Transcriptional regulator	Signal transduction	2
<b>Group B: membrane proteins</b>				
PSPPH_4907	<i>min79</i> , 93*	Outer membrane porin	Membrane protein	2
PSPPH_5137	<i>min107</i>	YeeE/YedE family protein	Putative membrane protein	1
<b>Group C: protein translation</b>				
<i>trmE</i>	<i>min47</i> , <i>L8</i>	tRNA modification GTPase	Translation	ND
<i>miaA</i>	<i>minL6</i>	tRNA modification isopentenyltransferase	translation	1
<b>Group D: metabolic genes</b>				
<i>cbiD</i>	<i>min83*</i>	Cobalamin biosynthesis protein	Porphyrin and metabolism	1
<i>cbiG</i>	<i>min75</i>	Precorrin-3B methyltransferase	Porphyrin and metabolism	2
<i>cobC</i>	<i>min87</i>	Cobalamin biosynthesis protein	Porphyrin and metabolism	1
<i>cobH</i>	<i>min103</i>	Precorrin-8X methylmutase	Porphyrin and metabolism	1
<i>cobM</i>	<i>min30</i> , 50, 55, 56	Precorrin-4 C11-methyltransferase	Porphyrin and metabolism	1
<i>cobN</i>	<i>min66</i> , 69	CobN/magnesium chelatase family protein	Porphyrin and metabolism	1.5
<i>cobQ</i>	<i>min51</i>	Cobyric acid synthase	Porphyrin and metabolism	1

PSPPH_3701	<i>min104</i> , 106	Cobyrinic acid synthase	a,c-diamide	Porphyrin and metabolism	chlorophyll	NA
PSPPH_2598	<i>min64</i> , 95	Tetrapyrrole methylase protein	family	Porphyrin and metabolism	chlorophyll	2
PSPPH_2227	<i>min77</i>	Magnesium chelatase	subunit ChID	Porphyrin and metabolism	chlorophyll	1
<i>bchI</i>	<i>min29</i> , 54	Magnesium chelatase, putative	subunit ChII,	Porphyrin and metabolism	chlorophyll	1.5
<i>argH</i>	<i>min10</i> , 13*, 45, 68	Argininosuccinate lyase		Arginine biosynthesis		1.5
<i>gltB</i>	<i>min04</i> *, 15, 20, 25, 26, 33, 40, 43, 52, 67, 99	Glutamate synthase, large subunit		Glutamate biosynthesis		1.7
<i>gltD</i>	<i>min22</i> , 28	Glutamate synthase, small subunit		Glutamate biosynthesis		2.5
<i>hisA</i>	<i>min38</i>	Phosphoribosylformimino-5-aminoimidazole ribotide isomerase	carboxamide	Histidine biosynthesis		1
<i>hisB</i>	<i>min59</i> , 74	Imidazoleglycerol-phosphate dehydratase		Histidine biosynthesis		2.5
<i>hisC</i>	<i>min05</i> , 11	Histidinol-phosphate aminotransferase		Histidine biosynthesis		2
<i>hisD</i>	<i>min37</i> , 76	Histidinol dehydrogenase		Histidine biosynthesis		1.5
<i>hisE</i>	<i>min53</i>	Phosphoribosyl-ATP pyrophosphatase		Histidine biosynthesis		3
<i>hisF</i>	<i>min24</i> *, 34	Imidazoleglycerol synthase, cyclase	phosphate	Histidine biosynthesis		0.5
<i>hisH</i>	<i>min35</i> , 36	Amidotransferase		Histidine biosynthesis		0
<i>ilvD</i>	<i>min32</i> *, 86	Dihydroxy-acid dehydratase		Valine and isoleucine biosynthesis		0
<i>leuA</i>	<i>min09</i> , 14*, 82	2-isopropylmalate synthase		Leucine biosynthesis		0.3
<i>leuB</i>	<i>min21</i> , 39, 61	3-isopropylmalate dehydrogenase		Leucine biosynthesis		0
<i>leuC</i>	<i>min60</i>	3-isopropylmalate dehydratase, large subunit	dehydratase,	Leucine biosynthesis		0
<i>metF</i>	<i>min49</i> *, 84	5,10-methylenetetrahydrofolate reductase	5,10-methylenetetrahydrofolate	Methionine biosynthesis		1.5

<i>meth</i>	<i>min92</i> , 108	5-methyltetrahydrofolate homocysteine methyltransferase	Methionine biosynthesis	1.5
<i>serA</i>	<i>min42</i> *, 73	D-3-phosphoglycerate dehydrogenase	L-serine biosynthesis	1
<i>trpA</i>	<i>min62</i> *	Tryptophan synthase, alpha subunit	Tryptophan biosynthesis	0
<i>trpD</i>	<i>min71</i>	Anthranilate phosphoribosyl transferase	Tryptophan biosynthesis	0
PSPPH_0847	<i>min65</i> , 98	Phosphoenolpyruvate-protein phosphotransferase, EI/HPPr/EIIA components	Fructose and mannose metabolism	2
<i>fruK</i>	<i>min18</i> *	1-phosphofructokinase	Fructose and mannose metabolism	2
PSPPH_0849	<i>min41</i>	Phosphotransferase system, fructose-specific IIBC component	Fructose and mannose metabolism	2
<i>purE</i>	<i>min78</i>	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	Purine metabolism	2
<i>purK</i>	<i>min27</i>	Phosphoribosylaminoimidazole carboxylase, atpase subunit	Purine metabolism	0
<i>eno-1</i>	<i>min23</i> *	Enolase	Glycolysis; gluconeogenesis	1
PSPPH_2878	<i>min85</i> *	Glycosyl hydrolase, family 15	Polysaccharide metabolism	1
<i>sypA</i>	<i>min63</i>	Putative peptide/siderophore synthetase		3
PSPPH_0569	<i>min02</i> *	ATP phosphoribosyltransferase subunit, putative		2

Mutants isolated with the *avrPto-luc* reporter are indicated by *min* followed by a number. Mutants isolated with the *hrpL-luc* reporter are indicated by *L* and a number. Genes with transposon insertions are named according to their homologs in the *Psph* 1448A strain. The mutants in boldface were shown with the *avrPto-luc* reporter activity in Fig. II-6. The mutants highlighted with \* were tested for *hrpL* RNA expression in Fig. II-1A. All the mutants were tested two times for

elicitation of disease symptoms on the bean plants and were assigned with a disease index according to the disease indices in Fig.

II-7.

**Table II- 2 HR assay of representative *min* mutants.**

WT	min4	min8	min12	min14	min16	min18	min21	min23	min24
	gltB	hrpS	rhpS	leuA	aeiR	fruK	leuB	eno-1	hisF
12h	+	-	-	+	+	+	+	-	-
14h	+	-	+	+	+	+	+	+	+
18h	+	-	+	+	+	+	+	+	+
min2	min32	min42	min47	min62	min71	min83	min85	min93	min107
7	ilvD	serA	trmE	trpA	trpD	cibD	PSPPH_0569	PSPPH_4907	PSPPH_5137
purK									
12h	-	+	+	-	-	+	+	+	-
14h	-	+	+	-	-	+	+	+	+
18h	-	+	+	-	-	+	+	+	+

The WT strain and *min* mutants at  $10^8$  CFU/mL were injected into the tobacco W38 leaves. The induction of non-host HR was documented at 12 hr, 14 hr, and 18 hr after inoculation. “+” indicates positive HR, “-” indicates lack of HR.

**Table II- 3 Plasmids**

Plasmids	Description	References
pBluescript-SK(+)	Cloning and sequencing	Stratagene, La Jolla, CA,
pGEM-7Z	Cloning and sequencing	Promega, Madison, WI,
pGEM-T	Cloning and sequencing	Promega, Madison, WI,
pML122	Broad-host plasmid	Labes et al. 1990
pHM2	Broad-host plasmid	Xiao et al. 2007
pHM2:: <i>avrPto-luc</i>	<i>avrPto-luc</i> reporter in pHM2	Xiao et al. 2007
pHM2:: <i>hrpL-luc</i>	<i>hrpL-luc</i> reporter in pHM2	Xiao et al. 2007
pPTE6:: <i>luc</i>	Firefly luciferase in pPTE6	Xiao et al. 2004
pBS- <i>hrpR</i>	Intermediate construct for <i>pLT-hrpR-luc</i>	This study
pPTE6:: <i>hrpR-luc</i>	<i>hrpR-luc</i> in pPTE6	This study
pLT:: <i>hrpR-luc</i>	Derived from pPTE6:: <i>hrpR-luc</i> by EZ-Tn5<TET-1> insertion	This study
pGEM-T-PSPPH_3244	Intermediate construct for <i>pML122::PSPPH_3244</i>	This study
pML122::PSPPH_3244	PSPPH_3244 in pML122 plasmid, under pNm promoter	This study
pBluescript-SK- <i>luc</i>	Firefly luciferase in pBluescript-SK(+)	This study
pBS-PSPPH_3244- <i>luc</i>	Intermediate construct for <i>pHM2-PSPPH_3244-luc</i>	This study
pBS-PSPPH_1614- <i>luc</i>	Intermediate construct for <i>pHM2-PSPPH_1614-luc</i>	This study
pHM2::PSPPH_3244- <i>luc</i>	PSPPH_3244- <i>luc</i> reporter in pHM2	This study
pHM2::PSPPH_1614- <i>luc</i>	PSPPH_1614- <i>luc</i> reporter in pHM2	This study
p7Z::PHPPH_3244FR	Intermediate construct for marker exchange	This study
p7Z::FKanR	Intermediate construct for marker exchange	This study
pHM1::PSPPH_3244-FKanR	For marker exchange	This study



**Table II- 4 Primers.**

Primer names	Sequences
pspph3244-O-F	TTCTCGAGTGGCAACAAGTAAACTGCTGA
pspph3244-O-R	TTATCGATGGGGCGTAGTACTCGACGTA
pspph3244-P-F	TTGAATTCGGTTTCCATCAGTGTCAG
pspph3244-P-R	TTGGATCCCATAGGTTTCGCTTCAAACGGA
pspph1614-P-F	TTGAATTCGCAGTATCTGGCCACTT
pspph1614-P-R	TTGGATCCCATATAAACTCCACTC
p3244LF	TTTCTAGAACGCAGGTATAGGACGCAGT
p3244LR	TTGGATCCGATGGATTCGCGTTTCTGAT
p3244RF	TTGGATCCGAACATGTTTCTGGGCTGGT
p3244RR	TTGAGCTCGCCAATCCACGTGATTTTCT
Kan-BF	TTGGATCCCATCGATGAATTGTGTCT
Kan-BR	TTGGATCCGGTGGACCAGTTGGTGAT
Psph-hrpR-PF	GAATTCGTTTTAAAGCCGGATGTATAG
Psph-hrpR-PR	TTGGATCCGTCCATATCCAGAAACGC
psph-hrpL-NF	GACTCTTCGTCTGCCGGTAT
psph-hrpL-NR	GGGTCAATCTGCTGCTTCAA
psph-gacA-NF	CATAGACGGTCTGCAGGTTG
psph-gacA-NR	GTGACGTACAGCGAGCAAAG
psph-rpoS-NF	AAGGAAGCGTCAAACGAGAA
psph-rpoS-NR	AGCCCGTTCTTTTCAAGGAT
psph-rpoN-NF	CGCCTTACTCCAGCTTTCCAC
psph-rpoN-NR	GTCGCCGTA CTCAAGAAAGC
psph-psrA-NF	CGTTGAACGCATTCTTGATG
psph-psrA-NR	GATCATGGTCGGGTCACTG
psph-ahlI-NF	GAGCGGGTTTGAGTTTCAGT
psph-ahlI-NR	AGCAGGTCATCCGTGACAG
psph-lonB-NF	GATTCGTGGCCCTGTACTGT
psph-lonB-NR	TGGATATGCGTGTCGTGTT
Kan2-SP1	GATAGATTGTCGCACCTGATTG
Kan2-SP2	AAGACGTTTCCCGTTGAATATG
Kan2-SP3	GCAATGTAACATCAGAGATTTTGAG
CEKG 2A	GGCCACGCGTCGACTAGTACNNNNNNNNNNNAGAG
CEKG 2B	GGCCACGCGTCGACTAGTACNNNNNNNNNNNACGCC
CEKG 2C	GGCCACGCGTCGACTAGTACNNNNNNNNNNNGATAT
CEKG 4	GGCCACGCGTCGACTAGTAC
HrpL-FLAG-F	TTCTCGAGGTGCTGTGGTCAGCCCGTG
HrpL-FLAG-R	CCTTCGAAGGCGAACGGGTCAATCTG

## CHAPTER 3

*Pseudomonas syringae* two component response regulator RhpR regulates  
promoters carrying an inverted repeat element

## ABSTRACT

The two component system RhpRS was identified in *Pseudomonas syringae* as a regulator of the genes encoding the type III secretion system (T3SS) and type III effector proteins (together called the T3 genes hereafter). In the absence of the sensor kinase RhpS, the response regulator RhpR represses the induction of the T3 gene regulatory cascade consisting of *hrpRS*, *hrpL*, and the T3 genes in a phosphorylation-dependent manner. The repressor activity of RhpR is inhibited by RhpS, which presumably acts as a phosphatase under the T3 gene inducing conditions. Here I show that RhpR binds and induces its own promoter in a phosphorylation-dependent manner. Deletion and mutagenesis analyses revealed an inverted repeat (IR) element GTATC-N6-GATAC in the *rhpR* promoter that confers the RhpR-dependent induction. Computational search of the *P. syringae* genomes for the putative IR elements and Northern blot analysis of the genes with a putative IR element in the promoter region uncovered five genes that were upregulated and two genes that were down-regulated in an RhpR-dependent manner. Two genes that were strongly induced by RhpR were assayed for the IR element activity in gene regulation, and in both cases the IR element mediated the RhpR-dependent gene induction. ChIP assays indicated that RhpR binds the promoters containing a putative IR element but not the *hrpR* and *hrpL* promoters that do not have an IR element, suggesting that RhpR indirectly regulates the transcriptional cascade of *hrpRS*, *hrpL*, and the T3 genes.

## INTRODUCTION

Bacteria primarily rely on two component systems (TCS) to sense and respond to environmental changes (Hoch 2000). A TCS usually consists of a sensor histidine kinase and a response regulator (Stock et al. 2000). In general, the sensor kinase, upon sensing a specific signal, 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 activates the response regulator, which in turn, stimulates or represses the transcription of its target genes (Stock et al. 2000). Many TCS sensor kinases also possess a phosphatase activity that can dephosphorylate the cognate response regulators and retain the later in an inactive state (Bijlsma and Groisman 2003). The relative kinase and phosphatase activities in bacterial cells are modulated by environmental stimuli and determine the outcome of signal transduction. Response regulators can be phosphorylated by unrelated sensor kinases or by small phosphate donor molecules such as acetyl phosphate in the absence of cognate sensor kinases (McCleary et al. 1993; Laub and Goulian 2007). As a result, many response regulators display a regulatory activity even in the absence of their cognate sensor kinases (Laub and Goulian 2007).

DNA binding response regulators usually bind directly to promoter elements to mediate gene regulation. Most response regulators bind to DNA elements consisting of direct or inverted repeats that are separated by a spacer of 2-11 base nucleotides (de Been et al. 2008). For example, response regulators in the OmpR family typically bind to direct repeat elements separated by a spacer of four or five nucleotides, whereas response regulators of the NarL family usually bind to inverted repeat elements separated by two to six nucleotides (de Been et al. 2008). A direct repeat element, (T/G)GTTTA-N<sub>5</sub>-

(T/G)GTTTA, is defined as the PhoP box (Groisman et al. 2001), whereas an imperfect inverted repeat (GCGGC-N<sub>5</sub>-GTCGC) is critical for DNA binding of the response regulator RegR of *Bradyrhizobium japonicum* (Emmerich et al. 2000). Response regulators are believed to form homodimers on the repeat elements (Blanco et al. 2002; Maris et al. 2002). Some promoters have several copies of the repeat elements, and response regulators often form an oligomer on such promoters (Maris et al. 2005).

Some response regulators are capable of positive as well as negative regulation of their target genes. For example, the phosphorylated response regulator OmpR (P-OmpR) of *E. coli* binds the promoters of *ompF* and *ompC* genes and regulates their expression in response to medium osmolarity (Head et al., 1998; Lan et al., 1998). There are four and three P-OmpR binding sites in the promoters of *ompF* and *ompC*, respectively (Yoshida et al. 2006). At low osmolarity, P-OmpR binds to two or three high affinity sites in the *ompF* promoter and activates *ompF*. Under this condition, only one site in the *ompC* promoter is occupied by P-OmpR, which is insufficient to activate *ompC*. At high osmolarity, P-OmpR occupies all the three sites in the *ompC* promoter to activate *ompC* and all the four sites in the *ompF* promoter to inhibit *ompF* (Yoshida et al. 2006). The response regulator CovR of *Streptococcus pyogenes* can also directly activate and repress its target genes (Churchward 2007). CovR represses its target genes via promoter occlusion, because the CovR binding site overlaps with the sigma 70 promoter and/or the transcriptional start site (Gao et al. 2005; Gusa et al. 2005; 2006). CovR activates the expression of the dipeptide permease gene *dppA* by interfering the binding of a repressor protein to the *dppA* gene promoter (Gusa et al. 2007).

In addition to regulating the downstream genes, many TCS response regulators are capable of self-regulation (Bijlsma and Groisman 2003). Self-regulation is mediated by direct binding of the response regulator protein to its own gene promoter (Bijlsma and Groisman 2003). Many response regulators autoactivate their expression (Soncini et al. 1995; Bang et al. 2002; Clarke and Sperandio, 2005; Gonzalo-Asensio et al. 2008), but a few response regulators such as CovR and TorR are capable of autoinhibition (Ansaldi et al. 2000; Gusa and Scott 2005). Direct self-regulation enables bacteria to respond more rapidly and efficiently to environmental changes (Hoffer et al. 2001; Shin et al. 2006).

The T3 genes of *Pseudomonas syringae* are repressed in rich medium but induced in the plant and minimal medium (MM; Xiao et al. 2004; Tang et al. 2006). In searching for genes regulating the induction of the T3 genes, we identified *rhpS*<sup>-</sup> mutation that severely inhibited the induction of the T3 genes in the plant and MM (Xiao et al. 2007; Deng et al. 2009). *rhpS* encodes a putative TCS sensor histidine kinase. *rhpS* is downstream of *rhpR* in an operon. *rhpR* encodes a putative TCS response regulator. Deletion of the whole *rhpRS* locus ( $\Delta$ *rhpRS*) restores the induction of the T3 genes, and overexpression of RhpR in the deletion mutant  $\Delta$ *rhpRS* suppresses the induction of the T3 genes in a phosphorylation-dependent manner (Xiao et al. 2007). It appears that RhpR is phosphorylated by an as-yet unknown factor in *rhpS*<sup>-</sup> mutant, the phosphorylated RhpR (P-RhpR) represses the T3 genes, and RhpS acts as a phosphatase and retains RhpR in a dephosphorylated state under conditions inducing the T3 genes.

Here I show that RhpR binds and activates its own promoter in a phosphorylation-dependent manner. An inverted repeat (IR) element was found in the *rhpR* promoter that mediates the RhpR-dependent regulation. Through genome-wide searching of the IR

element-containing promoters and Northern blot analysis of the corresponding genes, putative IR element-regulated genes were identified.

## RESULTS

### **The *rhpR* promoter is induced by RhpR.**

Our previous studies showed that RhpR represses the T3 genes in *rhpS* mutant, and the presence of RhpS derepresses the T3 genes in MM (Xiao et al. 2007). In an attempt to depict the regulatory pathway, I searched for the RhpR-regulated promoters. Bacterial TCS loci are often subject to direct autoregulation by the response regulators (Bijlsma and Groisman 2003). To determine if RhpR regulates the *rhpRS* expression, Northern hybridization was performed to compare the expression of *rhpR* RNA in WT DC3000 and the transposon insertion mutant of the *rhpS* gene. *rhpR* RNA was expressed at a much higher level in the *rhpS* mutant than in the WT strain in MM as well as in rich medium KB (Fig. III-1A).

To test if RhpR regulates the activity of the *rhpR* promoter, 540 bp *rhpR* promoter DNA (including the start codon ATG of the *rhpR* gene) was fused to the promoterless luciferase (*luc*) reporter gene, and the resulting plasmid pHM2::*rhpR*<sub>540</sub>-*luc* (Table III-2) was introduced into WT DC3000, *rhpS* mutant, and  $\Delta$ *rhpRS* mutant. The reporter activity was 10-fold higher in the *rhpS* mutant than in the WT and  $\Delta$ *rhpRS* mutant strains (Fig. III-1B), indicating an autoactivation of the *rhpR* promoter by RhpR.

### **RhpR requires the phosphorylation site to activate the *rhpR* promoter.**

RhpR requires the phosphorylation site for repression of the T3 genes in *P. syringae* strains (Xiao et al. 2007). To determine if the inducing activity of RhpR is also regulated by phosphorylation, the RhpR(D70A) mutant with the predicted phosphorylation site Asp70 substituted by alanine was tested for the activity to induce the *rhpR* promoter. HA-tagged wild type RhpR and RhpR(D70A) mutant proteins were expressed in the  $\Delta rhpRS$  mutant using a constitutive promoter harbored by the pML122 plasmid (Table III-2). Western blot analysis indicated that RhpR-HA and RhpR(D70A)-HA proteins were expressed at similar levels (Xiao et al. 2007). The expression of RhpR-HA, but not RhpR(D70A)-HA, in  $\Delta rhpRS$  mutant strongly induced the *rhpR*<sub>540</sub> promoter both in MM and in KB (Fig. III-2A), suggesting that phosphorylation of RhpR enhances its regulatory activity. These results also supported our hypothesis that RhpR is phosphorylated by unknown factors in *rhpS* mutant (Xiao et al. 2007).

