REGULATION OF TYPE III SECRETION SYSTEM IN *PSEUDOMONAS SYRINGAE*

by

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AN ABSTRACT OF A DISSERTATION

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Genetics Inter-department Program
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ABSTRACT

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A putative two-component sensor histidine kinase, RohS, is identified to be required for the induction of *avrPto-LUC* in MM and in plants. The *rohS* gene is in an operon containing a two-component response regulator gene *rohR*. Mutation of *rohS* in *P. s. phaseolicola* and *P. s. tomato* reduced the bacterial pathogenicity on hosts and HR-inducing activity on non-hosts. Our results suggested that RohS acts upstream of HrpR/HrpS. The phosphorylated RohR represses TTSS genes. It is likely that RohS acts as phosphatase of RohR in the TTSS-inducing conditions, and subsequently derepresses TTSS genes.

Simple sugars such as glucose, sucrose, and fructose are known to be inducers of the TTSS genes. Isolation of four *min* mutants defective in fructose-uptake enabled us to

study if sugars serve as extracellular signals or as essential nutrients. Our results suggest that fructose acts as an essential nutrient for the activation of type III genes. These mutants slightly compromised induction of *avrPto* promoter in *Arabidopsis* and pathogenicity on the host bean plant, but displayed normal HR elicitation on non-host plant tobacco. The reduced pathogenicity suggested that exploitation of fructose from the host tissue is an important means for pathogenesis of *P. s. phaseolicola*.

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CHAPTER I

LITERATURE REVIEW

Regulation of Type III Secretion System in *Pseudomonas Syringae*

INTRODUCTION

Pseudomonas syringae is an important Gram-negative bacterial pathogen that infects a wide variety of plants. *P. syringae* is classified into more than 50 pathovars based on its host specificity at plant species level, and some of the pathovars are further divided into races based on host range among different cultivars of the host species (Gonzalez *et al.,* 2000; Hirano and Upper, 2000). Like many other Gram-negative pathogenic bacteria, *P. syringae* employs the highly conserved type III secretion system (TTSS) for pathogenicity on hosts and hypersensitive reaction (HR) elicitation on nonhosts (Galan and Collmer, 1999; Mudgett and Staskawicz 1998; Staskawicz *et al.,* 2001). The bacteria rely on the TTSS to deliver an array of proteins directly into the host cells or intercelluar space to modulate the physiology of the host cells, which favors the bacterial growth and pathogenesis (Hueck, 1998; Jin and He, 2001; Jin *et al.,* 2003; Lee, 1997; Oguiza and Asensio, 2005). The genes encoding the components of the type III secretion system and a few effector proteins are clustered in an approximate 25 kb chromosomal region, while most of the effector genes are dispersed in the genome. Despite the location, genes encoding the TTSS and its effectors (hereafter referred to as TTSS genes collectively) are coordinately regulated and function as a regulon (Alfano and Collmer, 2004; Chang, 2005). The expression of the TTSS genes is under tight control in *P. syringae*. These genes are expressed at low levels in nutrient-rich medium and are induced to a high level upon contact with host cells (Xiao *et al.,* 1992; Xiao *et al.,* 1994; Wei *et al.,* 2005). The host signal(s) and the bacterial receptor for the signal(s) are still unknown. TTSS genes of *P. syringae* are also activated by defined minimal medium, which is believed to mimic the environment of intercellular space where the bacteria

proliferate (Lindgren, 1986; Xiao *et al.,* 1992). As TTSS is an essential determinant of bacterial pathogenesis, elucidation of the regulation of TTSS genes will be very important for understanding the molecular basis of bacterial pathogenesis and the microbe-plant interaction.

Type III secretion system and effectors

The type III secretion system is a specialized bacterial protein secretion system used by numerous Gram-negative bacterial pathogens of animals and plants to deliver proteins directly into the host cells. *P. syringae pv. phaseolicola* was the first plant pathogenic bacterium identified to possess a TTSS as an essential pathogenesis determinant (Lindgren *et al.,* 1986). The ability of TTSS to deliver effector proteins into host cells was first reported in the mammalian bacterial pathogen *Yersinia* (Pettersson *et al.,* 1996). Some animal bacterial pathogens have two distinguishable TTSSs, while plant bacterial pathogens only have one TTSS. The TTSS is a syringe needle-like translocation apparatus consisting of inner and outer membrane rings and a protruding filament called pilus. The rings are assembled from polypeptides highly conserved among diverse bacterial species. HrpA is the major structural protein of hrp pilus (Jin *et al.,* 2001; Kubori *et al.,* 1998; Hueck, 1998). The type III pilus functions as a conduit to guide the translocation of the effectors to the interior of the host cells (Jin *et al.,* 2001; Jin and He, 2001).

The type III secretion machinery in the phytopathogens is designated as the *hrp* (hypersensitive response and pathogenicity) system since the secretion mutants typically lose the ability to cause disease in hosts and to elicit HR in nonhosts completely

(Lindgren, 1997). The type III secretion apparatus is encoded by *hrp* genes, ten of which are highly conserved between diverse plant and animal pathogens and thus are renamed as *hrc* (hypersensitive response and conserved) genes. Proteins encoded by *hrc* genes form a core of the basal body of the TTSS apparatus, located in the bacterial inner membrane or in the cytoplasm loosely associated with the membrane. In addition to TTSS structural proteins, *hrp* genes also encode hrp regulatory system and some TTSS substrates. Most of *hrp/hrc* genes are clustered on a 20 to 30 kb chromosomal region consisting of more than 20 genes that are organized into multiple transcriptional units (Alfano and Collmer, 1997; Rahme *et al.,* 1991; Xiao *et al.,* 1992) (Fig. I-1). Some of the *hrp* genes such as *hrpM* are found in genomic regions that are physically distinct from the large *hrp* clusters (Niepold, 1985). Based on their genetic organization, sequence similarities and *hrp* regulatory systems, the *hrp/hrc* gene clusters of bacterial phytopathogens are divided into two groups. The *hrp* cluster of *P. syringae* belongs to group I , and the TTSS genes in this pathogen are activated by HrpL, an ECF (Extra Cytoplasmic Factor) type alternate RNA polymerase sigma factor (Alfano and Collmer, 1997).

TTSS can direct the translocation of proteins across the bacterial inner membrane and outer membrane to the extracelluar space or into host cells. Proteins that translocate through the *P. syringae* TTSS are called Hop (Hrp-dependent outer proteins) proteins. Some Hops are secreted into the intercellular space and function as TTSS accessory proteins. Another subset of Hops are secreted into and function in the host cells, and these Hops are designated as effectors. In *P. syringae*, type III effector genes, usually associated with mobile genetic elements, are dispersed throughout the genome, clustered

in a pathogenicity island (PAI), or located on a plasmid (Alfano *et al.,* 2002; Buell *et al.,* 2003). In *P. syringae,* the *hrp/hrc* gene cluster is flanked by the exchangeable effector locus (EEL) and conserved effector locus (CEL), forming a *hrp* PAI. Mutagenesis analysis demonstrated that the conserved effector locus of the DC3000 strain is essential for bacterial pathogenicity while the exchangeable effector locus only has minor effect on bacterial growth in host plants (Alfano *et al.,* 2000; Collmer *et al.,* 2000). Some effectors appear to be widely distributed among different strains, whereas others are shared by only a few strains (Alfano *et al.,* 2000; Chang *et al.,* 2005).

A number of TTSS effector genes are designated as *avr* genes because they were initially detected as proteins that induced resistance or avirulent reactions in the otherwise susceptible host plants (Alfano and Collmer, 2004; Leach and White, 1996). In the presence of cognate *R* genes in the resistant plants, the Avr proteins trigger the hypersensitive response (HR), a rapid and localized programmed cell death of plant cells associated with some defense responses. The HR, in some case, limits the growth and spread of the pathogen at the infection site. In the susceptible plants lacking corresponding *R* genes, the type III effectors function as virulent determinants. Inside the plant cells, the effectors interfere with the host defense signaling pathways, suppress resistance responses such as HR and modulate cell physiology to benefit the growth and multiplication of the pathogen, leading to diseases (Alfano and Collmer, 2004; Oguiza and Asensio, 2005). However, due to the redundancy or subtle effect on virulence of TTSS effectors, it is usually hard to detect a virulence function of individual effectors in plants.

A few effectors require TTSS chaperones for secretion. TTSS chaperones are small, acidic cytoplasmic proteins with an amphipathic region in their C-termini. During type III secretion, the chaperones specifically bind to individual TTSS effector proteins somewhere between residue 15-150 and may act as factors that: i. maintain the effector proteins unfolded or partially unfolded; ii. protect the effector proteins from the premature interaction with each other or with other proteins, thus prevent the aggregation or proteolysis of the cognate effectors in the bacterial cytoplasm; iii. function as type III secretion signals to direct the cognate effector to the type III apparatus (Losada and Hutcheson, 2005; Feldman and Cornelis, 2003). Genes encoding type III chaperones are usually linked with the genes encoding cognate effectors (Guo *et al.,* 2005; Guttman *et al.,* 2002; van Dijk *et al.,* 2002).

Identification of TTSS effectors in *Pseudomonas syringae*

In *P. syringae*, TTSS effector genes were first cloned based on their ability to convert a virulent strain to an avirulent strain and were designated as *avr* genes (Kobayashi *et al.,* 1989; Staskawicz *et al.,* 1984; Leach and White, 1996). Later studies revealed the *avr* genes encode TTSS effectors that function inside the plant cells (He, 1997). The initial effort to identify proteins secreted through the TTSS was to monitor extracellular proteins secreted in bacterial cultures in a TTSS-dependent manner (He *et al.,* 1993). However, the amounts of proteins secreted via TTSS in culture, even detectable by antibodies, are too low for systemic identification of TTSS effectors.

Sequencing of genomic regions flanking the *hrp/hrc* clusters in *P. s. tomato* DC3000, *P. s. syringae* B728a and *P. s. syringae* 61 revealed another number of TTSS

effectors. The *hrp/hrc* clusters of these strains are flanked by several TTSS effectors composing EELs and CELs (Alfano *et al.,* 2000; Fouts *et al.,* 2002). EELs are located on the left side of *hrp/hrc* clusters containing divergent sets of effector genes in the three strains. Genes in EELs appear to be exchanged at a high frequency. CELs are located on the right side of *hrp/hrc* clusters. The identities and arrangement of effector genes in CEL are highly conserved (Alfano *et al.,* 2000; Collmer *et al.,* 2000; Fouts *et al.,* 2002).

As the number of identified TTSS effectors increased, comparative studies unraveled several common features shared by TTSS effectors useful for TTSS effector identification: i. Genes encoding TTSS apparatus and most of the TTSS substrates possess a conserved *cis* element named *hrp* box with consensus sequence (GGAACC – $N_{15/16}$ – CCACNNA) in the promoter region and are regulated by HrpL (Hutcheson *et al.,* 2001; Innes *et al.,* 1993; Shen and Keen, 1993; Xiao and Hutcheson, 1994; Xiao *et al.,* 1994). ii. TTSS effector proteins appear to have a modular structure: the first 50 residues contains the targeting signals for secretion and translocation, and the C-terminal part has the effector activity. The N-terminal secretion signals and the C-terminal effector regions can be swapped between TTSS effectors (Guttman and Greenberg, 2001; Tampakaki *et al.,* 2004). i. Even though the N-termini of TTSS effectors do not show significant sequence similarity, they share some features important for secretion: an Ile, Leu, or Val at residue 3 or 4; amphipathicity and high Ser and Gln contents in the first 50 residues; and absence of acidic amino acid in the first 12 residues (Guttman *et al.,* 2002; Lloyd *et al.,* 2001, 2002; Petnichi- Ocwieja *et al.,* 2002; Tampakaki *et al.,* 2004).

The discoveries of these TTSS effectors' characteristics and completion of genome sequences of several *P. syringae* strains (Buell *et al.,* 2003; Feil *et al.,* 2005;

http://*Pseudomonas-syringae*.org) enabled the systematic identification or prediction of TTSS effectors via genomics and functional genomics approaches. For example, the modular structure of AvrRpt2 was widely used in identification of TTSS effectors. The C-terminal portion of AvrRpt2 is sufficient to trigger Rps2-dependent HR in *Arabidopsis thaliana*, and the N-terminal targeting signal of other TTSS effectors is sufficient to translocate the C-terminal part of AvrRpt2 or other peptides into plant cells in TTSS-dependent manner (Guttman & Greenberg, 2001). Thus AvrRpt2 lacking N-terminal targeting signal was used as reporters to identify genes containing type III secretion signals (Chang *et al.,* 2005; Guttman *et al.,* 2002). Similarly, the calmodulin-dependent adenylate cyclase domain (Cya) of the cyclolysin toxin from *Bordetella pertussis* is exploited as a reporter in functional assay to confirm novel TTSS effectors. Cya can be used as a reporter for TTSS-dependent secretion because it is not secreted by the TTSS and is not active in bacterial cytoplasm where it lacks calmodulin. When the N-terminal signal of an effector is fused to Cya, bacteria can deliver the resulting fusion protein into the host cells, where it can bind to calmodulin and produce cyclic AMP from ATP. Thus adenylate cyclase activity in plant tissue inoculated with bacteria specifically indicates the TTSS-dependent secretion of the fusion protein (Schechter *et al.,* 2004).

Based on the characteristic HrpL-dependent expression of TTSS genes, a HrpL-dependent promoter-trap assay was developed for large scale TTSS effector mining. *P. syringae* genomic library was constructed in promoterless *lacZ* vector and transformed to *E. coli* carrying an arabinose-inducible *hrpL* construct. The transformants that exhibited Lac phenotype in KB medium were further screened in the MacConkey lactose medium with and without arabinose. The colonies that contained HrpL-dependent promoter

fragments were selected based on the arabinose-inducible Lac phenotype. As a result, 22 HrpL-dependent promoter fragments were identified in *P. s. syringae* 61, one of which was shown to encode a new TTSS effector (Losada *et al.,* 2004). Similarly, Chang *et al.,* (2005) searched the hrpL-dependent promoters in *P. s.* pv. *tomato* DC3000 strain and *P. s.* pv. *phaseolicola* race 1448A using GFP as reporter. Bacterial genomic libraries were constructed in a broad host vector upstream of *avrRpt2*$_{\Delta 1\text{-}79}$::GFP and introduced to *P. s.* pv. *tomato* strain DC3000 *hrpL*$^{-}$ mutant carrying arabinose-inducible *hrpL* construct. Arabinose-inducible GFP is used to rapidly select bacteria that harbor *hrp* box-containing fragments by fluorescence-activated cell sorting. AvrRpt2$_{\Delta 1\text{-}79}$ is used to determine whether the *hrpL*-regulated genes encode TTSS effectors. The search yielded 43 HrpL-induced genes/operons in DC3000 and 41 genes/operons in 1448A, and 29 that in DC3000 and 19 that in 1448A were confirmed to encode TTSS effectors based on the bacterial ability to trigger RPS2-dependent HR in *Arabidopsis* (Chang *et al.,* 2005). As the TTSS effector genes often carry a *hrp* box in their promoter, several groups also searched the effector gene candidates downstream of *hrp*-box containing promoter using a genome-based computational approach (Fouts *et al.,* 2002; Zwiesler-Vollick *et al.,* 2002; Losada *et al.,* 2004).

The characteristic amino-acid pattern in the N-terminal regions of TTSS substrates also facilitates TTSS effectors mining by searching the genome for the ORFs with the putative N-terminal secretion signal (Guttman *et al.,* 2002; Petnicki-Ocwieja *et al.,* 2002). One hundred and twenty nine genes in the DC3000 genome appear to carry a putative type III N-terminal secretion signal sequence (Petnicki-Ocwieja *et al.,* 2002).

To date, more than 190 candidate effectors have been identified collectively in various *P. syringae* strains based on experimental or bioinformatic analysis (http://*Pseudomonas-syringae*.org). Forty effectors in *P. s.* pv. tomato strain DC3000 and 19 in *P. s.* pv. *phaseolicola* strain 6-1448a have been experimentally confirmed. A database of *P. syringae* TTSS effectors is available on the website http://*Pseudomonas-syringae*.org.

Host-associated signals regulating TTSS genes

In pathogen-host interactions, host environment detection is a crucial step for pathogen's establishment and successful infection. The host sensing leads to the activation of virulence genes, enabling the pathogen to overcome or tolerate host defense and exploit nutrients for their growth.

As the essential pathogenicity determinant, TTSS genes of *P. syringae* are modulated by host signal(s) (Rahme *et al.,* 1992; Xiao *et al.,* 1992; Xiao *et al.,* 2004). TTSS genes are repressed when bacteria are cultured in nutrient rich media but are induced to high levels when bacteria are inoculated into the plant (Rahme *et al.,* 1992; Xiao *et al.,* 2004). The *in planta* activation of TTSS genes is rapid. The expression of TTSS genes is detected as early as one hour after the bacteria are infiltrated into plant leaf tissues and peaks six hours after inoculation (Rahme *et al.,* 1992; Xiao *et al.,* 2004; Thwaites *et al.,* 2004). However, host signals that stimulate the expression of TTSS genes of *P. syringae* have not been characterized, and the nature of the signal(s) is not clearly defined. In *Ralstonia solanacearum*, the activation of TTSS genes in plants requires physical contact between bacteria and plant cells, suggesting that the plant-associated

inducer(s) is non-diffusible (Aldon *et al.,* 2000). Further, Aldon and associates characterized the inducing signal(s) and proved that the inducing signal(s) associated with cell wall is ubiquitous among plant species and is heat-stable and protease-resistant (Aldon *et al.,* 2000). It is likely that the cell wall-derived inducing signal(s) is a macromolecule(s). However, the possibility that the signal is related to the physical properties of the cell wall can not be excluded.

In addition to their induction in the plant apoplast, the expression of TTSS genes is also regulated by environmental factors such as temperature, nutrient sources, pH, and osmolarity (Huynh *et al.,* 1989; Rahme *et al.,* 1992). The expression of TTSS genes is strongly repressed in KB medium and is activated when the bacteria is cultured in minimal medium. The inhibitory activity of rich medium is exerted by high pH, osmolarity, and complex carbon/nitrogen nutrient sources. The TTSS gene-inducing minimal media are acidic, low osmotic, and with certain sugars such as fructose, mannitol, citrate or sucrose as the carbon source. Temperature also plays a role in modulating the TTSS genes. The optimal expression of TTSS genes can be obtained when the bacteria grow at 20 to 30 ºC. These environmental inducing conditions in minimal medium are thought to simulate the intercellular physiology environment encountered by bacteria during infection (Huynh *et al.,* 1989; Rahme *et al.,* 1992; Xiao *et al.,* 1992; van Dijk *et al.,* 1999). The factors influencing the TTSS gene expression in the inducing medium differ somewhat between different pathovars, which may reflect the differences between the apoplastic conditions of different host species. For example, the nature of carbon source plays a very important role for TTSS gene activation, and fructose and sucrose are the best inducers of *P. syringae* TTSS genes among the carbon sources tested (Huynh *et*

al., 1989). Minimal medium supplemented with mannitol induced the *avrB* gene almost as well as the medium supplemented with fructose in *P. s.* pv. *glycinea* (Huynh *et al.,* 1989), but mannitol is not able to induce *avrPto* in *P. s.* pv. *phaseolicola* strain NPS3121 (Xiao and Tang, unpublished results). It is thought that certain factors affecting TTSS gene expression in minimal medium are also present in the plant and contribute to the *in planta* activation of TTSS genes. However, several studies suggested that the TTSS genes are differentially regulated in minimal medium and in hosts (Aldon *et al.,* 2000; Brito *et al.,* 2002; Rahme *et al.,* 1992; Xiao and Tang, unpublished results), suggesting the existence of additional, possibly host-specific signaling pathways.

In a recent study attempting to identify host signals involved in TTSS gene regulation, an *Arabidopsis att1* loss-of-function mutant was isolated that supports much higher induction of TTSS genes than did the wild type plant (Xiao *et al.,* 2004). This observation suggests the existence of a plant-associated signal(s) that negatively regulates the expression of bacterial TTSS genes. *ATT1* encodes a cytochrome *P450* monooxygenase catalyzing fatty acid hydroxylation. In plants, hydroxylated fatty acids form extracellular polyesters that are major constituents of cutin, which envelopes the aerial part of the plant. The cutin content in *att1* mutant is reduced to 30% of the wild type level, and the composition of cutin monomers is also largely altered in the *att1* mutant compared with the wild type plant, indicating that ATT1 plays an important role in biosynthesis of cutin monomers. Certain commercially available fatty acids and plant cutin extracts can specifically suppress the induction of TTSS genes. Taken together, certain cutin monomer species may act as negative signals to regulate the bacterial TTSS genes *in planta*.

Evidence suggests that plant-associated signals regulate either positively or negatively the bacterial TTSS genes in *P. syringae* and other phytopathogens. However, it is still not understood how the plant pathogenic bacteria perceive these signals and how these signals are transferred to the regulatory system of the TTSS genes. The only exception is the regulation of TTSS genes in *Ralstonia solanacearum* by a non-diffusible plant-specific signal. The detection of the host-related non-diffusible signal was shown to require PrhA, an outer membrane protein sharing significant similarity with sideorphore receptors (Aldon *et al.,* 2000; Brito *et al.,* 2002). When bacteria contact with plant cells, PrhA perceives the non-diffusible signal and transduces this signal through the regulatory cascade integrated by PrhR, PrhI, PrhJ, HrpG and HrpB in order, resulting in the activation of the TTSS genes (Brito *et al.,* 2002). To date, no sensor has been identified in *P. syringae* that is involved in regulation of TTSS genes.

Regulation of TTSS genes in *P. syringae*

In *P. syringae*, TTSS genes are coordinately regulated in response to environmental conditions and in conditions in the plant apoplast. All TTSS genes are suppressed in rich medium but are activated when the bacteria grow in the plant apoplast or in defined inducing minimal medium (Huynh *et al.,* 1989; Rahme *et al.,* 1992; Xiao *et al.,* 1992; Xiao *et al.,* 2004). This regulation is dependent on HrpL, an alternate RNA polymerase sigma factor in the ECF family (Hutcheson *et al.,* 2001; Xiao *et al.,* 1994; Xiao and Hutcheson, 1994; Zwiesler-Vollick *et al.,* 2002). Even though the direct interaction between HrpL and *hrp* box has not been reported, one model proposes that

HrpL activates TTSS genes in response to inducing conditions via the interaction with the *hrp* box in the promoter region of these genes.