In addition to the RhpR-dependent induction, the *rhpR*<sub>540</sub>-*luc* reporter gene in WT DC3000, *rhpS* mutant, and  $\Delta rhpRS$  mutant displayed higher activities in MM than in KB medium (Fig. III-1B and Fig. III-2A), indicating an RhpR-independent induction of the *rhpR* promoter by MM .

The RhpR-dependent induction of *rhpR* promoter led us to test if RhpR directly regulates the *rhpR* promoter. Our attempts to purify the recombinant RhpR protein from *E. coli* were unsuccessful, which deterred the *in vitro* assays of RhpR interaction with the *rhpR* promoter. I thus tested if the expression of the recombinant RhpR protein can induce the *rhpR* promoter in *E. coli* cells. Plasmids expressing GST-RhpR and GST-



RhpR(D70A) fusion proteins were transformed into *E. coli* BL21 strain carrying the pHM2::*rhpR*<sub>540</sub>-*luc* plasmid. Consistent with the result in  $\Delta$ *rhpRS* mutant (Fig. III-2A), the *rhpR*<sub>540</sub>-*luc* reporter gene displayed a higher activity in BL21 strain expressing GST-RhpR protein than in BL21 strain expressing GST-RhpR(D70A) (plasmids generated by Dr. Xiao, Fig. III-2B), suggesting direct regulation of the *rhpR* promoter by RhpR.

Our lab showed previously that the expression of full length RhpS protein in *rhpS*<sup>-</sup> mutant restored the induction of T3 genes in MM (Xiao et al. 2007). Here I tested if signal input is necessary for the RhpS function. A partial RhpS protein without the N-terminal extracellular and transmembrane domains was expressed in the *rhpS*<sup>-</sup> mutant. The cytoplasmic domain of RhpS fully restored the induction of *avrPto-luc*, a reporter gene for the T3 genes, in MM and completely repressed the *rhpR*<sub>540</sub>-*luc* reporter gene activity (data not shown).

### **RhpR requires the phosphorylation site for maximal association with the *rhpR* promoter.**

Chromatin immuno-precipitation (ChIP) assay was performed to test if RhpR directly binds the *rhpR* promoter in *P. syringae*, and whether the binding activity of RhpR is affected by phosphorylation.  $\Delta$ *rhpRS* mutant strains expressing RhpR-HA and RhpR(D70A)-HA proteins were used for ChIP assay. RhpR-HA and RhpR(D70A)-HA were expressed at similar levels in the  $\Delta$ *rhpRS* mutant (Xiao et al. 2007).  $\Delta$ *rhpRS* mutant carrying the empty pML122 plasmid was used as a negative control. ChIP assay was performed with the anti-HA antibodies, and the enrichment of the selected promoter DNAs in the immunocomplexes was detected using quantitative real time PCR (qRT-

PCR). The primers used for amplification of the selected promoter DNAs are listed in Table III-3. ChIP assay was also performed without the use of the anti-HA antibodies to determine nonspecific precipitation of the promoter DNA. The amount of the promoter DNA precipitated by the anti-HA antibodies subtracted by the amount of the promoter DNA derived from nonspecific precipitation was regarded as the enrichment of the promoter DNA by the anti-HA antibodies. The *rhpR* promoter DNA was enriched by 2 fold by the anti-HA antibodies in the control strain carrying the empty pML122 vector (Fig. III-3). On the contrary, the *rhpR* promoter DNA was enriched by 215 fold in the strain expressing RhpR-HA (Fig. III-3). The large enrichment of the *rhpR* promoter DNA from the RhpR-HA expressing strain indicated a direct binding of RhpR-HA with the *rhpR* promoter. The *rhpR* promoter DNA was enriched by only 5 folds in the strain expressing RhpR(D70A)-HA (Fig. III-3). The much larger enrichment of the *rhpR* promoter DNA from the RhpR-HA expressing strain than the RhpR(D70A)-HA expressing strain (215 verses 5 folds) was consistent with the strong induction of *rhpR* promoter by RhpR but not by RhpR(D70A) in the  $\Delta rhpRS$  mutant, suggesting that phosphorylation of RhpR increases its interaction with the *rhpR* promoter. The differential enrichment of the promoter DNA by the anti-HA antibodies was not detected to PSPTO1489 (Fig. III-3), a house-keeping gene that is not regulated by RhpR (L. Lan and X. Tang, unpublished data).

#### **Identification of the RhpR-regulated element in the *rhpR* promoter.**

The 540 bp *rhpR* promoter in the pHM2::*rhpR*<sub>540</sub>-*luc* plasmid was deleted to 170, 120, 80, and 40 bp upstream of the *rhpR* start codon ATG, and the resulting deletions were assayed for the promoter activities in the *rhpS*<sup>-</sup> mutant (Fig. III-4A). The 170 bp promoter

(*rhpR*<sub>170</sub>) had ~60% of the activity relative to *rhpR*<sub>540</sub>. However, the 120 bp promoter (*rhpR*<sub>120</sub>) had only 7% of the activity. Further deletion to 40 bp completely eliminated the promoter activity. *rhpR*<sub>170</sub> and *rhpR*<sub>120</sub> promoters displayed low activities at the same level in the  $\Delta$ *rhpRS* mutant (Fig. III-1B). These indicated the presence of an RhpR-dependent promoter element in the region between -170 to -120.

The region between -170 and -120 has a perfect 5 bp inverted repeat (IR) sequence (between -147 and -132) with a 6 bp spacer, GTATC-N6-GATAC (Fig. III-4C). To test if this IR element has a role in the RhpR-dependent regulation, two additional deletions were generated in the region between -170 and -120. The 147 bp promoter carrying the IR element displayed a strong activity, while the 132 bp promoter without the IR element exhibited a low activity in the *rhpS*<sup>-</sup> mutant (Fig. III-4A). These results indicated that the IR element mediates the RhpR-dependent induction of the *rhpR* promoter.

The inverted repeat sequences of the IR element are perfectly conserved in the promoters of *rhpR* orthologs in *P. s. pv. tomato* (*Pst*), *P. s. pv. syringae* (*Psy*), and *P. s. pv. phaseolicola* (*Pph*) (Fig. III-4D). However, the 6-bp spacers are variable in sequence (Fig. III-4D). All the IR elements are 132 bp upstream of their corresponding *rhpR* open reading frames. Additional element similar to this IR element was not found in the *rhpR* promoters.

#### **Determination of the *rhpR* transcriptional start site.**

To define the position of the IR element in *rhpR* promoter, 5' rapid amplification of cDNA ends (RACE) reaction was performed with RNA samples prepared from WT DC3000 and *rhpS*<sup>-</sup> mutant to determine the transcriptional start site of *rhpR*. Four clones

containing the PCR products derived from each 5' RACE reaction were sequenced. All the clones derived from WT DC3000 showed that the 5' end of *rhpR* RNA starts at T<sub>165</sub>, which is 165 bp upstream of the *rhpR* open reading frame (Fig. III-4C). All the clones derived from the *rhpS*<sup>-</sup> mutant showed that the *rhpR* RNA starts at G<sub>87</sub>, which is 87 bp upstream of the *rhpR* open reading frame (Fig. III-4C). G<sub>87</sub> is conserved in *Pst* and *Psy* but not in *Pph*, while T<sub>165</sub> is not conserved in the three sequenced *P. syringae* genomes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). A putative sigma 70 promoter element was predicted at the -10 and -35 regions upstream of T<sub>165</sub>, and a sigma 54 promoter element of poor homology to the consensus sequence was predicted between the IR element and G<sub>87</sub> (Fig. III-4C). This sigma 54 element is moderately conserved in the sequenced *P. syringae* genomes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). Although the promoter reporter assay suggested a transcriptional start site in the region between -40 bp and -80 bp, a transcript starting at this region was not identified by the RACE analysis. It is common that TCSs have more than one transcriptional start sites controlled by different promoters (Bijlsma and Groisman, 2003). For example, three transcriptional start sites have been reported for the *E. coli* TCS locus *qseBC* (Clarke and Sperandio, 2005).

### **Mutagenesis analysis of the IR element.**

To determine if the sequence of the IR element is important to the *rhpR* promoter activity, mutations (G to T, T to G, A to C, and C to A) were generated to each nucleotide of the IR modules in the pHM2::*rhpR*<sub>147</sub>-*luc* reporter plasmid, and the resulting mutant plasmids were introduced into the *rhpS*<sup>-</sup> mutant. Each mutation reduced the promoter activity but not in a uniform fashion (Fig. III-4E). Generally, mutants of the upstream 5-

bp module (-147 to -143) showed better promoter activity than mutants of the downstream 5-bp IR module (-136 to -132), indicating that the downstream IR module is more important in regulating the promoter activity.

To determine if the spacer length between the two inverted repeat modules is critical for the promoter activity, four mutants were generated to the spacer in the pHM2::*rhpR*<sub>147</sub>-*luc* plasmid: one mutant carries deletion of one base pair (-C<sub>142</sub>); one mutant carries deletion of two base pairs (-C<sub>142</sub> and -G<sub>141</sub>); one mutant carries insertion of one adenine between -C<sub>142</sub> and -G<sub>141</sub>; and one mutant carries insertion of four adenines between -C<sub>142</sub> and -G<sub>141</sub> (Fig. III-4E). Promoter activity assay showed that any change of the spacer length inactivated the promoter (Fig. III-4E), indicating that the 6 bp length of the spacer is required for the activity of the IR element.

### **Genome-wide search of the genes regulated by the putative IR elements.**

The identification of RhpR-regulated IR element enabled us to search for putative RhpR-regulated promoters in *P. syringae*. The DC3000 genome was searched for the perfect IR sequence GTATC-N<sub>6</sub>-GATAC using the pattern discovery function in the RSAT (<http://rsat.ulb.ac.be/rsat/>), which uncovered only the *rhpR* promoter. Because mutant IR elements with one nucleotide substitution exhibited partial activities, I also searched the DC3000 genome for putative IR sequences carrying one variable nucleotide in the repeat modules (NTATC-N<sub>6</sub>-GATAC, GNATC-N<sub>6</sub>-GATAC, GTNTC-N<sub>6</sub>-GATAC, GTANC-N<sub>6</sub>-GATAC, GTATN-N<sub>6</sub>-GATAC, GTATC-N<sub>6</sub>-NATAC, GTATC-N<sub>6</sub>-GNTAC, GTATC-N<sub>6</sub>-GANAC, GTATC-N<sub>6</sub>-GATNC, and GTATC-N<sub>6</sub>-GATAN). The search produced 44 hits (Table III-1).

Eighteen genes downstream of these putative IR elements were analyzed using RNA blotting for their expression in WT DC3000, *rhpS* mutant, and  $\Delta$ *rhpRS* mutant (Fig. III-5). These genes were selected because, according to a microarray analysis, they displayed a differential expression in WT DC3000 and *rhpS* mutant cultured in MM (L. Lan and X. Tang, unpublished data). The PSPTO2036 gene promoter contains a putative IR element (GTATC-N<sub>6</sub>-CTTAC) with two variable nucleotides (underlined) in the downstream IR module. This gene was also selected for RNA blot analysis, because it was expressed at a much higher level in *rhpS* mutant than in WT DC3000 according to the microarray analysis (unpublished data). RNA blot analysis indicated that five genes, including PSPTO2767, PSPTO2036, PSPTO3477, PSPTO3574, and PSPTO3600, displayed the same expression pattern as that of the *rhpR* promoter, i.e., more transcripts in *rhpS* mutant than in WT DC3000 and  $\Delta$ *rhpRS* mutant (Fig. III-5). These genes are probably induced by RhpR. Two genes, PSPTO0536 and PSPTO0897, were expressed at lower levels in *rhpS* mutant than in WT DC3000 and  $\Delta$ *rhpRS* mutant (Fig. III-5). These genes may be suppressed by RhpR. The putative functions of these genes are summarized in Table III-1. The remaining genes displayed an expression pattern independent of RhpR.

#### **Function of the putative IR elements in PSPTO2767 and PSPTO2036 promoters.**

The PSPTO2767 and PSPTO2036 transcripts were strongly induced in the *rhpS* mutant. The putative IR elements were analyzed for their roles in regulating PSPTO2767 and PSPTO2036 promoters. PSPTO2767 and PSPTO2036 promoters with the IR element (238 bp upstream of PSPTO2767 orf; 109 bp upstream of PSPTO2036 orf) and without the IR element (222 bp upstream of the PSPTO2767 orf; 93 bp upstream of the

PSPTO2036 orf) were fused with the *luc* reporter gene and assayed in WT DC3000, *rhpS* mutant, and  $\Delta$ *rhpRS* mutant (Fig. III-6, A and B). Both promoters with the IR element showed higher LUC activity in *rhpS* mutant than in WT DC3000 and  $\Delta$ *rhpRS* mutant. However, both promoters without the IR element showed low activities in all three strains. These results indicated that the putative IR elements in promoters of PSPTO2767 and PSPTO2036 conferred the RhpR-dependent induction of these genes in *rhpS* mutant.

PSPTO2767 encodes a lipopolysaccharide core biosynthesis domain protein. The ortholog of PSPTO2767 in *P. s. pv. syringae*, Psy\_2496, has two putative IR elements: one is identical to the PSPTO2767 IR element in the repeat modules, the other is identical to the IR element in the promoter of PSPPH\_2653, the ortholog of PSPTO2767 in *P. s. pv. phaseolicola* (Fig. III-6C). There is no additional IR element in the DC3000 genome identical to the IR element of PSPTO2767 in the repeat modules.

PSPTO2036 encodes a putative small lipoprotein, and its orthologs in the *P. s. pv. phaseolicola* and *P. s. pv. syringae* genomes were not annotated (Feil et al. 2005; Joardar et al. 2005). Tblastn search identified a small ORF (named PSPPH\_1805^6) between PSPPH1805 and PSPPH1806 in the *P. s. pv. phaseolicola* genome and a small ORF (named Psy\_1846^7) between Psy\_1846 and Psy\_1847 in the *P. s. pv. syringae* genome (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). Proteins encoded by these small genes are identical in the N-terminal signal peptide but variable in C-terminal portion following the lipid modification site (data not shown). The IR elements of these genes are identical in the repeat modules (Fig. III-6C). No additional IR element was found in the

DC3000 genome identical to the IR element of PSPTO2036 in the repeat modules. Northern blot analysis indicated that PSPPH\_1805<sup>6</sup> was expressed at a higher level in the *rhpS* mutant than in the WT *P. s. pv. phaseolicola* strain (data not shown). The promoters of PSPTO2767 and PSPTO2036 and their orthologous genes in *P. s. pv. syringae* and *P. s. pv. phaseolicola* all carry a putative sigma 54 element downstream of the IR element (data not shown).

### **RhpR binds the promoters containing a putative IR element.**

ChIP and qRT-PCR assays were performed to test if RhpR binds the promoters carrying a putative IR element. The promoters of eleven genes were tested. Three of these genes (PSPTO2767, PSPTO2036, and PSPTO3477) displayed an RhpR-dependent up-regulation; two genes (PSPTO0536 and PSPTO0897) displayed an RhpR-dependent down-regulation; and six genes (PSPTO0898, PSPTO0406, PSPTO1066, PSPTO5198, PSPTO5200, and PSPTO3659) displayed an RhpR-independent expression. Except the promoter DNA of PSPTO3659, the remaining 10 promoter DNAs all exhibited a clear RhpR-dependent enrichment in ChIP assay (Fig. III-3), even though some of the corresponding genes did not show an RhpR-dependent regulation in Northern blot analysis (Fig. III-5).

Because RhpR represses the induction of genes in the T3 gene regulatory cascade in MM, I also tested if RhpR binds the promoters of the known T3 regulatory genes including *hrpR*, *hrpL*, and *rpoN* (Xiao and Hutcheson 1994; Xiao et al. 1994; Hendrickson et al. 2000). The promoters of these genes do not contain a putative IR element (Buell et al. 2003). The promoter DNA of these genes did not exhibit an RhpR-



dependent enrichment in ChIP assay (Fig. III-3), indicating that RhpR does not bind these promoters.

**Mutation or overexpression of PSPTO2036, PSPTO2767, PSPTO0536, and PSPTO0897 does not affect bacterial pathogenicity.**

The differential expression of PSPTO2036, PSPTO2767, PSPTO0536, and PSPTO0897 in the WT strain and *rhpS*<sup>-</sup> mutant raised the question if these genes connect RhpR and the *hrpRS-hrpL*-T3 gene regulatory cascade. Mutants were generated for the four genes either by double crossover recombination (for PSPTO2036) or single crossover inactivation (for PSPTO2767, PSPTO0536, and PSPTO0897). The mutations were confirmed by Southern hybridization (data not shown). The four mutant strains and the WT strain were infiltrated into the host tomato plant, and the bacterial growth was measured four days after inoculation. All the mutants were undistinguishable from the WT in bacterial growth (Fig. III-7, A and B). An *rhpS* PSPTO2036 double mutant was also generated. Bacterial growth assay indicated that *rhpS* PSPTO2036 double mutant and *rhpS*<sup>-</sup> mutant were similarly compromised in bacterial pathogenicity (Fig. III-7B).

Given that the transcription levels of PSPTO2036 and PSPTO2767 are much higher in the *rhpS*<sup>-</sup> mutant compared with the WT, we hypothesized that the elevated transcription of PSPTO2036 or PSPTO2767 accounts for the abolished T3 gene induction in MM. To test this hypothesis, PSPTO2036 and PSPTO2767 were constitutively expressed in the WT strain using the pNm promoter in the pML plasmid (Fig. III-8A). Compared to the WT strain containing an empty pML vector, the overexpression of

PSPTO2036 or PSPTO2767 in the WT strain did not reduce the *hrpL* expression in MM and bacterial pathogenicity in tomato plants (Fig. III-7C, III-8A).

Since the transcription of PSPTO0536 and PSPTO0897 was significantly reduced in the *rhpS* mutant compared to the WT, we proposed that the two genes act as positive regulators of the T3SS genes. However, pathogenicity assay indicated that neither mutation reduced the bacterial growth in tomato plants, which did not support the hypothesis. In addition, the two genes were constitutively overexpressed in the *rhpS* mutant using the pML plasmid. Compared with the *rhpS* mutant, overexpression of neither gene in the *rhpS* mutant enhanced the *hrpL* expression in MM (Fig. III-8B). These results suggested that PSPTO2036, PSPTO2767, PSPTO0536, and PSPTO0897 are unlikely the genes downstream of RhpR that regulate the *hrpRS-hrpL*-T3 gene regulatory cascade.

## DISCUSSION

Like many bacterial TCS genes, the *rhpRS* locus is subject to positive autoregulation by RhpR. ChIP and qRT-PCR assays indicated that RhpR directly binds to the *rhpR* promoter. RhpR regulates the *rhpR* promoter in a phosphorylation-dependent manner. Mutation of the putative phosphorylation site in RhpR protein (D70A) almost abolished its regulatory activity and association with the *rhpR* promoter. Based on these results, we propose that phosphorylation of RhpR facilitates its interaction with the *rhpR* promoter. The *rhpR*<sub>540</sub>-*luc* reporter gene displayed a low activity in  $\Delta$ *rhpRS* mutant both in MM and in KB, indicating an RhpR-independent basal expression of the *rhpRS* locus. Results from our previous study as well as this study both suggested that, in KB medium, MM,

and in the plants, RhpS serves as a phosphatase to retain RhpR in an unphosphorylated state (Xiao et al. 2007). Similar interactions between a sensor kinase and the cognate response regulator have been reported to several TCSs including QseBC of *E. coli* (Kostakioti et al. 2009), CovRS of *Streptococcus pyogenes* (Dalton et al. 2004), and VanRS of *Streptomyces coelicolor* (Hutchings et al. 2006). In this study, we further showed that the RhpS protein without the extracellular and transmembrane domains could still suppress the RhpR activities to induce its own promoter and to repress the induction of the T3 genes in MM, suggesting that the phosphatase activity of RhpS is constitutive and does not require signal input. This result also implied that the kinase activity of RhpS is probably regulated by signal input. Based on these results, we propose that, upon signal perception, the RhpS kinase activity is stimulated, which in turn phosphorylates RhpR, and the phosphorylated RhpR (P-RhpR) binds to the *rhpR* promoter and activates the expression of *rhpRS*, leading to rapid accumulation of RhpS and RhpR proteins and quick response to the signal.

Deletion analysis revealed a perfect IR element in the *rhpR* promoter that confers the RhpR-dependent gene regulation. I was unable to demonstrate the direct interaction of RhpR protein with the IR element due to the failure to obtain purified RhpR protein. However, based on the requirement of the IR element for the RhpR-dependent induction of *rhpR* promoter, we propose that P-RhpR protein forms a homodimer on the IR element. Point mutations of the repeat modules reduced but did not abolish the *rhpR* promoter activity, suggesting that P-RhpR can dimerize on an imperfect IR element. However, alteration of the spacer length between the repeats completely abolished the promoter activity, suggesting that the space between the two repeat modules is crucial either for

dimerization of P-RhpR on the IR element or the engagement of P-RhpR protein with the RNA polymerase complex. This result also suggested that a single repeat module sequence in the promoter is unlikely to have an RhpR-dependent regulatory activity.

The identification of the IR element enabled computational search of the RhpR-regulated genes in the DC3000 genome. The IR element in the *rhpR* promoter is the only perfect IR element in DC3000. Because mutagenesis analysis indicated that point mutations of the repeat modules only reduced the IR activity, our initial search focused on putative IR elements with one nucleotide mismatch. 44 putative IR elements of this type were identified. Many of these 44 putative IR elements are in the promoters of conserved genes in the three sequenced *P. syringae* genomes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). Some of these IR elements may be functional, because the RhpR proteins in *P. syringae* strains are >98% identical (Xiao et al. 2007), and they are likely to regulate conserved functions.