Transcription of the *hrpL* gene is under the control of σ^{54}-dependent promoter in an alternate sigma factor RpoN-dependent manner (Alarcon-Chaidez *et al.,* 2003; Chatterjee *et al.,* 2003; Hendrickson *et al.,* 2000). Activation of *hrpL* also requires HrpR and HrpS, the enhancer-binding proteins in the NtrC family of two-component regulatory proteins (Grimm *et al.,* 1995; Hutcheson *et al.,* 2001; Xiao *et al.,* 1994). HrpR and HrpS-coding genes are located on the right end the *hrp/hrc* gene cluster (Alfano and Collmer, 1997) (Fig.I-1). Both HrpR and HrpS contain a motif that functions in interaction with σ^{54}- RNA polymerase holoenzyme and an enhancer-binding domain. Unlike most of the other two-component regulatory proteins, HrpR and HrpS lack the receiver domain that functions in phosphorylation-dependent modulation of response regulator activity (Hutcheson *et al.,* 2001). The mechanism by which HrpR and HrpS regulate *hrpL* transcription differs in different strains. In *P. s. syringae* strain 61 and *P. s. tomato* strain DC3000, *hrpR* and *hrpS* are expressed as an operon from a promoter on the upstream of *hrpR*. The maximum activation of hrpL requires both HrpR and HrpS. Under TTSS gene-inducing conditions, HrpR and HrpS form a heterodimer to activate *hrpL* expression by interacting with the *hrpL* promoter (Chatterjee *et al.,* 2003; Hutcheson *et al.,* 2001; Xiao *et al.,* 1994). In *P. s. phaseolicola*, *hrpL* is activated by HrpS alone. Expression of hrpS requires HrpR. Upstream of the *hrpS* start codon and in the *hrpR* coding region, there is a σ^{54} promoter consensus sequence (HrpR box) that was shown to be able to interact with HrpR, and the expression of hrpS requires HrpR. In this strain, *hrpS* transcription

appeared to initiate near the HrpR box. Overexpression of *hrpS* can complement *hrpR* mutation to activate *hrpL* and other TTSS genes (Grimm *et al.,* 1995).

The fact that HrpR and HrpS lack the receiver domain suggests that the activity of HrpR and HrpS may not be controlled directly by any signal via a two component sensor (Hutcheson *et al.,* 2001). Thus the pool size of HrpR and HrpS may play a key role in the regulation of TTSS gene expression. In *P. s. phaseolicola,* the expression of *hrpRS* is low when the bacteria are grown in rich medium but is rapidly induced in minimal medium or in plants (Rahme, 1992; Thwaites *et al.,* 2004; Xiao *et al.,* 1992). However, some controversy exists regarding the transcriptional regulation of *hrpR* and *hrpS*. In *P. s. syringae* 6 and *P. s. tomato* DC3000 strains, it was observed that *hrpR* and *hrpS* are constitutively transcribed and HrpR and HrpS is regulated at the post-transcriptional level (Bretz *et al.,* 2002).

Expression of TTSS genes is influenced by a global regulatory system GacS/GacA as well (Chatterjee *et al.,* 2003). The GacS/GacA system is a two-component regulatory system found in many Gram-negative bacteria controlling a variety of phenotypes (reviewed by Heeb and Haas, 2001). GacS is the sensory histidine kinase, containing a periplasmic domain and cytoplasmic receiver and output domains. GacA is the cognate response regulator of GacS, carrying the response regulatory domain at the N-terminus and a HTH motif of the LuxR family at the C-terminus. In response to environmental stimuli, GacS autophosphorylates and subsequently transfers the phosphoryl group to GacA. In turn, GacA activates the target genes (Zuber *et al.,* 2003). The GacS/GacA system controls expression of TTSS genes through the HrpR/HrpS-HrpL regulatory cascade in *P. s. tomato* strain DC3000. GacA-deficiency attenuates the

transcription levels of *hrpRS*, *rpoN* and *hrpL* significantly (Chatterjee *et al.,* 2003), suggesting that GacA positively regulates the transcript levels of *hrpL*, most likely due to its effects on *hrpRS* and *rpoN*. Consequently, GacA positively regulates the HrpRS or HrpL-dependent TTSS gene expression. The nature of signals perceived by GacS and the mechanism by which GacA regulates the expression of *hrpRS*, *rpoN* and *hrpL* are not known thus far. Nevertheless, the GacS/GacA system is activated during transition from exponential to stationary phase (Chatterjee *et al.,* 2003; Heeb and Haas, 2001). The fact that the activation of GacS/GacA system does not require the presence of a plant cell suggests that the TTSS genes are controlled by other sensory/regulatory system specific to plant-associated signals.

Several proteins have been identified that affect expression of the TTSS genes. One of them is HrpA, the major subunit of *hrp* pilus (Wei *et al.,* 2000). HrpA is required for full expression of all TTSS genes. In *hrpA* mutant, TTSS gene transcriptional induction was repressed in inducing medium and overexpression of *hrpRS* restored the TTSS gene induction. However, the mechanism by which HrpA regulates TTSS genes is still unknown.

Another protein affecting expression of the TTSS genes is Lon, an ATP-dependent protease that negatively regulates the TTSS genes. *lon* mutants showed increased HrpR stability and higher transcription of *hrpL* and TTSS effector genes under repressive conditions. This suggests that Lon regulates the expression of TTSS genes through proteolysis of HrpR under repressive conditions (Bretz *et al.,* 2002). A recent study showed that Lon protease also regulates the type III secretion at posttranslational levels via proteolysis of effectors prior to secretion. The half-lives of the effectors tested

in this study were significantly higher in *lon* null mutants than in wild type strain (Losada and Hutcheson, 2005).

HrpV and HrpG regulate TTSS genes by a mechanism similar to anti-anti-activator mechanism in *P. aeruginosa*. In *P. aeruginosa*, expression of TTSS genes is controlled at the transcriptional level by ExsA, an AraC-like transcriptional activator. The anti-activator ExsD interacts with ExsA in repressive conditions and thus represses the transcription of the TTSS genes. When the bacteria are grown in the inducing conditions, a third regulator, ExsC, can interact with ExsD and release ExsA from inhibition by ExsD, which, in turn, activates the TTSS gene expression (Dasgupta, 2004). In this model, ExsD acts as an anti-activator of ExsA, and ExsC acts as an anti-anti-activator. In *P. syringae*, *hrpG* and *hrpV* genes are located in the *hrcC* operon and are specific to group I *hrp/hrc* gene cluster (See Fig.I-1). HrpV is a negative regulator of TTSS genes upstream of HrpR/HrpS (Preston *et al.,* 1998). In *hrp*-inducing minimal medium, overexpression of the *hrpV* gene down-regulates *TTSS* gene expression, while *hrp* genes are expressed at a moderately elevated level in *hrpV* mutants. HrpG, a chaperone-like protein can derepress the TTSS gene expression in *hrpV*-overexpression strain without affecting the *hrpV* transcription (Wei *et al.,* 2005). It has been further demonstrated that HrpV interactes with HrpS and HrpG *in vivo*. Thus it is likely that HrpG suppresses HrpV via a protein-protein interaction to free HrpS when the bacteria are grown in *hrp*-inducing conditions. In this hypothesis, HrpV acts as anti-activator of HrpS, and HrpG is an anti- anti-activator.

CONCLUSION

As the key determinant of pathogenesis in *P. syringae*, the TTSS has been extensively studied. The structure of TTSS apparatus and its function in secretion of virulent factors, identities of TTSS effectors, the mechanism of TTSS in pathogenesis and regulation of TTSS have been elucidated somewhat. However, many questions remain to be answered. As for the regulation of TTSS, the key puzzles are the identity and nature of the environmental signals that regulate bacterial TTSS gene expression, the bacterial sensory protein(s) perceiving the signals, and the link between the signal sensor to TTSS gene expression. The possible cross talk between TTSS and other biochemical processes in bacteria is also a potential topic to be exploited.

REFERENCES

Alarcon-Chaidez F. J., Keith L., Zhao Y., Bender C. L., 2003. RpoN (σ^{54}) is required for plasmid-encoded coronatine biosynthesis in *Pseudomonas syringae*. *Plasmid* 49: 106-117.

Aldon D., Brito B., Boucher C., Genin S., 2000. A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. *EMBO J.* 19: 2304-2314.

Alfano J. R., Charkowski A. O., Deng W. L., Badel J. L., Petnicki-Ocwieja T., *et al.,* 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl Acad. Sci. USA*. 97: 4856–4861.

Alfano J. R., Collmer A., 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J. Bacteriol.* 179: 5655-5662.

Alfano J. R., Collmer A., 2004. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* 42: 385-414.

Bre*Tz* J., Losada L., Lisboa K., Hutcheson S. W., 2002. Lon protease functions as a negative regulator of type III protein secretion in *Pseudomonas syringae*. *Mol. Microbiol.* 45: 397-409.

Brito B., Aldon D., Barberis P., Boucher C., Genin S., 2002. A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum hrp* genes. *Mol. Plant Microbe Interact.* 15: 109-119.

Buell C. R., Joardar V., Lindeberg M., Selengut J., Paulsen I. T., Gwinn M. L. *et al.,* 2003. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. USA* 100: 10181-10186.

Chang J. H., Urbach J. M., Law T. F., Arnold L. W., Hu A., Gombar S., Grant S. R., Ausubel F. M., Dangl J. L., 2005. A high-throughput, near-saturating screen for type III effector genes from *Pseudomonas syringae*. *Proc. Natl. Acad. Sci. USA* 102: 2549-2554.

Chatterjee A., Cui Y., Yang H., Collmer A., Alfano J. R., Chatterjee A. K., 2003. GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. tomato DC3000 by controlling regulatory RNA,

transcriptional activators, and alternate sigma factors. *Mol. Plant Microbe Interact.* 16: 1106-1117.

Collmer A., Badel J. L., Charkowski A. O., Deng W. L., Fouts D. E., Ramos A. R., Rehm A. H., Anderson D. M., Schneewind O., van Dijk K., Alfano J. R., 2000. *Pseudomonas syringae* Hrp type III secretion system and effector proteins. *Proc. Natl. Acad. Sci. USA* 97: 8770-8777.

Feil H., Feil W. S., Chain P., Larimer F., Dibartolo G., Copeland A., Lykidis A., Trong S., Nolan M., Goltsman E., Thiel J., Malfatti S., Loper J. E., Lapidus A., Detter J. C., Land M., Richardson P. M., Kyrpides N. C., Ivanova N., Lindow S. E., 2005. Comparison of the complete genome sequences of *Pseudomonas syringae* pv. *syringae* B728a and pv. tomato DC3000. *Proc. Natl. Acad. Sci. USA* 102: 11064-11069

Feldman M. F., Cornelis G. R., 2003. The multitalented type III chaperones: all you can do with 15 kDa. *FEMS Microbiol. Lett.* 219: 151–158.

Fouts D. E., Abramovitch R. B., Alfano J. R., Baldo A. M., Buell C. R., Cartinhour S., Chatterjee A. K., D'Ascenzo M., Gwinn M. L., Lazarowi*Tz* S. G., Lin N. C., Martin G. B., Rehm A. H., Schneider D. J., van Dijk K., Tang X., and Collmer A., 2002. Genomewide identification of *Pseudomonas syringae* pv. tomato DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc. Natl. Acad. Sci. USA* 99: 2275–2280.

Galan J. E. and Collmer A., 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* 284: 1322-1328.

Gonzalez A. J., Landeras E., Mendoza M. C., 2000. Pathovars of *Pseudomonas syringae* causing bacterial brown spot and halo blight in *phaseolus vulgaris* L. are distinguishable by ribotyping. *Appl. Environ. Microbiol.* 66: 850-854.

Grimm C., Aufsa*Tz* W., Panopoulos N. J., 1995. The hrpRS locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. *Mol. Microbiol.* 15: 155-165.

Guo M., Chancey S.T., Tian F., Ge Z., Jamir Y., Alfano J. R., 2005. *Pseudomonas syringae* type III chaperones ShcO1, ShcS1, and ShcS2 facilitate translocation of their cognate effectors and can substitute for each other in the secretion of HopO1-1. *J. Bacteriol.* 187: 4257-4269.

Guttman D. S., Greenberg J. T., 2001. Functional analysis of the type III effectors AvrRpt2 and AvrRpm1 of *Pseudomonas syringae* with the use of a single-copy genomic integration system. *Mol. Plant Microbe Interact.* 14: 145-155.

Guttman D. S., Vina*Tz*er B. A., Sarkar S. F., Ranall M. V., Kettler G., Greenberg J. T., 2002. A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. *Science* 295: 1722-1726.

He S. Y., 1997. Hrp-controlled interkingdom protein transport: learning from flagellar assembly? *Trends Microbiol.* 5: 489-495.

He S. Y., Huang H. C., Collmer A.,1993. *Pseudomonas syringae* pv. *syringae* harpin Pss: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* 73: 1255-1266.

Heeb S., Haas D., 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Mol. Plant Microbe Interact.* 14: 1351-1363.

Hendrickson E. L., Guevera P., and Ausubel F. M., 2000. The alternative sigma factor RpoN is required for hrp activity in *Pseudomonas syringae* pv. *maculicola* and acts at the level of *hrpL* transcription. *J. Bacteriol.* 182: 3508–3516.

Hirano S. S. and Upper C. D., 2000. Bacteria in the Leaf Ecosystem with emphasis on *Pseudomonas syringae*—a Pathogen, Ice Nucleus, and Epiphyte Microbiol. *Mol. Biol. Rev.* 64: 624–653

Hutcheson S. W., Bre*Tz* J., Sussan T., Jin S., Pak K., 2001. Enhancer-binding proteins HrpR and HrpS interact to regulate hrp-encoded type III protein secretion in *Pseudomonas syringae* strains. *J. Bacteriol.* 183: 5589-5598.

Hueck C. J., 1998. Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants. Microbiol. *Mol. Biol. Rev.* 62: 379-433.

Huynh T. V., Dahlbeck D., and Staskawicz B. J., 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* 245: 1374–1377.

Innes R. W., Bent A. F., Kunkel B. N., Bisgrove S. R., and Staskawicz B. J., 1993. Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J. Bacteriol.* 175: 4859–4869.

Jin Q., and He S.Y., 2001. Role of the Hrp pilus in type III protein secretion in *Pseudomonas syringae*. *Science* 294: 2556-2558.

Jin Q., Hu W., Brown I., McGhee G., Hart P., Jones A.L., He S.Y., 2001. Visualization of secreted Hrp and Avr proteins along the Hrp pilus during type III secretion in Erwinia amylovora and *Pseudomonas syringae*. *Mol. Microbiol.* 40: 1129-1139.

Jin Q., Thilmony R., Zwiesler-Vollick J., He S.Y.,2003. Type III protein secretion in *Pseudomonas syringae*. *Microbes Infect* 5: 301-310.

Kobayashi D.Y., Tamaki S. J., Keen N. T., 1989. Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. tomato confer cultivar specificity on soybean. *Proc. Natl. Acad. Sci. USA* 86: 157–161.

Kubori T., Matsushima Y., Nakamura D., Uralil J., Lara-Tejero M., Sukhan A., Galan J. E., Aizawa S. I., 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* 280: 602-605.

Leach J. E., White F. F., 1996. Bacterial avirulence genes. *Annu. Rev. Phytopathol.* 34: 153-179.

Lee C. A., 1997. Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells? *Trends Microbiol.* 5: 149-156.

Lindgren P.B., 1997. The role of hrp genes during plant-bacterial interactions. *Annu. Rev. Phytopathol.* 35: 129-152.

Lindgren P. B., Peet R. C., Panopoulos N. J., 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity on bean plants and hypersensitivity on nonhost plants. *J. Bacteriol.* 168: 512–522

Lloyd S., Norman M., Rosqvist R., Wolf-Wa*Tz* H., 2001. *Yersinia* YopE is targeted for type III secretion by Nterminal, not mRNA, signals. *Mol. Microbiol.* 39: 520–531.

Lloyd S., Sjostrom M., Anderson S., Wolf-Wa*Tz* H., 2002. Molecular characterization of the type III secretion signals *via* analysis of synthetic N-terminal amino acid sequences. *Mol. Microbiol.* 43: 51–59.

Losada L. C., Hutcheson S. W., 2005. Type III secretion chaperones of *Pseudomonas syringae* protect effectors from Lon-associated degradation. *Mol. Microbiol.* 55: 941-953.

Losada L., Sussan T., Pak K., Zeyad S., Rozenbaum I., Hutcheson S. W., 2004. Identification of a novel *Pseudomonas syringae* Psy61 effector with virulence and avirulence functions by a HrpL-dependent promoter-trap assay. *Mol. Plant Microbe Interact.* 17: 254-262.

Mudgett M. B., Staskawicz B. J., 1998. Protein signaling *via* type III secretion pathways in phytopathogenic bacteria. *Curr. Opin. Microbiol.* 1: 109–114.

Niepold F., Anderson D., Mills D.. 1985. Cloning determinants of pathogenesis from *Pseudomonas syringae* pathovar *syringae*. *Proc. Natl. Acad. Sci. USA.* 82: 406–410

Oguiza J. A., Asensio A. C., 2005. The VirPphA/AvrPtoB family of type III effectors in *Pseudomonas syringae*. *Res. Microbiol.* 156: 298-303.

Petnicki-Ocwieja T., Schneider D. J., Tam V. C., Chancey S. T., Shan L., Jamir Y., Schechter L. M., Janes M. D., Buell C. R., Tang X., Collmer A., Alfano J. R., 2002. Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. USA* 99: 7652-7657.

Pettersson J., Nordfelth R., Dubinina E., Bergman T., Gustafsson M., Magnusson K. E., Wolf-Wa*Tz* H., 1996. Modulation of virulence factor expression by pathogen target cell contact. *Science* 273: 1231-1123.

Preston G., Deng W. L., Huang H. C., Collmer A., 1998. Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J. Bacteriol.* 180: 4532–4537.

Rahme L.G., Mindrinos M.N., Panopoulos N.J., 1991. Genetic and transcriptional organization of the hrp cluster of *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 173: 575-586.

Rahme L. G., Mindrinos M. N., Panopoulos N. J., 1992. Plant and environmental sensory signals control the expression of hrp genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 174: 3499-3507.

Schechter L. M., Roberts K. A., Jamir Y., Alfano J. R., Collmer A., 2004. *Pseudomonas syringae* type III secretion system targeting signals and novel effectors studied with a Cya translocation reporter. *J. Bacteriol.* 186: 543-555.

Shen H., Keen N. T., 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. tomato. *J. Bacteriol.* 175: 5916–5924.

Staskawicz B. J., Dahlbeck D., Keen N. T., 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. glycinea determines race-specific incompatibility on Glycine max (L.) Merr. *Proc. Natl. Acad. Sci. USA* 81: 6024–6028.

Staskawicz B. J., Mudgett M. B., Dangl J. L., Galan J. E., 2001. Common and contrasting themes of plant and animal diseases. *Science* 292: 2285–2289.

Tampakaki A. P., Fadouloglou V. E., Gazi A. D., Panopoulos N. J., Kokkinidis M., 2004. Conserved features of type III secretion. *Cell Microbiol*. 6: 805-816.

Thwaites R., Spanu P. D., Panopoulos N. J., Stevens C., Mansfield J. W., 2004. Transcriptional regulation of components of the type III secretion system and effectors in *Pseudomonas syringae* pv. *phaseolicola*. *Mol. Plant Microbe Interact.* 17: 1250-1258.

van Dijk K., Fouts D. E., Rehm A. H., Hill A. R., Collmer A., Alfano J. R., 1999. The Avr (effector) proteins HrmA (HopPsyA) and AvrPto are secreted in culture from *Pseudomonas syringae* pathovars via the Hrp (type III) protein secretion system in a temperature- and pH-sensitive manner. *J. Bacteriol.* 181: 4790-4797.

van Dijk K., Tam V. C., Records A. R., Petnicki-Ocwieja T., Alfano J. R., 2002. The ShcA protein is a molecular chaperone that assists in the secretion of the HopPsyA effector from the type III (Hrp) protein secretion system of *Pseudomonas syringae*. *Mol. Microbiol.* 44: 1469-1481.

Wei C. F., Deng W. L., Huang H. C., 2005. A chaperone-like HrpG protein acts as a suppressor of HrpV in regulation of the *Pseudomonas syringae* pv. *syringae* type III secretion system. *Mol. Microbiol.* 57: 520-536.

Wei W., Plovanich-Jones A., Deng W. L., Jin Q. L., Collmer A., Huang H. C., and He S. Y., 2000. The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. tomato. *Proc. Natl. Acad. Sci. USA* 97: 2247–2252.

Xiao F., Goodwin S. M., Xiao Y., Sun Z., Baker D., Tang X., Jenks M. A., Zhou J. M., 2004. Arabidopsis CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development. *EMBO J.* 23: 2903-2913.

Xiao Y., Heu S., Yi J., Lu Y., and Hutcheson S. W., 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* 176: 1025-1036.

Xiao Y., Hutcheson S. W., 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. *J. Bacteriol.* 176: 3089-3091.

Xiao Y., Lu Y., Heu S., Hutcheson S. W., 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 hrp cluster. *J. Bacteriol.* 174: 1734-1741.

Zuber, S., F. Carruthers, C. Keel, A. Mattart, C. Blumer, G. Pessi, C. Gigot-Bonnefoy, U. Schnider-Keel, S. Heeb, C. Reimmann, D. Haas. 2003. GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. *Mol. Plant Microbe. Interact.* 16: 634–644.

Zwiesler-Vollick J., Plovanich-Jones A. E., Nomura K., Bandyopadhyay S., Joardar V., Kunkel B. N., He S. Y., 2002. Identification of novel hrp-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. tomato DC3000 genome. *Mol. Microbiol.* 45: 1207-1218.

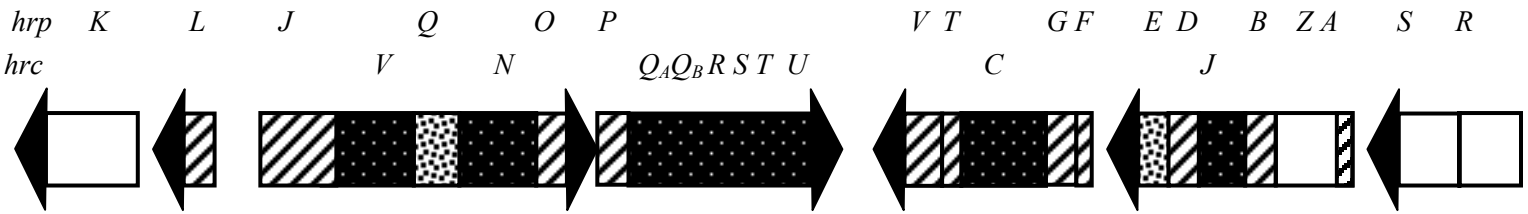


Figure I-1. Organization of the *hrp* gene cluster in *P. syringae* pv. *tomato* strain DC3000

Arrowheads indicate the direction of transcription for each operon. Widely conserved *hrp* genes (*hrc*) are dark shaded; genes that are conserved between phytopathogens but show weaker similarity to *Yersinia ysc* genes than *hrc* genes are stippled; genes common to group I *hrp* gene cluster are hatched; gene organization is similar in all sequenced *P. syringae* strains (Buell *et al.,* 2003; Feil *et al.,* 2005; http://*Pseudomonas-syringae*.org).

CHAPTER II

Genetic Screening for the *Pseudomonas syringae* Genes Involved in Exogenous Signal Sensing and Transduction

SUMMARY

Pseudomonas syringae TTSS genes are repressed when bacteria grow in the rich medium but induced to a high level within 6 hours after growth in inductive minimal medium or in plants. The induction of the *avrPto* promoter in minimal medium can be specifically suppressed by the fatty acid cis-9, 10-epoxystearic acid (ESA), a precursor of plant cutin and a candidate negative signal for TTSS gene induction in plants. To understand how *P. syringae* senses the environmental signals to regulate TTSS genes, an EZ::TN^{TM} transposon insertion (*Tz*) mutagenesis was performed on *P. s. phaseolicola* (*Psph*) strain NPS3121 carrying a plasmid borne *avrPto-LUC*, a luciferase (LUC) reporter gene driven by the promoter of the TTSS effector gene *avrPto*. The resulting library (of size 11,872 mutants) was screened for the mutants showing altered expression of the *avrPto-LUC*. Four categories of mutants were obtained that impacted *avrPto* promoter activities in various conditions. Transposon insertions (*Tz*) in mutants were mapped by two-stage semidegenerate PCR and sequence analysis. For the selected mutants, *Tz* positions were verified by regular PCR and sequencing analysis or electrophoresis, and the identities of several *pin* (plant-signal insensitive) and *min* (minimal medium insensitive) mutant genes were characterized.