Further characterization of these putative IR elements was guided by the data from a microarray analysis that was designed to compare gene expression in WT DC3000 and *rhpS* mutant cultured in MM. Nineteen genes (including one gene with two nucleotide mismatches) that displayed a differential expression in the microarray analysis were assayed for their expression in WT DC3000, *rhpS* mutant, and  $\Delta rhpRS$  mutant using RNA blot analysis. This assay identified five genes that showed an RhpR-dependent induction and two genes that showed an RhpR-dependent suppression in *rhpS* mutant. The IR elements of PSPTO2036 and PSPTO2767 were further assayed for their activities to regulate the corresponding promoters. Both IR elements were found to be required for the RhpR-dependent induction of the respective promoters. The remaining IR elements

have not been characterized for their activities to regulate their corresponding promoters. Further characterization of these IR elements will show if these IR elements indeed mediate the RhpR-dependent induction or suppression of their corresponding genes. It should be noted that the IR element in the PSPTO2036 promoter has two nucleotide mismatches in one of the repeat modules, but this IR element still confers a strong RhpR-dependent induction, suggesting that a functional IR element can tolerate more than one variable nucleotide in the repeat modules. Thus, future studies of the IR element should test how mutation of two or more nucleotides in the repeat modules affects the activity of the IR element. Such information is crucial for computational identification of the RhpR-regulated genes. All the three confirmed IR elements are upstream of putative sigma 54 binding site, suggesting that P-RhpR may interact with the sigma 54 protein to activate the transcription of the corresponding genes. Position of response regulator binding site relative to the sigma factor binding site is crucial to its regulatory activities. In general, response regulators that bind upstream of the sigma factor-binding site positively regulate gene transcription (Bijlsma and Groisman 2003). The upstream position of the IR elements relative to the putative sigma 54 binding site is consistent with the positive role of RhpR in regulating the corresponding genes.

PSTTO2767 encodes a putative lipopolysaccharide core biosynthesis domain protein, while PSPTO2036 encodes a putative small lipoprotein (Buell et al. 2003). These genes encode conserved functions in the sequenced *P. syringae* genomes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005). It is interesting that both genes seem to have a bacterial cell wall-related function. The opposite expression patterns of the two cell wall-related

genes and the T3 genes suggested coordination of the T3 gene expression with a cell wall-related function.

ChIP assay was used to determine if RhpR indeed interacts with the promoters carrying a putative IR element. This assay confirmed that RhpR interacts with the three promoters carrying an RhpR-induced IR element (*rhpR*, PSPTO2767, and PSPTO2036). RhpR also interacts with the promoters of the two genes repressed by RhpR (PSPTO0536 and PSPTO0897). Yet, it remains to be determined if RhpR binds the putative IR element in these promoters, and if the binding of RhpR with these promoters mediates the negative regulation of the corresponding genes. Surprisingly, RhpR also interacts with the putative IR promoters that have a putative IR element but did not show an RhpR-dependent regulation. The result is unlikely an artifact of the ChIP assay, because the interaction is specific to the promoters without the IR element, and promoters without the IR element did not show any interaction with the RhpR protein. It is possible that RhpR indeed interacts with these promoters, and the regulation of these promoters requires the function of RhpR. However, activation or suppression of these promoters requires additional proteins that were not present in the test growth conditions (i.e., culture in MM and KB medium). Similar results have been reported to the TCS response regulator CovR that interacts with specific sites not found to be regulated by CovR (Churchward et al. 2009).

Although RhpR has been identified as a suppressor of the *P. syringae* T3 genes, ChIP assays indicated that RhpR does not bind directly to the promoters of the T3 regulatory genes including *hrpR*, *hrpL*, and *rpoN*. These regulatory genes do not have a putative IR element in their promoters. These results suggested that RhpR indirectly regulates the T3

regulatory cascade consisting of *hrpRS*, *hrpL*, and the T3 genes. One or more of the genes directly regulated by RhpR may serve as the link to connect RhpR and the *hrpRS-hrpL*-T3 gene transcriptional cascade.

Among all putative IR-containing genes confirmed by Northern hybridization, PSPTO2036, PSPTO2767, PSPTO0536, and PSPTO0897 displayed the biggest changes in transcript level between the *rhpS*<sup>-</sup> mutant and WT strain. We hypothesized that one or more of these genes may serve as the link to connect RhpR and the *hrpRS-hrpL*-T3 gene transcriptional cascade. However, genetic and molecular analyses of the four genes did not support this hypothesis. Nonetheless, we could not rule out the possibility that two or more of the four genes work in concert to regulate the *hrpRS-hrpL*-T3 gene transcriptional cascade. In this scenario, double or triple mutant of these genes should be studied. In addition, genetic analysis of other genes directly downstream of RhpR should be performed to test if they have a role in regulating the *hrpRS-hrpL*-T3 gene transcriptional cascade. Given the fact that PSPTO2036 carries two mismatches in its putative IR element but still displayed direct regulation by RhpR, it is also possible that some genes carrying two or more mismatches in their putative IRs represent the link between RhpR and the *hrp* transcriptional cascade.

## **MATERIALS AND METHODS**

### **Bacterial strains and media.**

Bacterial strains used in this study were *Pseudomonas syringae* pv. *tomato* DC3000 (Buell et al. 2003) and the *rhpS*<sup>-</sup> and the  $\Delta$ *hrpRS* mutant strains derived from DC3000 (Xiao et al. 2007). *E. coli* DH5 $\alpha$  was used for constructing all plasmids. *E. coli* BL21

strain was used for testing of RhpR-mediated induction of *rhpR* promoter. DC3000 and its derivatives were grown at room temperature in KB (King et al. 1954) containing appropriate antibiotics. *E. coli* strains were cultured in LB at 37°C. Antibiotics (in mg/L) for selection of *P. syringae* strains are: rifampicin, 25; kanamycin, 10; spectinomycin, 50; tetracycline, 10; and gentamycin, 10. Antibiotics (in mg/L) for selection of *E. coli* are: ampicillin, 100; kanamycin, 50; spectinomycin, 100; and gentamycin, 20.

### **Construction of plasmids for promoter analysis.**

All promoter DNA fragments were PCR-amplified using the DC3000 genomic DNA as a template. Primers used for PCR amplifications are listed in Table III-3. To facilitate cloning, all forward primers were added with an *EcoRI* site, and all reverse primers were added with a *BamHI* site.

For *rhpR* promoter deletion analysis, reverse primer rhpR-proR was used with one of the following forward primers, rhpR-pro540F, rhpR-pro170F, rhpR-pro147F, rhpR-pro132F, rhpR-pro120F, rhpR80-pro120F and rhpR-pro40F, to PCR-amplify the *rhpR* promoter fragments of 540, 300, 170, 147, 132, 120, and 40 bps upstream of the *rhpR* orf.

To create point mutations in the IR element of *rhpR* promoter, respective forward PCR primers rhpR-pro147G-TF (-147G to T), rhpR-pro146T-GF (-146T to G), rhpR-pro145A-CF (-145A to C), rhpR-pro144T-GF (-144T to G), rhpR-pro143C-AF (-143C to A), rhpR-pro136G-TF (-136G to T), rhpR-pro135A-CF (-135A to C), rhpR-pro134T-GF (-134T to G), rhpR-pro133A-CF (-133A to C), and rhpR-pro132C-AF (132C to A) were used with the reverse primer rhpR-proR in PCR amplifications.



To insert or delete nucleotide in the spacer of the IR element of *rhpR* promoter, the forward primers rhpR-pro141I1F (one adenine insertion between -141 and -142), rhpR-pro141I4F (four adenine insertion between -141 and -142), rhpR-pro141DF (deletion of -141), and RhpR-pro141-142DF (deletion of both -141 and -142) were used in PCR with the reverse primer rhpR-proR.

A 238-bp fragment (with the putative IR element) and a 222-bp fragment (without the putative IR element) of the PSPTO2767 promoter were PCR-amplified using the forward primers PSPTO2767-pro238F and PSPTO2767-pro222F in combination with the reverse primer PSPTO2767-proR.

A 109-bp fragment (with the putative IR element) and a 93-bp fragment (without the putative IR element) of the PSPTO2036 promoter were PCR-amplified using the forward primers PSPTO2036-pro109F and PSPTO2036-pro93F, respectively, with the reverse primer PSPTO2036-proR.

The PCR products were digested with *EcoRI* and *BamHI*, cloned into pBluescript-SK-*luc* (Deng et al. 2009), sequence-verified, and subsequently cloned into the broad host pHM2 plasmid (Xiao et al. 2007). The resulting plasmids (Table III-2) were introduced into WT DC3000, *rhpS* mutant, and  $\Delta$ *rhpRS* mutant by eletroperation.

To determine the promoter activities, bacteria containing the promoter reporter genes were grown in KB medium to an optical density at 600 nm of 2 ( $OD_{600}=2$ ), washed twice with MM, resuspended in MM to  $OD_{600} = 0.2$ , and cultured at 28°C with constant shaking for 0 and 6 h before the measurement of reporter gene activities. 100  $\mu$ l of bacterial culture was mixed with 1  $\mu$ l of 1 mM luciferin in a 96-well plate, and the

luciferase activities were determined using a cooled CCD camera (Roper Scientific, Trenton, NJ).

#### **Analysis of RhpR-mediated regulation of *rhpR* promoter in *E. coli*.**

pGST::*rhpR* and pGST::*rhpR(D70A)* were constructed to express GST-RhpR and GST-RhpR(D70A) proteins in *E. coli* BL21 strain. The pML122::*rhpR-HA* and pML122::*rhpR-D70A-HA* plasmids (Xiao et al. 2007) were used as template DNA for PCR-amplification of *rhpR* and *rhpR(D70A)*, respectively, with rhpR-GST-F (containing an *XbaI* site) and rhpR-GST-R (containing a *HindIII* site) as primers (Table III-3). The PCR products were digested with *XbaI* and *HindIII*, cloned into pGEX-KG (Guan and Dixon 1991), and sequence-verified. pGST::*rhpR* and pGST::*rhpR-D70A* were transformed into *E. coli* BL21 strain containing the reporter plasmid pHM2::*rhpR<sub>540</sub>-luc*. To determine the LUC activities, bacterial strains were grown at 37°C in LB medium containing spectinomycin and ampicillin to OD<sub>600</sub> = 1. IPTG was added into the cultures to a final concentration of 1mM to induce the GST fusion protein production. One hr after IPTG-induction, 100µl culture was mixed with 1 µl of 1 mM luciferin in a 96-well plate, and the luciferase activity was determined using a cooled CCD camera (Roper Scientific, Trenton, NJ).

#### **Determination of the *rhpR* transcriptional start site.**

5' rapid amplification of cDNA ends (5'-RACE) was performed using the 5'-Full RACE core set (Takara, Japan) and total RNAs prepared from WT DC3000 and *rhpS* mutant following the manufacturer's instructions. First strand cDNAs were prepared from 1µg total RNA with the 5'-phosphorylated reverse transcription primer, DC-rhpR-RTP

(Table III-3), and avian myeloblastosis virus (AMV) reverse transcriptase. The template RNAs were then digested by RNase H, and the cDNAs were circulated by ligation. The circulated cDNAs was then amplified by nested PCR with two pairs of primers: DC-rhpR-PE1 and DC-rhpR-S1 as the first pair, and DC-rhpR-PE2 and DC-rhpR-S2 as the second pair (Table III-3). The PCR products were cloned into the pGEM-T vector (Promega, Wisconsin, WI) and sequenced. Homology of the trapped sequences was searched with the BLASTn program.

### **RNA isolation and Northern blotting.**

Procedures described by Lan and associates (2006) were used for RNA extraction and Northern blotting. The bacterial strains were grown in KB broth (King et al. 1954) to approximately  $OD_{600}=2$  before being harvested for RNA extraction. For gene expression analysis in MM, the bacteria first were grown in KB to  $OD_{600}=2$ , then centrifuged, washed twice with MM (Huynh et al. 1989), resuspended in MM to  $OD_{600}=0.3$  CFU/ml, and cultured for different periods before RNA extraction. Primers that were used to amplify probe sequences are listed in Table III-3. The PCR products were radio-labeled with  $^{32}P$ -dCTP using the Random Primed DNA Labeling kit (Ambion, Austin, TX) as probes.

### **Chromosome immuno-precipitation (ChIP) and quantitative real-time PCR (qRT-PCR).**

The ChIP experiments were performed using the ChIP-IT Express kit (Active Motif, Carlsbad, CA) according to the manufacturer protocol, and the procedures were modified according to Bruscella et al (2008). *P. syringae* bacteria were grown in KB containing

gentamycin overnight. Cross-linking was performed by adding formaldehyde (final concentration 1%) to the medium for 10 min. The reaction was terminated by adding glycine Stop-Fix solution (Active Motif, Carlsbad, CA) and incubating for 10 min at room temperature with gentle agitation. Bacteria were centrifuged for 1 min at 12,000g, washed twice with ice-cold phosphate-buffered saline, resuspended in 1 ml of lysis solution supplemented with 5  $\mu$ l of phenylmethylsulfonyl fluoride (PMSF) and 5  $\mu$ l of protease inhibitor cocktail, and incubated on ice for 30 min. One ml of digestion buffer containing 5  $\mu$ l of PMSF and 5  $\mu$ l of protease inhibitor cocktail was added to the lysate and then heated for 5 min at 37°C. Thirty microliters of an enzymatic shearing mixture (200 U/ml) was added to the digestion mixture. After incubation for 25 min at 37°C with agitation, the reaction was stopped by the addition of 20  $\mu$ l of 0.5 M EDTA and incubation for 10 min on ice. After centrifugation at 15,000g and 4°C for 10 min, the supernatant was recovered, and the shearing efficiency was examined as described by the manufacturer. Preclearing of chromatin samples, input recovery, immunoprecipitation with or without the anti-HA antibody, addition of Protein G beads, washing, elution of DNA-protein complexes, reverse cross-linking, RNA removal, and proteinase K treatment were performed by following the manufacturer's instructions.

The eluted DNA samples from ChIP assay were used for qRT-PCR experiments using Bio-Rad icycler IQ (Bio-Rad, Hercules, CA). PCR primers (Table III-3) for amplification of promoter regions were designed by using the Primer3 software (<http://frodo.wi.mit.edu>). The PSPTO1489 gene (encoding a putative xenobiotic reductase) that is equally expressed in WT DC3000 and *rhpS*<sup>-</sup> mutant (L. Lan and X. Tang, unpublished data) was used for normalization. The SYBR green PCR mixture

(Bio-Rad, Hercules, CA) was mixed with appropriate amounts of ChIP samples for qRT-PCR. The amount of PCR product was estimated for different promoter regions using the input DNA (the total sheared DNA prior to ChIP) and the immunoprecipitated DNA with and without the anti-HA antibodies as the matrix. Threshold cycle (Ct) values were obtained for all samples. The ChIP enrichments were determined by the fold change of amplification between the immunoprecipitated DNA with the antibodies (AB) and the immunoprecipitated DNA without the antibodies, and these was calculated by  $2^{-\Delta Ct}$  ( $\Delta Ct = Ct_{AB} - Ct_{No\ AB}$ ). A standard curve and a melt curve were drawn for each primer pair. The slope of the standard curve was used to calculate the primer efficiency for each primer pair. Results were collected only from the reactions showing primer efficiencies between 95% and 105%. A melt curve was drawn for each primer pair to ensure that only one specific PCR product was obtained. The results for all reactions were obtained from at least two independent experiments.

**Construction of  $\Delta PSPTO2036$ ,  $\Delta PSPTO2036 rhpS$ ,  $PSPTO2767$ ,  $PSPTO0536$ , and  $PSPTO0897$  mutants.**

To construct  $\Delta PSPTO2036$  and  $\Delta PSPTO2036 rhpS$ , an 1.8-kb DNA fragment upstream of  $PSPTO2036$  was PCR-amplified using primers PSPTO2036LF and PSPTO2036LR (Table III-3, *KpnI* and *BamHI* sites are underlined). An 1.8-kb DNA fragment downstream of  $PSPTO2036$  was PCR-amplified using primers PSPTO2036RF and PSPTO2036RR (Table III-3, *BamHI* and *SacI* sites are underlined). The PCR products were digested with *KpnI* and *BamHI* and *BamHI* and *SacI*, respectively, and cloned into the *XbaI* and *SacI* sites of pGEM-7Z, resulting in p7Z-2036FR. A DNA fragment containing the kanamycin resistance gene was PCR-amplified from EZ::Tn<

KAN-2> (Epicentre, Wisconsin, MD), using primers Kan-BF and Kan-BR (Table III-3, *Bam*HI sites underlined), digested with *Bam*HI, and cloned into the *Bam*HI site of p7Z-2036FR, resulting in p7Z-2036FkanR. The *Kpn*I and *Sac*I fragment in p7Z-2036FkanR was cloned into pHM1, and the resulting pHM1::2036FkanR plasmid was introduced into DC3000 WT or *rhpS* strain for marker exchange. Colonies sensitive to spectinomycin but resistant to kanamycin were further verified by PCR and Southern blotting using DNA probes derived from the PSPTO2036 coding region.

To construct PSPTO2767, PSPTO0536, and PSPTO0897 knockout mutants, truncated coding sequences of PSPTO2767, PSPTO0536, and PSPTO0897 were PCR-amplified using corresponding primers (PSPTO2767MF and PSPTO2767MF, PSPTO0536MF and PSPTO0536MF, PSPTO0897MF and PSPTO0897MR, respectively) and inserted into the PCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). The resulting plasmid was introduced into the WT DC3000 strain, and kanamycin resistant clones were verified by PCR and Southern hybridization. The resulting mutants carry truncated coding regions of PSPTO2767, PSPTO0536, and PSPTO0897 lacking the N-terminal 80, 220, and 40 amino acids, respectively, and the C-terminal 47, 409, and 27 amino acids, respectively.

### **Construction of plasmids overexpressing PSPTO2036, PSPTO2767, PSPTO0536, and PSPTO0897.**

The pML122 plasmid (Labes et al. 1990) was used to express PSPTO2036, PSPTO2767, PSPTO0536, and PSPTO0897 in *P. syringae* strains. PSPTO2036, PSPTO2767, PSPTO0536, and PSPTO0897 were amplified by PCR from the DC3000

strain using the primer pairs PSPTO2036OXF and PSPTO2036OXR, PSPTO2767OXF and PSPTO2767OXR, PSPTO0536OXF and PSPTO0536OXR, and PSPTO0897OXF and PSPTO0897OXR, respectively. The PCR products were digested with *Hind*III and *Nhe*I and cloned into pBluescript-HA plasmid between *Hind*III and *Nhe*I. After sequence confirmation, the inserts were released by *Hind*III/*Bam*HI digestion and cloned into the pML122 plasmid predigested with the same enzymes.

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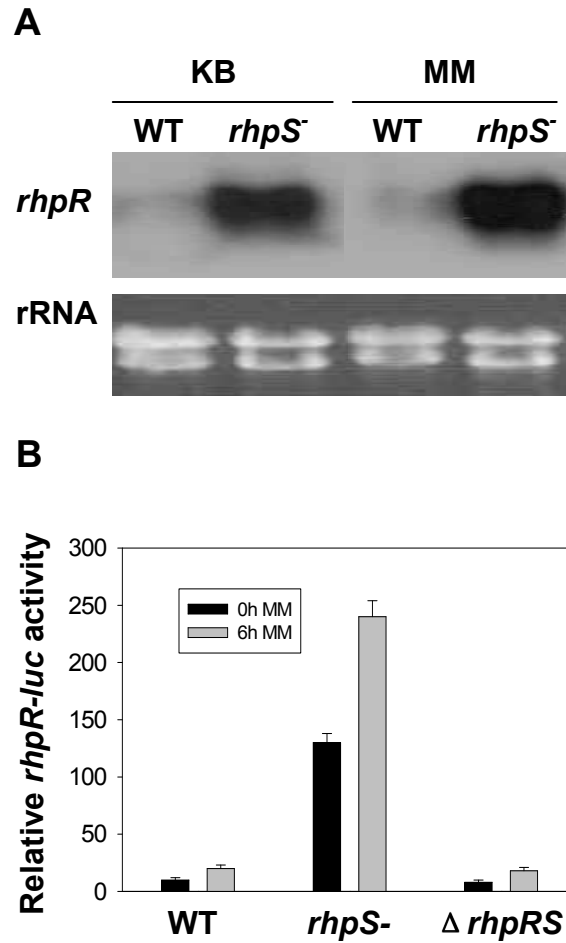
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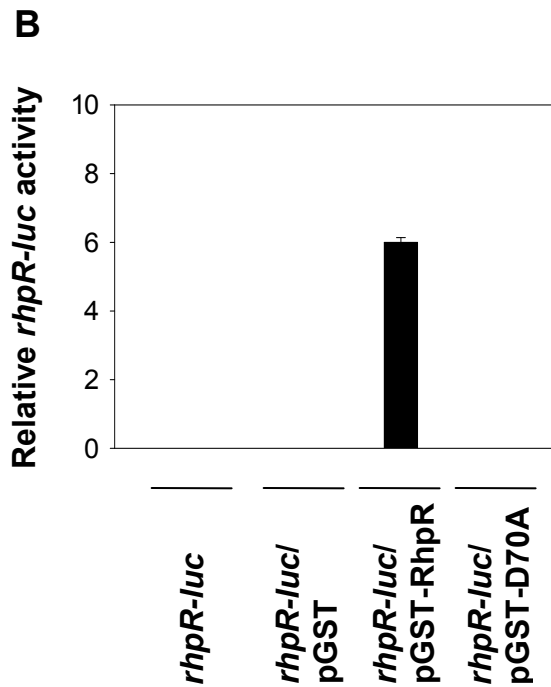
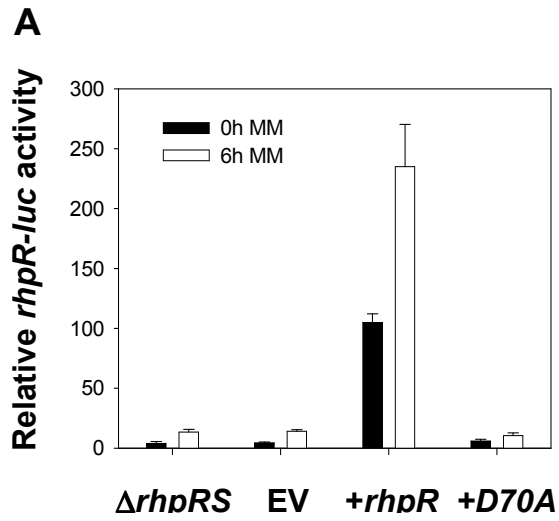


**Fig. III- 1 *rhpR* is highly induced in *rhpS*<sup>-</sup> mutant.**

**A**, Wild type DC3000 and *rhpS*<sup>-</sup> mutant were grown in KB medium and subsequently induced in MM for 6 h before RNA extraction. Total RNA (10  $\mu$ g per sample) was analyzed by RNA blotting with the radio labeled *rhpR* probes. The ethidium bromide-stained RNA gel indicates the loading of RNA samples. **B**, Wild type DC3000, *rhpS*<sup>-</sup> mutant, and  $\Delta rhpRS$  mutant carrying the pHM2::*rhpR*<sub>540</sub>-*luc* reporter gene were cultured in KB and then induced in MM. The luciferase activities were measured at 0 and 6 h after

induction in MM. Each data point represents the average of three measurements. Error bars indicate standard deviations.

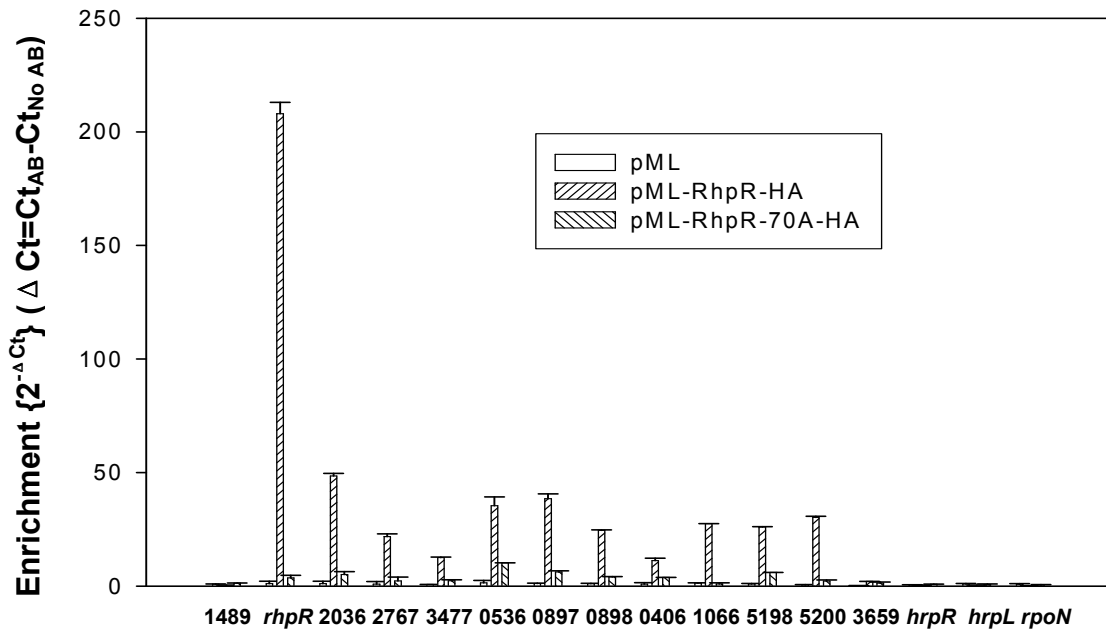




**Fig. III- 2 RhpR requires the phosphorylation site for the induction of *rhpR* promoter.**

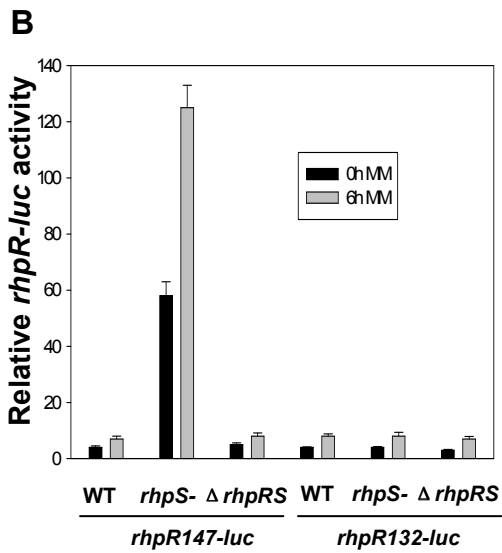
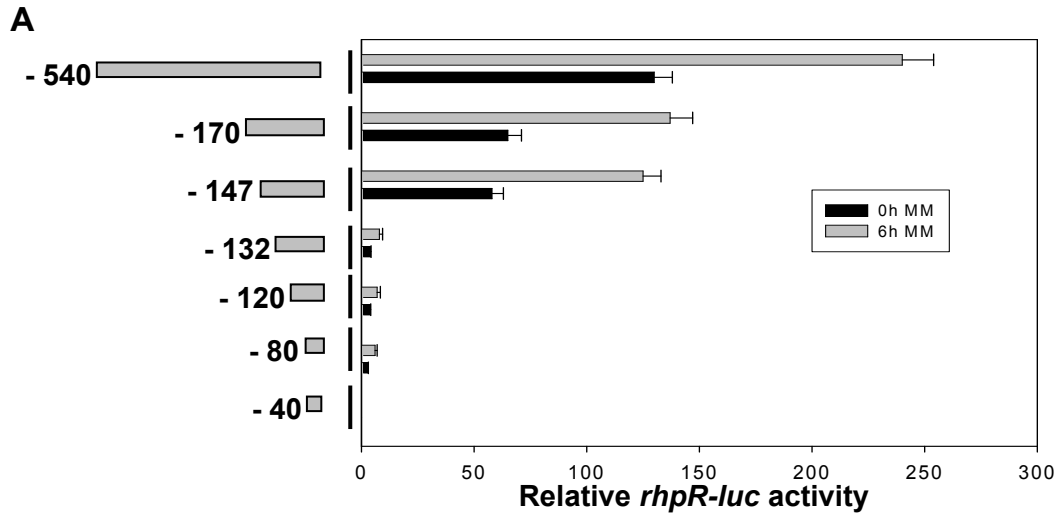
**A**, HA-tagged *rhpR* and *rhpR(D70A)* genes under the constitutive pNm promoter in the pML122 plasmid were expressed in the  $\Delta rhpRS$  mutant carrying the pHM2::*rhpR*<sub>540</sub>-*luc*

reporter.  $\Delta rhpRS$  strain carrying no plasmid and  $\Delta rhpRS$  strain carrying the empty pML122 vector (EV) were included as control. Bacteria were cultured in KB and then induced in MM. The luciferase activities were measured at 0 (KB) and 6h (MM) after incubation in MM. **B**, *rhpR* and *rhpR(D70A)* genes were cloned into the pGST plasmid and introduced into the *E. coli* BL21 strain carrying the pHM2::*rhpR*<sub>540</sub>-*luc* reporter. Bacterial strains were cultured in LB medium and induced with IPTG for the production of GST-RhpR and GST-RhpR(D70A) fusion proteins. Luciferase activities were measured 1 hr after IPTG-induction. Each data point represents the average of three measurements. Error bars indicate standard deviations.



**Fig. III- 3 ChIP-qRT-PCR assay of *in vivo* RhpR binding with promoters.**

*ΔrhpRS* strains containing the pML122 empty vector, pML122::*rhpR-HA* and pML122::*rhpR(D70A)-HA* were used in ChIP assay with and without the anti-HA antibodies. The strains were grown in KB medium. Promoter regions of the selected genes in the immunocomplexes were examined by qRT-PCR. Enrichments of promoter DNAs in the immunocomplexes by the anti-HA antibodies (expressed as fold changes) were calculated as  $2^{-\Delta Ct}$  ( $\Delta Ct = Ct_{AB} - Ct_{No AB}$ ). The results are from three independent experiments. Error bars indicate standard deviations.



**C**

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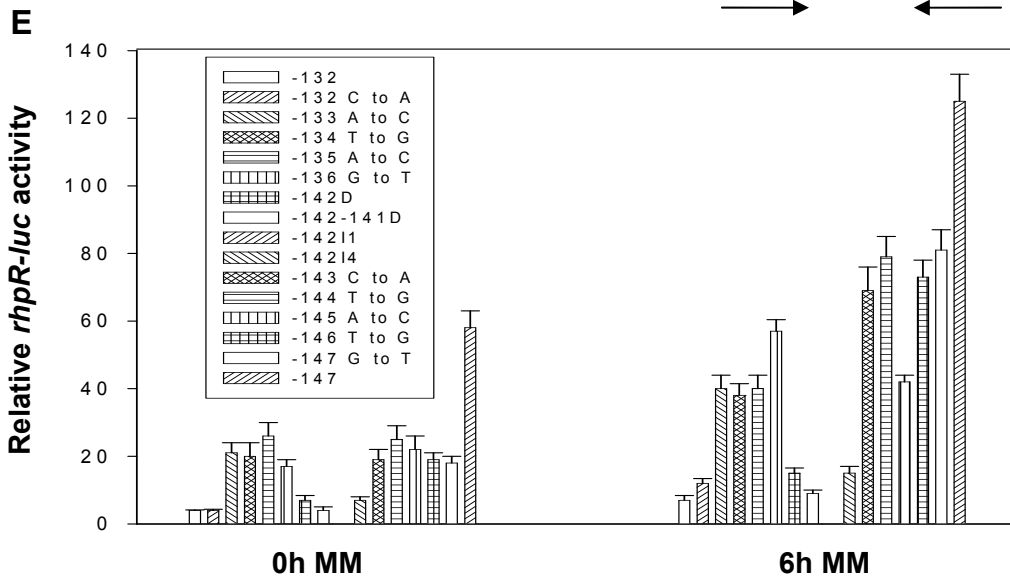
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CGAAGTAGCATGAGCGGCAGCGTTG
ATCTTCAACAGCGGCCAGACAACGG
CGGCACACGCAGGCGACAGACGCAC
TATG

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**D**

$IR_{pst}$	AT	<b>GTATC</b>	CGTATC	<b>GATAC</b>	AT
$IR_{psy}$	TC	<b>GTATC</b>	AATCTA	<b>GATAC</b>	AT
$IR_{pph}$	TT	<b>GTATC</b>	AGGCTG	<b>GATAC</b>	AT

→
←



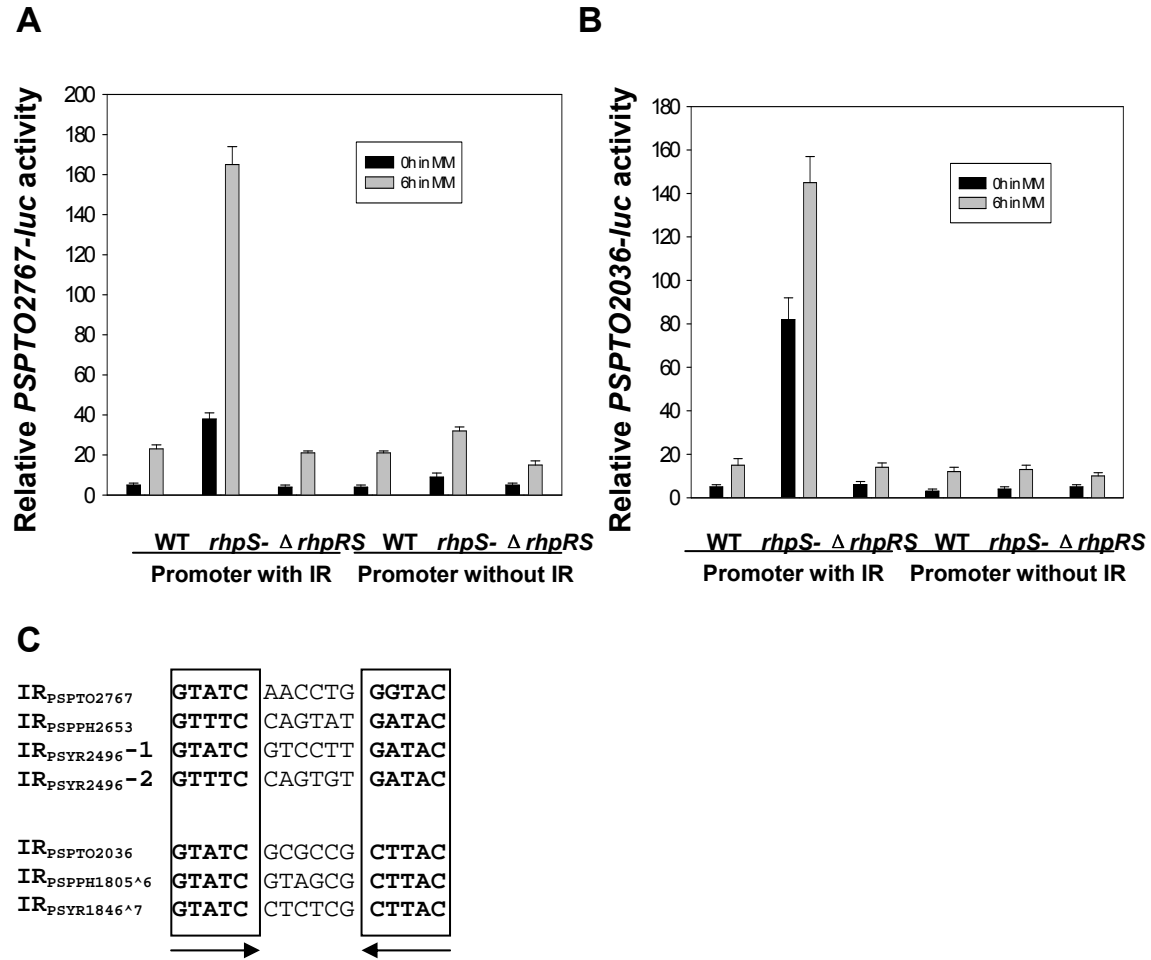
**Fig. III- 4 Identification and mutagenesis analysis of the IR element in the *rhpR* promoter.**

**A,** The *rhpR*<sub>540</sub> promoters of 540, 170, 147, 132, 120, 80, and 40 bp were cloned into the pHM2-*luc* plasmid. The resulting constructs were introduced into the *rhpS*<sup>-</sup> mutant. Bacteria were cultured in KB medium and then incubated in MM for 6 hr before the measurement of luciferase activities. Gray bars in the left indicate the length of promoter deletions. Black and gray bars in the plot indicate the luciferase activities in MM and KB, respectively. **B,** Reporter genes for the 147 and 132 bp *rhpR* promoters were introduced into the *rhpS*<sup>-</sup> and  $\Delta$ *rhpRS* mutants, and the activities were measured as described in A. **C,** Sequence features of the *rhpR* promoter. Translational start codon ATG of *rhpR* is bold. The transcriptional start sites G87 and T165 are underlined. The IR element is bold and underlined. The putative sigma 70 site is italic underlined, and the putative sigma 54 site is bold italic. **D,** Alignment of the IR elements in the *rhpR* promoters of *P. s. pv. tomato* DC3000 (*Pst*), *P. s. pv. syringae* B728a (*Psy*), and *P. s. pv. phaseolicola* 1448A (*Pph*) strains. The inverted repeat modules are in boxes. **E,** Mutagenesis analysis of the IR element. The length of the promoters and the mutations of the inverted repeat modules are indicated by the numbers and letters in the plot legends. 142D and 141-2D represent the deletion of one nucleotide (C<sub>142</sub>) and two nucleotides (G<sub>141</sub> and C<sub>142</sub>) in the spacer, respectively. 142I1 and 142I4 represent the insertion of one adenine and four adenines between G<sub>141</sub> and C<sub>142</sub>, respectively. The mutant promoters were cloned into the pHM2-*luc* reporter plasmid. The activities of the mutant promoters were assayed as described in A. Error bars indicate standard deviations.

PSPTO number	Northern						putative IR		
	WT		<i>rhpS</i> -		$\Delta$ <i>rhpRS</i>				
	KB	MM	KB	MM	KB	MM			
2036							GTATC	GCGCCG	CTTAC
2767							GTATC	AACCTG	GGTAC
3477							GTATC	GCCGCT	GCTAC
3574							GTTTC	AAGACT	GATAC
3660							GTATC	GCAACC	GATGC
0536							GTATC	ACCCCG	GACAC
0897							GTAAC	ACAGAC	GATAC
0406							GTATC	CGACCA	GTTAC
0898							GTATC	GTCTGT	GTTAC
1065							GTTTC	AATGGC	GATAC
1066							GTATC	GCCATT	GAAAC
1543							GTAAC	GTATTT	GATAC
1903							GTATA	CGAGGC	GATAC
2749							GTATC	GTCGTT	GACAC
3099							GTATA	TTTCCG	GATAC
3659							GCATC	GGTTGC	GATAC
3796							GTATC	ATTTCG	GATTC
5198							GTATC	TGCCGT	GACAC
5200							GTGTC	ACGGCA	GATAC
rRNA									

**Fig. III- 5 Northern blot analysis of genes carrying a putative IR element in the promoter.**

Wild type DC3000, *rhpS* mutant, and  $\Delta$ *rhpRS* mutant were grown in KB medium and then incubated in MM for 6 h before RNA extraction. Total RNA (10  $\mu$ g per sample) was analyzed by RNA blotting with radio-labeled probes derived from the coding regions of the corresponding genes. The ethidium bromide-stained RNA gel indicates the loading of RNA samples. The sequences of the putative IR elements in the gene promoters are shown in the right column.

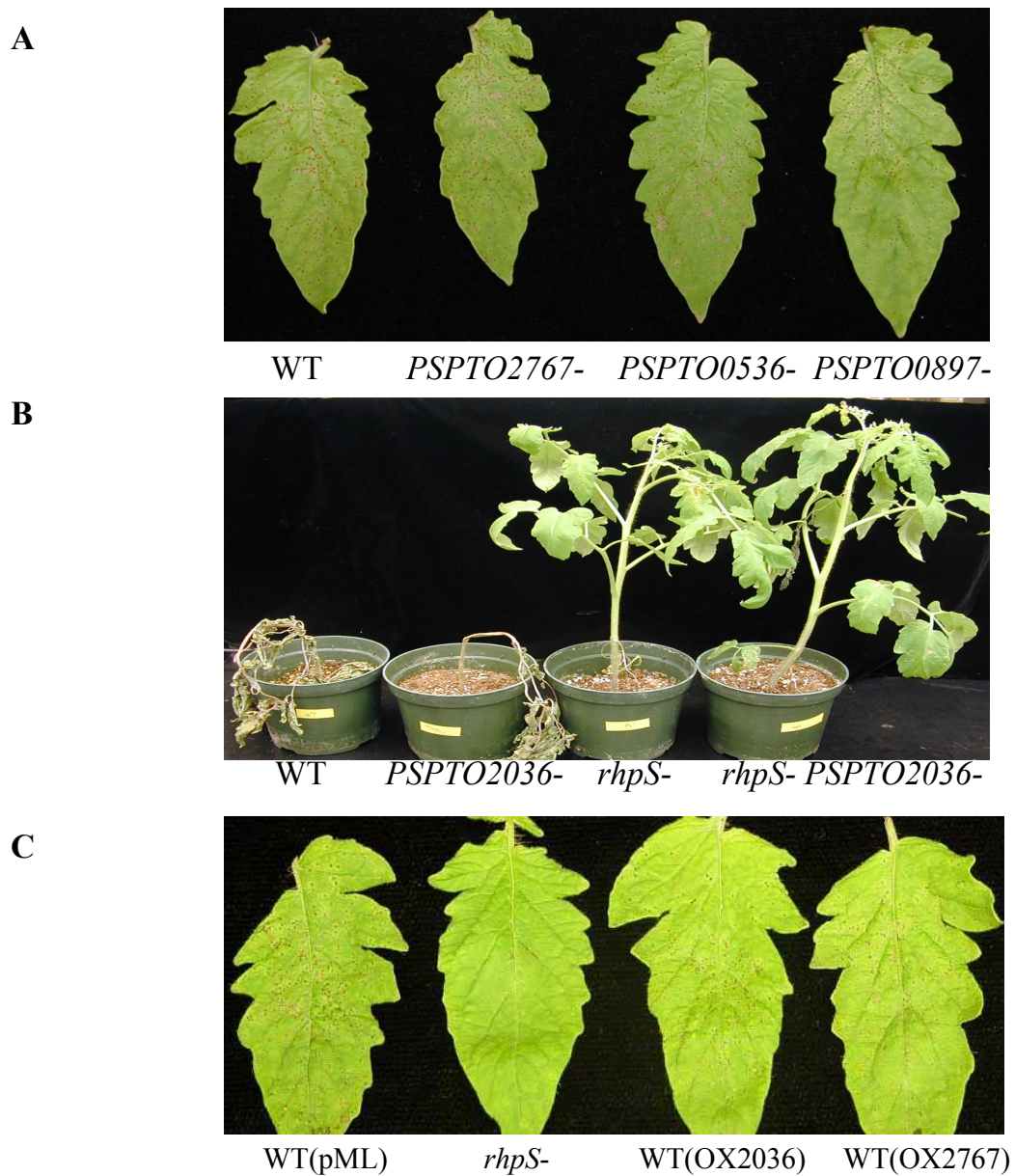


**Fig. III- 6 Characterization of the putative IR elements in the promoters of PSPTO2767 and PSPTO2036 genes.**

PSPTO2767 promoters (**A**) and PSPTO2036 promoters (**B**) with and without the IR element in the pHM2-*luc* plasmid were introduced into wild type DC3000, *rhpS* mutant, and  $\Delta rhpRS$  mutant. The bacterial strains were cultured in KB medium and then incubated in MM. The luciferase activities were measured at 0 and 6 h after incubation in MM. Error bars indicate standard deviations. **C**, Sequences of the putative IR elements