INTRODUCTION

Pseudomonas syringae can infect a wide variety of plants and produce chlorotic and necrotic lesions on leaves or fruits (Gonzalez *et al.,* 2000; Hirano and Upper, 2000). Pathogenicity of *P. syringae* relies on the type III secretion system (TTSS) that is highly conserved among many Gram-negative bacterial pathogens. The TTSS is a syringe

needle-like structure through which bacteria deliver an array of effector proteins directly into host cells (Collmer *et al.,* 2000; Galan and Collmer, 1999; Hueck, 1998; Staskawicz *et al.,* 2001). The TTSS of *P. syringae* is encoded by a cluster of genes designated as *hrp* (HR and pathogenicity) genes, because defects in these genes abolish both bacterial pathogenicity on hosts and elicitation of HR on nonhosts (Galan and Collmer, 1999; Lindgren, 1997; Wei *et al.,* 2000).

hrp genes and TTSS effector genes (collectively called TTSS genes) are coordinately regulated by environmental signals in *P. syringae*. TTSS genes are repressed when bacteria grow in nutrient rich media such as KB (King'sB) and LB (Luria-Bertani) media and are induced to high levels in the plant apoplast or in defined TTSS-inducing minimal medium that is thought to mimic the plant apoplast environment (Rahme *et al.,* 1992; Thwaites *et al.,* 2004; Xiao *et al.,* 1992; Xiao *et al.,* 1994). The induction of TTSS genes in the plant apoplast implicates the existence of positive signals in plants. However, the identities and nature of the plant-associated positive signals are unclear. The factors in minimal medium that affect TTSS expression may provide some clues for identifying the plant-associated signals. Recent studies in our laboratory suggest the existence of a negative plant-derived signal for *P. syringae* TTSS gene regulation (Xiao *et al.,* 2004). The loss-of-function *att1* mutant of *Arabidopsis* supports significantly higher induction of TTSS genes compared with the wild type plants. *ATT1* encodes a cytochrome *P450* monooxyenase catalyzing fatty acid hydroxylation and is involved in the biogenesis of plant cutin. Certain fatty acids and plant cutin monomers can specifically suppress the induction of TTSS genes, suggesting that cutin monomer species act as a negative signal to regulate the *P. syringae* TTSS genes *in planta*.

Activation of *P. syringae* TTSS genes is mediated by a *cis* element, termed *hrp* box, in the promoter region (Innes *et al.,* 1993; Shen and Keen, 1993). Activation of TTSS genes in response to the inducing conditions requires HrpL, an alternate sigma factor in ECF family, through interaction with the *hrp* box (Hutcheson *et al.,* 2001; Xiao *et al.,* 1994; Xiao and Hutcheson, 1994; Zwiesler-Vollick *et al.,* 2002). Transcription of *hrpL* is under the control of a σ^{54}-dependent promoter in an alternate sigma factor RpoN-dependent manner (Alarcon-Chaidez *et al.,* 2003; Chatterjee *et al.,* 2003; Hendrickson *et al.,* 2000). Activation of *hrpL* also requires HrpR and HrpS, two highly homologous enhancer-binding proteins in the NtrC family of two-component regulatory proteins (Grimm *et al.,* 1995; Hutcheson *et al.,* 2001; Xiao *et al.,* 1994). In pathovars *syringae* and *tomato*, HrpR and HrpS form a heterodimer that binds the promoter of *hrpL* to activate the *hrpL* transcription (Hutcheson *et al.,* 2001; Xiao *et al.,* 1994). In pathovar *phaseolicola*, however, HrpS alone was shown to activate *hrpL* transcription, and the expression of *hrpS* requires HrpR (Grimm *et al.,* 1995).The GacS/GacA system, a global two-component regulatory system, also participates in regulation of TTSS genes by positively regulating the transcription of *rpoN* and *hrpR* (Chatterjee *et al.,* 2003; Heeb and Haas, 2001). HrpA, the major subunit of hrp pilus is also required for optimal expression of all TTSS genes. In a *hrpA* mutant, the transcriptional induction of TTSS genes was repressed in inducing medium (Wei *et al.,* 2000). The mechanism by which HrpA regulates TTSS genes is still unknown. Another protein affecting expression of the TTSS genes is Lon, an ATP-dependent protease. Lon negatively regulates the TTSS genes through proteolysis of HrpR (Bretz *et al.,* 2002). Additional proteins that regulate TTSS genes include HrpV and HrpG. HrpV is a negative regulator of TTSS genes

upstream of HrpR/HrpS (Preston *et al.,* 1998; Wei *et al.,* 2005). In *hrp*-inducing minimal medium, overexpression of *hrpV* down-regulates TTSS gene expression, while in *hrpV* mutants *hrp* genes are expressed at a moderately elevated level. HrpG, a chaperone-like protein can derepress the TTSS gene expression in *hrpV*-overexpression strain (Wei *et al.,* 2005). It is proposed that HrpG derepresses TTSS genes via protein-protein interaction to free HrpS from HrpV in inducing conditions.

Although it is known that expression of TTSS genes is regulated by plant-associated signals and influenced by environmental factors, the bacterial sensors for the exogenous signals and the mechanisms by which the signals are transferred to the known regulators are still not known. To identify the *P. syringae* bacterial genes required for exogenous signal sensing and transduction, EZ::TN^{TM} transposon insertion (*Tz*) mutagenesis was performed on *P. s. phaseolicola* (*Psph*) strain NPS3121 carrying a plasmid borne, HrpL-dependent *avrPto* promoter fused to firefly luciferase (LUC) gene. The mutant library (11,872 mutants) was screened for the mutants showing altered expression of TTSS genes, as indicated by the LUC activity. In the wild type parental strain, *avrPto* promoter is repressed when the bacteria are grown in a rich medium such as King'sB (KB) broth, but induced to a high level within 6 hours after growth in inductive minimal medium or in plants. The induction of *avrPto* promoter in minimal medium can be specifically suppressed by the fatty acid cis-9, 10-epoxystearic acid (ESA), a precursor of plant cutin. We screened for mutants that showed aberrant *avrPto* promoter activity in various conditions: i, active constitutively in KB (KB mutants); ii, inactive or less active in the inductive minimal medium (termed as *min* mutants standing for minimal medium insensitive); iii, inactive or less active in plant (termed as *pin*

mutants to stand for plant insensitive); iv, less sensitive to the ESA suppression in minimal medium (fin, fatty acid insensitive; these mutants showed higher LUC activity in minimal medium supplemented with ESA than did the parental strain). In mutants with the desired phenotypes, genes disrupted by *Tz* were amplified by two-stage semidegenerate PCR and sequenced using a transposon-specific primer. Identity of the genes was determined by blasting the genome of *P. s.* pv. *tomato* DC3000 or *P. s. phaseolicola* 1448A in the GenBank database. Selected genes of interest were further confirmed by regular PCR and sequencing analysis or electrophoresis. A few of the *pin* and *min* mutants were preliminarily characterized.

RESULTS

Construction of Pseudomonas syringae pv. phaeseolicola NPS 3121 mutant library

The *avrPto* gene of *P. syringae* pv. *tomato str.* DC3000 has a typical *hrp* box in its promotor region. Like many other TTSS genes, *avrPto* expresses at a minimal level when bacteria grow in KB medium and is rapidly and strongly induced in minimal medium and *in planta* (Salmeron and Staskawicz, 1993; Xiao *et al.,* 2004). To non-intrusively monitor the transcription of TTSS genes, we constructed an *avrPto–luc* reporter on broad host plasmid pHM2 by fusing a DNA fragment encompassing the *avrPto* promoter and the start codon in frame to the open reading frame of the firefly luciferase gene (*luc*) without start codon ATG. The construct was introduced to *P. syringae* pv. *phaeseolicola* NPS 3121. The resulting reporter strain displayed low LUC activity in KB but high LUC activity in minimal medium and in *Arabidopsis att1* plants

(Fig. II-1), that can be detected by a cooled CCD camera (Roper Scientific, Trenton, NJ; Xiao *et al.,* 2004).

To identify bacterial genes required for the signal perception or transduction in regulation of type III genes, EZ::TN^{TM}<Kan-2> transposon insertion mutagenesis was performed on *P. syringae* pv. *phaseolicola* NPS 3121 carrying the plasmid borne *avrPto–luc* reporter. The resulting mutant library contains 11, 872 clones, providing approximately 2× coverage of the *P. syringae* genome. The library was subject to genetic screen for mutants that display altered LUC activity compared with the parental strain under different conditions.

Isolation of KB mutants

TTSS genes in the parental strain are suppressed when bacteria are grown in KB medium. To identify the genes involved in the suppression of TTSS genes, all mutants were tested for the suppression of *avrPto-LUC* in KB medium. From the whole library, 15 mutants that displayed higher LUC activities in KB medium than did the parental strain were isolated (Fig. II-2, A). These mutants are designated as KB mutants. The transposon insertions in these mutants were mapped to eight genes/ORFs using a two-step semigenerated PCR (Table II-1). Three mutants were mapped to *lon1*, which encodes an ATP-dependent protease La (Lon), a known negative regulator of TTSS genes in both *P. syringae* (Bretz *et al.,* 2002) and *Samlonella enterica* serovar *Typhimurium* (Takaya *et al.,* 2002). The *Tz* insertion sites in *lon1*$^{-}$ mutants were confirmed by regular PCR and gel electrophoresis (Fig. II-2, B and C). *envZ*$^{-}$ and *ompR*$^{-}$ were also isolated as KB mutants. *envZ* and *ompR* encode the two-component histidine kinase sensor and response regulator, respectively, regulating bacterial response to

osmolarity. In *Salmonella typhimurium*, the EnvZ/OmpR system has a role in regulating the SPI-2 TTSS genes in responding to the change of Ca^{2+} concentration, osmolarity, and pH (Garmendia *et al.,* 2003).

Tz insertions in *KB11* and *KB12* were mapped to the gene PSPPH_0858, which encodes a putative histidine kinase sensor. The ligand of this sensor is not known. *PSPPH_B0005* (*KB9*) encodes transcriptional regulators in Cro/CI family, a xenobiotic response element family of transcriptional regulators (Marchler-Bauer *et al.,* 2005). *PSPPH4014* (*KB8*) encodes a multidrug/solvent transporter in AcrB/AcrD/AcrF family, which may be involved in transporting small molecules across the bacterial membrane. *PSPPH4014* (*KB7*) and *SucD* (*KB5*) appear to be metabolic genes that are involved in starch and sucrose metabolism and tricarboxylic acid (TCA) cycle, respectively (Buell *et al.,* 2003; Joardar *et al.,* 2005). The role of these genes in TTSS gene regulation has not been reported.

Isolation of min mutants that are insensitive to minimal medium induction

To understand how the bacteria sense the minimal medium signals to induce expression of TTSS genes, the library was screened for mutants with compromised induction of *avrPto-LUC* in minimal medium. From the library, 102 mutants that exhibited lower *avrPto-LUC* activities than the parental strain did in minimal medium were recovered (Fig. II-3) and are named as minimal medium insenstitive (*min*) mutants. As listed in Table II-2, these mutants are distributed on 51 loci.

Based on the function of the gene products, *min* mutants can be divided into three groups. The genes in the first group are involved in the signal perception and

transduction. *PSPPH_2003* (*min12* and *min80*) and *envZ* (*min58*) encode sensor histidine kinases of bacterial two-component regulatory systems (TCRS). EnvZ together with OmpR coordinates the bacterial activity to the change of osmolarity and plays a role in TTSS gene regulation in *Salmonella typhimurium* (Garmendia *et al.,* 2003). Mutation of this gene also derepresses *avrPto* expression in KB medium. Mutant of *PSPPH_2003* was also identified in isolation of *pin* mutants (a more detailed characterization of this gene is presented in Chapter III). *hrpR* (*min94*, *min96* and *min102*) encodes an enhancer-binding protein of the NtrC family, a well-defined regulator of TTSS genes in *P. syringae* (Xiao *et al.,* 1994; Grimm *et al.,* 1995). *PSPPH_3244* (*min16, min17*) encodes a transcriptional regulator in TetR family, whose target genes are not known.

The genes in the second group encode membrane proteins (Table II-2). *oprE* (*min79*) and *PSPPH_4907* (*min93* and *min100*) encode porin proteins. OprE is an anaerobically induced porin of unknown function (Hancock and Brinkman, 2002). *PSPPH_4907* encodes a porin protein in OprD family that functions in trafficking of small molecules across the outer membrane (Hancock and Brinkman, 2002). *PSPPH_1921* (*min06*) encodes a peptidase, an iron-regulated membrane protein. *PSPPH_0578* (*min01*) encodes a putative membrane protein of unknown function. *PSPPH_5137* (*min107*) encodes a putative YeeE/YedE family protein. The function of this protein family is not known.

The genes in the third group encode proteins that are required for basic metabolism (Table II-2). *PSPPH_2878* (*min85*) encodes a glycosyl hydrolase in family 15 that is involved in the metabolism of polyscaccharide, the major component of bacterial cell wall. *cbiD* (*min83*), *cbiG* (*min75*), *cobC* (*min87*), *cobH* (*min103*), *cobI*

(*min110*), *cobM* (*min30, min50, min55, min56*), *cobN* (*min66, min69*), *cobQ* (*min51*), *PSPPH_3701* (*min104, min106*), *PSPPH_2598* (*min64, min95, min88*), *PSPPH_2227* (*min77*) and *bchI* (*min29, min54*) are involved in the porphyrin and chlorophyll metabolism (Buell *et al.,* 2003; Joardar *et al.,* 2005). *PSPPH_0847* (*min65* and *min98*), *fruK* (*min18*), *PSPPH_* 0849 (*min41*) are in an operon required for fructose and mannose uptake and metabolism. These four mutants are further characterized in Chapter IV. *PSPPH_0542* (*min46*) encodes 4-hydroxybenzoyl-coa thioesterase domain protein that is probably involved in fatty acid metabolism (Dillon and Bateman, 2004). The remaining genes in this group are involved in biosynthesis of basic amino acids. Mutants in the third group may affect the basic metabolism of bacterial cells in nutrient-limited conditions.

Since a large proportion of *min* mutants appeared to be defective in amino acid biosynthesis, we tested the *avrPto-LUC* activity in *min* mutants in minimal medium supplemented with arginine, histidine, glutamate or combination of five amino acids (arginine, histidine, glutamate, leucine and serine). As listed in Table II-3, *avrPto-LUC* activity in the mutants with defects in amino acid biosynthesis could be restored by corresponding amino acids. For example, glutamate can partially restore the *avrPto-LUC* activity in *gltB*$^-$ and *gltD*$^-$ mutants, and histidine can restore the *avrPto-LUC* activity in *hisA*$^-$, *hisB*$^-$, *hisC*$^-$, *hisD*$^-$, *hisE*$^-$, *hisF*$^-$ and *hisH*$^-$ mutants. *avrPto-LUC* activity in leucine biosynthesis mutants *leuA*$^-$, *leuB*$^-$ and *leuC*$^-$ can be restored by none of the three single amino acid treatments, but can be restored by the leucine-containing five amino acid treatment.

We also tested the *in planta* induction of *avrPto-LUC* in some of the mutants defective in basic metabolism. Results showed that *avrPto-LUC* activities in the mutants

with defects in biosynthesis of arginine, histidine, glutamate and porphyrin and chlorophyll metabolism are as high as that in parental strain in *att1* plants, while the mutants that exhibit defects in biosynthesis of isoleucine, leucine, methionine, serine, tryptophan, valine and purine exhibited low *avrPto-LUC* activities in *att1* plants. This observation may reflect the nutrition profile, especially the abundances of different amino acids, in the apoplast fluid of *att1* plants.

Isolation of pin mutants that are insensitive to induction in planta

To investigate how *P. syringae* bacteria sense and transduce positive plant signals to activate TTSS genes, we screened the NPS 3121 mutant library for strains defective in *avrPto-LUC* induction in plants. *Arabidopsis att1* mutant was used as plant material since it supported higher TTSS gene induction and provided a sensitive system to study the TTSS gene regulation (Xiao *et al.,* 2004). From ~6, 000 mutants, 62 were isolated as plant signals insensitive (*pin*) mutants that displayed reduced or abolished *avrPto-LUC* induction in *att1* plants (Fig. II-4A; Table II-4). *pin* mutants are distributed on 41 *loci*. In agreement with the *min* mutant data, the mutants defective in biosynthesis of isoleucine, leucine, methionine, purine, serine, tryptophan, and valine were also isolated as *pin* mutants (Table II-3, 4).

Based on the function of gene products, the *pin* mutants can be divided into four groups (Table II-4). The most striking group includes genes that are involved in signaling. Besides *PSPPH_2003* (*pin9* and *pin19*), another sensor histidine kinase mutant *phoQ* (*pin18*) was isolated as a *pin* mutant. PhoQ is the sensory component of PhoQ/PhoP system. In *Salmonella spp.*, the PhoQ/PhoP system regulates SPI-1 TTSS genes in response to low concentrations of extracellular Mg^{2+} (Garcia *et al.,* 1996). *hrpR* (*pin22*)

and *hrpS* (*pin20*) encode two highly homologous enhancer-binding proteins required for the activation of TTSS genes (Grimm *et al.,* 1995; Hutcheson *et al.,* 2001). *gidA* (*pin17* and *pin30*) encodes a global regulator required for *P. syringae* virulence (Kinscherf and Willis, 2002). *exsB* (*pin01*) encodes a transcription regulator related protein. In *P. aeruginosa*, *exsB* appeared to be untranslated, but the deletion of *exsB* affected the expression of the downstream gene *exsA*, resulting in the reduction of ExoS production. It is likely that *exsB* RNA contains a region that facilities the translation of ExsA or stabilities of the mRNA of *exsA* (Goranson *et al.,* 1997). *pin01* only partially loses the induction of *avrPto-LUC* in minimal medium and in plant. *tonB* (*pin35*) and *PSPPH_1736* (*pin59*) encode TonB and TonB-dependent siderophore receptor, respectively. TonB and TonB-dependent siderophore receptor are not only required for ferric iron uptake and are involved in signal transduction from cell surface to cytoplasm (Braun, 1995). In *Xanthomonas campestris* pv. *campestris*, *tonB* is required for HR elicitation on nonhost plant pepper (Wiggerich and Pühler, 2000). In *Ralstonia solanacearum*, PrhA, a homolog of TonB-dependent siderophore receptor, acts as bacterial receptor for a plant-derived nondiffusible signal and plays an important role in *in planta* activation of TTSS genes (Brito *et al.,* 2002; Marenda *et al.,* 1998). Functions of these two genes in regulation of TTSS genes in *P. syringae* have not been reported.

The *Tz*-harboring genes in second group of *pin* mutants encode proteins involved in bacterial cell wall/membrane biogenesis. Among these mutants, *PSPPH_0519* (*pin49*) and *PSPPH_0520* (*pin50*) are located in an operon and encode glycosyl transferase and carbamoyltransferase family protein, respectively. *PSPPH_3810* (*pin21*) encodes lipoprotein NlpD containing a LysM domain that may have a general peptidoglygan

binding function and a peptidase M37 domain. However, no proteolytic activity has been demonstrated for NlpD (Marchler-Bauer *et al.,* 2005). *galU* (*pin36*) encodes UTP-glucose-1-phosphate uridylyltransferase. In *P. aeruginosa*, *galU* is required for lipopolysaccharide core synthesis (Dean and Goldberg, 2002). *oprF* (*pin57* and *pin80*) encodes the major structural porin OprF in *Pseudomonas spp.* (Hancock and Brinkman, 2002).

The third group is composed of those involved in stress response. *oxyR* (*pin47*) encodes an oxidative stress regulatory protein OxyR in LysR family. *PSPPH_* 0671 (*pin79*) encodes a DNA-directed DNA polymerase in ImpB/mucB/samB family that functions in UV protecting. *PSPPH_0667* (*pin58*) encodes an OsmC/OhR family protein that likely confers to adaption to atypical conditions and resistance to tert-butyl hydroperoxide killing. *hptX* (*pin44*) encodes a heat shock protein involved in posttranslational modification or protein turnover or acts as chaperone. *nuoM* (*pin72*) encodes the M subunit of NADH dehydrogenase I that is involved in the biosynthesis of ubiquinone.

The fourth group of *pin* mutants has the *Tz* insertion in the metabolic genes, including those that are defective in biosynthesis of isoleucine, leucine, methionine, purine, serine, tryptophan, or valine. This group also includes *pgi* (*pin37*), *pgk* (*pin39*), *PSPPH_3261* (*pin60*), and *PSPPH_4463* (*pin42*). *pgi* and *pgk* are involved in glycolysis/gluconeogenesis and encode glucose-6-phosphate isomerase and phosphoglycerate kinase/phosphotransferase, respectively. *PSPPH_3261* encodes a hypothetical protein homologous to the flavoprotein subunit of succinate dehydrogenase/fumarate reductase that may be involved in tricarboxylic acid cycle.

PSPPH_4463 encodes a putative precorrin-6X reductase that may be involved in porphyrin and chlorophyll metabolism.

There are other *pin* mutants including *rplI* (*pin52*), *rph* (*pin41*), *PSPPH_4907* (*pin27*), *PSPPH_2198* (*pin24*) and *ispZ* (*pin46*), which fall into none of the four groups mentioned above. *rplI* encodes ribosomal protein L9 that is involved in protein biosynthesis and functions in restricting peptidyl-tRNA slippage (Herr *et al.,* 2001). *rph* encodes ribonuclease PH that plays an important role in splicing and maturation of CCA-containing tRNA. The maturation process of the longer precursor of tRNA into a functional form is essential for bacterial growth and viability (Choi *et al.,* 2004). *PSPPH_4907* encodes an outer membrane porin in OprD family. *PSPPH_2198* encodes a conserved hypothetical protein and is located 22 bp downstream of *prt* gene that encoding a metalloprotease (Joardar *et al.,* 2005). The close localization of the two genes suggests that they may belong to the same operon and are functionally related. Prt is homologous to the C-terminal of LasB protease in *Pseudomonas aeruginosa*, which is critical to the bacterial virulence (McIver *et al.,* 2004). *ispZ* encodes intracellular septation protein A. In *Shigella flexneri*, *ispA*, a homolog of *ispZ*, is believed to play a role in cell division and spreading throughout an epithelial cell monolayer. Mutation of *ispA* in *Shigella flexneri* renders the bacterium avirulent (Mac Siomoin *et al.,* 1996).