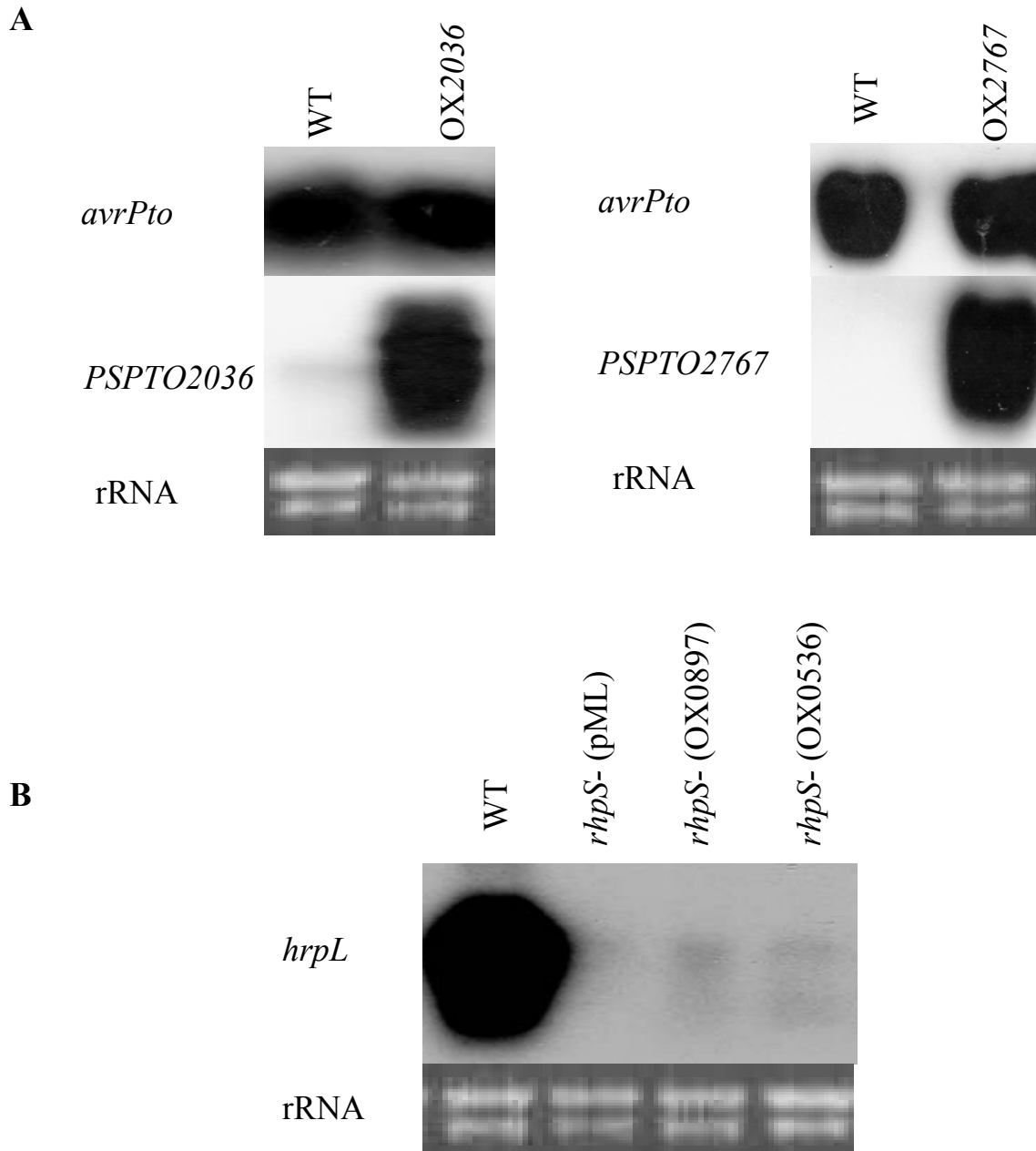
in the promoters of PSPTO2767 and PSPTO2036 and their orthologs in *P. s. pv. syringae* B728a (*Psy*) and *P. s. pv. phaseolicola* 1448A (*Pph*) strains.



**Fig. III- 7 Mutation or overexpression of four RhpR-regulated genes.**

All bacterial strains were grown in rich medium King's B to an optical density at 600 nm of 2.0 to 3.0, washed twice with sterile water, and resuspended in water (plus silwet L-77 at 10  $\mu$ l/liter) to the final concentration of  $2 \times 10^4$  CFU/ml. The bacteria were vacuum infiltrated into tomato. A. Pathogenicity assay of the *PSPTO2767*, *PSPTO0536*, and *PSPTO0897* mutants. The mutations were generated by single crossover in WT DC3000.

Tomato leaves were photographed at 5 days after inoculation. B. Pathogenicity assay of the *PSPTO2036*-, *rhpS* and *rhpS PSPTO2036* mutants. The mutation of *PSPTO2036* was generated by double crossover in WT DC3000 and *rhpS* mutant. Tomato plants were photographed at 10 days after inoculation. C. Pathogenicity assay of WT DC3000 strains overexpressing *PSPTO2036* and *PSPTO2767*. Tomato leaves were photographed at 5 days after inoculation.



**Fig. III- 8 Overexpression of four *rhpRS*-regulated genes does not alter the induction of *avrPto* and *hrpL* in MM.**

A. *avrPto*, *PSPTO2036* and *PSPTO2767* RNA. WT DC3000 strain, WT DC3000 strains overexpressing *PSPTO2036* and *PSPTO2767* were grown in KB medium and induced in

MM for 6 h. Total RNA (10 µg) from each sample was subjected to electrophoresis in a denaturing agarose gel. The blot was hybridized with DNA probes derived from the *avrPto*, PSPTO2036, and PSPTO2767 coding regions. Loading of RNA samples is indicated by rRNA. B. *hrpL* RNA. WT DC3000, *rhpS*<sup>-</sup> mutant, *rhpS*<sup>-</sup> mutant overexpressing PSPTO0897, and *rhpS*<sup>-</sup> mutant strain overexpressing PSPTO0536 were grown in KB medium and induced in MM for 4 h. Total RNA (10 µg) from each sample was subjected to electrophoresis in a denaturing agarose gel. The blot was hybridized with DNA probes derived from the *hrpL* coding region. Loading of RNA samples is indicated by rRNA.

**Table III- 1 Genes containing a putative IR element in their promoters.**

Gene	Function	Position	IR sequence
Group I upregulated genes in the <i>rhpS</i> - mutant			
PSPTO2036	Lipoprotein, putative	-109 to -94	GTATCGCGCCGCTTAC
PSPTO2223	RhpR	-147 to -132	GTATCCGTATCGATAAC
PSPTO2767	LPS core biosynthesis domain protein	-238 to -222	GTATCAACCTGGGTAC
PSPTO3477	Hypothetical TonB-dependent	-290 to -275	GTATCGCCGCTGCTAC
PSPTO3574	siderophore receptor, putative	-103 to -88	GTTTCAAGACTGATAAC
PSPTO3660	Xanthine dehydrogenase	-231 to -216	GTATCGCAACCGATGC
Group II downregulated genes in the <i>rhpS</i> - mutant			
PSPTO0536	Sensory box/GGDEF domain/EAL domain protein	-99 to -84	GTATCACCCCGGACAC
PSPTO0897	DNA-binding response regulator, LuxR family	-68 to -53	GTAACACAGACGATAAC
Group III unchanged genes in the <i>rhpS</i> - mutant			
PSPTO0406	Sensory box/GGDEF domain/EAL domain protein	-163 to -148	GTATCCGACCAGTTAC
PSPTO0898	Sensor histidine kinase/response regulator	-221 to -206	GTATCGTCTGTGTTAC
PSPTO1065	DnaJ domain protein	-248 to -223	GTTTCAATGGCGATAAC
PSPTO1066	Methyl-accepting chemotaxis protein	-111 to -96	GTATCGCCATTGAAAC
PSPTO1543	Outer membrane protein OmpH	-272 to -257	GTAACGTATTTGATAAC
PSPTO1903	Hypothetical	-109 to -94	GTATACGAGGCGATAAC
PSPTO2749	Hypothetical	-217 to -202	GTATCGTCGTTGACAC
PSPTO3099	MexE, multidrug efflux membrane fusion protein	-288 to -273	GTATATTTCCGGGATAAC
PSPTO3659	Transcriptional regulator, GntR family	-59 to -44	GCATCGGTTGCGATAAC
PSPTO3796	GGDEF domain protein	-306 to -291	GTATCATTCGTGATTC
PSPTO5198	Dioxygenase, TauD/TfdA family	-153 to -138	GTATCTGCCGTGACAC
PSPTO5200(2)	Autotransporter, putative	-218 to -203 -105 to -90	GTGTCACGGCAGATAAC GTGTCGTCCCTGATAAC
Group IV untested genes			

PSPTO0076	Hypothetical	-326 to -311	GTTTCATCTGGGATAC
PSPTO0095	Phospholipase D family protein	-71 to -56	GTATCGTGGGCGAGAC
PSPTO0189	Nitrilase, putative	-154 to -139	GTATCGAAAAAGATGC
PSPTO0769	ABC transporter, ATP-binding protein	-78 to -63	GTATCGCGCCTGAAAC
PSPTO1468	PurT, phosphoribosylglycinamide formyltransferase 2	-255 to -240	GCATCCCGCTGGATAC
PSPTO1566	Hypothetical	-36 to -21	GTCTCACCCCTCGATAC
PSPTO1567	ISPsy6, transposase	-367 to -352	GTATCGAGGGTGAGAC
PSPTO2053	Hypothetical	-354 to -339	GTAACGTATCAGATAC
PSPTO2055	SpeE, spermidine synthase	-206 to -191	GTATCTGATACGTTAC
PSPTO2185	EtfB-2, electron transfer flavoprotein	-281 to -266	GTAACGGTCAAGATAC
PSPTO2224	Hypothetical	-250 to -235	GTATCGATACGGATAC
PSPTO2292	Phosphoenolpyruvate synthase	-246 to -231	GTTTCGGCGGTGATAC
PSPTO2794	Hypothetical	-57 to -42	GTATAGCCGTCGATAC
PSPTO2885	Transposase_34	-148 to -133	GTATCGAGTGCGATAG
PSPTO3098	Methyl-accepting chemotaxis protein	-29 to -14	GTATCCCGAAATATAC
PSPTO3254	Transcriptional regulator, GntR family	-44 to -29	GTATCGCAGCCTATAC
PSPTO3266	Phosphate ABC transporter	-232 to -217	GTATCACCGGCGAAAC
PSPTO3478	Hypothetical	-230 to -215	GTAGCAGCGGCGATAC
PSPTO3797	Hypothetical	-107 to -92	GAATCACGAATGATAC
PSPTO3903	Hypothetical	-150 to -135	TTATCAGCGTAGATAC
PSPTO3913	Hypothetical	-347 to -332	GTATCGGTGCAGAAAC
PSPTO4154	Hypothetical	-334 to -319	GTATCAAAACAGATGC
PSPTO4588	HopS2, type three effector	-226 to -211	GTATCGCGCTGGATAT
PSPTO5482	Response regulator	-252 to -237	GTATCTCGAGCGAAAC

**Table III- 2 Plasmids.**

plasmids	description	reference
pML122::rhpR-HA	rhpR in pML122 plasmid, under pNm promoter	Xiao et al. 2007
pML122::rhpR(D70A)-HA	Derived from pML122::rhpR-HA, with Asp70 replaced by Ala	Xiao et al. 2007
pML122	Broad-host plasmid	Labes et al. 1990
pHM2	Broad-host plasmid	Xiao et al. 2007
pBluescript-SK-luc	firefly luc in pBluescript-SK(+)	Deng et al. 2009
pGEX-KG	plasmid to produce GST-fusion protein	
pHM2::rhpR-pro-540-luc	rhpR-luc (-540 from ATG) reporter in pHM2	this study
pHM2::rhpR-pro-300-luc	rhpR-luc (-300 from ATG) reporter in pHM2	this study
pHM2::rhpR-pro-170-luc	rhpR-luc (-170 from ATG) reporter in pHM2	this study
pHM2::rhpR-pro-147-luc,*	rhpR-luc (-147 from ATG) reporter in pHM2	this study
pHM2::rhpR-pro-132-luc	rhpR-luc (-132 from ATG) reporter in pHM2	this study
pHM2::rhpR-pro-120-luc	rhpR-luc (-120 from ATG) reporter in pHM2	this study
pHM2::rhpR-pro-80-luc	rhpR-luc (-80 from ATG) reporter in pHM2	this study
pHM2::rhpR-pro-40-luc	rhpR-luc (-40 from ATG) reporter in pHM2	this study
pHM2::rhpR-pro-147GT-luc	Derived from *, with -147G replaced by T	this study
pHM2::rhpR-pro-146TG-luc	Derived from *, with -146T replaced by G	this study
pHM2::rhpR-pro-145AC-luc	Derived from *, with -145A replaced by C	this study
pHM2::rhpR-pro-144TG-luc	Derived from *, with -144T replaced by G	this study
pHM2::rhpR-pro-143CA-luc	Derived from *, with -143C replaced by A	this study
pHM2::rhpR-pro-136GT-luc	Derived from *, with -136G replaced by T	this study
pHM2::rhpR-pro-135AC-luc	Derived from *, with -135A replaced by C	this study
pHM2::rhpR-pro-134TG-luc	Derived from *, with -134T replaced by G	this study



luc	G	
pHM2::rhpR-pro-133AC-luc	Derived from *, with -133A replaced by C	this study
pHM2::rhpR-pro-132CA-luc	Derived from *, with -132C replaced by A	this study
pHM2::rhpR-pro-141D-luc	Derived from *, with -141C deleted	this study
pHM2::rhpR-pro-141-142D-luc	Derived from *, with -141C and -142G deleted	this study
pHM2::rhpR-pro-141I1-luc	Derived from *, with 1 A inserted between -141C and -142G	this study
pHM2::rhpR-pro-141I4-luc	Derived from *, with 4 A inserted between -141C and -142G	this study
pHM2::PSPTO2767-pro-238-luc	PSPTO2767-luc (-238 from ATG, contating IR) reporter in pHM2	this study
pHM2::PSPTO2767-pro-222-luc	PSPTO2767-luc (-222 from ATG, without IR) reporter in pHM2	this study
pHM2::PSPTO2036-pro-109-luc	PSPTO2036-luc (-109 from ATG, containing IR) reporter in pHM2	this study
pHM2::PSPTO2036-pro-93-luc	PSPTO2036-luc (-93 from ATG, without IR) reporter in pHM2	this study
pGST-rhpR	<i>rhpR</i> in pGEX-KG plasmid	this study
pGST-rhpR-D70A	Derived from pGST-rhpR, with Asp70 replaced by Ala	this study
p7Z::PSPTO2036FR	Intermediate construct for marker exchange	this study
p7Z::PSPTO2036FkanR	Intermediate construct for marker exchange	this study
pHM1::PSPTO2036FkanR	for marker exchange	this study
TOPO-PSPTO2767mid	for single crossover	this study
TOPO-PSPTO0536mid	for single crossover	this study
TOPO-PSPTO0897mid	for single crossover	this study
pML122::PSPTO2036-HA	PSPTO2036 in pML122 plasmid, under pNm promoter	this study
pML122::PSPTO2767-HA	PSPTO2767 in pML122 plasmid, under pNm promoter	this study
pML122::PSPTO0536-HA	PSPTO0536 in pML122 plasmid, under pNm promoter	this study
pML122::PSPTO0897-HA	PSPTO0897 in pML122 plasmid, under pNm promoter	this study

**Table III- 3 Primers.**

Primer	Sequence
DC-rhpR-F	AACATATGATGCAAGCACTTCCCGAC
DC-rhpR-R	AAGGATCCACCCAGCTCCCTGGCATC
DC-rhpR-pro-540F	TTGAATTCAAACGTCCTTGTTC AACG
DC-rhpR-pro-300F	TTGAATTC TTTAAGCCAGCCGAAACC
DC-rhpR-pro-170F	TTGAATTCGTGGTTCGTTCACTCATC
DC-rhpR-pro-147F	TTGAATTCGTATCCGTATCGATACATTC
DC-rhpR-pro-132F	TTGAATTCATTCACGACATCCGCCTG
DC-rhpR-pro-120F	TTGAATTC CCGCCTGACATCGGCCAG
DC-rhpR-pro-80F	TTGAATTCACGGCGAAGTAGCATGAG
DC-rhpR-pro-40F	TTGAATTC CCGGCCAGACAACGGCGGC
DC-rhpR-pro-R	TTGGATCCCATAGTGCGTCTGTGCGCC
DC-rhpR-proF147GT	TTGAATTC TTATCCGTATCGATACATTC
DC-rhpR-proF146TG	TTGAATTC CGGATCCGTATCGATACATTC
DC-rhpR-proF145AC	TTGAATTC GTCTCCGTATCGATACATTC
DC-rhpR-proF144TG	TTGAATTC GTAGCCGTATCGATACATTC
DC-rhpR-proF143CA	TTGAATTC GTATACGTATCGATACATTC
DC-rhpR-proF136GT	TTGAATTC GTATCCGTATCTATACATTC
DC-rhpR-proF135AC	TTGAATTC GTATCCGTATCGCTACATTC
DC-rhpR-proF134TG	TTGAATTC GTATCCGTATCGAGACATTC
DC-rhpR-proF133AC	TTGAATTC GTATCCGTATCGATCCATTC
DC-rhpR-proF132CA	TTGAATTC GTATCCGTATCGATAAATTC
DC-rhpR-proF142D	TTGAATTC GTATCGTATCGATACATTC
DC-rhpR-proF142-141D	TTGAATTC GTATCTATCGATACATTC
DC-rhpR-proF14211	TTGAATTC GTATCACGTATCGATACATTC
DC-rhpR-proF14214	TTGAATTC GTATCAAACGTATCGATACATTC
DC-rhpR-RTP	(P)ATCAGGTCGAGCAC
DC-rhpR-PE1	CATAGTGCGTCTGTGCGCC
DC-rhpR-PE2	GGTCGATGTGGCATCAAG
DC-rhpR-S1	TGACCGACGGTTCGCAGATG
DC-rhpR-S2	CAGGCACTCACCGATGAAAC
rhpR-GST-F	AATGTCTAGAACGCACTATGCAAGCAC
rhpR-GST-R	AACTAAGCTTGATCAACCCAGCTCCCTG
PSPTO1489-pro101F	GCAGGAACCCCTCTCGTTATC
PSPTO1489-pro21R	GGCAACCTCTCGTAATGAAAA
PSPTO2223-pro188F	TTTAAAGGCGTAAGCGTCGT
PSPTO2223-pro12+8R	GCTTGCATAGTGCGTCTGTC

PSPTO2036-pro232F	CTGTGTTTCCGTGTGGGTTT
PSPTO2036-pro97R	ATCTGGTCGCCAACCTGTAA
PSPTO2767-pro400F	TCATTCCGGGCTATCTGAAG
PSPTO2767-pro184R	AATAGCGGGGCTAAGTCGAT
PSPTO3477-pro283F	CGCTGCTACGACACTGATGT
PSPTO3477-pro305R	GCAGGCAGTAGCACAGGTTA
PSPTO0536-pro147F	TCAGACATTGGTCTGGTTGC
PSPTO0536-pro22R	GTGGCTCAAGTCCGTGTTTG
PSPTO0897-pro114F	TCTTTCCCGAACGTCGATAC
PSPTO0897-pro21R	CCTCTGCTTCCGGTATTTTA
PSPTO0898-pro179F	GTATCGACGTTCCGGGAAAGA
PSPTO0898-pro54R	TTAAGGCTCCAGGCTCATTG
PSPTO0406-pro240F	CCTCTCGAGAAGCTTGAACC
PSPTO0406-pro111R	GAGACCACAGTGGCTTAGTGC
PSPTO1066-pro167F	ACTTGCTACGAAAGCGATCC
PSPTO1066-pro58R	AAACAGAGGCGATGCATTTT
PSPTO5198-pro127F	CGACAAAGTATGCGGACGTA
PSPTO5198-pro27R	TCCA ACTCCAGAACAGTGTGA
PSPTO5200-pro175F	GGTCTGCGCCTTATTCAAAC
PSPTO5200-pro52R	TGATCCTGTCGTCACCTGAG
PSPTO3659-pro217F	GCCAATTGGGTCAATTTGTT
PSPTO3659-pro110R	GCACGAAATACGCAAAACCT
DChrpR-pro166F	AGCCTGAGTCTATCGGTAGGG
DChrpR-pro36R	GGGTGGCAAGCGGAGTATTA
DC-hrpL-pro178F	AGCTGACCGATGTTTTTGTG
DC-hrpL-pro58R	CGATAACCATGCCAGCTTAAA
DCRpoN-pro110F	ACTCTAGGCAAAGGCACAGG
DCRpoN-pro20R	GGCAGGGGCTAAACACCTTA
PSPTO2036-F	ATGTTGAGTCGAGTAGCAAG
PSPTO2036-R	TTAGCGCTCGCCGCCACCC
PSPTO2767-F	ATGACTAGTCCATCTATCATTG
PSPTO2767-R	TTAATGACCGATTACTGCGTC
PSPTO3477-F	TTGGTCAAGCAGTTCCAATC
PSPTO3477-R	TTACTCGCTGGCCTTGAAGC
PSPTO3574-F	TTGAGCATCTCCTCCCAACG
PSPTO3574-R	TTAAAACGTGACGCTGGCGC
PSPTO3660-F	GTGATTCAGTTCCTTTTG
PSPTO3660-R	TCAGACATAGTCGGTCAC
PSPTO0536-F	ATGGCTGGATTAATGATCGA
PSPTO0536-R	TTATAACGGCGGTTTCGCGG
PSPTO0897-F	ATGTCGTGCAGAATCATAGTG

PSPTO0897-R	TTACCTGATAGCCAGGCTGG
PSPTO0406-F	ATGAAAAGCCAAACCGATGC
PSPTO0406-R	ACGCGTCAGTTTTTCGATCAG
PSPTO0898-F	ATGAAAGACCGGAAAAACGC
PSPTO0898-R	TTTCGCGAACCGACAGCAGGTTCA
PSPTO1065-F	ATGACCACCCCGCCCGCG
PSPTO1065-R	TATCGGAACAGGGTGTC
PSPTO1066-F	ATGAACAGTCTTTTGTCACC
PSPTO1066-R	CGCCGTATTGTCATTCAGCG
PSPTO1543-F	GTGCGTAAGTTGACTCAATTG
PSPTO1543-R	TTACTTCAGCTGGTTCATGC
PSPTO1903-F	ATGACTCAGCTAGAAAAAGC
PSPTO1903-R	CTACTTCCAGTTCGAGGCCTTC
PSPTO2749-F	ATGAAGCTTTTCAGACTA
PSPTO2749-R	TCACAGTTCGGCCCCAT
PSPTO3099-F	ATGACCCAGACACTCAGCC
PSPTO3099-R	GGAGGCGCTGGCGATTTTGAC
PSPTO3659-F	ATGACGTTCAAGGCCCCG
PSPTO3659-R	TCAGCTCGCTTCCAGAGC
PSPTO3796-F	ATGAATGGTGAAATGCAGAC
PSPTO3796-R	AACCTCTTCGCCCTCACCCATC
PSPTO5198-F	ATGCCAGCAGCCTCCCTC
PSPTO5198-R	TCAAAACGGCGCAGTGCC
PSPTO5200-F	TTGTGGACCACCGGCGCG
PSPTO5200-R	TTAAAACGCCAACGTCAC
PSPTO2767-pro238F	TTGAATTCGTATCAACCTGGGTACAA
PSPTO2767-pro222F	TTGAATTCAACTAGTGGCCAAACAAT
PSPTO2767-proR	TTGGATCCCATGACTACCCTGTGAGCAC
PSPTO2036-pro109F	TTGAATTCGTATCGCGCCGCTTACAG
PSPTO2036-pro93F	TTGAATTCAGGTTGGCGACCAGATCG
PSPTO2036-proR	TTGGATCCCATCCGGATTCACTCTCTC
PSPTO2036LF	TTCAGTGGTACCCATCGCCTTGTATGCCTA
PSPTO2036LR	TCAGTTCCCGGGCCGGATTCACTCTCTCGA
PSPTO2036RF	TCAGTTCCCGGGAAGTGCATGCTGCCCGTG
PSPTO2036RR	TTCTGAGAGCTCAGAGGACAATGTGCGAAA
PSPTO2767MF	TTCTATGTGTGCTCTGAC
PSPTO2767MR	GACTACATGCTTTGCCAT
PSPTO0536MF	CTTCGCAATGTCAATGGC
PSPTO0536MR	AAGGCTGCCGGACTCTTC
PSPTO0897MF	CTGATGCTTGCGCGCTCG
PSPTO0897MR	TATACGCACGGTGAAGGG

PSPTO2036OXF	TTAAGCTTATGTTGAGCTGAGTAGC
PSPTO2036OXR	CTAGCTAGCGCGCTCGCCGCCACCCGG
PSPTO2767OXF	TT AAGCTTAGTGCTCACAGGGTAGTC
PSPTO2767OXR	CTAGCTAGCATGACCGATTACTGCGTC
PSPTO0536OXF	TTAAGCTTAACACGGACTTGAGCCACTT
PSPTO0536OXR	CTAGCTAGCTAACGGCGGTTTCGCGGCTG
PSPTO0897OXF	TTAAGCTTAAAATACCGGAAGCAGAGGC
PSPTO0897OXR	CTAGCTAGCCCTGATAGCCAGGCTGGAAA

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## Chapter 4

**Lon protease and a putative sigma 70 family protein are suppressors of the  
*Pseudomonas syringae rhpS* mutant.**

## ABSTRACT

*Pseudomonas syringae* bacteria depend on the type III secretion system (T3SS) to translocate effector proteins into host cells. The T3SS and T3SS effector genes (together called T3 genes hereafter) are repressed in rich medium King's broth (KB) but rapidly induced after the bacteria are transferred into minimal medium (MM) or infiltrated into the plant. A transposon insertion mutant of the two component system sensor kinase *rhpS* was isolated previously that has repressed the induction of the T3 genes in MM and in the plant. The inhibition is mediated by *rhpR*, the cognate response regulator gene of *rhpS*. *rhpR* is immediately upstream of *rhpS*, and the two genes are co-transcribed as a polycistronic RNA. RhpR directly activates the *rhpR* promoter and a few promoters carrying an inverted repeat (IR) element. RhpR represses the T3 genes and activates the IR element promoters in a phosphorylation-dependent manner. To identify additional genes involved in the *rhpR*-mediated repression of the T3 genes, suppressor mutants were screened that restored the induction of the T3 reporter gene *avrPto-luc* in *rhpS* mutant in MM. Determination of the transposon-insertion sites led to the identification of *rhpR*, *lon*, sigma 70 family protein gene P3PPH1909, and a few metabolic genes. A *lon*<sup>-</sup> *rhpS*<sup>-</sup> double mutant exhibited phenotypes typical of a *lon*<sup>-</sup> mutant, suggesting that *rhpS* acts with or through *lon*. The expression of *lon* was elevated in *rhpS*<sup>-</sup> and other T3-deficient mutants, indicating a negative feedback mechanism. *hrpL* is expressed at higher level in the *lon*<sup>-</sup> *rhpS*<sup>-</sup> and P3PPH1909<sup>-</sup> *rhpS*<sup>-</sup> double mutant than the *rhpS*<sup>-</sup> mutant in MM.

## INTRODUCTION

*Pseudomonas syringae*, a Gram-negative plant pathogen, relies on the type III secretion system (T3SS) for successful infection of host plants (Jin et al. 2003) and elicitation of hypersensitive response in the resistant plants and nonhost plants. Through the T3SS, *P. syringae* secretes an array of type III effector proteins into the plant cells. Genes encoding the T3SS and effectors (hereafter called the T3 genes) are repressed in rich medium such as King's B (KB) medium (King et al. 1954) but induced in minimal medium (MM) and in the plant (Tang et al. 2006). Induction of the T3 genes is directly regulated by HrpL, an alternate sigma factor that is essential for the induction of genes carrying a *hrp* box in their promoters (Xiao et al. 1994). The *hrpL*-based induction depends on another alternate sigma factor, RpoN ( $\sigma^{54}$ ), and two NtrC-family transcription factors, HrpR and HrpS (Hendrickson et al. 2000; Hutcheson et al. 2001; Xiao et al. 1994). HrpR and HrpS physically interact with and activate the RpoN-dependent *hrpL* promoter (Hutcheson et al. 2001).

The HrpR protein is degraded by Lon, an ATP-dependent protease that also degrades unstable or misfolded proteins of various biological functions (Bretz et al. 2002). HrpR is unstable in KB but is stabilized in the *lon*<sup>-</sup> mutant, leading to elevated expression of the T3 genes in KB medium (Bretz et al. 2002; Lan et al. 2007). In addition, the *lon*<sup>-</sup> mutant hypersecretes T3 effectors, suggesting a Lon-associated degradation of these effectors. The effectors have been shown to be protected from Lon degradation by their cognate chaperones prior to secretion (Losada and Hutcheson. 2005). In *lon*<sup>-</sup> mutant, the expression of *hrpL* exhibits a dynamic change in MM. *hrpL* is transcribed at a higher level in the *lon*<sup>-</sup> mutant than in the wild-type strain shortly after



induction in MM, but it is more abundant in the WT strain at later time points (Lan et al. 2007).

The *hrpRS* operon is regulated by two two-component systems, GacAS and RhpRS (Chatterjee et al. 2003; Xiao et al. 2007). In the strain *P. syringae* pv. *tomato* DC3000, a mutation in the response regulator gene *gacA* significantly reduces the transcription of *hrpRS*, *rpoN*, and *hrpL* (Chatterjee et al. 2003). A transposon insertion in the sensor kinase gene *rhpS* abolishes the induction of T3 genes in MM and in the plant (Xiao et al. 2007; Deng et al. 2009). However, disruption of the cognate response regulator gene *rhpR* in the *rhpS*<sup>-</sup> mutant completely restores the *hrpRS* induction, suggesting that RhpR is a negative regulator of *hrpRS*. Overexpression of RhpR in the deletion mutant  $\Delta$ *rhpRS* suppresses the induction of the T3 genes in a phosphorylation-dependent manner (Xiao et al. 2007). *rhpR* regulates itself and other downstream genes under an inverted repeat element promoter (Deng et al. submitted). Based on these observations, we propose that RhpR is phosphorylated by an unknown factor in *rhpS*<sup>-</sup> mutants and the phosphorylated RhpR represses the T3 genes. In wild-type bacteria, RhpS acts as a phosphatase and retains RhpR in a dephosphorylated state under the T3 gene-inducing conditions.

To further dissect the signaling pathway connecting RhpR and the T3 genes, suppressor mutants were screened in the *rhpS*<sup>-</sup> background that restored the induction *avrPto-luc*, a reporter gene of the T3 genes, in MM. This chapter describes the isolation and characterization of *lon* and PSPPH1909, two suppressors of the *rhpS*<sup>-</sup> mutant.

## RESULTS

### Isolation of suppressor mutant of *rhpS*<sup>-</sup> using the *avrPto-luc* reporter.

Our previous studies indicated that the induction of the *hrpRS-hrpL*-T3 cascade is repressed in *rhpS*<sup>-</sup> mutant (Xiao et al. 2007). To understand the molecular mechanism underlying the repression, mutants were isolated that can restore the induction of the T3 genes in *rhpS*<sup>-</sup> mutant. The LUC activity derived from *avrPto-luc* is ~20 fold lower in the *rhpS*<sup>-</sup> mutant than in the WT strain in MM (Xiao et al. 2007). The *rhpS*<sup>-</sup> mutant carrying *avrPto-luc* was subjected to EZ::TN<Tet-1> transposon insertion mutagenesis. 15,000 double mutant clones were screened for higher LUC activity than that in the *rhpS*<sup>-</sup> mutant strain 6 h after induction in MM. 13 mutants were recovered from the screen (Table IV-1).

Transposon insertion sites in these mutants were determined using a two-stage semi-degenerate PCR (Jacobs et al. 2003), and the flanking sequences were searched against the *PspH* 1448A genomic sequence (Joardar et al. 2005). Thirteen mutants are distributed in 10 loci, including 3 regulatory genes (*rhpR*, *lon*, and PSPPH1909) (Table IV-1; Fig. IV-1). Three mutants were derived from independent insertions in *rhpR*. The isolation of *rhpR* as a suppressor of the *rhpS*<sup>-</sup> mutant was expected given the previous observations that the deletion mutant of the *rhpRS* locus and the WT strain showed similar levels of the induction of the T3 genes (Xiao et al. 2007).

Two mutants were of the *lon* gene encoding an ATP-dependent protease. Lon negatively regulates the *P. syringae* T3SS in rich medium by degrading the protein HrpR (Bretz et al. 2002). Compared to the WT strain, *lon*<sup>-</sup> mutant displayed higher induction of

the T3 genes within 2 hr after induction in MM, but the induction of the T3 genes turned lower 6 hr after induction in MM (Lan et al. 2007). In MM, *lon<sup>-</sup> rhpS<sup>-</sup>* double mutant and the WT strain displayed similar levels of LUC activities 6 hr after induction in MM (Table IV-1, Fig. IV-1).

The third regulatory gene is PSPPH1909 that encodes a putative sigma 70 family protein. There are 15 genes encoding putative sigma 70 family proteins in *P. syringae* genome. PSPPH1909 is the one that is most similar to PvdS, the major iron starvation sigma factor of *Pseudomonas aeruginosa*; the two proteins share 87% identity (Joardar et al. 2005; Tiburzi et al. 2008) In *P. aeruginosa*, PvdS regulates the transcription of pyoverdine and virulence genes under iron limitation by competing with the major sigma factor RpoD (Tiburzi et al. 2008). After induction in MM for 6 hr, the LUC activity derived from *avrPto-luc* in the *PSPPH1909<sup>-</sup> rhpS<sup>-</sup>* double mutant was about 3-fold higher than that in the parent *rhpS<sup>-</sup>* mutant (Table IV-1; Fig. IV-1).

The remaining 7 mutant genes encode a putative membrane protein (PSPPH3067) and 6 metabolic enzymes, including exodeoxyribonuclease V, pyoverdine sidechain peptide synthetase IV, NADH-quinone oxidoreductase (A, K, and M subunit), and phosphoheptose isomerase. These double mutants displayed ~ 2-fold increase in the LUC activity compared to the *rhpS<sup>-</sup>* mutant (Table IV-1).

**The *lon<sup>-</sup> rhpS<sup>-</sup>* double mutant and the *lon<sup>-</sup>* mutant exhibited similar phenotypes in MM and *in planta*.**

To confirm the effect of the *lon<sup>-</sup> rhpS<sup>-</sup>* mutations on the induction of the T3 genes, we examined the *hrpL* RNA in WT *Psph*, *rhpS<sup>-</sup>* mutant, *lon<sup>-</sup>* mutant, and *lon<sup>-</sup> rhpS<sup>-</sup>* double

mutant strains by Northern hybridization. Consistent with previous reports, the *lon*<sup>-</sup> mutant exhibited a marginal increase of the *hrpL* RNA levels in KB media and reduced *hrpL* RNA in MM 4 h after induction in MM (Bretz et al. 2002; Lan et al. 2007). After 4 h of incubation in MM, the *rhpS*<sup>-</sup> mutant severely reduced the level of *hrpL* RNA, while the *lon*<sup>-</sup> *rhpS*<sup>-</sup> double mutant largely restored this defect in MM, indicating that the RNA level of *hrpL* correlates well with the LUC activity derived from the *avrPto-luc* reporter in the *lon*<sup>-</sup> *rhpS*<sup>-</sup> double mutant (Fig. IV-2A).

To determine if there is a correlation between the T3 gene expression in MM and pathogenicity in host plants for the *lon*<sup>-</sup> *rhpS*<sup>-</sup> double mutant, four strains (WT *PspH*, *rhpS*<sup>-</sup> mutant, *lon*<sup>-</sup> mutant, and *lon*<sup>-</sup> *rhpS*<sup>-</sup> double mutant) were infiltrated at 2×10<sup>4</sup> CFU/mL into the primary leaves of host bean plants. After 6 days of growth in the plants, the WT strain exhibited the most concentrated specks and the highest bacterial population. The *lon* mutation showed slightly fewer specks and 2~3-fold less growth than did the WT strain. The *rhpS*<sup>-</sup> mutant was symptom-free and exhibited more than 100-fold less growth than did the WT bacteria (Fig. IV-2B). Compared to the *rhpS*<sup>-</sup> mutant, the *lon*<sup>-</sup> *rhpS*<sup>-</sup> double mutant restored the symptoms and bacterial growth to the same levels as that of the *lon*<sup>-</sup> mutant (Fig. IV-2C). These data confirmed that *lon* is a suppressor of the *rhpS*<sup>-</sup> mutant.

#### ***lon* expression was induced in the *rhpS*<sup>-</sup> mutant and other T3-deficient mutants.**

Given that *lon* is positive regulator of *hrpL* in WT strain and a negative regulator of *hrpL* in *rhpS*<sup>-</sup> mutant after 4 hr incubation in MM, we proposed that the *rhpS*<sup>-</sup> mutation alters the expression of *lon* under the same condition. To test this possibility, Northern

blots were performed on *P. s. pv. phaseolicola* WT strain and the *rhpS*<sup>-</sup> mutant to compare the *lon* RNA levels. The level of *lon* RNA was severely reduced in MM compared to KB medium in the WT strain (Fig. IV-3, A, B, and C). The *lon* reduction would elevate the level of HrpR, allowing the induction of T3 genes in MM. Compared with the WT strain, the *rhpS*<sup>-</sup> mutant clearly increased the *lon* RNA levels in MM (Fig. IV-3A). Western blots further confirmed that more Lon protein was produced in the *rhpS*<sup>-</sup> mutant than in WT bacteria in MM (Fig. IV-3B). The elevated Lon protease in the *rhpS*<sup>-</sup> mutant explains its negative role in regulating the T3 genes in MM. To determine whether this is a general phenomenon, the *P. s. pv. tomato* DC3000 *lon*<sup>-</sup> mutant was tested, and this mutant exhibited similar results (Fig. IV-3C). We propose that RhpR in the *rhpS*<sup>-</sup> mutant directly or indirectly upregulates the *lon* expression, which leads to degradation of HrpR and reduced expression of the T3 genes in MM.

### **RhpR is associated with the *lon* gene promoter.**

Chromatin immunoprecipitation (ChIP) assay showed that RhpR binds to a few IR-containing promoters (Deng et al. submitted). Because *lon* expression was induced in the *rhpS*<sup>-</sup> mutant, ChIP assay was performed to test whether if RhpR is associated with the *lon* promoter. *rhpRS* mutant strains expressing RhpR-HA and RhpR-D70A-HA (containing a mutation in the predicted phosphorylation site) proteins were used for the ChIP assay. Mutation of D70A disrupts the activities of RhpR to repress the induction of the T3 genes in MM and to bind the IR element promoters (Xiao et al., 2007; Deng et al. submitted). Our previous analysis indicated that RhpR-HA and RhpR-D70A-HA were expressed at similar levels (Xiao et al. 2007). The *rhpRS* mutant carrying the empty pML122 plasmid was used as a control. ChIP was performed with the anti-HA antibody.

Quantitative PCR (qPCR) was used to detect the *lon* promoter DNA in the immunocomplexes. More *lon* promoter DNA was detected in association with RhpR than with RhpR-D70A, suggesting that RhpR is associated with the *lon* promoter (Fig. IV-3D).

**The *lon* RNA is feedback-regulated by other T3 gene regulators.**

The level of *lon* RNA was examined in several T3-deficient mutants, including *hrpS*<sup>-</sup> and *hrpR*<sup>-</sup> mutants of *P. s. pv. phaseolicola*, and *hrpS*<sup>-</sup>, *hrpR*<sup>-</sup>, *hrpL*<sup>-</sup>, and *gacA*<sup>-</sup> mutants of *P. s. pv. tomato* DC3000 grown in MM. Surprisingly, *lon* RNA was induced in all mutants in MM, as compared to the WT strains (Fig. IV-3A; Fig. IV-3C), suggesting a negative feedback regulation of *lon* by other T3 gene regulators.

**Mutation of PSPPH1909 elevated the *hrpL* RNA and reduced the *rhpR* RNA in *rhpS*<sup>-</sup> mutant in MM.**

To verify the effect of *PSPPH1909*<sup>-</sup> *rhpS*<sup>-</sup> mutation on the expression of the T3 genes, *hrpL* RNA was examined in WT *Psph*, *rhpS*<sup>-</sup> mutant, and *PSPPH1909*<sup>-</sup> *rhpS*<sup>-</sup> double mutant using Northern blot. After 4 h of incubation in MM, the *hrpL* RNA was low in the *rhpS*<sup>-</sup> mutant but slightly elevated in the *PSPPH1909*<sup>-</sup> *rhpS*<sup>-</sup> double mutant, indicating that the level of *hrpL* RNA was well correlated with the LUC activity derived from the *avrPto-luc* reporter (Fig. IV-2A).

RhpR is known to be responsible for the repression of the T3 genes in *rhpS*<sup>-</sup> mutant in MM. To determine if the elevated induction of *hrpL* RNA in the *PSPPH1909*<sup>-</sup> *rhpS*<sup>-</sup> double mutant was associated with reduced level of *rhpR* RNA in the mutant, *rhpR* RNA

was examined in WT *PspH*, *rhpS*<sup>-</sup> mutant, and *PSPPH1909*<sup>-</sup> *rhpS*<sup>-</sup> double mutant using Northern blot. The *rhpR* RNA was slightly higher in the *rhpS*<sup>-</sup> mutant than in the *PSPPH1909*<sup>-</sup> *rhpS*<sup>-</sup> double mutant (Fig. IV-2A), which is consistent with the role of RhpR in suppressing the T3 genes.