Preliminary characterization of some pin mutants

To determine if the *pin* mutants specifically affect *avrPto-LUC* induction *in planta*, we tested induction of *avrPto-LUC* in minimal medium for *pin* mutants. Based on the phenotypes, the *pin* mutants can be separated into two groups. The mutants of one group show the same level of reduction of *avrPto-LUC* induction in minimal medium as

that in *att1* plant (Fig. II-4B), while those in the other group display normal or slightly reduced induction of *avrPto-LUC* in minimal medium but lower or abolished induction of *avrPto-LUC* in *att1* plant compared with the wild type parental strain (Fig. II-5). Most of the *pin* mutants, including the metabolic mutants, belong to the former group (Fig. II-4B, and data not shown). Further characterization of the second group revealed that the mutants involved in the cell wall biogenesis and outer membrane proteins, including *pin49*, *pin50*, *pin36*, *pin57* and *pin80,* affect the bacterial viability in plants. Inoculated at the same level as the parental strain into plant, the recoverable populations of these mutants are significantly smaller than that of the parental strain (Table II-5). Thus, the reduction of the *avrPto-LUC* induction may simply result from the reduced population size.

To test the correlation of the *avrPto-LUC* induction and the elicitation of HR and pathogenicity of the *pin* mutants, we chose 14 mutants including *hrpR*$^-$ (*pin29*) and conducted the HR assay on nonhost plant tobacco *W38* and pathogenicity analysis on host plant Red Kidney bean. For the HR assay, bacteria were inoculated at 10^8 CFU/ml levels and scored at 20 hours after inoculation. Among the selected mutants, only *pin60* and wild type parental strain showed an HR in 20 hours on *W38* plant (Table II-5). For pathogenicity analysis, the bacteria were inoculated at 2×10^5 CFU/ml and 2×10^4 CFU/ml levels into the primary leaves of 10-day old Red Kidney plants for disease symptom and bacterial growth assays, respectively. The disease symptoms were scored at 9 days after inoculation using a 1-5 disease index (see the notes of Table II-5) or were photographed (Fig. II-6). As a result, *pin60* showed symptoms as severe as the wild type parental strain, while the others were less virulent. Among those mutants, *phoQ*$^-$ (*pin18*), *rohS*$^-$ (*pin9* and

pin19), *PSPPH_2198*$^{-}$ (*pin24*), *galU*$^{-}$ (*pin36*) were symptom-free. The bacterial growth assay revealed that the reduced virulence in mutants *pin18*, *pin21*, *pin36*, *pin49*, *pin50*, *pin57,* and *pin80* was at least partially due to the reduced bacterial viability and growth on plants. Inoculated at the same levels, these mutants exhibited significantly smaller recoverable population size at 0 day and lower growth rate as indicated by ratio of population size on 6 dpi (day post inoculation) to that on 0 day compared to the wild type strain (Table II-5). However, disease symptoms were well correlated with the bacterial growth rate of these mutants. While in the *pin* mutants that do not affect the bacterial viabilities, *avrPto-LUC* induction is positively correlated with bacterial pathogenicity and HR elicitation (Table II-5; Fig. II-6).

Plant cutin extract and cis-9,10-epoxystearic acid specifically suppresses the induction of avrPto-LUC activity in minimum media

In our recent study attempting to identify plant signals that regulate bacterial TTSS genes, we isolated an *Arabidopsis att1* mutant that supports enhanced induction of bacterial TTSS genes (Xiao *et al.,* 2004). *ATT1* encodes a cytochrome P450 monooxygenase catalyzing fatty acid hydroxylation. Hydroxylated fatty acids form extracellular polyesters that are major constituents of cutin that envelops aerial parts of the plants (Bonaventure *et al.,* 2004). The cutin content in *att1* mutant is reduced to 30% of that in the wild type plant (Xiao *et al.,* 2004), indicating that ATT1 plays an important role in biosynthesis of cutin or cutin monomers. The reduced cutin content and enhanced induction of bacterial TTSS genes in *att1* mutant suggested that cutin or cutin monomers are negative signals for bacterial TTSS gene regulation. To test this, we examined the *avrPto-LUC* induction in minimal medium supplemented with plant cutin extract. Plant

cutin extract significantly inhibited the *avrPto-LUC* induction in minimal medium but not the metabolic gene *trp* promoter activity (Fig. II-7, A and B). The cutin extract-suspension in minimal medium at levels as low as 0.3 mg/ml exhibited significant inhibitory activity to *avrPto-LUC* induction. This result demonstrated that cutin extract specifically inhibits TTSS gene induction.

The major cutin monomers are ω-hydroxy C16 and C18 fatty acids (Bonaventure *et al.,* 2004). We tested the effect of several C16 and C18 fatty acids obtained from commercial sources on the induction of *avrPto-LUC*, while all solid fatty acids tested including 9,10-dihydroxy-octadecanoic acid, 12-hydroxy stearic acid, *cis*-9,10-epoxystearic acid, oleic acid, palmitic acid, stearic acid and threo-9,10-dihydroxystearic acid suppressed the induction of *avrPto-LUC* at different levels, *cis*-9,10-epoxystearic acid (referred to as ESA hereafter) showed the strongest inhibitory activity (data not shown). The addition of ESA in minimal medium strongly repressed the induction of *avrPto-LUC* (Fig. II-7, C). As low as 0.1 mM ESA suspension exhibited maximum inhibitory activity. Bacterial growth assays and measurements on the effect of ESA on expression of *trp-LUC* demonstrated that the reduced *avrPto-LUC* activity was not due to the reduced bacterial growth or overall cellular activity (Fig. II-7, D). In contrast, better bacterial growth was detected in minimal medium supplemented with ESA. These results suggested that a variety of fatty acids specifically repress bacterial TTSS gene expression, and certain species of fatty acids such as ESA are more potent than the others in this inhibition.

Isolation of fin (Fatty acid insensitive) mutants that are insensitive to fatty acid ESA

To better understand how bacteria sense the cutin-related signals, the NPS3121 *Tz* transposon insertion library was screened for the mutants that are less sensitive to the ESA inhibition compared to the parental strain. In total, 60 independent mutants that showed higher *avrPto-LUC* activity cultured in minimal medium supplemented with 0.1 mmol/L ESA for 6 to 12 hours than parental strain did (Fig. II-8) were isolated and designated as *fin* (fatty acid insensitive) mutants. *Tz*-mapping results showed these mutants represent 24 *loci* (Table II-6). The *Tz* localization in all *fin* mutants was further confirmed by sequencing the PCR products amplified using primers specific to the *Tz*-flanking gene toward 5′ end of *Tz* and the *Tz*-specific primer Kan2-RP (Table II-7).

Eleven *fin* mutants that represent three *loci* displayed higher basal level of *avrPto-LUC* activity when cultured in KB medium than did parental strain (Table II-6, Fig. II-8). These 11 *fin* mutants were also isolated as *KB* mutants (Table II-1). The three *loci* are *lonB* (*fin01*, *fin02*, *fin03,* and *fin04*), *ompR* (*fin05*) and *PSPPH_4014* (*fin06*, *fin07*, *fin08*, *fin09*, *fin10,* and *fin11*). The other *fin* mutants showed similar basal *avrPto-LUC* activity in KB medium and higher *avrPto-LUC* activity in minimal medium supplemented with 0.1 mmol/L ESA than did the wild type control (Fig. II-8). These mutants can be classified into two groups based on their functions. The genes in one group are involved in fatty acid metabolism (Table II-6). These genes encode FadA, FadB, FadD-1, FadE, a FadL-like fatty acid transport protein (*PSPPH_3689*) and an oxidoreductase in short-chain dehydrogenase/reductase family (*PSPPH_2612*). These proteins are known to be involved in the fatty acid transport or metabolism (http://www.genome.jp/kegg/pathway/psp/psp00071.html). *PSPPH_2613*, encoding an

aminoglycoside phosphotransferase, is located 28 bp upstream of *PSPPH_2612* in the same orientation. The close localization suggests that the two genes are likely to be in the same operon and are functionally related, thus mutants of *PSPPH_2613* may impact fatty acid metabolism as well. *PSPPH_2631* encodes a transcriptional regulator in LysR family and is located 184 bp upstream of *PSPPH_2632* encoding an acyl-CoA dehydrogenase family protein that has the same function as FadE. It is not known if the interruption of *PSPPH_*2631 affects the transcription of *PSPPH_2632*. This *fin* mutant is also grouped into the fatty acid metabolism group because genes flanking the transposon insertion site are involved in fatty acid metabolism.

The *Tz*-harboring genes in the other group of *fin* mutants are involved in the Type IV pilus biogenesis, consisting of *pilB*, *pilF*, *pilM*, *pilQ*, *pilR*, *pilS* and *pilY1*. These genes or their counterparts in other bacteria are known to be required for Type IV pilus biogenesis (Nudleman and Kaiser, 2004; Roine *et al.,* 1996 and 1998). *PSPPH_1317* (*fin37* and *fin41*) encodes a radical SAM protein in Cfr family and is 26 bp upstream of *pilF* translation start codon. The gene *PSPPH_0783* (*fin57*) encodes a hypothetical protein and is located 10 bp upstream of *pilS*. Their close localization with *pilF* or *pilS* suggests that genes *PSPPH_1317* and *PSPPH_0783* are likely to be involved in Type IV pilus biogenesis as well. It is also possible that the *Tz* insertions in these two genes impact Type IV pilus biogenesis by interrupting their downstream genes *pilF* or *pilS*.

In the remaining two *fin* mutants, the *Tz* transposon insertions were mapped to two closely located genes *PSPPH_3205* (*fin15*) and *PSPPH_3206* (*fin54*). These two genes encode two conserved hypothetical proteins of unknown function.

DISCUSSION

It is advantageous for plant pathogenic bacteria to activate virulence genes only upon detection of and in response to host-associated signals since gene expression is a highly energy-consuming process. A classical example is the regulation of *Agrobacterium* virulence (*vir*) genes that encode proteins required for plant cell recognition and T-DNA transfer from the bacterium to the host cell. *Agrobacterium vir* genes are not expressed in saprophytic bacteria, but are induced by plant-released signals including specific phenolic compounds and monosaccharides (Ankenbauer and Nester, 1990; Melchers *et al.,* 1989; Spencer and Towers, 1988). Induction of *vir* genes is also affected by pH and temperature (Alt-Moerbe *et al.,* 1988; Jin *et al.,* 1993; Li *et al.,* 2002; Stachel, 1986). Activation of *vir* genes in response to plant-released signals requires the two-component regulatory system VirA/VirG (Zhu *et al.,* 2000). VirA is the sensor histidine kinase that detects phenolics and acidic pH by its linker domain. A chromosomally encoded sugar binding protein ChvE detects and binds sugar and contacts with VirA periplasmic domain, resulting in the enhanced phenolic-induced VirA activity (Peng *et al.,* 1998; Shimoda *et al.,* 1993). In the presence of plant-released signals, VirA is autophosphorylated and subsequently transfers the phosphate group to the response regulator VirG, which in turn activates the transcription of *vir* genes including *virA* and *virG*.

For many Gram-negative plant pathogenic bacteria, TTSSs are their essential pathogenesis determinants. The bacteria rely on TTSSs to deliver an array of effectors directly into the host cells for successful infection (Alfano and Collmer, 1997; Galan and Collmer 1999; He, 1997; Hueck, 1998). The majority of the TTSS genes that encodes the

TTS apparatus and its substrates are regulated by environmental factors and host-associated signals (Arlat *et al.,* 1992; Huynh *et al.,* 1989; Rahme *et al.,* 1992; Schulte and Bonas, 1992; Xiao *et al.,* 1992). Unfortunately, even though some downstream components of the regulatory pathways of TTSS genes have been studied in detail (Alfano and Collmer, 1997; He, 1997; Hueck, 1998; See chapter I), it is poorly understood what signal(s) regulate TTSS genes in plants and how the signals are transferred to the known pathway. In *Ralstonia solanacearum*, full induction of TTSS genes requires an unknown plant-specific signal. PrhA, a sideorphore receptor-like outer membrane protein detects and transfers this signal to HrpB, the activator of TTSS genes, through the regulatory cascade integrated by PrhR, PrhI, PrhJ, and HrpG in order (Aldon *et al.,* 2000; Brito *et al.,* 2002).

In this study, we have uncovered numerous functionally diverse proteins affecting the regulation of *P. syringae* TTSS genes. The reduced induction of TTSS genes in Group C *min* mutants and Group D *pin* mutants in inductive conditions may simply result from altered metabolism or reduced overall cellular activities because these genes are involved in basic metabolism. The reduced induction of TTSS genes in Group B *pin* mutants may result from the significantly reduced viability of bacteria in plant. The others may be directly or indirectly involved in the TTSS gene regulation. The genes *envZ*, ompR, *PSPPH_0858*, Group A *Min* genes and Group A *pin* genes encode either bacterial sensory proteins or regulatory proteins, which are usually involved in signal sensing or transduction. Group B *Min* genes and *oprF* encode membrane proteins. These genes also have potential roles in signaling. The membrane protein may directly detect signals or function in the transporting of the signals across the cell membrane. It is also

possible membrane proteins participate in TTSS gene regulation by exporting the regulator(s). Group C *Fin* genes are required for biogenesis of Type IV pili. In *P. syringae*, type IV pili are required for bacterial attachment and twitching motility on the leaf surface (Romantschuk and Bamford, 1986; Romantschuk *et al.,* 1993). Type IV pili also play an important role in bacterial epiphytic fitness (Roine *et al.,* 1998). These results suggest that *P. syringae* TTSS genes are controlled by a complex array of host signals. The detection of these signals enables the bacteria to differentiate the environments inside or outside plants, and sequentially decide whether or not to activate TTSS genes. Consistent with this hypothesis, the epiphytic bacteria do not activate TTSS genes but the endophytic bacteria do (Xiao *et al.,* 2004).

Cutin monomers are likely to act as negative signals in bacterial TTSS gene regulation. ATT1 is involved in cutin biosynthesis and negatively regulates bacterial TTSS genes (Xiao *et al.,* 2004). The negative effect of ATT1 on the expression of bacterial TTSS genes could be mediated either by cuticle membrane structure or by cutin monomers synthesized by ATT1. However, the *wax2* mutant, which has a similar alteration in cuticle membrane structure, supports normal bacterial TTSS gene activation (Xiao *et al.,* 2004), suggesting that cuticle membrane *per se* is not the bacterial signal. Additionally, in *vitro* assays showed that plant cutin extract and the synthetic fatty acids specifically inhibit bacterial TTSS gene induction in minimal medium. These results suggest that cutin monomers and/or their analogs could be negative signals regulating TTSS genes. Type IV pili are involved in sensing the cutin monomer signals probably by direct binding. Consistent with this speculation, *P. syringae* Type IV pili are required for bacterial adherence to leaf surface and binding to stomata (Romantschuk and Bamford,

1986; Romantschuk *et al.,* 1993), where ATT1 is exclusively expressed (Xiao *et al.,* 2004) and cutin monomers are presumably most abundant. The physical binding of type IV pili tips with cutin monomers may generate negative signals for bacterial TTSS genes. It is also possible that the binding facilitates the uptake of cutin monomers, and the metabolism of the cutin monomers generates negative signals. In supporting this, several mutants defective in fatty acid metabolism were identified as *fin* mutants (Group B *fin* mutants).

The environmental factors that are known to influence *P. syringae* TTSS genes include osmolarity and pH. High osmolarity and basic pH value suppresses TTSS genes, while low osmolarity and acidic pH value induces TTSS genes. Consistent with this, we isolated *envZ*$^-$, *ompR*$^-$ as the *KB* mutants and *envZ*$^-$ as the *min* mutants. EnvZ-OmpR system is responsible for both activation and repression of gene expression, in response to changes in osmolarity and pH in *Escherichia coli* (Heyde & Portalier, 1987) and plays a role in activation of SP-2 TTSS gene in response to low osmolarity and acidic pH (Garmendia *et al.,* 2003). Isolation of *phoQ*$^-$ as *pin* mutants suggested that low concentration of Mg^{2+} in plant apoplast is also a positive signal for *P. syringae* TTSS gene regulation. Other positive host-related signals are not known.

Transposon mutagenesis combined with genetic screening enabled us to isolate an array of mutants that function in either negative or positive regulation of *P. syringae* TTSS genes. This provides us a good opportunity to better understand how the bacteria coordinate virulence gene expression to the change of the environment. On the other hand, the transposon mutagenesis and genetic screen employed here also highlights several limations: 1. This collection of mutants does not cover all the genes required for

the bacterial TTSS gene regulation because the mutant screening is not saturated. For example, *hrpL*$^{-}$ mutants were not isolated in this study. 2. The mutants that affect the basic cellular metabolism or bacterial viability are likely to be isolated in screening for genes involved in the positive TTSS gene regulation, such as the group C *min* mutants and group C and D *pin* mutants. 3. Transposome mutagenesis may introduce multiple *Tz* insertions to the bacteria, however, we conducted southern blotting assay on randomly selected 17 *pin* mutants and the result showed that all the 17 mutants have single *Tz* insertion (data not shown). 4. Because many bacterial genes are organized as operons, we should be cautious of the possible polar mutations, i.e., the phenotypes of the mutants are caused by the interruption of the downstream gene(s) other than the *Tz*-harboring genes. 5. The phenotype of the mutants may be caused by secondary spontaneous mutation taken place during the process of mutagenesis. Concerning the last two points, after the confirmation of *Tz* mapping results and prior to further studies to characterize the mutant genes, complementation tests should be carried out to identify polar mutation and to rule out the the possibility that desired phenotypes are caused by a secondary spontaneous mutation.

With the collection of the *KB*/*pin*/*min*/*fin* mutants, the ongoing work is to genetically position the *KB*/*pin*/*min*/*fin* genes relative to each other and to the known regulators such as HrpR/S, HrpL, Lon, RpoN. First, epistasis analysis will be carried out between *KB*/*Pin*/*Min*/*Fin* and known regulators. The plasmid-borne reporters *hrpL-luc, hrpR-luc, rpoN-luc, lon-luc* will be introduced to the mutants cured of the *avrPto-LUC*, and the expression of the new reporters will be tested in different conditions. This approach will identify the *KB*/*pin*/*min*/*fin* genes required for the transcription of the

known regulators. *KB*/*pin*/*min*/*fin* genes may regulate the known TTSS gene regulatory genes at posttranslational level, and this can be determined by examining the known regulatory proteins in the *KB*/*pin*/*min*/*fin* mutants by western blot assay or GFP-fusion proteins. Similar experiments will be conducted to determine the epistasis relationship among the *KB*/*pin*/*min*/*fin* genes. We will test the expression of the cloned *KB*/*pin*/*min*/*fin* genes in wild type bacterium in KB medium, plant, minimum medium or minimum medium supplemented with ESA. For the genes show a change of expression pattern in response to plant or media signals, further studies will be perfomed to test whether mutations of other *KB*/*pin*/*min*/*fin* genes alter the expression pattern. Overall, we are expecting to construct a genetic framework of the TTSS gene regulation and to gain better understanding how the bacteria integrate various environmental signals to coordinately regulate TTSS gene expression.

MATERIALS AND METHODS

Construction of the reporter plasmid pHM2::avrPto-LUC

The *avrPto-LUC* reporter genes were previously constructed into the pPTE6 plasmid by Xiao *et al.* (2004). To generate the pHM2:: *avrPto-LUC* plasmid, the pHM1 plasmid (Zhu *et al.,* 1999) was first digested with BamHI to eliminate the *lacZ* promoter and multiple cloning sites. The vector was then partially filled-in with Klenow and dATP/dGTP and subsequently ligated with the adaptor DNA formed by association of the oligo nucleotides Adaptor-F and Adaptor-R (Table 3), resulting in pHM2 plasmid. The *avrPto-LUC* expression cassette was released from the pPTE6 plasmid digested by

restriction digestion with EcoRI and XbaI, and was subsequently cloned into the EcoRI- and XbaI- digested pHM2 plasmid, resulting in pHM2::*avrPto-LUC* plasmid.

Transposon mutagenesis

Transposon mutagenesis was conducted using an EZ-*Tz*^{TM}<KAN-2> transposon mutagenesis kit (Epicentre Technologies, Wisconsin, WI). *P. syringae* pv. *phaseolicola* NPS3121 carrying pHM2::*avrPto-LUC* plasmid was used as recipient cell. In brief, electrocompetent recipient cells were mixed with EZ-*Tz*^{TM}<KAN-2> transposon and transposase as instructed by manufacturer. Following electroperation, the bacteria were plated on KB medium containing 10 mg/L kanamycin and 10 mg/L spectinomycin to recover the mutant bacteria carrying the EZ::TN^{TM}<KAN-2> transposon and the pHM2::*avrPto-LUC* reporter plasmid. The resulting mutant library was grown and stored on 96-well plates.

Mutant screening

The mutant bacterial colonies were first grown in liquid KB medium containing 30 mg/L rifampicillin, 25 mg/L spectinomycin and 10 mg/L kanamycin, collected by centrifuge, and washed twice with sterile water. To screen for *KB* mutants and *min* mutants, the bacteria were resuspended in minimum medium (pH5.7) to OD_{600}=0.5. From each sample, 100 µl cell suspension was pippeted to 96 well plate, mixed with 2ul 1mM luciferin and measured the luciferase activity using a cooled CCD (Roper Scientific, Trenton, NJ). The mutants that showed significantly higher level of luciferase activity than the wild type did were selected as *KB* mutants. The remaining bacterial suspensions were cultured at room temperature for 6 hours and the luciferase activities were

determined as above. The mutants that showed at least 50% reduction of luciferase activities were selected as *min* mutants. To screen for *pin* mutants, the washed overnight cultures were resuspended in sterile water to OD_{600}=0.5 and injected into *att1* mutant plants. Six hours after inoculation, the inoculated leaves were sprayed with 1mM luciferin dissolved in 0.01% Tween-20. The luciferase activity was determined using the cooled CCD. Putative bacterial mutants with <30% of the wild type luciferase activity were selected and confirmed. To screen for *fin* mutants, the washed overnight cultures were resuspended in minimum medium (pH5.7) to OD_{600}=1. Cell suspensions were mixed with equal volume of 0.2 mM ESA. The luciferase activity was measured at 0 hour and 6 hours after culture at room temperature. The mutants that displayed at least 2 fold of the luciferase activity of parental strain were selected as *fin* mutants and confirmed.

Determination and confirmation of transposon insertion sites

The transposon insertion sites were determined by a two-stage semidegenerate PCR according to procedures described by Jacobs *et al.* (2003) using two transposon-specific primers (Kan2-SP1 and Kan2-SP2) and four degenerated primers (CEKG 2A, CEKG 2B, CEKG 2C, and CEKG) (Table II-7). The PCR product was sequenced using the third transposon-specific primer Kan2-SP3 (Table II-7). Blastn program was used to search for the homologous sequence in GenBank database.

To confirm the transposon insertion sites in selected mutants, a gene specific primer and a transposon-specific primer were used to PCR-amplify the transposon-flanking DNA. The PCR products were fractionated on agarose gel and sequenced.

Amino acid complementation assay on min mutants

Bacterial overnight culture in KB medium supplemented with appropriate antibiotics were collected by centrifuge and washed twice with sterile water. The bacteria were resuspended in minimum medium supplemented with 10 mM amino acid (for 5 amino acids treatment, individual amino acid were added at 2mM each) (pH5.7) to $0.5OD_{600}$ and cultured at room temperature with 250rpm shaking for 6 hours. Hundred micro liter cell suspension was mixed with 2ul 1mM luciferin in 96-well plate for luciferase activity measurement using the cooled immediately.