## DISCUSSION

To identify additional players in the pathway underlying the RhpRS-mediated regulation of the T3 genes, a transposon-insertion mutant library was constructed in the *PspH* NPS3121 *rhpS*<sup>-</sup> mutant and screened for suppressors of the *rhpS*<sup>-</sup> mutant based on the *avrPto-luc* reporter activity in MM. The *rhpS*<sup>-</sup> mutation severely inhibits *avrPto-luc* induction in MM, providing a clean background for the suppressor screening (Xiao et al. 2007). From a total of ~15,000 mutants, 10 mutant genes were isolated. The screening was ~2.5x coverage of the ~6 Mb *PspH* genome (Joardar et al. 2005), assuming that average bacterial genes are 1 kb. Some of the mutants may be polar, because the mutant gene is organized in an operon with other genes. Six of the 10 mutant genes encode metabolic enzymes; three genes encode regulatory functions, and one gene encodes a membrane protein. Characterization of the reporter gene activities and *hrpL* RNA expression in various mutant strains indicated that the reporter gene activities reflected the T3 gene expression, indicating that the reporting systems are valid. The isolation of *rhpR*<sup>-</sup> *rhpS*<sup>-</sup> double mutant was consistent with our previous finding the RhpR is a negative regulator of the T3 genes in *rhpS*<sup>-</sup> mutant. Here we focused on characterization of the *lon*<sup>-</sup> *rhpS*<sup>-</sup> and *PSPPH1909*<sup>-</sup> *rhpS*<sup>-</sup> double mutants. *lon* mutation largely restored the *avrPto-luc* induction, while *PSPPH1909* mutation partially restored the *avrPto-luc* induction in *rhpS*<sup>-</sup> mutant in MM.

The bacterial Lon protein is a stress-induced ATP-dependent protease that participates in a variety of biological processes by degrading a number of abnormal regulatory proteins under stringent conditions (Tsilibaris et al. 2006). Lon has been reported to downregulate the T3SS in *Salmonella enterica* serovar Typhimurium, but upregulate it in *Yersinia pestis*. In *Salmonella* spp., Lon degrades HilC and HilD, two positive transcriptional regulators of the T3 genes in pathogenicity island 1 (Takaya et al. 2005). However, in *Yersinia* spp., Lon cleaves YmoA, a histone-like protein that represses the expression of the T3 genes (Jackson et al. 2004). In *Pseudomonas syringae*, Lon degrades HrpR in rich medium (Bretz et al. 2002). HrpR is stabilized in a *lon*<sup>-</sup> mutant, leading to elevated expression of the T3 genes in KB medium (Bretz et al. 2002; Lan et al. 2007). In MM, however, the *lon* mutation causes a dynamic change on *hrpL* expression. *hrpL* is expressed at a higher level in the *lon*<sup>-</sup> mutant than in the WT strain shortly after induction in MM, but is more abundant in the WT strain at later time points (Lan et al. 2007). It is possible that the changes in the Lon protease catalytic activity at the later time points lead to more efficient degradation of a different protein (presumably a negative regulator of the T3 gene) rather than HrpR, allowing optimal induction of the T3 genes.

We assume that the *lon* gene plays a major role in degradation of HrpR in KB medium but not so when bacteria are grown in MM (Bretz et al. 2002; Lan et al. 2007). Consistent with this assumption, we found that the *lon* RNA as well as the Lon protein are expressed at a much higher level in the WT *P. syringae* strains when grown in the KB medium rather than in MM. The higher level of Lon protease in KB medium presumably retains the HrpR protein at a low level, which is insufficient to activate the transcription



of *hrpL* and downstream T3 genes. The Lon protease is much reduced when the WT *P. syringae* bacteria are cultured in the MM. The reduced Lon protease presumably allows the stabilization of the HrpR protein and thus the activation of the downstream *hrpL* and T3 genes.

The reduced *hrpL* expression in *lon*<sup>-</sup> mutant at later time points in MM indicated that Lon plays a positive role in regulating the T3 genes. As such, how could mutation of a positive regulator in *rhpS* mutant lead to elevated expression of the T3 genes in the *lon*<sup>-</sup> *rhpS* double mutant in MM? Our analyses of the *lon* RNA and Lon protein in the WT strain and *rhpS* mutant provided clues to this question. We found that Lon was elevated in the *rhpS* mutant compared to the WT strain in MM. The elevated Lon in *rhpS* mutant probably serves as a negative regulator of the T3 gene in MM, as it does in the WT strain in KB medium, to destabilize HrpR, and consequently to suppress the T3 genes. Thus, mutation of *lon* in the *rhpS* mutant would remove the negative regulator, and therefore lead to elevated induction of the T3 genes. Such results suggest a possibility that bacteria use RhpS to sense the nutrient level in environment. In the absence of RhpS, *P. syringae* bacteria are blind to the nutrient poor condition such as MM, and the Lon protease remains active in the degradation of HrpR, and thus keeps the downstream *hrpL* and T3 genes inactive even at the T3 gene-inducing conditions. Consistent with this assumption, gene expression profiles between the *rhpS* mutant and WT DC3000 treated with MM were found to be highly similar to the gene expression profiles between the WT DC3000 in KB and WT DC3000 in MM with microarray analyses (Lan and Tang, unpublished).

Our previous results indicated that RhpR is responsible for the suppression of the T3 genes in *rhpS* mutant (Xiao et al. 2007; Deng et al. submitted). Three pieces of evidence

suggested that *lon* acts downstream of RhpR. (1) the *lon*<sup>-</sup> *rhpS*<sup>-</sup> double mutant was almost indistinguishable from the *lon*<sup>-</sup> single mutant, suggesting that RhpR acts through *lon*. (2) *lon* was clearly induced in the *rhpS*<sup>-</sup> mutant when grown in MM, as compared to the wild type strain. The enhanced *lon* expression in the *rhpS*<sup>-</sup> mutant may account for the T3-repressing phenotype. (3) ChIP-RT-qPCR assay indicated that RhpR associated with the *lon* promoter. RhpR was shown to interact with promoters carrying an IR motif (GTATC-N<sub>6</sub>-GATAC) or variants with one or two mismatches (Deng et al. submitted). Although an IR element with one or two mismatches was not found in the *lon* promoter, a motif containing four variations (GTTTC-N<sub>6</sub>-GCTTG) was found in the *lon* promoter. This motif may play a role in RhpR-mediated induction of *lon* expression in the *rhpS*<sup>-</sup> mutant by direct binding with the RhpR protein. Alternatively, RhpR may associate with the *lon* promoter indirectly via a second protein. However, we can not rule out the possibility that an unknown feedback mechanism is responsible for the higher expression of *lon* RNA in the *rhpS*<sup>-</sup> mutant than in the WT strain in MM. A higher level of *lon* RNA was detected in multiple T3-deficient mutants, strongly suggesting a feedback regulation of the *lon* RNA by the T3 gene expression. The *lon* gene of *Salmonella* has been demonstrated to be involved in a negative feedback regulatory loop mediated by *rpoH* ( $\sigma^{32}$ ) (Matsui et al. 2008). *HilD*, a critical gene of the *Salmonella* T3 regulatory loop, is specifically degraded by *lon*, which is in turn induced by  $\sigma^{32}$ .  $\sigma^{32}$  senses the cellular protein folding environment through negative feedback control mediated by molecular chaperones such as DnaKJ and GroELS. Our previous microarray analysis indicated that  $\sigma^{32}$  (PSPTO0430, *rpoH*) is induced 3-fold in an *hrpRS*<sup>-</sup> mutant as compared to the WT strain, suggesting

that  $\sigma^{32}$  could be the regulator that is responsible for enhanced *lon* expression in the T3-deficient mutants (Lan et al. 2007).

PSPPH1909, a sigma 70 family protein, also plays a role in suppressing the T3 genes in the *rhpS* mutant. Northern blot analysis indicated that *rhpR* RNA was slightly reduced in the *rhpSPSPPH1909* double mutant than in the *rhpS* mutant, suggesting that PSPPH1909 may regulate the *rhpR* expression, which in turn regulates the T3 genes. Interestingly, among the 15 sigma 70 family proteins in *P. syringae*, PSPPH1909 is the one most homologous to PvdS, the major iron starvation sigma factor of *Pseudomonas aeruginosa* (Joardar et al. 2005; Tiburzi et al. 2008), suggesting that PSPPH1909 is probably an ortholog of PvdS. In *P. aeruginosa*, PvdS regulates the transcription of pyoverdine and virulence genes under iron limitation by competing with the major sigma factor RpoD (Tiburzi et al. 2008). Interestingly, among the six metabolic genes identified in our screening, one encodes the pyoverdine side chain peptide synthetase IV, suggesting that pyoverdine synthesis may play a role in repressing the T3 genes in the *rhpS* mutant. Pyoverdine is a siderophore that plays a major role in iron uptake (Taguchi et al. 2009). Iron is a major virulence factor in many pathogenic bacteria. The results suggested that T3 genes in *P. syringae* are regulated by availability of iron.

## **MATERIALS AND METHODS**

### **Plant materials, bacterial strains, culture media and plasmids.**

Bean (*Phaseolus vulgaris* cv. Red Kidney) plants and tomato (*Lycopersicon esculentum* cv. Rio-Grande PtoS) plants were used for assays of disease symptoms and bacterial growth. Plant materials were grown in a greenhouse as described previously

(Xiao et al. 2007). The bacterial strains used in this study were the wild-type *P. s. pv. phaseolicola* NPS3121 and the *rhpS*<sup>-</sup>, *lon*<sup>-</sup>, *lon*<sup>-</sup> *rhpS*<sup>-</sup>, *hrpS*<sup>-</sup> and *hrpR*<sup>-</sup> mutant strains derived from *PspH* NPS3121, *P. s. pv. tomato* DC3000 and the *rhpS*<sup>-</sup>,  $\Delta$ *rhpRS*, *rhpS*<sup>-</sup>, *hrpR*<sup>-</sup>, *hrpL*<sup>-</sup> and *gacA*<sup>-</sup> mutant strains derived from DC3000 (Xiao et al. 2007). These strains were grown in KB medium (King et al. 1954) containing the appropriate antibiotics to an optical density at 600 nm (OD<sub>600</sub>) of 2.0. The bacteria were centrifuged, washed twice with MM (Huynh et al. 1989), resuspended in MM to an OD<sub>600</sub> of 0.2 and cultured in MM for 6 h before extracting RNA or measuring of luciferase activities. Antibiotics (in mg/L) used for the selection of *P. syringae* strains were: rifampicin, 25; kanamycin, 10; spectinomycin, 50; tetracycline, 10; and gentamycin, 10. The plasmids and primers used are listed in Tables IV-2 and IV-3.

### **Screen for suppressor mutants.**

The transposon insertion mutant library was constructed in the *P. s. pv. phaseolicola* NPS3121 *rhpS*<sup>-</sup> mutant carrying the pHM2::*avrPto-luc* reporter plasmid as described previously (Xiao et al. 2007). Mutant colonies grown on KB plates containing rifampicin, kanamycin, spectinomycin and tetracycline were transferred with sterile toothpicks into 100  $\mu$ l of liquid KB media containing the same antibiotics in 96-well plates and cultured for 36 h until complete saturation. The 96-well plates were centrifuged, and the bacteria were washed twice with MM and resuspended in 500  $\mu$ l of MM. After induction in MM for 6 h, 100  $\mu$ l of cell suspension was transferred from each sample to a new 96-well plate and mixed with 10  $\mu$ l of 0.1 mM luciferin. LUC activity was measured using a cooled charge-coupled device (CCD, Roper Scientific, Trenton, NJ, U.S.A.). Mutants displaying more than 5-fold LUC activity relative to the other clones on the same plate

were selected as putative suppressor mutants. These mutants were confirmed for the induction of reporter genes in MM as described previously (Deng et al. 2009).

### **Measurement of reporter gene activities in MM.**

Bacteria were grown in liquid KB medium containing rifampicin and spectinomycin to an OD600 between 2.0 to 2.5. To induce the reporter genes in MM, bacteria were washed twice with MM, resuspended in MM to an OD600 of 0.1, and incubated for 6 h to allow for the induction of *avrPto-luc*. The cell suspension (100 µl) was mixed with 10 µl of 0.1 mM luciferin, and the LUC activity was measured using a cooled CCD (Roper Scientific). After LUC measurement, the bacteria were diluted and plated on TSA plates in order to count CFUs. The relative LUC activity was normalized to the number of bacteria in the MM.

### **Mapping transposon insertion sites.**

The transposon insertion sites were determined by a two stage semi-degenerate PCR according to Jacobs and associates (2003) using two transposon-specific primers (Tet1-SP1 and Tet1-SP2) and four degenerate primers (CEKG 2A, CEEK2B, CEEK 2C and CEEK). The PCR products were sequenced using a third transposon-specific primer, Tet3-SP3. Sequences flanking the transposon DNA were searched against the *P. s. pv. phaseolicola* 1448A genome sequence using the BLASTn program from NCBI.

### **Infiltration inoculation.**

The preparation of bacteria to inoculate plants has been described previously (Shan et al. 2000). Bacteria at a concentration of  $2 \times 10^4$  CFU/ml were hand injected into the

primary leaves of 2-week-old bean plants or tomato leaves for the symptom assays and bacterial growth assays. For the bacterial growth assays, leaf disks (1 cm<sup>2</sup>) were removed at 0 and 6 days after inoculation and ground in sterile water. Bacteria were diluted to the proper concentration and plated on a TSA plate containing rifampicin at 25 mg/L (Xiao et al. 2007) in order to count the bacteria.

### **RNA isolation and Northern blotting.**

The procedures described by Lan and associates (2006) were used for RNA extraction and Northern blotting. The bacterial strains were grown in KB medium (King et al. 1954) to approximately  $1 \times 10^9$  CFU/ml before being harvested for RNA extraction. For gene expression analysis in MM, the bacteria were first grown in KB medium to  $1 \times 10^9$  CFU/ml, then centrifuged, washed twice with MM (Huynh et al. 1989), resuspended in MM to  $3 \times 10^8$  CFU/ml, and cultured for different periods of time before RNA extraction. The primers that were used to amplify the probe sequences are listed in Table IV-3. The PCR products were radio-labeled with <sup>32</sup>P-dCTP using the Random Primed DNA Labeling kit (Ambion, Austin, TX, USA) to create probes.

### **ChIP-qRT-PCR.**

Procedures were as described by Deng and associates (submitted). The ChIP experiments were performed using a ChIP-IT Express kit (Active Motif, CA, USA), with a few modifications (Bruscella et al. 2008). After culture overnight in KB media, 1 ml (OD<sub>600</sub>=1.0) *P. syringae* bacterial cultures were cross-linked with formaldehyde and enzymatically sheared. The following steps were performed following the kit instructions: clearing of the chromatins, input collection, IP with or without anti-HA

antibodies, culture with protein G beads, washing, elution of DNA-protein complexes, reverse cross-linking and RNA and protein digestion. Final DNA samples were tested by quantitative real-time PCR (qRT-PCR) assays (Bio-Rad icycler IQ, CA, USA). The PCR primers used to amplify the *lon* promoter region were designed using Primer3 software (<http://frodo.wi.mit.edu/>) (listed in Table IV-3). The SYBR green PCR mixture (Applied Biosystems, CA, USA) was mixed with the ChIP samples. Threshold cycle (Ct) values were obtained for all samples. ChIP Enrichments were determined by the fold change in amplification between immunoprecipitated DNA with antibodies (AB) and immunoprecipitated DNA without antibodies:  $2^{-\Delta Ct} (\Delta Ct = Ct_{AB} - Ct_{No AB})$ . The results for all samples were obtained from four independently repeated experiments.

#### **Western blot analysis.**

Bacteria grown in KB medium and MM were adjusted with the corresponding media to an OD600 of 1. The bacteria (30  $\mu$ l) were boiled in 1 $\times$  sodium dodecyl sulfate (SDS) sample buffer, loaded onto an SDS polyacrylamide gel and subjected to electrophoresis. A Western blot was performed as described (Shan et al. 2000) with the monoclonal anti-HA antibodies (Sigma, St Louis, MO).

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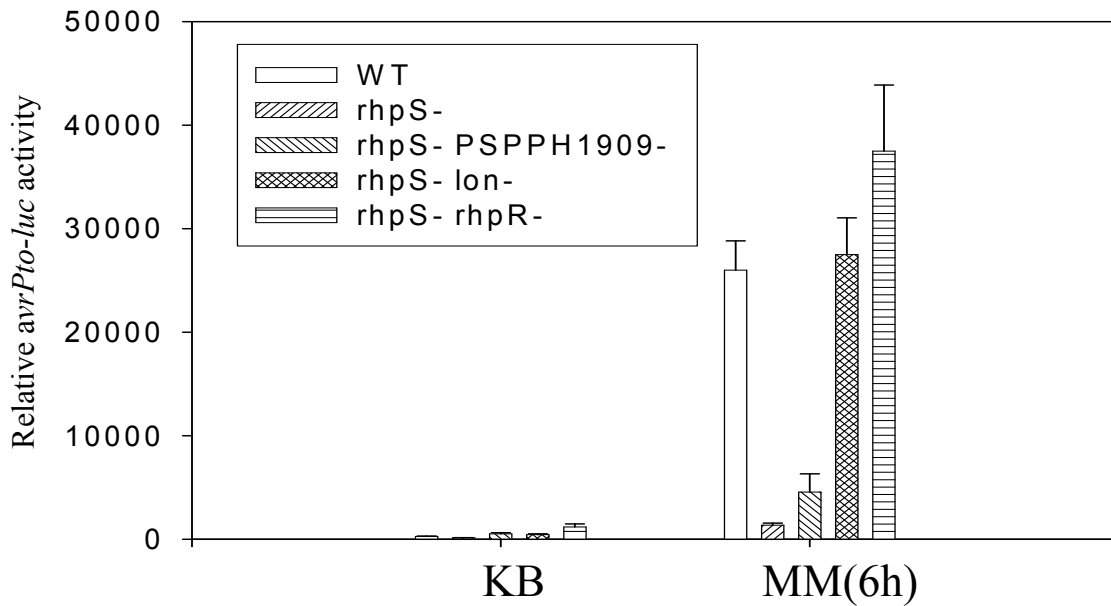
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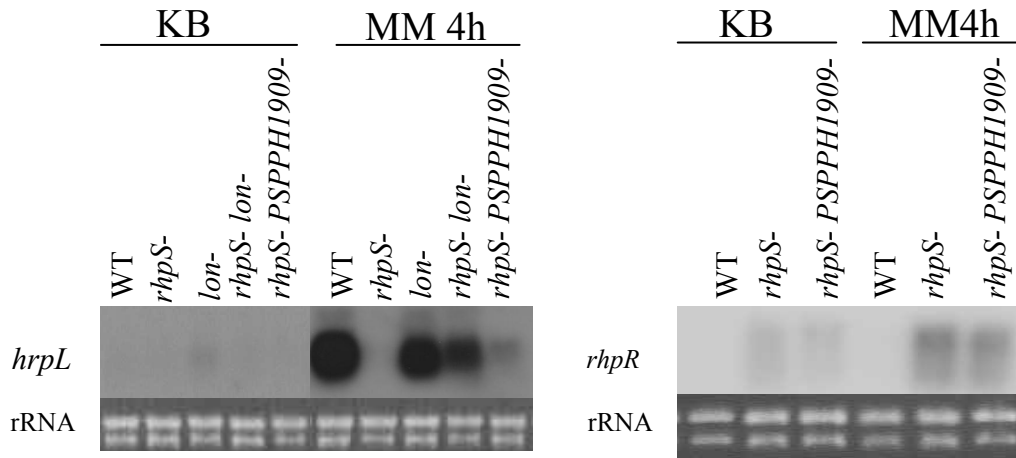
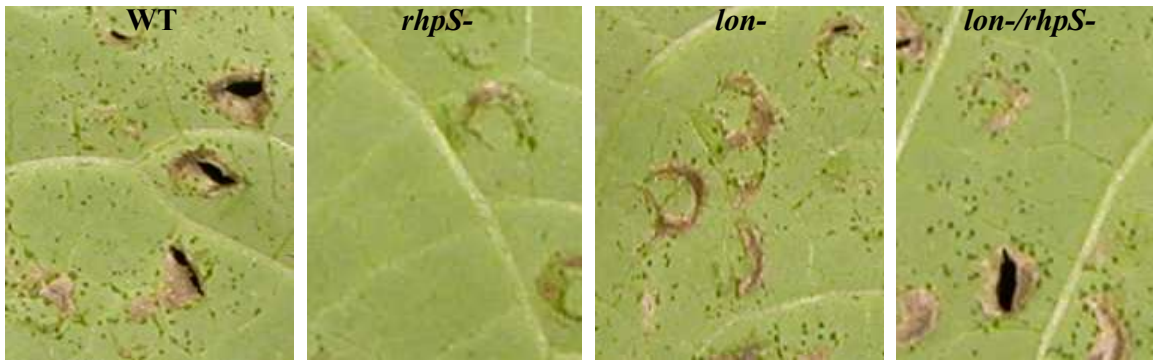
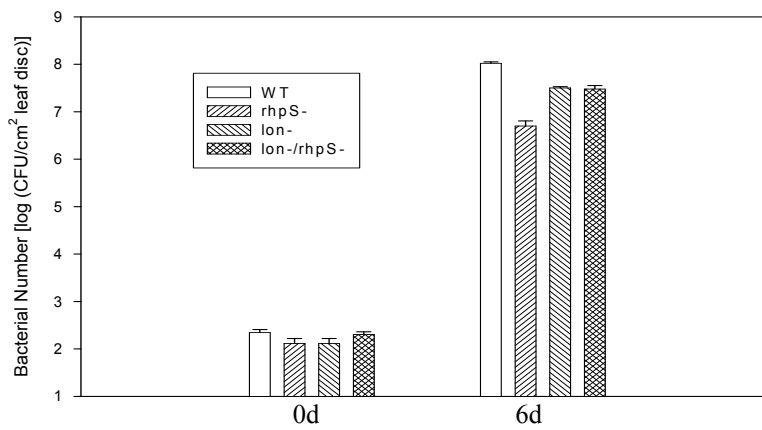
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**Fig. IV- 1 Identification of *rhpR*, *lon*, and *PSPH1909* as suppressors of the *rhpS* mutant.**