Bacterial growth, disease symptom, and non-host HR assays

Bacteria were cultured in liquid KB medium containing appropriate antibiotics overnight at room temperature. To prepare the inocula, the bacterial cells were centrifuged at 4000g, washed twice with sterile water, and resuspended in 10mM $MgCl_2$. For HR assay, 10^8 CFU/ml were inoculated into young, fully expanded tobacco W38 leaves by hand injection, and the HR were visually examined every 2 hrs after inoculation. To monitor the bacterial growth in bean plants, bacteria at $2x10^4$ CFU/ml or $5x10^4$ CFU/ml were inoculated using a needle-less syringe into the primary leaves of 10-day old Red Kidney bean plants. The bacterial number was determined by grinding 1 cm^2 leaf disc in sterile water, plating the diluted suspensions on TSA agar plate (10g/L Bacto tryptone, 10g/L sucrose, and 1g/L glutamic acid) containing appropriate antibiotics and counting colony-forming units. Disease symptoms on the inoculated bean leaves were scored or photographed 9 days after inoculation.

REFERENCES

Alarcon-Chaidez F. J., Keith L., Zhao Y., Bender C. L., 2003. RpoN (sigma(54)) is required for plasmid-encoded coronatine biosynthesis in *Pseudomonas syringae*. *Plasmid* 49: 106-117.

Arlat M., Gough C. L., Zischek C., Barberis P. A., Trigalet A., Boucher C. A., 1992. Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. *Mol. Plant Microbe. Interact.* 5: 187–193.

Aldon D., Brito B., Boucher C., Genin S., 2000. A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. *EMBO J.* 19: 2304-2314.

Alfano J. R., Collmer A., 1997. The type III (Hrp) secretion pathway of plant-pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J. Bacteriol.* 179: 5655–5662.

Alt-Moerbe J., Neddermann P., Vonlintig J., Weiler E. W., Schroder J., 1988. Temperature sensitive step in Ti plasmid *vir* region induction and correlation with cytokinin secretion by agrobacteria. *Mol. Gen. Genet.* 213: 1–8.

Ankenbauer R. G., Nester E. W., 1990. Sugar-mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides. *J. Bacteriol.* 172: 6442–6446.

Bonaventure G., Beisson F., Ohlrogge J., Pollard M., 2004. Analysis of the aliphatic monomer composition of polyesters associated with Arabidopsis epidermis: occurrence of octadeca-*cis*-6, *cis*-9-diene-1,18-dioate as the major component. *Plant J.* 40: 920-930.

Braun V., 1995. Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. *FEMS Microbiol. Rev.* 16: 295-307.

Bretz J., Losada L., Lisboa K., Hutcheson S. W., 2002. Lon protease functions as a negative regulator of type III protein secretion in *Pseudomonas syringae*. *Mol. Microbiol.* 45: 397-409.

Brito B., Aldon D., Barberis P., Boucher C., Genin S., 2002. A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum hrp* genes. *Mol. Plant Microbe Interact.* 15: 109-119.

Buell C. R., Joardar V., Lindeberg M., Selengut J., Paulsen I. T., Gwinn M. L. *et al.,* 2003. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. USA* 100: 10181-10186.

Chatterjee A., Cui Y., Yang H., Collmer A., Alfano J. R., Chatterjee A. K., 2003. GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol. Plant Microbe Interact.* 16: 1106-1117.

Choi J. M., Park E. Y., Kim J. H., Chang S. K., Cho Y., 2004. Probing the functional importance of the hexameric ring structure of RNase PH. *J. Biol. Chem.* 279: 755-764.

Collmer A., Badel J. L., Charkowski A. O., Deng W. L., Fouts D. E., Ramos A. R., Rehm A. H., Anderson D. M., Schneewind O., van Dijk K., Alfano J. R., 2000. *Pseudomonas syringae* Hrp type III secretion system and effector proteins. *Proc. Natl. Acad. Sci. USA* 97: 8770-8777.

Dean C. R., Goldberg J. B., 2002. *Pseudomonas aeruginosa galU* is required for a complete lipopolysaccharide core and repairs a secondary mutation in a PA103 (serogroup O11) wbpM mutant. *FEMS Microbiol. Lett*. 210: 277-283.

Dillon S. C., Bateman A., 2004. The Hotdog fold: wrapping up a superfamily of thioesterases and dehydratases. *BMC Bioinformatics*. 5: 109.

Fouts D. E., Abramovitch R. B., Alfano J. R., Baldo A. M., Buell C. R., Cartinhour S., *et al.,* 2002. Genomewide identification of *Pseudomonas syringae* pv. tomato DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc. Natl. Acad. Sci. USA* 99: 2275–2280.

Galan J. E. and Collmer A., 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* 284: 1322-1328.

Garcia Vescovi E., Soncini F. C., Groisman E. A., 1996. Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* 84: 165-174.

Garmendia J, Beuzon C. R., Ruiz-Albert J., Holden D. W., 2003. The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the *Salmonella typhimurium* SPI-2 type III secretion system. *Microbiology* 149: 2385-2396.

Gonzalez A. J., Landeras E., Mendoza M. C., 2000. Pathovars of *Pseudomonas syringae* causing bacterial brown spot and halo blight in *phaseolus vulgaris* L. are distinguishable by ribotyping. *Appl. Environ. Microbiol.* 66: 850-854.

Goranson J., Hovey A. K., Frank D. W., 1997. Functional analysis of *exsC* and *exsB* in regulation of exoenzyme S production by *Pseudomonas aeruginosa*. *J. Bacteriol.* 179: 1646-1654.

Grimm C., Aufsa*Tz* W., Panopoulos N. J., 1995. The hrpRS locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. *Mol. Microbiol.* 15: 155-165.

Hancock R. E., Brinkman F. S., 2002. Function of *Pseudomonas* porins in uptake and efflux. *Annu. Rev. Microbiol.* 56: 17-38.

Heeb S., Haas D., 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Mol. Plant Microbe Interact.* 14: 1351-63.

He S. Y. 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. *Annu. Rev. Phytopathol.* 36: 363–392.

Hendrickson E. L., Guevera P., and Ausubel F. M., 2000. The alternative sigma factor RpoN is required for hrp activity in *Pseudomonas syringae* pv. maculicola and acts at the level of *hrpL* transcription. *J. Bacteriol.* 182: 3508–3516.

Herr A. J., Nelson C. C., Wills N. M., Gesteland R. F., Atkins J. F., 2001. Analysis of the roles of tRNA structure, ribosomal protein L9, and the bacteriophage T4 gene 60 bypassing signals during ribosome slippage on mRNA. *J. Mol. Biol.* 309: 1029-1048.

Heyde M. and Portalier R., 1987. Regulation of major outer membrane porin proteins of *Escherichia coli* K12 by pH. *Mol. Gen. Genet.* 208: 511–517.

Hirano S. S., Upper C. D., 2000. Bacteria in the Leaf Ecosystem with emphasis on *Pseudomonas syringae*—a Pathogen, Ice Nucleus, and Epiphyte Microbiol. *Mol. Biol. Rev.* 64: 624–653.

Hutcheson S.W., Bre*Tz* J., Sussan T., Jin S., Pak K., 2001. Enhancer-binding proteins HrpR and HrpS interact to regulate hrp-encoded type III protein secretion in *Pseudomonas syringae* strains. *J. Bacteriol.* 183: 5589-5598.

Hueck C. J., 1998. Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants. *Microbiol. Mol. Biol. Rev.* 62: 379-433.

Huynh T. V., Dahlbeck D., and Staskawicz B. J., 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* 245: 1374–1377.

Innes R. W., Bent A. F., Kunkel B. N., Bisgrove S. R., Staskawicz B. J.. 1993. Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J. Bacteriol.* 175: 4859–4869.

Jacobs M. A., Alwood A., Thaipisuttikul I., Spencer D., Haugen E., Ernst S., Will O., Kaul R., Raymond C., Levy R., Chun-Rong L., Guenthner D., Bovee D., Olson M. V., Manoil C. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 100: 14339-14344.

Jin S. G., Song Y. N., Deng W. Y., Gordon M. P., Nester E. W., 1993. The regulatory VirA protein of *Agrobacterium tumefaciens* does not function at elevated temperatures. *J. Bacteriol.* 175: 6830–6835.

Joardar V., Lindeberg M., Jackson R., Selengut J., Dodson R., Brinkac L.M., *et al.,* 2005. Whole-Genome Sequence Analysis of *Pseudomonas syringae* pv. *phaseolicola* 1448A reveals divergence among pathovars in genes involved in virulence and transposition. *J. Bacteriol.* 187: 6488-6498.

Kinscherf T. G., Willis D. K., 2002. Global regulation by *gidA* in *Pseudomonas syringae*. *J. Bacteriol.* 184: 2281-2286.

Li L., Jia Y., Hou Q., Charles T. C., Nester E. W., Pan S. Q., 2002. A global pH sensor: *Agrobacterium* sensor protein ChvG regulates acid-inducible genes on its two chromosomes and Ti plasmid.*Proc. Natl. Acad. Sci. USA* 99: 12369-12374.

Lindgren P.B., 1997. The role of *hrp* genes during plant-bacterial interactions. *Annu. Rev. Phytopathol.* 35:129-152.

Mac Siomoin R. A., Nakata N., Murai T., Yoshikawa M., Tsuji H., Sasakawa C., 1996. Identification and characterization of *ispA*, a *Shigella flexneri* chromosomal gene essential for normal in vivo cell division and intracellular spreading. *Mol. Microbiol.* 19: 599-609.

Marchler-Bauer A., Anderson J. B., Cherukuri P. F., DeWeese-Scott C., Geer L. Y., Gwadz M., *et al.,* 2005. CDD: a Conserved Domain Database for protein classification. *Nucleic Acids Res.* 33(Database issue): D192-196.

Marenda M., Brito B., Callard D., Genin S., Barberis P., Boucher C., Arlat M., 1998. PrhA controls a novel regulatory pathway required for the specific induction of *Ralstonia solanacearum hrp* genes in the presence of plant cells. *Mol. Microbiol.* 27: 437-453.

McIver K. S., Kessler E., Ohman D. E., 2004. Identification of residues in the *Pseudomonas aeruginosa* elastase propeptide required for chaperone and secretion activities. *Microbiology* 150: 3969-3977.

Melchers L. S., Regensburgtuink A. J. G., Schilperoort R. A., Hooykaas P. J. J., 1989. Specificity of signal molecules in the activation of *Agrobacterium* virulence gene expression. *Mol. Microbiol.* 3: 969–977.

Peng W. T., Lee Y. W., Nester E. W., 1998. The phenolic recognition profiles of the *Agrobacterium tumefaciens* VirA protein are broadened by a high level of the sugar binding protein ChvE. *J. Bacteriol.* 180: 5632-5638.

Preston G., Deng W. L., Huang H. C., Collmer A., 1998. Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J. Bacteriol.* 180: 4532–4537.

Rahme L.G., Mindrinos M.N., Panopoulos N.J., 1992. Plant and environmental sensory signals control the expression of hrp genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 174: 3499-3507.

Roine E., Nunn D. N., Paulin L., Romantschuk M., 1996. Characterization of genes required for pilus expression in *Pseudomonas syringae* pathovar *phaseolicola*. *J. Bacteriol.* 178: 410-417.

Roine E., Raineri D.M., Romantschuk M., Wilson M., Nunn D.N., 1998. Characterization of type IV pilus genes in *Pseudomonas syringae* pv. tomato DC3000. *Mol. Plant Microbe Interact.* 11: 1048-1056.

Romantschuk, M., Bamford D. H., 1986. The causal agent of halo blight in bean, *Pseudomonas syringae* pv. *phaseolicola*, attaches to stomata via its pili. *Microb. Pathog.* 1: 139–148.

Romantschuk M., Nurmiaho-Lassila E. L., Roine E., Suoniemi A., 1993. Pilus-mediated adsorption of *Pseudomonas syringae* to host and nonhost plant leaves. *J. Gen. Microbiol.* 139: 2251–2260.

Salmeron J.M., Staskawicz B.J., 1993. Molecular characterization and *hrp* dependence of the avirulence gene avrPto from *Pseudomonas syringae* pv. tomato. *Mol. Gen. Genet.* 239: 6-16.

Schulte R., Bonas U., 1992. A *Xanthomonas* pathogenicity locus is induced by sucrose and sulfur-containing amino acids. *Plant Cell* 4: 79–86.

Shen H., Keen N. T., 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. tomato. *J. Bacteriol.* 175: 5916–5924.

Shimoda N., Toyoda-Yamamoto A., Aoki S., Machida Y., 1993. Genetic evidence for an interaction between the VirA sensor protein and the ChvE sugar-binding protein of *Agrobacterium*. *J. Biol. Chem.* 268: 26552-26558.

Spencer P. A., Towers G. H. N., 1988. Specificity of signal compounds detected by *Agrobacterium tumefaciens*. *Phytochemistry* 27: 2781–2785.

Stachel S. E., Nester E. W., Zambryski P. C., 1986. A plant-cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proc. Natl. Acad. Sci. USA* 83: 379–383.

Staskawicz B.J., Mudgett M.B., Dangl J.L., Galan J.E., 2001. Common and contrasting themes of plant and animal diseases. *Science* 292: 2285–2289.

Takaya A., Tomoyasu T., Tokumitsu A., Morioka M., Yamamoto T., 2002. The ATP-dependent *lon* protease of *Salmonella enterica* serovar *Typhimurium* regulates

invasion and expression of genes carried on *Salmonella* pathogenicity island I. *J. Bacteriol.* 184: 224-232.

Thwaites R., Spanu P. D., Panopoulos N. J., Stevens C., Mansfield J. W., 2004. Transcriptional regulation of components of the type III secretion system and effectors in *Pseudomonas syringae* pv. *phaseolicola*. *Mol. Plant Microbe Interact.* 17: 1250-1258.

Wei C. F., Deng W. L., Huang H. C., 2005. A chaperone-like HrpG protein acts as a suppressor of HrpV in regulation of the *Pseudomonas syringae* pv. *syringae* type III secretion system. *Mol. Microbiol.* 57: 520-536.

Wei W., Plovanich-Jones A., Deng W. L., Jin Q. L., Collmer A., Huang H. C., He S. Y., 2000. The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. tomato. *Proc. Natl. Acad. Sci. USA* 97: 2247–2252.

Wiggerich H. G., Pühler A., 2000. The *exbD2* gene as well as the iron-uptake genes *tonB*, *exbB* and *exbD1* of *Xanthomonas campestris* pv. *campestris* are essential for the induction of a hypersensitive response on pepper (*Capsicum annuum*). *Microbiology* 146: 1053-1060.

Xiao F., Goodwin S.M., Xiao Y., Sun Z., Baker D., Tang X., Jenks M.A., Zhou J.M., 2004. Arabidopsis CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development. *EMBO J.* 23: 2903-2913.

Xiao Y., Heu S., Yi J., Lu Y., Hutcheson S. W., 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade

controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* 176: 1025-1036.

Xiao Y., Hutcheson S. W., 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. *J. Bacteriol.* 176: 3089-3091.

Xiao Y., Lu Y., Heu S., Hutcheson S. W., 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 hrp cluster. *J. Bacteriol.* 174: 1734-1741.

Zhu W., Magbanua M. M., White F. F., 1999. Identification of two novel *hrp*-associated genes in the *hrp* gene cluster of *Xanthomonas oryzae* pv. *oryzae*. *J. Bacteriol.* 182: 1844-1853

Zhu J., Oger P. M., Schrammeijer B., Hooykaas P. J., Farrand S. K., Winans S. C., 2000. The bases of crown gall tumorigenesis. *J. Bacteriol.* 182: 3885-3895.

Zwiesler-Vollick J., Plovanich-Jones A.E., Nomura K., Bandyopadhyay S., Joardar V., Kunkel B.N., He S.Y., 2002. Identification of novel *hrp*-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. tomato DC3000 genome. *Mol. Microbiol.* 45: 1207-1218.

Table II-1 *KB* Mutants that exhibit constitutive expression of *avrPto-LUC* in KB medium

Gene	Mutant	Gene product
envZ	*KB2, KB3, KB6, KB13*	Two component sensor protein
ompR	*KB1, KB16*	DNA binding response regulator
PSPPH_0858	*KB11, KB12*	Sensor histidine kinase
lon1	*KB4, KB10, KB14*	ATP-dependent protease
PSPPH_B0005	*KB9*	Transcriptional regulator, Cro/CI family
PSPPH_3346	*KB7*	Copper-translocating P-type ATPase
PSPPH4014	*KB8*	Multidrug/solvent transporter, AcrB/AcrD/AcrF family
SucD	*KB5*	Succinyl-CoA synthetase, a subunit

Table II-2. Mutants that are insensitive to minimal media signals (*min* mutants)

Gene	Mutant	Gene product	Pathway or biological process
Group A: signal sensor or transcriptional regulator			
PSPPH_2003	*Min12, Min80*	Sensor histidine kinase	Signal transduction mechanisms
envZ	*Min58*	Osmolarity sensor protein EnvZ	Signal transduction mechanisms
hrpR	*Min94, Min96, Min102,*	Type III transcriptional regulator HrpR	Signal transduction mechanisms
PSPPH_3244	*Min16, Min17*	Transcriptional regulator, TetR family	Signal transduction mechanisms
Group B: genes encode membrane proteins			
oprE	*Min79*	Outer membrane porin OprE	Membrane protein
PSPPH_1921	Min06	Peptidase, iron-regulated membrane protein	Putative membrane protein
PSPPH_4907	*Min93, Min100*	Outer membrane porin, OprD family	Membrane protein
PSPPH_0578	*Min01*	Membrane protein, putative	Putative membrane protein
PSPPH_5137	*Min107*	YeeE/YedE family protein	Putative membrane protein
Group C: Metabolic genes			
PSPPH_2878	*Min85*	Glycosyl hydrolase, family 15	Polysaccharide metabolism
cbiD	*Min83*	Cobalamin biosynthesis protein CbiD	Porphyrin and chlorophyll metabolism
cbiG	*Min75*	CbiG protein/precorrin-3B C17-methyltransferase	Porphyrin and chlorophyll metabolism
cobC	*Min87*	Cobalamin biosynthesis protein CobC	Porphyrin and chlorophyll metabolism
cobH	*Min103*	Precorrin-8X methylmutase	Porphyrin and chlorophyll metabolism
cobI	*Min110*	Precorrin-2 C20-methyltransferase	Porphyrin and chlorophyll metabolism
cobM	*Min30, Min50, Min55, Min56*	Precorrin-4 C11-methyltransferase	Porphyrin and chlorophyll metabolism
cobN	*Min66, Min69*	CobN/magnesium chelatase family protein	Porphyrin and chlorophyll metabolism; cobalamin biosynthesis
cobQ	*Min51*	Cobyric acid synthase	Porphyrin and chlorophyll metabolism
PSPPH_3701	*Min104, Min106*	Cobyrinic acid a,c-diamide synthase family protein	Porphyrin and chlorophyll metabolism
PSPPH_2598	*Min64, Min95, Min88*	Tetrapyrrole methylase family protein	Porphyrin and chlorophyll metabolism

(*To be continued*)

Table II-2 (*Continued*)

Gene	Mutant	Gene product	Pathway or biological process
PSPPH_2227	*Min77*	Magnesium chelatase subunit ChlD	Porphyrin and chlorophyll metabolism
bchI	*Min29, Min54*	Magnesium chelatase, subunit ChiI, putative	Porphyrin and chlorophyll metabolism
argH	*Min13, Min44, Min45, Min68,*	Argininosuccinate lyase	Arginine biosynthesis
eno-1	*Min23*	Enolase, glycolysis / gluconeogenesis	Glycolysis; Phenylalanine, tyrosine and tryptophan biosynthesis;
gltB	*Min04, Min10, Min15, Min20, Min25, Min26, Min31, Min33, Min40, Min43, Min52, Min67, Min72 Min99*	Glutamate synthase, large subunit	Glutamate biosynthesis
gltD	*Min22, Min28*	Glutamate synthase, small subunit	Glutamate biosynthesis
hisA	*Min38*	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	Histidine biosynthesis
hisB	*Min59, Min74*	Imidazoleglycerol-phosphate dehydratase;	Histidine biosynthesis
hisC	*Min05, Min11*	Histidinol-phosphate aminotransferase	Histidine biosynthesis
hisD	*Min37, Min76*	Histidinol dehydrogenase	Histidine biosynthesis
hisE	*Min47, Min53, Min57*	Phosphoribosyl-ATP pyrophosphatase	Histidine biosynthesis
hisF	*Min24, Min34*	Imidazoleglycerol phosphate synthase, cyclase subunit	Histidine biosynthesis
hisH	*Min35, Min36*	Amidotransferase HisH	Histidine biosynthesis
ilvD	*Min32, Min81, Min86, Min91*	Dihydroxy-acid dehydratase	Valine and isoleucine biosynthesis
leuA	*Min09, Min14, Min82*	2-isopropylmalate synthase	Leucine biosynthesis
leuB	*Min21, Min39, Min61*	3-isopropylmalate dehydrogenase	Leucine biosynthesis
leuC	*Min08,Min60, Min111*	3-isopropylmalate dehydratase, large subunit	Leucine biosynthesis
metF	*Min48, Min49, Min84*	5,10-methylenetetrahydrofolate reductase	Methionine biosynthesis

(To be continued)

Table II-2 (*Continued*)

Gene	Mutant	Gene product	Pathway or biological process
metH	*Min89, Min92, Min108*	5-methyltetrahydrofolate--homocysteine methyltransferase	Methionine biosynthesis
PSPPH_0847	*Min65, Min98*	Phosphoenolpyruvate-protein phosphotransferase,EI/HPr/EIIA components	Fructose and mannose metabolism
fruK	*Min18*	1-phosphofructokinase	Fructose and mannose metabolism
PSPPH_0849	*Min41*	Phosphotransferase system, fructose-specific IIBC component	Fructose and mannose metabolism
purE	*Min78*	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	Purine metabolism; purine ribonucleotide biosynthesis
purK	Min27	Phosphoribosylaminoimidazole carboxylase, atpase subunit	Purine metabolism; purine ribonucleotide biosynthesis
serA	*Min42, Min73*	D-3-phosphoglycerate dehydrogenase	L-serine biosynthesis
sypA	*Min63*	Putative peptide/siderophore synthetase	
trpA	*Min62*	Tryptophan synthase, alpha subunit	Tryptophan biosynthesis
trpD	*Min71*	Anthranilate phosphoribosyltransferase	Tryptophan biosynthesis
PSPPH_0569	*Min02*	ATP phosphoribosyltransferase regulatory subunit, putative	
PSPPH_0452	*Min46*	4-hydroxybenzoyl-coA thioesterase domain protein	Metabolism

Table II-3. *avrPto-LUC* activity in *min* mutants in minimal medium supplemented with different amino acid(s) or in *att1* plants (contributed by Dr. Xiaoyan Tang)