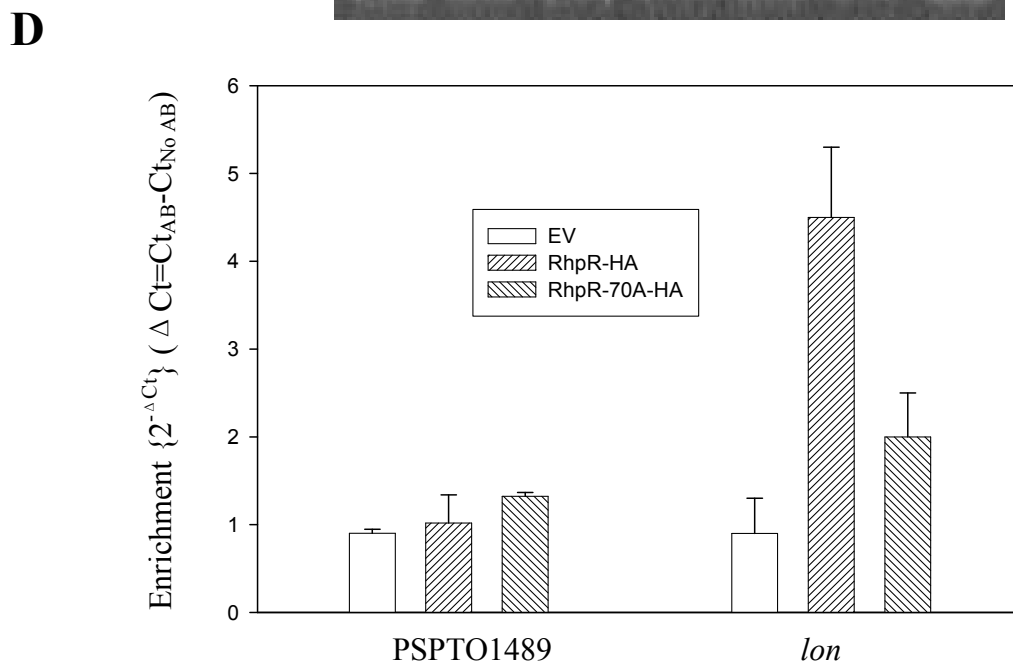
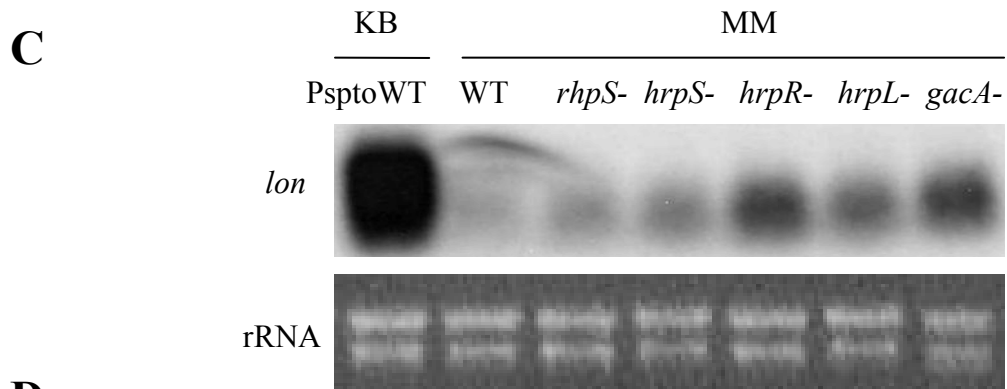
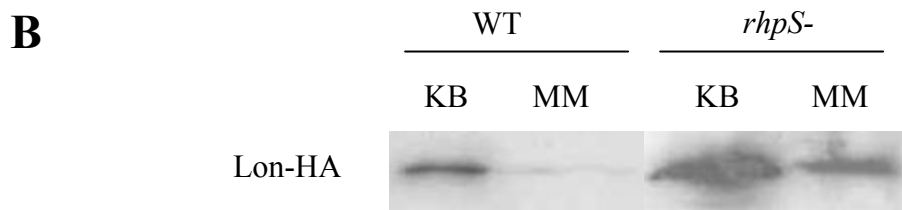
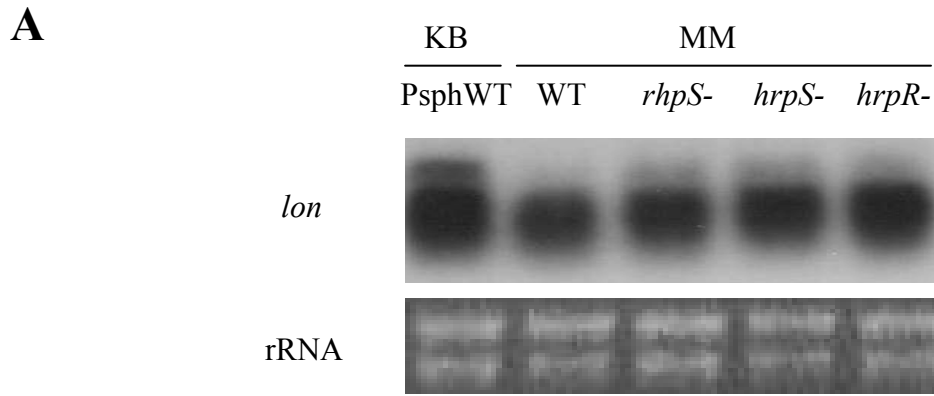
Luciferase (LUC) activity was derived from the *avrPto-luc* reporter. Bacteria were grown in King's B (KB) media and induced in minimal media (MM) for 6 h. LUC activity was measured with a cooled charge-coupled device. Each data point represents three replicates. Error bars indicate standard error.

**A****B****C**

**Fig. IV- 2 The *Psph lon*<sup>-</sup> *rhpS* double mutant and the *lon*<sup>-</sup> mutant exhibited similar phenotypes.**



A. Expression of *hrpL* and *rhpR* RNAs. Wild-type (WT) *P. s. pv. phaseolicola* (*Psph*) NPS3121, the *rhpS* mutant, the *lon*<sup>-</sup> mutant, the *lon*<sup>-</sup> *rhpS* double mutant, and the *PSPPH1909*<sup>-</sup> *rhpS* double mutant were grown in KB medium and induced in MM for 4 h. Total RNA (10 µg) from each sample was subjected to electrophoresis in a denaturing agarose gel. The blot was hybridized with DNA probes derived from the *hrpL* or *rhpR* coding region. Loading of RNA samples is indicated by the presence of rRNA. B. Disease symptoms elicited by infiltrating  $2 \times 10^4$  CFU/ml of wild-type (WT) *Psph* NPS3121, the *rhpS* mutant, the *lon*<sup>-</sup> mutant, and the *lon*<sup>-</sup> *rhpS* double mutant into the primary leaves of 2-week-old bean plants. Disease symptoms were photographed 5 days after inoculation. C. Bacterial growth in host bean plants. Bacteria at a concentration of  $2 \times 10^4$  CFU/ml were injected into primary bean leaves. For each data point, three leaf disks (1 cm<sup>2</sup>) were removed at 0 and 6 days after inoculation and ground separately in sterile water for counting of colony forming units. Error bars represent standard error. The experiment was repeated three times with similar results.



**Fig. IV- 3 *lon* expression was induced in the *rhpS*<sup>-</sup> mutant and other T3-deficient mutants.**

A. Wild-type (WT) *PspH* NPS3121, the *rhpS*<sup>-</sup> mutant, the *hrpS*<sup>-</sup> mutant and the *hrpR*<sup>-</sup> mutant were grown in King's B (KB) medium and induced in minimal medium (MM) for 4 h. Total RNA (10 µg) from each sample was subjected to electrophoresis in a denaturing agarose gel. The blot was hybridized with DNA probes derived from either the *lon* coding region. Loading of the RNA samples is indicated by the presence of rRNA. B. Lon-HA protein derived from a pHM2::*lon*-HA reporter. The *PspH* WT and *rhpS*<sup>-</sup> mutant strains carrying a pHM2::*lon*-HA reporter were grown in KB medium and induced in MM for 4 h. The expression of *lon*-HA in the plasmid is driven by the native *lon* gene promoter. Bacteria were harvested by centrifugation and resuspended in MM to an optical density at 600 nm of 1. Bacteria (30µl) were boiled in 1× sodium dodecyl sulfate (SDS) sample buffer, loaded onto an SDS polyacrylamide gel and subjected to electrophoresis. A Western blot was performed using anti-HA antibodies. C. Wild-type (WT) *P. s. pv. tomato* DC3000, the *rhpS*<sup>-</sup> mutant, the *hrpS*<sup>-</sup> mutant, the *hrpR*<sup>-</sup> mutant, the *hrpL*<sup>-</sup> mutant and the *gacA*<sup>-</sup> mutant were grown in KB medium and induced in MM for 4 h. Total RNA (10 µg) from each sample was subjected to electrophoresis in a denaturing agarose gel. The blot was hybridized with DNA probes derived from either the *lon* coding region. Loading of RNA samples is indicated by the presence of rRNA. D. ChIP-RT-qPCR analysis of *in vivo* binding of RhpR to the *lon* promoter. The *lon* promoter region was examined by performing qRT-PCR with immunoprecipitated samples of *P. syringae* *pv. tomato* DC3000  $\Delta$ *rhpRS* strains (containing either the pML122 empty vector, EV, pML122::*rhpR*-HA or pML122::*rhpRD70A*-HA). The strains were grown in

KB medium. Enrichments (expressed as fold changes) were calculated as  $2^{-\Delta Ct}$  ( $\Delta Ct = Ct_{AB} - Ct_{No\ AB}$ ). The error bars indicate standard deviations. AB, antibody. The control PSPTO1489 gene promoter is not regulated by RhpR.

**Table IV- 1 *rhpS*- suppressors.**

Strain	Protein	<i>avrPto-luc</i> 0h	<i>avrPto-luc</i> 6h
WT		275+/-35	26000+/-2800
<i>rhpS</i> -		140+/-15	1350+/-215
<i>rhpR</i> -/ <i>rhpS</i> -	Response regulator	450+/-70	27500+/-3500
<i>lon</i> -/ <i>rhpS</i> -	ATP-dependent protease	1200+/-280	37500+/-6300
<i>PSPPH0694</i> -/ <i>rhpS</i> -	Exodeoxyribonuclease V, gamma subunit	220+/-30	2400+/-100
<i>PSPPH1909</i> -/ <i>rhpS</i> -	Sigma 70 family protein	525+/-106	4550+/-300
<i>PSPPH1926</i> -/ <i>rhpS</i> -	Pyoverdine sidechain peptide synthetase IV	190+/-30	2650+/-120
<i>PSPPH3067</i> -/ <i>rhpS</i> -	Putative member protein	640+/-80	3400+/-150
<i>PSPPH3109</i> -/ <i>rhpS</i> -	NADH-quinone oxidoreductase, A subunit	900+/-100	3100+/-110
<i>PSPPH3118</i> -/ <i>rhpS</i> -	NADH-quinone oxidoreductase, K subunit	915+/-80	3000+/-180
<i>PSPPH3120</i> -/ <i>rhpS</i> -	NADH-quinone oxidoreductase, M subunit	330+/-40	2700+/-260
<i>PSPPH4121</i> -/ <i>rhpS</i> -	Phosphoheptose isomerase	230+/-50	2900+/-240

**Table IV- 2 Plasmids.**

Plasmid	Description	Reference
pML122:: <i>rhpR</i> -HA	<i>rhpR</i> in pML122 plasmid, under pNm promoter	Xiao et al. 2007
pML122:: <i>rhpR</i> (D70A)-HA	Derived from pML122:: <i>rhpR</i> -HA, with Asp70 replaced by Ala	Xiao et al. 2007
pML122	Broad-host plasmid	Labes et al. 1990
pHM2:: <i>avrPto-luc</i>	<i>avrPto-luc</i> reporter in pHM2	Xiao et al. 2007
pHM2:: <i>lon</i> -HA	<i>lon</i> -HA in pHM2	Lan et al. 2007

**Table IV- 3 Primers.**

Primer	Sequence
Tet1-SP1	TGAGCGCATTGTTAGATTTC
Tet1-SP2	GCTGTCAAACATGAGAATTAC
Tet1-SP3	TAAGATGATCCCCGGGTACC
CEKG 2A	GGCCACGCGTCGACTAGTACNNNNNNNNNNNAGAG
CEKG 2B	GGCCACGCGTCGACTAGTACNNNNNNNNNNNACGCC
CEKG 2C	GGCCACGCGTCGACTAGTACNNNNNNNNNNNGATAT
CEKG 4	GGCCACGCGTCGACTAGTAC
DC-lon-pro195F	TTGCGTGTGAAGTGACACAA
DC-lon-pro110R	GAGAAACACCACGCCAAGAT
DC-hrpL-F	TTGTGATCCTCGACTCAACC
DC-hrpL-R	GGGTCGATTTGCTGCTTG
DC-hrpR-F	GTCGAGGTATCGAGCGTCTG
DC-hrpR-R	AGCGATACCCTGGGTGAACT
PH-hrpL-F	GACTCTTCGTCTGCCGGTAT
PH-hrpL-R	GGGTCAATCTGCTGCTTCAA
PH-hrpR-F	TAATGAACAGCGCGTTTCTG
PH-hrpR-R	AGCAACTCCCAACTCCTTCA
DC-lon-F	TGCGTGATGTCGTGGTTTAT
DC-lon-R	CGCACAAACACTTCCGATT
PH-lon-F	GATTCGTGGCCCTGTACTGT
PH-lon-R	TGGATATGCGTGTCGTGTTT

## FINAL CONCLUSIONS

Several new regulators upstream of *hrpRS* have been identified in *Pseudomonas syringae* that regulates the T3SS genes (Fig. V). The *Psph* screen identified two novel T3-regulatory genes, *aefR* and *rhpS*. *AefR<sub>Psph</sub>*, which encodes a transcription regulator, is homologous to *AefR*, a regulator of the quorum sensing signal and epiphytic traits that was not known previously to regulate the T3 genes in *P. syringae* pv. *syringae* (*Psy*) B728a. *AefR<sub>Psph</sub>* and *AefR<sub>Psy</sub>* are similar in regulating the quorum sensing signal in liquid medium but different in regulating epiphytic traits such as swarming motility, entry into leaves, and survival on the leaf surface. *AefR* positively regulates the transcription of *ahII*, which encodes the AHL synthase. *AefR* also acts upstream of *hrpRS* to stimulate the T3 genes expression.

The two component system RhpRS was identified in *Pseudomonas syringae* as a regulator of the T3 genes (Xiao et al. 2007). In the *rhpS* mutant, the response regulator RhpR represses the induction of the T3 gene regulatory cascade, but induces its own promoter in a phosphorylation-dependent manner. An inverted repeat (IR) element GTATC-N<sub>6</sub>-GATAC in the *rhpR* promoter confers the RhpR-dependent induction. Computational search of the *P. syringae* genomes for the putative IR elements and Northern blot analysis of the genes with a putative IR element in the promoter region identified five genes that were up-regulated and two genes that were down-regulated in an RhpR-dependent manner. RhpR binds the promoters containing a putative IR element but not the *hrpR* and *hrpL* promoters that do not have an IR element, suggesting that RhpR indirectly regulates the transcriptional cascade of *hrpRS*, *hrpL*, and the T3 genes. In T3-repressing conditions or in *rhpS* mutant, RhpR is probably phosphorylated and act



upstream of *hrpRS* to suppress the T3 gene expression. In T3-inducing conditions, RhpS is proposed to act as a phosphatase to dephosphorylate P-RhpR into RhpR, thus removes the P-RhpR-dependent repression of the T3SS.

To identify additional genes involved in the *rhpRS* pathway, suppressor mutants were screened that restored the induction of the *avrPto-luc* reporter gene in the *rhpS*<sup>-</sup> mutant. The suppressor screen identified three regulatory genes: *rhpR*, *lon* encoding an ATP-dependent protease, and PSPPH1909 encoding a putative sigma 70 family protein. The expression of *lon* was elevated in *rhpS*<sup>-</sup> and other T3-deficient mutants, indicating a negative feedback mechanism. RhpR interacts with the *lon* promoter, suggesting that *lon* is a downstream gene of *rhpR*. *PSPPH1909 rhpS*<sup>-</sup> double mutant displayed enhanced transcription of *rhpR* in MM than did the *rhpS*<sup>-</sup> mutant, suggesting that PSPPH1909 positively regulates the transcription of *rhpR*.

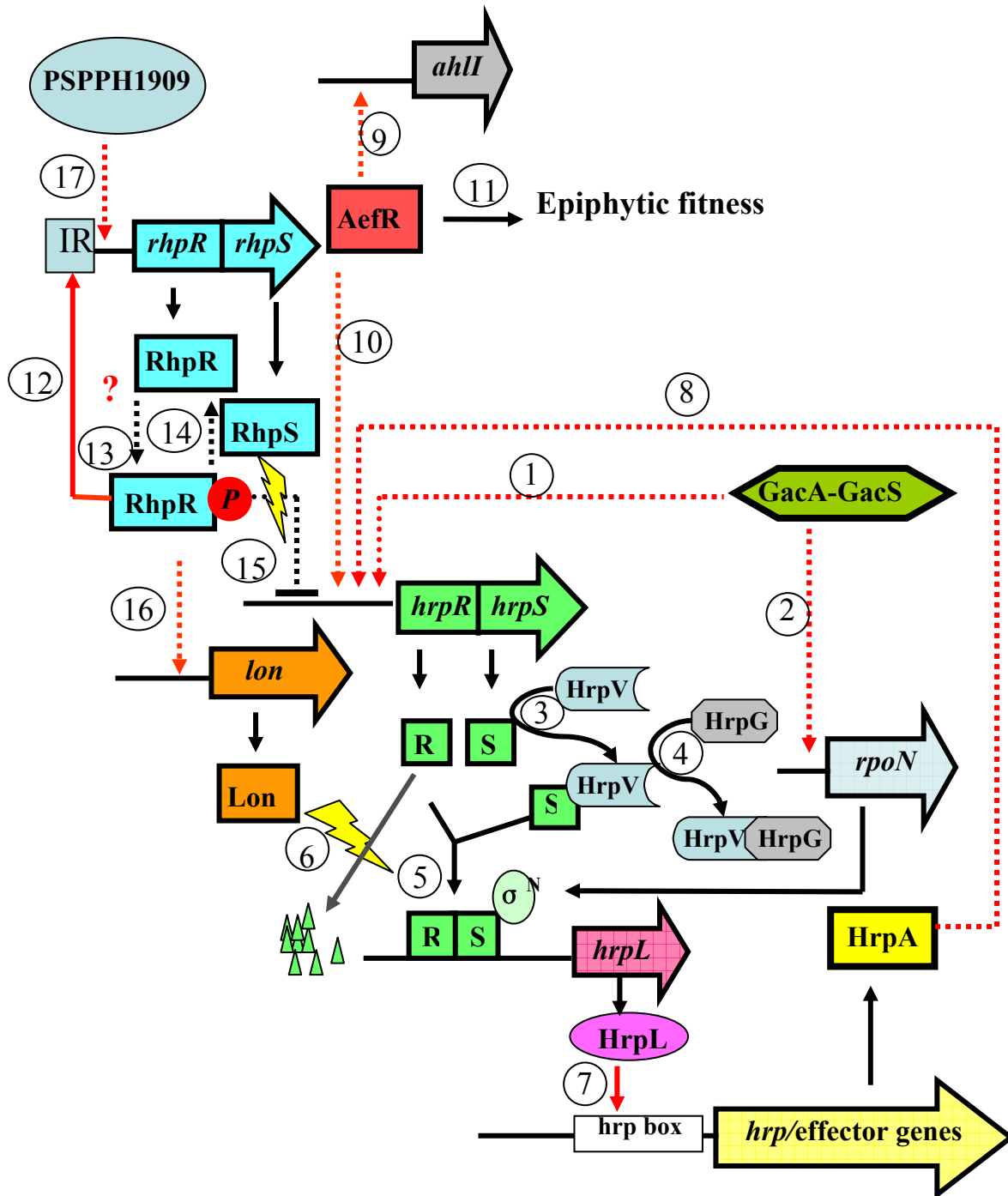


Fig V- 1 Models of T3SS gene regulation in *Pseudomonas syringae*.

1, GacS/GacA activates the transcription of *hrpRS*. 2, GacS/GacA activates the transcription of *rpoN*. 3, HrpS is repressed by HrpV via protein–protein interaction. 4,

HrpG interacts with HrpV and derepresses HrpS. 5, HrpS and HrpR proteins form heterodimer and associate with RpoN in the *hrpL* promoter to activate the *hrpL* expression. 6, HrpR protein is degraded by Lon protease. 7, HrpL recognizes the hrp box promoter and activates the transcription of *hrp*/effector genes. 8, HrpA acts upstream of *hrpRS* transcription to stimulate the T3 gene expression. 9, AefR activates *ahlI* transcription. 10, AefR acts upstream of *hrpRS* transcription to activate the T3 gene expression. 11, AefR controls epiphytic fitness. 12, RhpR activates its own promoter by interacting with an inverted repeat (IR) motif. 13, In T3-repressing conditions or in *rhpS*-mutant, RhpR is proposed to be phosphorylated by unknown donor(s). 14, In T3-inducing conditions, RhpS is proposed to act as a phosphatase to dephosphorylate P-RhpR into RhpR. 15, In T3-repressing conditions, P-RhpR is proposed to act upstream of *hrpRS* to suppress the T3 gene expression, while in T3-inducing conditions, RhpS can derepress P-RhpR. 16, RhpR activates the transcription of *lon*. 17, PSPPH1909 activates the transcription of *rhpR*.