Gene	MM+Glu	MM+Arg	MM+His	MM+Arg, Leu, Glu, His, Ser	MM	*att1* plant
gltB	Med	Low	Low	High	Low	High
purE	High	High	High	High	Low	High
sypA	High	High	High	High	Low	High
argH	Low	High	Low	High	Low	High
cbiD	Low	Low	Low	Low	Low	High
cbiG	Low	Low	Low	Med	Low	High
cobC	Low	Low	Low	Low	Low	High
cobH	Low	Low	Low	Low	Low	High
cobL	Low	Low	Low	Low	Low	High
cobM	Low	Low	Low	Low	Low	High
cobN	Low	Low	Low	Low	Low	High
cobQ	Low	Low	Low	Low	Low	High
gltD	Med	Low	Low	High	Low	High
hisA	Low	Low	High	High	Low	High
hisB	Low	Low	High	High	Low	High
hisC	Low	Low	High	High	Low	High
hisD	Low	Low	High	High	Low	High
hisE	Low	Low	High	High	Low	High
hisF	Low	Low	High	High	Low	High
hisH	Low	Low	High	High	Low	High
PSPPH_2878	Low	Low	Low	Low	Low	High
PSPPH_2227	Low	Low	Low	Low	Low	High
bchI	Low	Low	Low	Low	Low	High
PSPPH_0452	Low	Low	Low	High	Low	High
PSPPH_4907	High	High	High	Low	Med	High
PSPPH_0847	High	Low	Low	Low	Low	Med
fruK	High	Low	Low	Low	Low	Med
PSPPH_0849	High	Low	Low	Low	Low	Med
eno-1	High	Low	Low	High	Low	Med
PSPPH_3701	High	Low	High	Med	Low	N.D.
serA	High	Low	Low	Low	Low	Low
ilvD	Low	Low	Low	Low	Low	Low
leuA	Low	Low	Low	High	Low	Low
leuB	Low	Low	Low	High	Low	Low
leuC	Low	Low	Low	High	Low	Low
metF	Low	Low	Low	Low	Low	Low
metH	Low	Low	Low	Low	*Low*	*Low*
PSPPH_2598	N.D.	N.D.	N.D.	Low	Low	N.D.
PSPPH_0569	Low	Low	High	High	Low	Low
purK	Low	Low	Low	Low	Low	Low

(To be continued)

Table II-3 (*Continued*)

Gene	MM+Glu	MM+Arg	MM+His	MM+Arg, Leu, Glu, His, Ser	MM	*Att1* plant
trpA	Low	Low	Low	Low	Low	Low
trpD	Low	Low	Low	Low	Low	Low
PSPPH_5137	Low	Low	Low	Low	Low	Low
PSPPH_3241	High	Low	Low	Low	Med	Low
PSPPH_0578	High	High	Low	High	Med	Low
PSPPH_1921	Low	Low	Low	High	Med	Low
PSPPH_3244	Low	Low	Low	High	Med	Low
PSPPH_2003	Low	Low	Low	Low	Low	Low
hrpR	Low	Low	Low	Low	Low	Low

Table II-4. *pin* mutants that are insensitive to plant signals

gene	***Mutant***	**Gene products**	**Pathway or biological pathway**
Group A: Signal sensor or transcriptional regulator			
PSPPH_2003	*Pin09, Pin19*	Sensor histidine kinase	Signal transduction mechanisms
phoQ	*Pin18*	Sensor protein PhoQ	Signal transduction mechanisms
exsB	*Pin01*	ExsB protein	Transcription regulator related protein
hrpR	*Pin29*	Transcriptional regulator	Signal transduction mechanisms
hrpS	*Pin20*	Transcriptional regulator	Signal transduction mechanisms
gidA	*Pin17, Pin30*	Glucose-inhibited division protein A	Cell division, global regulator
tonB	*Pin35*	TonB protein; siderophore-iron transporter activity	Iron-siderophore transport, signal transduction mechanisms
PSPPH_1736	*Pin59*	TonB-dependent siderophore receptor	Signal transduction mechanisms
Group B: genes involved in Cell wall/membrane biogenesis			
PSPPH_0520	*Pin50*	Glycosyl transferase, group 1 family protein	Cell wall/membrane biogenesis
PSPPH_0519	*Pin49*	Carbamoyltransferase family protein	Cell wall/membrane biogenesis
PSPPH_3810	*Pin21*	Lipoprotein NlpD, putative	Cell wall/membrane biogenesis
oprF	*Pin57, Pin80*	Outer membrane porin OprF	Cell wall/membrane biogenesis
galU	*Pin36*	UTP-glucose-1-phosphate uridylyltransferase	Capsular Polysaccharide Biosynthesis
Group C: genes involved in stress response			
oxyR	*Pin47*	Oxidative stress regulatory protein OxyR, LysR family	
PSPPH_0671	*Pin79*	ImpB/mucB/samB family protein, DNA-directed DNA polymerase	DNA polymerase, involved in UV protecting
htpX	*Pin44*	Heat shock protein htpx	Posttranslational modification, protein turnover, chaperones
ParB	*Pin68*	Chromosome partitioning protein ParB	Posttranslational modification, protein turnover, chaperones
PSPPH_0677	*Pin58*	OsmC/OhR family protein	Posttranslational modification, protein turnover, chaperones

(To be continued)

Table II-4 (*continued*)

gene	*Mutant*	Gene products	Pathway or biological pathway
Group D: metabolic genes			
ilvD	*Pin10, Pin15, Pin54, Pin62, Pin64, Pin76*	Dihydroxy-acid dehydratase	Valine and isoleucine biosynthesis
leuA	*Pin07, Pin16, Pin70*	2-isopropylmalate synthase	Leucine biosynthesis
leuB	*Pin06*	3-isopropylmalate dehydrogenase,	Leucine biosynthesis
leuC	*Pin03, Pin31, Pin43*	3-isopropylmalate dehydratase, large subunit	Leucine biosynthesis
metF	*Pin45*	5,10-methylenetetrahydrofolate reductase	Methionine biosynthesis
metH	*Pin05*	5-methyltetrahydrofolate--homocysteine methyltransferase	Methionine biosynthesis
meTz	*Pin11, Pin55*	O-succinylhomoserine sulfhydrylase	Methionine biosynthesis
purD	*Pin51, Pin69*	Phosphoribosylamine--glycine ligase	Purine ribonucleotide biosynthesis
purE	*Pin02, Pin12, Pin13, Pin38*	Phosphoribosylaminomidazole carboxylase	Purine ribonucleotide biosynthesis
purK	*Pin04, Pin26*	Phosphoribosylaminoimidazole carboxylase, ATPase subunit	Purine ribonucleotide biosynthesis
pyrB	*Pin25, Pin75*	Aspartate carbamoyltransferase	Pyrimidine ribonucleotide biosynthesis
serA	*Pin08, Pin14*	D-3-phosphoglycerate dehydrogenase	L-serine biosynthesis
trpA	*Pin32*	Tryptophan synthase, alpha subunit	Tryptophan biosynthesis
nuoM	*Pin72*	NADH dehydrogenase I, M subunit	Oxidative phosphorylation; Ubiquinone biosynthesis
pgi	*Pin37*	Glucose-6-phosphate isomerase	Glycolysis / Gluconeogenesis
pgk	*Pin39*	Phosphoglycerate kinase, phosphotransferase	Glycolysis / Gluconeogenesis
PSPPH_3261	*Pin60*	Hypothetical protein, Succinate dehydrogenase/fumarate reductase, flavoprotein subunit	Tricarboxylic acid cycle
PSPPH_4463	*Pin42*	Precorrin-6X reductase, putative	Porphyrin and chlorophyll metabolism

(To be continued)

Table II-4 (*continued*)

gene	*Mutant*	Gene products	Pathway or biological pathway
Others:			
rplI	*Pin52*	Ribosomal protein L9	Protein biosynthesis
rph	*Pin41*	Ribonuclease PH	Maturation of tRNA
PSPPH_4907	*Pin27*	Outer membrane porin, OprD family	Transportation of small molecules
PSPPH_2198	*Pin24*	Hypothetical protein	
ispZ	*Pin46*	Intracellular septation protein A	Cell division

Table II-5. HR and pathogenicity tests of selected *pin* mutants

Mutant	Gene	HR in 20 hours[a]	Disease Index[b]	No. of bacteria recovered from 1cm^2 leaf disc[c]	Increase fold of bacterial no. in 6 days[d]
WT	Positive CK	+	5	1250 ± 117.3	1.87×10^6
Pin22	*hrpR*	-	1	1097 ± 203.5	168.0
Pin18	*phoQ*	-	1	0	N.D.
Pin9	*rohS*	-	1	847.5 ± 128.2	1.41×10^5
Pin19	*rohS*	-	1	1290 ± 113.6	1.30×10^5
Pin24	*PSPPH_2198*	-	1	1250 ± 238.7	6.24×10^4
Pin44	*htpX*	-	3	1475 ± 250.0	1.94×10^5
Pin46	*ispZ*	-	4	1350 ± 574.4	2.69×10^5
Pin60	PSPPH_3261	+	5	235 ± 115.6	6.67×10^6
Pin21	*PSPPH_3810*	-	2	300 ± 100.3	3.87×10^5
Pin36	*galU*	-	1	12.5 ± 5.0	5.48×10^3
Pin49	*PSPPH_0519*	-	3	112.5 ± 33.0	1.03×10^6
Pin50	*PSPPH_0520*	-	3	0	N.D.
Pin57	*oprF*	-	3	0	N.D.
Pin80	*oprF*	-	3	20 ± 14.1	1.22×10^6

Notes: a. Non-host HR on tobacco W38 plants. W38 plants were inoculated with 10^8 CFU/ml bacteria. The non-host HR was scored at 20 hr after inoculation. +, HR; -, No HR.

b. Disease test on Red Kidney bean plants. Bacteria were inoculated into primary leaves of 10-day old bean plant at 2×10^4 CFU/ml. Disease was scored on 9 days after inoculation at 1 to 5 indice: 1, symptom free; 2, slight chlorotic lesion with scattered specks; 3, chlorotic lesion with concentrated specks; 4, chlorotic and necrotic lesions; 5. large necrotic lesions.

c. d. Bean plants were inoculated with 5×10^5 CFU/ml bacteria, bacterial number were measured at 3 hours (0 day) (mean ± standard deviation) and 6 days after inoculation.

Table II-6. Mutants that are insensitive to fatty acid (ESA) suppression of *avrPto-LUC* in minimal medium

Gene	Mutant	Gene product	Pathway or biological pathway
Group A: Negative regulators of TTSS genes			
lonB	*Fin01, Fin02, Fin03, Fin04,*	ATP-dependent protease	Signal transduction mechanisms
OmpR	*Fin05*	DNA binding response regulator	Signal transduction mechanisms
PSPPH_4014	*Fin06, Fin07, Fin08, Fin09, Fin10, Fin11*	multidrug/solvent tranporter, AcrB/AcrD/AcrF family protein	
Group B: Mutants defect in fatty acid metabolism			
fadA	*Fin20, Fin22 Fin24, Fin26, Fin47, Fin48, Fin49*	3-ketoacyl-CoA thiolase	Fatty acid metabolism
fadB	*Fin21, Fin23, Fin46, Fin52,*	Fatty oxidation complex, alpha subunit	Fatty acid metabolism
fadD-1	*Fin18, Fin19, Fin25,*	Long-chain-fatty-acid--CoA ligase	Fatty acid metabolism
fadE	*Fin34, Fin42, Fin50, Fin51, Fin53, Fin60*	Acyl-CoA dehydrogenase family protein	Fatty acid metabolism
PSPPH_3689	*Fin31*	Outer membrane protein transport protein, FadL like protein	Aromatic hydrocarbon degradation
PSPPH_2612	*Fin28*	Oxidoreductase, short-chain dehydrogenase/reductase family	
PSPPH_2613	*Fin16, Fin29, Fin30, Fin39, Fin40, Fin44, Fin45,*	Aminoglycoside phosphotransferase, Phosphotransferase enzyme family	
PSPPH_2631	*Fin12, Fin14, Fin35, Fin36, Fin38*	Transcriptional regulator, LysR family	Information processing
nuoL	*Fin17*	NADH dehydrogenase I, L subunit	Energy production and conversion

(*To be continued*)

Table II-6 (*continued*)

Gene	Mutant	Product	Pathway or biological pathway
Group C: mutants defect in type IV pilus biogenesis			
pilB	*Fin59*	Type IV pilus biogenesis protein PilB	Type II secretion system, Cell motility
pilF	*Fin32*	Type IV pilus biogenesis protein PilF	Type II secretion system, Cell motility
pilM	*Fin27, Fin58*	Type IV pilus biogenesis protein PilM	Type II secretion system, Cell motility
pilQ	*Fin13, Fin55*	Type IV pilus biogenesis protein PilQ	Type II secretion system, Cell motility
pilY1	*Fin33*	Type IV pilus-associated protein	Type II secretion system, Cell motility
pilR	*Fin43,*	Type IV fimbriae expression regulatory protein PilR	Type II secretion system, Signal transduction mechanisms
pilS	*Fin56*	Sensor protein PilS	Type II secretion system, Signal transduction mechanisms
PSPPH_1317	*Fin37, Fin41*	Radical SAM enzyme, Cfr family,	Upstream of *pilF*
PSPPH_0738	*Fin57*	Hypothetical protein	Upstream of *pilS*
Others			
PSPPH_3205	*Fin15*	Conserved hypothetical protein	
PSPPH_3206	*Fin54*	Conserved hypothetical protein	

Table II-7. Primers used in this study

Name	5′ sequence 3′	Description
CEKG 2A	ggccacgcgtcgactagtacnnnnnnnnnnagag	For *Psph* semiconservative PCR
CEKG 2B	ggccacgcgtcgactagtacnnnnnnnnnnacgcc	For *Psph* semiconservative PCR
CEKG 2C	ggccacgcgtcgactagtacnnnnnnnnnngatat	For *Psph* semiconservative PCR
CEKG 4	ggccacgcgtcgactagtac	For *Psph* semiconservative PCR
Kan2-SP1	gatagattgtcgcacctgattg	For *Psph* semiconservative PCR
Kan2-SP2	aagacgtttcccgttgaatatg	For *Psph* semiconservative PCR
Kan2-SP3	gcaatgtaacatcagagattttgag	Sequencing semiconservative PCR products
Kan2-R	ctacctttgccatgtttcag	Verify *Tz* insertion in *argH*⁻ mutant
ArgH1	tcagcgcccggccagca	Verify *Tz* insertion in *argH*⁻ mutant
ArgH2	cgccacgatgctggcca	Verify *Tz* insertion in *argH*⁻ mutant
GltB1	acaggttcaggccaccg	Verify *Tz* insertion in *gltB*⁻ mutant
GltB2	tccttaatcaggacgacg	Verify *Tz* insertion in *gltB*⁻ mutant
LeuB-FP	ctacacgtttcatcgcaac	Verify *Tz* insertion in *leuB*⁻ mutant
Kan2-RP	ctacctttgccatgtttcag	Verify *Tz* insertion in *leuB*⁻ mutant
exsB-P	gtcgccatggcccgcgcag	Verify *Tz* insertion in *exsB*⁻ mutant
Lon-FP	gagcgtttcatcgaagtc	Verify *Tz* insertion in *lon*⁻ mutant
phoQ-P	tccttgaccagccgccagac	Verify *Tz* insertion in *phoQ*⁻ mutant
rohS-P	gagaatgcgcaggacaatgg	Verify *Tz* insertion in *rohS*⁻ mutant
tonB-P	caagcgcagtcctccaaag	Verify *Tz* insertion in *tonB*⁻ mutant
rph-P	gtacttcaatgaagccgcctg	Verify *Tz* insertion in *rph*⁻ mutant
PSPPH0519	aatggcgtttccctgaacac	Verify *Tz* insertion in *PSPPH_0519*⁻ mutant
pilB-R	tctctagatgccttgctggccatgc	Verify *Tz* insertion in *pilB*⁻ mutant
pilM-F	ttgaattcctcgagcgagcagaataccgc	Verify *Tz* insertion in *pilM*⁻ mutant
pilM-R	tttctagatggcgcggatcaactt	Verify *Tz* insertion in *pilM*⁻ mutant
pliQ-F	ttgaattcctcgagaaagaacgctcatgaagtg	Verify *Tz* insertion in *pilQ*⁻ mutant
pliQ-R	tttctagaatcgcctggttgttcatg	Verify *Tz* insertion in *pilQ*⁻ mutant
PSPPH0738-F	ttggatcccctgggtctgttcgactga	Verify *Tz* insertion in *PSPPH_0738*⁻ mutant
PSPPH0736-R	cctctagatcaatccagscccagyttc	Verify *Tz* insertion in *PSPPH_0738*⁻ mutant
PSPPH0737-R	tctctagatcagctcgttttgaacgg	Verify *Tz* insertion in *PSPPH_0738*⁻ mutant
PSPPH0736-F	ttggatccaaaaggagctgagcatgagccaac	Verify *Tz* insertion in *PSPPH_0738*⁻ mutant
PSPPH1317-F	ttggatccgaagtaaccgctcgctaag	Verify *Tz* insertion in *PSPPH_1317*⁻ mutant
PSPPH1318-R	tttctagagtttctccagggttcacg	Verify *Tz* insertion in *PSPPH_1318*⁻ mutant
PSPPH0730-F	tttcccgtcaggcagcgtg	Verify *Tz* insertion in *PSPPH_0730*⁻ mutant
FadA-R	tcagacgcgttcgaaaac	Verify *Tz* insertion in *fadA*⁻ mutant
FadA-F	atgagccttaatccaagag	Verify *Tz* insertion in *fadA*⁻ mutant
FadB-R	tcatacctcggaagacgc	Verify *Tz* insertion in *FadB*⁻ mutant

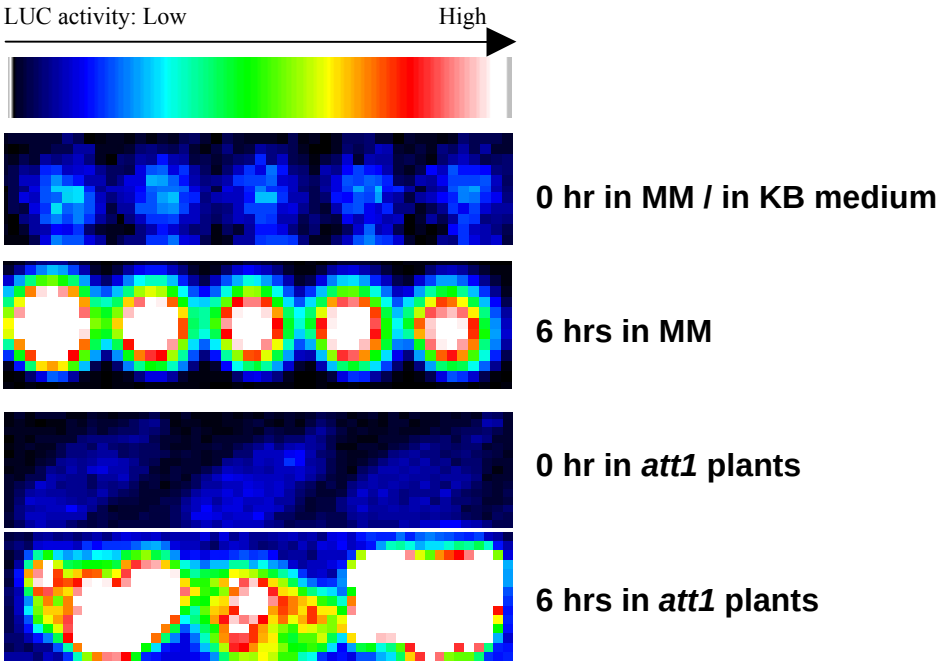


Figure II-1. *LUC* activities in wild type *P. syringae* pv. *phaseolicola* NPS3121 carrying *avrPto-LUC* reporter.

Overnight culture of wild type *P. syringae* pv. *phaseolicola* NPS3121 carrying *avrPto-LUC* reporter in KB medium were collected by centrifugation and washed twice with sterile water. The bacteria were diluted in MM to OD_{600}=0.02 for induction assay of *avrPto-LUC* by MM. The bacteria were diluted in 10mM $MgCl_2$ to OD_{600}=0.5 and injected to the leaves of 5-6 week-old *att1* plants for induction of *avrPto-LUC* by plants. The images were captured using the cooled CCD camera at the indicated time points. The *LUC* activity in MM at 0 hour indicates the *avrPto-LUC* activity in KB medium. Synthetic color from left to right in bar above indicates low to high LUC activity

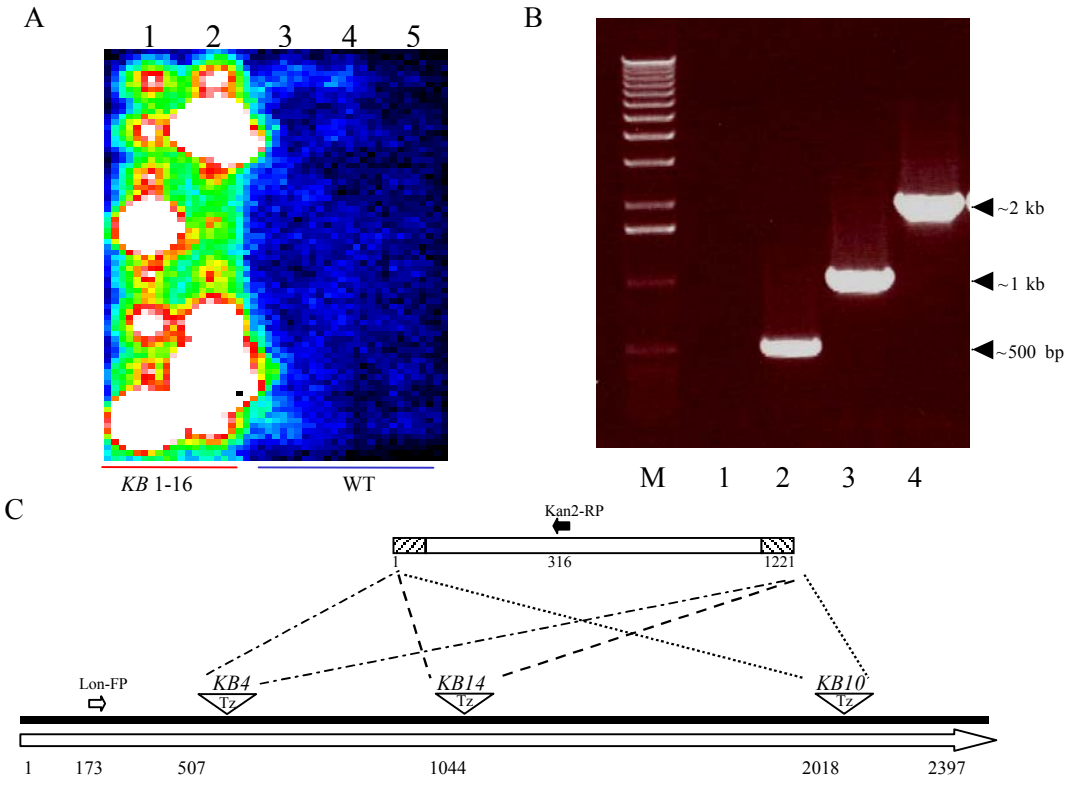


Figure II-2. Phenotype of KB mutants and confirmation of *Tz* mapping in *lon1*$^{-}$ mutants

A. *avrPto-LUC* activity in KB medium. *KB* mutants displayed higher *avrPto-LUC* activities in KB medium than did the wild type parental strain (WT, column 3-5), in column 1 and 2, each cell represents a mutant strain (Contributed by Dr. Xiaoyan Tang);

B. Confirmation of Tz-mapping in *lon*$^{-}$ mutants by gel fractionating the PCR products from the genomic DNA using primers Lon-RP and Kan2-RP. *lon1*$^{-}$ mutants KB4 (lane 2), KB14 (lane 3), and KB10 (lane4) showed the band of expected size. Wild type strain (lane 1) did not;

C. Schematic diagram of Tz insertion and the primers sites in *lon1*$^{-}$ mutants. Open arrow denotes the Lon ORF and gene orientation. The number under the line indicates the locations relative to translation initiation site (1). Open Triangles denote the *Tz* insertion.

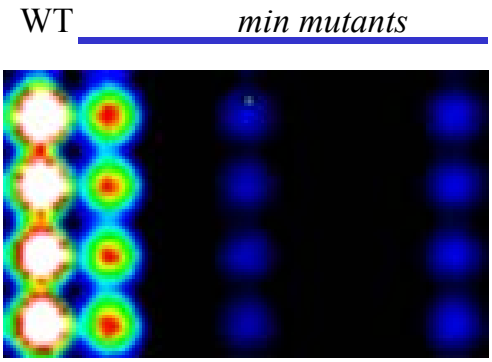


Figure II-3. *avrPto-LUC* induction in *min* mutants in minimal medium

Mutants displaying lower LUC in MM than parental strain (WT) were isolated as *min* mutants. Each column represents a strain, each cell in a column represents a replication.

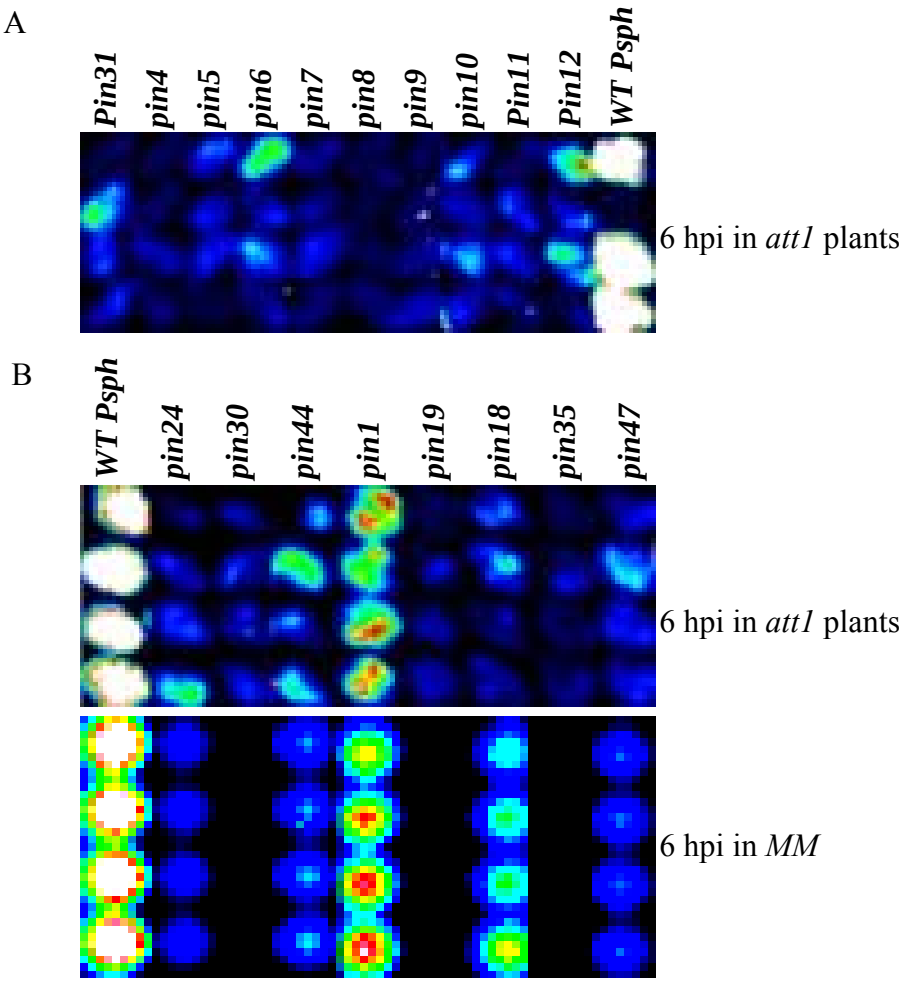


Figure II-4. Phenotype of *pin* mutants and the *pin* mutants with similar *avrPto-LUC* induction in minimal medium as in plant

A. *pin* mutants displayed lower *avrPto-LUC* induction in att1 plants than did the parental strain (WT *Psph*).

B. The *pin* mutants that displayed similar reduction of *avrPto-LUC* induction in *att1* plants (upper picture) as in MM (lower picture).

* hpi = hour(s) post inoculation; Each column represented a strain, and each cell in a column represents a replication

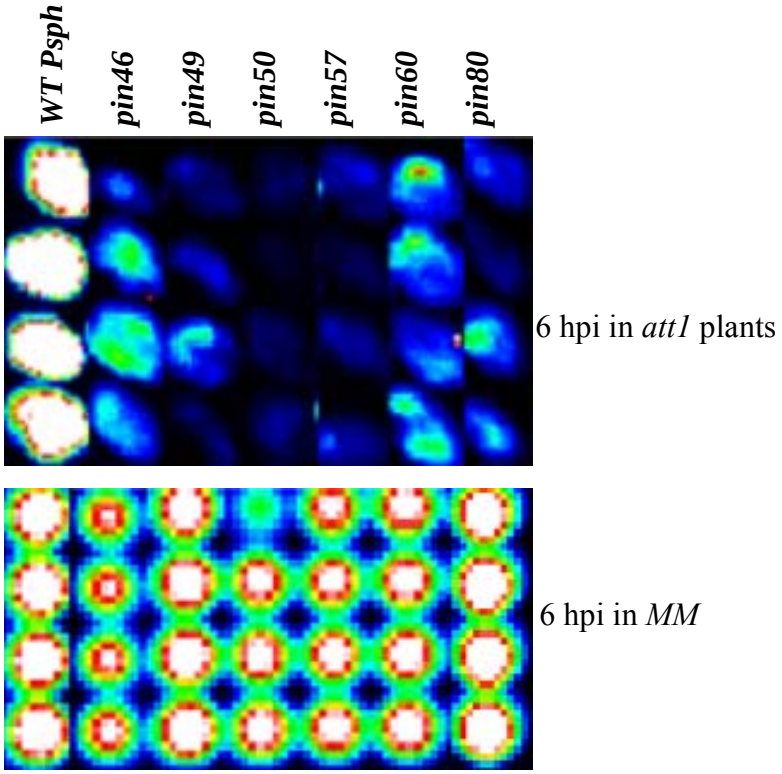


Figure II-5. Phenotype of *pin* mutants with normal *avrPto-LUC* induction in minimal medium

The *pin* mutants that displayed lower *avrPto-LUC* induction in *att1* plants than parental strain (upper picture) while showed normal *avrPto-LUC* induction in MM (lower picture). Each column represents a strain, and each cell in a column represents a replication

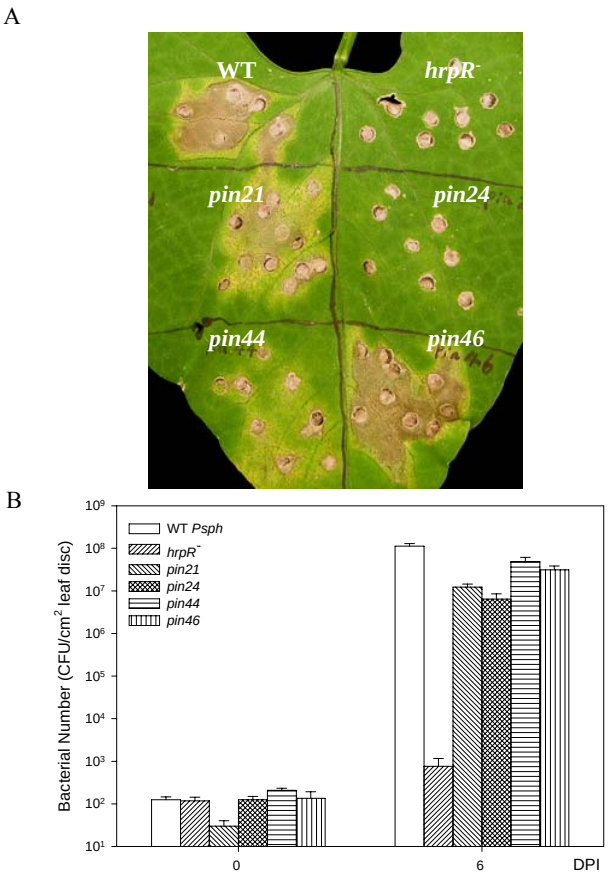


Figure II-6. Selected *pin* mutants compromised bacterial pathogenicity on host bean plants

A. Disease symptoms caused by the *pin* mutants on bean plants. Bacteria were inoculated at 2×10^5 CFU/ml into primary leaves of 10-day old Red Kidney bean plants. Disease symptoms were documented 9 days after inoculation. WT (Parental strain) is used as the postitive control and *hrpR*$^-$ (*pin22*) mutant as the negative control.

B. Bacterial growth assay on the *pin* mutants. Bacteria were inoculated at 2×10^4 CFU/ml into Red Kidney bean leaves. Bacterial numbers were measured at the indicated time. Error bar indicated standard error. Experiment was repeated with similar results.

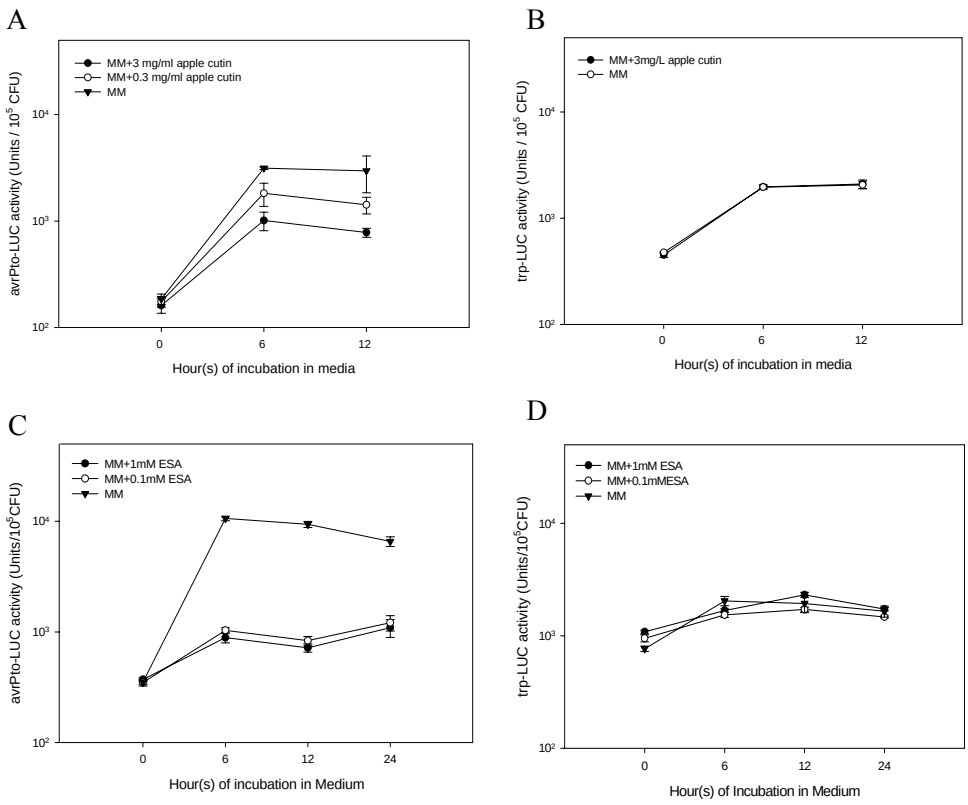
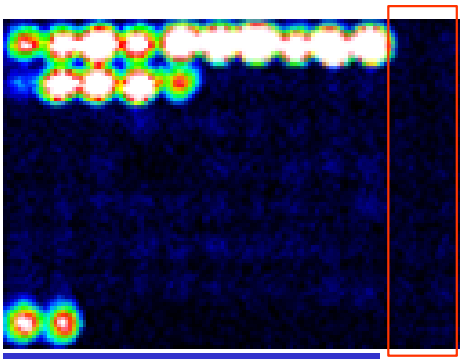


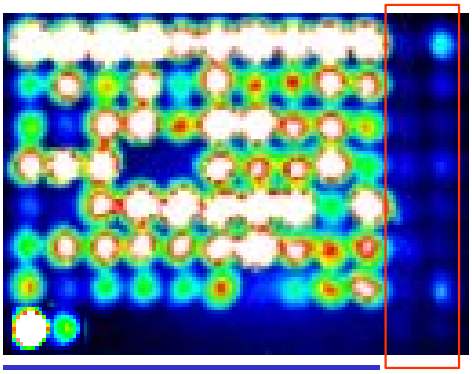
Figure II-7. Effect of plant cutin extract and ESA on *avrPto-LUC* induction in minimal medium.

Overnight cultures of wild type NPS3121 strain carrying *avrPto-LUC* or *trp-LUC* reporter in KB medium were collected by centrifugation and washed twice with distilled H_2O and diluted to 0.02 OD_{600} with MM supplemented with plant cutin extract (A and B) or ESA (C and D) at the indicated concentrations. LUC activities were measured at the indicated time. Plant cutin extract and ESA inhibit induction of *avrPto-LUC* in MM (A and C) but do not affect *trp-LUC* activity in MM (B and D).



fin mutants WT

Incubated in MM+1mM ESA at 0 hr (0-200)



fin mutants WT

Incubated in MM+1mM ESA for 12 hr (0-1000)

Figure II-8. Phenotype of *fin* mutants

Overnight cultures of parental strain (WT) and *fin* mutants in KB medium were collected by centrifugation, washed twice with distilled H_2O and diluted to 0.02 OD_{600} with MM supplemented with 1 mM ESA. LUC activities were measured at the indicated time. The *fin* mutants that were also isolated as *KB* mutants displayed higher LUC activities in KB medium than did parental strain, while the others did not affect LUC activities in KB medium (upper picture). All *fin* mutants displayed higher LUC activities in the presence of ESA in MM (lower picture) than did parental strain. Number in parenthesis indicates the contrast of the pictures. Each cell in the first ten columns represents a mutant strain.

CHAPTER III

A Novel Two-Component Sensor Histidine Kinase Is Required for *Pseudomonas syringae hrp* Promoter Induction and Pathogenicity

SUMMARY

The type III secretion system (TTSS) of *Pseudomonas syringae* is induced by contact with the plant cell and by growth in minimal medium (MM). The induction of TTSS genes is controlled by HrpL, an alternate sigma factor that binds the conserved *hrp* box motif in the promoter of TTSS structure and effector genes, which in turn is activated by HrpR and HrpS, two homologous enhancer binding proteins in NtrC family. To understand how *P. syringae* senses the inducing signal, we screened a *P. syringae* pv. *phaseolicola* transposon insertion library for mutants defective in induction of the promoter of type III effector gene *avrPto*, and identified a mutant of *rohS*, encoding a putative two-component sensor histidine kinase that is required for the induction of *avrPto-LUC* in plants and in MM. The *rohS* gene is in an operon containing the two-component response regulator gene *rohR*. The RohS and RohR proteins are highly conserved in different *P. syringae* pathovars. Mutation of *rohS* in *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tomato* reduced the bacterial pathogenicity on host plants and HR-inducing activity on non-host plants. Expression of the wild type *rohS* but not the *rohS* mutant of the autophosphorylation site H147 in the *rohS*$^-$ mutant strain restored the *avrPto* gene induction and bacterial pathogenicity, indicating the requirement of phosphoryl-transfer between RohS and RohR in activation of TTSS genes. However, deletion of the whole *rohR/S* locus did not alter *avrPto* induction in the plant or MM and did not affect bacterial pathogenicity. In contrast, overexpression of RohR in the *rohR/S* deletion strain completely repressed the induction of *avrPto*, indicating that RohR acts as negative regulator of TTSS genes. Overexpression of RohR(D70A), a mutant unable to be phosphorylated, in the *rohR/S* deletion mutant strain only slightly repressed the *avrPto*

expression in MM, indicating that the phosphorylated RohR (P-RohR) is much more efficient in repressing TTSS genes. It is possible that RohS has both the RohR kinase and the P-RohR phosphatase activities, and in the TTSS-inducing conditions, the phosphatase activity is dominant and prevents the accumulation of P-RohR.

INTRODUCTION

Pseudomonas syringae consists of a large group of phytopathogens that are divided into numerous pathovars, each specialized on one or several plant species (Hirano and Upper, 2000). The ability of *P. syringae* to infect their host plants is linked to a cluster of genes, termed hypersensitive response and pathogenicity (*hrp*) genes, that are also essential for induction of hypersensitive response (HR) on resistant plants (Collmer *et al.*, 2000). Hrp genes encode a type III secretion system (TTSS) that is conserved in numerous Gram-negative bacterial pathogens of plants and animals (Hueck, 1998; Galan and Collmer, 1999). TTSS is a syringe needle-like structure consisting of inner and outer membrane rings and a protruding filament called pilus (Kubori *et al.*, 1998; Marlovits *et al.*, 2004). The type III pilus functions as a conduit to deliver an array of virulence proteins, termed type III effectors, into host cells (Jin and He, 2001; Jin *et al.*, 2001 and 2003). Virulence functions have been demonstrated for a number of type III effectors of animal bacterial pathogens. Many type III effectors of plant bacteria were originally discovered as avirulence proteins because of their function in eliciting HR and disease resistance in plants carrying cognate disease resistant genes (Leach and White, 1996). A number of avirulence proteins have since been shown to confer a virulence function in plants lacking the disease resistance gene (Alfano and Collmer, 2004).

Like that in most pathogenic bacteria, the expression of TTSS and effector genes (hereafter called type III genes collectively) in *P. syringae* is coordinately regulated by various environmental and host factors (Huynh *et al.*, 1989; Rahme *et al.*, 1992; Xiao *et al.*, 1992; van Dijk *et al.*, 1999; Francis *et al.*, 2002). When grown in nutrient rich medium such as KB (King *et al.*, 1954), the expression of type III genes is minimal. However, these genes are rapidly induced after the bacteria are infiltrated into plants or cultured in MM (Huynh *et al.*, 1989; Rahme *et al.*, 1992; Xiao *et al.*, 1992). Studies in *Ralstonia solanacearum* suggest that the induction of type III genes in plants involves a contact-dependent signal (Marenda *et al.*, 1998; Aldon *et al.*, 2000). The perception of the contact-dependent signal is mediated by PrhA, a membrane protein with significant similarities to siderophore receptors (Aldon *et al.*, 2000). However, it is not known if the same contact-dependent signal also activates *P. syringae* type III genes in plants. The MM is believed to resemble the environment of plant intercellular spaces where bacteria proliferate (Huynh *et al.*, 1989). It is possible that certain MM signals are also present in plants and contributes to the regulation of *P. syringae* type III genes *in planta*. However, the biochemical nature of the signal, the pathway for signal perception and transduction, and the significance of the signal to bacterial pathogenicity remain completely unknown.

A few downstream signal transduction components regulating *P. syringae* type III genes have been identified. HrpL, a member of the ECF family alternate σ factors, recognizes the "*hrp* box" motif conserved in the promoter of many type III genes and activates their transcription (Xiao and Hutcheson, 1994; Fouts *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002). Transcription of *hrpL* gene is under the control of σ^{54}-dependent promoter in an alternate sigma factor RpoN-dependent manner (Alarcon-Chaidez *et al.*,

2003; Chatterjee *et al.*, 2003; Hendrickson *et al.*, 2000a and 2000b). Activation of *hrpL* also requires HrpR and HrpS, two homologous DNA binding proteins similar to the NtrC family regulators but lack the modulating receiver domain (Xiao *et al.*, 1994; Grimm *et al.,* 1995; Hutcheson *et al.*, 2001). HrpR and HrpS are encoded by the *hrpRS* operon, and the two proteins form a heterodimer that binds to the *hrpL* promoter to activate *hrpL* transcription (Hutcheson *et al.*, 2001). The stability of HrpR protein is negatively controlled by LonB, an ATP-dependent protease (Bretz *et al.*, 2002). Additional type III regulatory proteins in *P. syringae* includeHrpA, HrpV and HrpG. HrpA is a pilus structural protein, but the *hrpA* mutation down-regulates the *hrpR/S* genes, resulting in reduced type III gene expression in MM (Wei *et al.*, 2000). HrpV negatively regulates type III genes (Preston *et al.*, 1998) and can physically interact with HrpR and HrpG (Wei *et al.*, 2005). HrpG can derepress the TTSS gene expression in the presence of HrpG probably through freeing HrpR from interaction with HrpV (Wei *et al.*, 2005).

We have been using the interaction of *P. syringae* and *Arabidopsis* as a model system to study plant signals and bacterial mechanisms regulating type III genes. Recently we have identified a genetic locus in *Arabidopsis*, *ATT1* (for *aberrant* induction of *type three* genes), which suppresses type III gene expression (Xiao *et al.*, 2004). *ATT1* encodes a fatty acid ω-hydroxylase involved in cutin biosynthesis, suggesting cutin as a negative signal for bacterial type III gene expression. The *att1* mutant plants provide a sensitive system for type III gene induction analysis *in vivo*.

To investigate how *P. syringae* perceives positive host signals to activate type III genes, we screened for *P. syringae* pv. *phaseolicola* mutants unable to or partially induce a "*hrp* box" promoter in *att1* mutant plants and/or in minimum medium. Two mutants on

a novel two-component sensor histidine kinase gene designated as *rohS* were identified that poorly induced type III genes *in planta*, as well as in MM. *rohS* is immediately downstream of a two-component response regulator gene term *rohR,* and the two genes appear to be organized in an operon.

A bacterial two-component system (TCS) usually consists of a histidine kinase and a response regulator (Stock *et al*., 2000). In general, the histidine kinase autophosphorylates at a highly conserved histidine residue in the transmitter domain in response to a stimulus and subsequently transfers the phosphoryl group to an aspartate residue in the receiver domain of its cognate response regulator. Most of the known phosphorylated response regulators either stimulate or repress the transcription of their target genes (Graham *et al*., 2005; Stock *et al*., 2000). Besides the kinase activity, many histidine kinases possess a phosphatase activity that enables them to dephosphorylate the phosphorylated cognate response regulators. The equilibrium between the kinase and the phosphatase activities determines the outcome of downstream responses (Boyd and Lory, 1996; Stock *et al*., 2000).

In this study, we attempt to elucidate the role of RohS and RohR in regulation of TTSS genes in *Pseudomonas syringae.* Our results suggest that phosphorylated RohR acts as suppressor of TTSS genes. It is likely that RohS derepresses TTSS genes in inducing conditions through dephosphorylating the phosphorylated RohR.

RESULTS

Isolation of the P. syringae pv. phaseolicola min12 and pin19 mutants

Type III genes are expressed at minimal level when bacteria grow in nutrient rich medium such as KB and LB medium. The *avrPto* gene of *P. syringae* pv. *tomato* has a typical *hrp* box in its promoter and is strongly induced in MM and *in planta* (Salmeron and Staskawicz, 1993; Xiao *et al.*, 2004). To understand the signal transduction events involved in *hrp* promoter activation, the *avrPto* promoter was fused to the coding region of the firefly luciferase gene *LUC*, the resulting *avrPto-LUC* reporter gene was introduced into *P. syringae* pv. *phaseolicola* NPS3121 strain, and the strain was subject to EZ-Tn5^{TM}<Kan-2> transposon mutagenesis. The mutant library were screened for mutants defective in *avrPto-LUC* induction in *att1* mutant plants and in minimum medium. The isolated mutants were designated *pin* (*p*lant *in*sensitive) and *min* (*m*inimum medium *in*sensitive) mutants, respectively. Sixty three out of 6, 000 Kan^{R} mutants were isolated as *pin* mutants, and 102 out of 11, 872 mutants were isolated as *min* mutants (See Chapter II). Transposon insertion sites in these mutants were determined by a two-stage semidegenerate PCR (Jacobs *et al.,* 2003) and sequence analysis. Among these mutants, *min12* and *pin*19 (*min80*) carry transposon inserted in a putative two-component sensor histidine kinase gene (Fig. III-1A). A mutant carrying transposon in the *hrpR* gene was also isolated (Fig. III-1B), and this mutant was used as reference in *avrPto-LUC* induction and pathogenicity assays. As shown in Fig. III-2, the *pin19* mutant displayed severely reduced induction of the *avrPto-LUC* gene in the non-host *Arabidopsis* plants, the host bean plants, and MM. *Min12* mutant displayed the same phenotype as did the *pin19* mutant (data not shown). We named the two-component sensor histidine kinase

gene *rohS* (regulator of *hrp* genes sensory component) to reflect the requirement of this gene for the induction of a *hrp* promoter. Immediately upstream of *rohS* is a putative two-component response regulator gene that is designated here as *rohR* (regulator of *hrp* genes response regulator component). To determine if *rohS* is specifically involved in the regulation of TTSS genes, we tested how the *rohS*⁻ mutation affects the activity of a constitutive promoter *pro2*. As shown in Fig. III-3, the Pro2 promoter displayed the same activity in *rohS*⁻ mutant and the wild type strain in MM.

Characteristics of the RohS/RohR two-component system

rohS and *rohR* are organized in an operon that is controlled by the promoter upstream of *rohR*. *rohS* and *rohR* are highly homologous to *PSPTO2222* and *PSPTO2223*, respectively, in the *P. syringae* pv. *tomato* DC3000 strain. According to the DC3000 genome annotation, the coding regions of the two genes overlap 4 bps.

As predicted by TopPred II (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), $RohS_{Psph}$ protein possesses two transmembrane domains (aa 12-32 and aa 62-80) flanking a short periplasmic domain (*Fig.* III-4, A and B). Alignment of RohS orthologs from three strains representing three different *P. syringae* pathovars (*P. syringae* pv. *syringae* B728a, *P. syringae* pv. *tomato* DC3000, and *P. syringae* pv. *phaseolicola* 1448A) revealed that RohS proteins are highly conserved in the transmembrane domains and cytoplasmic region but variable in the periplasmic domain. Although variable in sequence, the periplasmic domains of all *RohS* orthologs are rich in proline and charged amino acid residues. No conserved receptor motif was identified in the periplasmic region.

The analysis of the deduced amino acid sequence indicated that RohS belongs to the sensor histidine kinase group represented by EnvZ (Park *et al.*, 1998). Pfam analysis

revealed that the putative *RohS* protein has conserved HAMP domain (Histidine kinases, Adenylyl cyclases, Methyl binding proteins, Phosphatases, aa 84-133), HisKA (dimerization/ phosphoacceptor domain, aa 135-199), and a HATPase_C (Histidine kinase-like ATPase, aa 238-338) domain (*Fig.* III-4A). HisKA and HATPase_c work together as the signal transmitter (Dutta *et al.*, 1999; Stock *et al.*, 2000). The HAMP domain is not only a structural linker between the signal receptor and the transmitter, but also plays an important role in the signal transduction between these two regions (Zhu and Inouye, 2004). In the HisKA domain of $RohS_{Psph}$, H147 is predicted to be the phosphorylation site. In the presence of the signal, H147 will be autophosphorylated, and the phosphate is subsequently transferred to the conserved aspartic acid residue of the response regulator with the energy provided by HATPase_C domain.

The orthologs of RohR in the three *P. syringae* pathovars are almost identical. $RohR_{Psph}$ and $RohR_{Psto}$ share 99% identity, while $RohR_{Psph}$ and $RohR_{Pss}$ share 98% identity. Pfam analysis indicated that RohR proteins possess two conserved domains: the N-terminal CheY-like signal receiver domain (REC) (Volz, 1993) and C-terminal winged-helix DNA-binding effector domain (trans_reg_C) as represented by the OmpR protein (Martinez-Hackert and Stock, 1997; Kenney, 2002) (*Fig.* III-4C). The Asp70 residue in the receiver domain is presumably the phosphoacceptor site that is phosphorylated by histidine kinase. Phosphorylation of the receiver domain may alter the DNA binding affinity or specificity, which in turn alters the expression of target genes and thus triggers the cellular response.

BlastP analysis indicated that a highly conserved locus also exists in *Pseudomonas fluorescens* PfO-1 strain that does not have a functional TTSS. In PfO-1,

the putative two-component sensor histidine kinase (Pflu02003275) and response regulator (Pflu02003274) are 60% and 86% identical to $RohS_{Psph}$ and $RohR_{Psph}$, respectively. The function of this putative two-component system in *P. fluorescens* is not known.

Mutation of $rohS_{Psph}$ compromised the bacterial pathogenicity and elicitation of HR

To determine if the reduced *hrp* promoter activity in the $rohS_{psph}^-$ mutants decreases bacterial pathogenicity, the *pin19* mutant was inoculated to the susceptible host, Red Kidney bean. The wild type *P. syringae* pv. *phaseolicola* NPS3121 caused severe disease on bean in 9 days, but the mutant *pin19* only caused very weak symptoms (*Fig. III-5*A). Consistent with the weak disease symptoms, the bacterial population of *pin19* mutant was significantly smaller than that of the wild type strain but much larger than that of the $hrpR^-$ mutant 6 days after inoculation (P value < 0.01) (*Fig. III-5*B). The *min12* mutant also showed the same phenotype as did *pin19* (data not shown). The $rohS_{Psph}^-$ mutants were also tested for the induction of non-host HR on tobacco W38 plants (*Fig. III-5*C). The wild type NPS3121 triggered HR in 12 hours, but the *pin19* mutant and $hrpR^-$ mutant did not cause HR of the infiltrated leaf area in 2 days. These results indicated that $rohS_{Psph}$ mutation reduced bacterial virulence on host plants and eliminated the HR on non-host plants.

Complementation of the $rohS_{Psph}^-$ mutant

Complementation analysis was performed to rule out the possibility that the phenotype of the $rohS_{Psph}^-$ mutants were caused by an unrelated mutation. Three versions of the $rohS_{Psph}$ gene differing in the translation initiation codon were used in

complementation test: *rohS$_{psph}$ (TTG)* was identical to the genomic sequence in the NPS3121 strain; *rohS$_{psph}$ (ATG)* and *rohS$_{psph}$ (TTC)* were identical to *rohS$_{psph}$ (TTG)* except the putative translation initiation codon was changed from TTG to ATG and TTC, respectively. TTC was never reported to act as a start codon in any organisms (http://www3.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi?mode=c). These genes were cloned under a strong constitutive promoter P_{Nm} in the low copy number plasmid pML123 (Labes *et al.*, 1990) and introduced into the *pin19* mutant. Compared with the wild type NSP3121 strain, *pin19* mutant expressing *rohS(ATG)* displayed the same induction of *avrPto-LUC* in MM and *in planta* (Fig. 2, A, B, and C), full virulence on Red Kidney plants (*Fig. III-5*, A and B), and complete restoration of the HR-inducing activity on tobacco W38 plants (*Fig. III-5*C). However, *rohS$_{psph}$(TTG)* and *rohS$_{psph}$(TTC)* did not complement the *avrPto-LUC* induction and other mutant phenotypes (data not shown), suggesting TTG could not direct effective translation of the *RohS* protein if it is not in the polycistronic context. Complementation of the mutant by the *rohS$_{psph}$(ATG)* gene demonstrated that *rohS$_{psph}$* is responsible for the observed phenotype. Interestingly, constitutive expression of *rohS$_{psph}$(ATG)* did not alter the *avrPto-LUC* expression in KB medium, suggesting that the sensor protein is active only in the presence of its specific signal.

rohS is functionally conserved in P. syringae pv. tomato DC3000

Because the orthologs of *rohS* and *rohR* from three *P. syringae* pathovars are highly homologous, we speculated that the *RohS* proteins are functionally conserved among the pathovars. We thus screened a *P. syringae* pv. *tomato* DC3000 transposon insertion library by PCR and identified a mutant with transposon inserted in the *rohS$_{Psto}$*

gene (Fig. 1C). This mutant was tested for the *avrPto-LUC* induction, bacterial pathogenicity, and ability to elicit non-host HR on tobacco W38 plants. The wild type DC3000 strain showed high induction of *avrPto-LUC* in MM and *in planta*, but the *rohS*$_{Psto}^{-}$ mutant showed low induction in MM and in tomato PtoS plants (*Fig. III-6*, A and B). When inoculated to the tomato PtoS plants, the wild type DC3000 strain caused severe disease lesions and multiplied to a high level, but the *rohS*$_{Psto}^{-}$ mutant only caused scattered disease lesions, and the bacterial growth is 20 to 30 fold lower than that of the wild type strain (*Fig. III-6*, C and D). Besides the reduced pathogenicity on host plants, the *rohS*$_{Psto}^{-}$ mutant displayed delayed HR on the non-host plants tobacco W38 (*Fig. III-6*E). These results indicated that the *rohS* genes are functionally conserved among *P. syringae* pathovars.

We further tested if *rohS*$_{Psto}$ from DC3000 can complement the NPS3121 *rohS*$_{Psph}^{-}$ mutant. Table III-3 shows that *rohS*$_{Psto}$ was able to restore the full induction of *avrPto-LUC* in MM but only half of the induction both in bean and in *Arabidopsis*. Consistent with the partial *hrp* promoter induction *in planta*, ectopic expression *rohS*$_{Psto}$ in *rohS*$_{Psph}^{-}$ mutant only recovered partially the bacterial pathogenicity (data not shown). The partial complementation of the *avrPto-LUC* induction *in planta* and bacterial pathogenicity of the *rohS*$_{Psph}^{-}$ mutant by the *rohS*$_{Psto}$ gene could result from the poor expression of the RohS$_{Psto}$ protein or "non-perfect" compatibility of the RohS$_{Psto}$ protein with the *P. syringae pv. phaseolicola* NSP3121 strain. We can not differentiate these possibilities because no RohS antibodies are available to determine the levels of RohS protein in these bacterial strains. Nevertheless, even partial complementation of the

rohS$_{Psph}^{-}$ mutant by the *rohS*$_{Psto}$ gene still indicated that the *rohS* genes from different *P. syringae* pathovars are functionally conserved.

The conserved histidine residue is required for signaling transduction in TTSS genes regulation

All two-component sensor kinases have a histidine residue in a highly conserved domain that serves as the autophosphorylation site. Mutation of the site disrupts the phosphorelay between the sensor kinase and the response regulator that is essential for signal transduction. The conserved His residue in RohS is His147. To determine if this site is important for RohS to regulate TTSS genes, site-directed mutagenesis was used to change this residue to alanine in plasmids pML123::*rohR/S*$_{Psph}$ and pML123::*rohS*$_{Psph}$. Plasmids carrying the wild type *rohS* gene and the H147A mutant were introduced to the *rohS*$^{-}$ mutant, and the resulting strains were tested for induction of the *avrPto-LUC* in MM and *in planta*. The wild type *rohS* gene alone fully restored the induction of the *avrPto-LUC* activity, but *rohS(H147A)* did not complement any of the *avrPto-LUC* induction. Interestingly, the wild type *rohR-rohS* locus only partially restored the *avrPto-LUC* induction, while the *rohR-rohS(H147A)* did not restore any of the *avrPto-LUC* induction (Table III-3). These results indicated that RohS regulates TTSS genes through phosphoryl transfer.

Epistatic relationship between rohS and hrpR, hrpS, and hrpL

In *P. syringae*, HrpR, HrpS, and HrpL are known to be involved in the activation of the *hrp* promoters. To determine the relationship between RohS and these known type III regulators, we conducted epistatic analysis between RohS, HrpR/S, and HrpL.

As shown in *Fig. III-7*A and B, the activation of the *hrpL* promoter requires *RohS*. In the wild-type NPS3121, the expression of the *hrpL-luc* reporter was dramatically induced in MM and *in planta*, but this induction was attenuated in the $rohS_{Psph}^{-}$ mutant. This result indicated that *rohS* acts upstream of *hrpL*. To further test this relationship, the wild type *hrpL* gene was expressed under a constitutive promoter in wild type NPS3121, $rohS_{Psph}^{-}$ mutant, and $hrpR^{-}$ mutant. All the strains constitutively expressing HrpL displayed strong *avrPto-LUC* expression even in the non-inductive KB medium (*Fig. III-7*C) and severely reduced bacterial growth on KB agar plates and liquid medium (data not shown). The growth defect suggested that constitutive expression of TTSS genes is deleterious to the bacterium.

Mutation of $rohS_{Psto}$ impacted the accumulation of *hrpR* RNA both in KB medium and in MM (*Fig. III-8*). A low level *hrpR* RNA was detected in the wild type strain grown in KB medium, and the abundance of the transcripts are further induced in MM. This induction of the *hrpR* RNA in MM is consistent with *hrpR* promoter assay of others and ours that showed ~3 fold induction of the *hrpR* promoter by MM (Rahme *et al.*, 1992). However, *hrpR* RNA was not detectable in the $rohS_{Psto}^{-}$ mutant grown in either KB medium or MM. The lower *hrpR* RNA abundance in the *rohS* mutant was contributed at least partially by the reduced *hrpR* promoter activity in the mutant, because a lower *hrpR-luc* expression was detected in the $rohS_{Psph}^{-}$ mutant strain than in the wild type strain both in KB medium and in MM (*Fig. III-9*, A). However, the *hrpR* promoter displayed an induction in MM and in planta in the $rohS_{Psph}^{-}$ mutant (*Fig. III-9*, A and B). These data indicated the requirement of *rohS* for the basal *hrpR* expression. We also tested if constitutive expression of HrpR or HrpS could restore the expression of *avrPto-*

LUC in *rohS* mutant. As shown in *Fig. III-9* (C and D), constitutive expression of HrpR elevated the basal *avrPto-LUC* expression both in the wild type strain and the $rohS_{Psph}^{-}$ mutant in KB medium (*Fig. III-9*, C and D, 0hr). Upon induction in the MM and *in planta*, the *avrPto-LUC* expression was further increased in all the strains (*Fig. III-9*, C and D, 0hr). Likewise, constitutive expression of HrpS displayed the same effect as constitutive expression of HrpR in the wild type and $rohS_{Psph}^{-}$ mutant (data not shown). However, constitutive expression of the $rohS_{Psph}$ in $hrpR^{-}$ mutant did not restore the induction of the *avrPto-LUC* expression gene (*Fig. III-9*, E and F). These results suggested that *RohS* acts upstream of HrpR/HrpS.

Phosphorylated rohR inhibits the induction of avrPto-LUC

rohS and *rohR* appear to be located in an operon and are functionally related. As we failed to isolate $rohR^{-}$ mutant in *min* and *pin* mutant screening, we speculated that our mutant screen was not saturated, or *rohR* is essential for bacterial growth. To test this, we screened the *P. s.* pv. *phaseolicola* NPS3121 and *P. s.* pv. *tomato* DC3000 transposon insertion libraries by PCR and identified the mutant with transposon inserted in the *rohR* gene (*rohR*::*Tz*). We tested the *avrPto-LUC* induction for *rohR*::*Tz* mutant strains. Surprisingly, the *rohR*::*Tz* mutants displayed similar *avrPto-LUC* induction as did parental strains (data not shown). Additionally, in attempt to complement the *avrPto-LUC* induction in the $rohS_{Psph}^{-}$ mutant, we observed that overexpression of *rohS* alone fully restored the *avrPto-LUC* induction in the $rohS_{Psph}^{-}$ mutant, but overexpression of the *rohRS* locus only partially restored the *avrPto-LUC* induction, and overexpression of *rohR* alone in the $rohS_{Psph}^{-}$ mutant even reduced the *avrPto-LUC* expression to a level lower than that in the $rohS^{-}$ mutant (Table III-3). These observations proposed that *rohR*

acts as a repressor in TTSS gene regulation and RohS derepresses TTSS genes in inducing conditions in some way. To test this, we measured the *avrPto-LUC* induction in wild type *P. s. phaseolicola* NPS3121 carrying *avrPto-LUC* and overexpressing *rohS*, *rohR/S*, *rohR/S* H147A, or *rohR*. As a result, overexpression of *rohS* alone did not interfere with the induction of *avrPto-LUC* both in MM and *in planta*, while overexpression of *rohR/S*, *rohR/S* H147A and *rohR* inhibited the induction of *avrPto-LUC* in the wild type strain (Table III-3). It is noticeable that overexpression of *rohR* or *rohR/S* H147A was more inhibitory than was *rohR/S* overexpression.

To further investigate how RohR negatively regulates bacterial TTSS genes, we generated a $rohRS_{Psto}$ deletion (Δ*rohRS*) mutant by marker exchange. The Δ*rohRS* mutant did not affect the induction of *avrPto* in MM (*Fig. III-10*). However, overexpression of the wild type *rohR* in the Δ*rohRS* mutant strain completely repressed the *avrPto-luc* induction in MM (*Fig. III-10*). To test if this inhibitory activity of RohR is phosphorelay-dependent, RohR(D70A) mutant with phosphoacceptor site (aspartate 70) substituted by alaline was overexpressed in the Δ*rohRS* mutant strains. As a result, overexpression of RohR (D70A) only slightly reduced the *avrPto-LUC* induction in MM compared to the parental stain and Δ*rohRS* mutant (*Fig. III-10*), suggesting that phosphorylated RohR is more efficient than the non-phosphorylated RohR in repression of TTSS genes.

DISCUSSION

In searching for the *P. syringae* genes required for induction of type III genes in plant, we identified RohS, a putative two-component sensor histidine kinase, as a critical component for the activation of a *hrp* promoter both in plant and in MM. Mutation of

RohS attenuated not only the induction of type III genes but also bacterial pathogenicity and elicitation of HR on non-host plants. Sequence analysis indicated that *rohS* is in the same operon with *rohR* encoding a putative two-component response regulator. Mutational analysis indicated that RohR in the phosphorylated form is a negative regulator of TTSS genes. Epistatic analysis indicated that RohS/RohR functions upstream of HrpR/HrpS.

Bacteria rely primarily on two-component systems to sense and respond to environmental changes. Typically, a two-component system consists of a sensor histidine kinase and a response regulator (Reviewed by Stock *et al.*, 2000). The sensor histidine kinases perceive the environmental stimuli via the signal input domain, and the interaction of signal with the input domain leads to activation of the histidine kinase and autophosphorylation of a specific histidine residue in the cytoplasmic transmitter domain. The phosphorylation of the sensor enables it to interact with specific response regulators and then transfer the phosphoryl group to a conserved aspartate residue on the receiver domain of the response regulator. The receiver domain of the response regulator is usually connected with its output domain that generally has DNA-binding activity. The phosphorylation of the receiver domain alters the DNA-binding affinity of response regulator and thus the transcription of its target gene(s). Many histidine kinases possess both autophosphorylation activity and phosphatase activity (Boyd and Lory, 1996; Stock *et al.*, 2000). RohS and RohR have the typical structural features as represented by the bacterial two-component system EnvZ and OmpR (Martinez-Hackert and Stock, 1997; Park *et al.*, 1998; Kenney, 2002). Pfam analysis indicated that RohS has a periplasmic signal input domain that is highly variable among the RohS proteins in different *P.*

syringae pathovars; this variation may be the result of bacterial adaptation to specific host plants. Despite the great variation on the primary sequences, the RohS protein from *P. syringae* pv. *tomato* DC3000 strain can complement the $rohS_{Psph}^{-}$ mutant of *P. syringae* pv. *phaseolicola* NPS3121 strain, suggesting a conserved tertiary structure formed by periplasmic domains that can perceive the same signal. The signal input domain is connected via a transmembrane linker to the cytoplasmic signal transmitter region that is highly conserved among the various RohS proteins. The RohR proteins consist of a CheY-like receiver domain and a winged-helix DNA-binding domain. The extremely high degree of sequence identity among the RohS cytoplasmic signal transmitter regions and the RohR proteins suggests the conserved signal transduction mechanism controlled by this two-component system in different *P. syringae* pathovars.

Several pieces of experimental evidence indicated that RohS acts upstream of HrpR/HrpS. First, Northern blot analysis indicated that *RohS* affects the accumulation of the *hrpR* RNA both in rich medium and in MM. Northern blotting in *Fig. III-8* indicated that the *hrpR* RNA is expressed at a low level in rich medium but increased several folds in MM. The increased *hrpR* RNA in MM is contributed at least partially by the *hrpR* promoter activity that also displayed an induction in MM (Rahme *et al.*, 1992; *Fig. III-9A*). The *hrpR* promoter activity and the accumulation of the *hrpR* RNA were both reduced in the $rohS^{-}$ mutant. It remains to be tested if the transcription of *hrpR/S* operon is under the direct control of the *RohS*/*RohR* two-component system. Second, overexpression of *hrpR*, *hrpS*, or *hrpL* rendered *rohS* dispensable for a high level of *avrPto-LUC* expression even in the KB medium, suggesting that *rohS* regulates the *avrPto* promoter activity through these intermediate regulators. The reduced *hrpR, hrpL,*

and *avrPto* promoter activities in the *rohS*$^{-}$ mutant suggest a transcriptional regulation cascade downstream of RohS. Third, overexpression of RohS in *hrpR*$^{-}$ mutant did not restore the induction of *avrPto-LUC* in MM, indicating the requirement of HrpR protein for signal transduction from *RohS* to the induction of downstream *hrp* promoters.

Our results suggested that phosphorylated RohR (P-RohR) represses TTSS genes and RohS derepresses TTSS genes in inducing conditions through dephosphorylating P-RohR. First, interruption of *rohS* alone abolished *avrPto-LUC* induction in MM and in the plant, while Tz insertion in *rohR* or deletion of *rohRS* did not affect the induction of *avrPto-LUC*, indicating that RohS works through RohR to regulate TTSS genes. Second, overexpression of wild type *RohR* gene in wild type bacterium and in *rohRS* deletion mutant strain inhibited *avrPto-LUC* induction, suggesting RohR acts as repressor of TTSS genes. Third, overexpression of the *RohR(D70A)* mutant gene in wild type bacterium and in *rohRS* deletion mutant strain only slightly reduced *avrPto-LUC* induction, suggesting that the phosphylated form of RohR (P-RohR) is responsible for repression of TTSS genes in the *RohS*$^{-}$ mutant strain. Finally, the wild type *rohS* gene fully complemented the phenotype of *rohS* transposon insertion mutant, while the *rohS(H147A)* mutant gene did not. To most two-component sensor kinases, mutation of the autophosphorylation site disrupts not only the kinase but also the phosphatase activity. It is likely that in the *rohS*$^{-}$ mutant, RohR is phosphorylated by another TCS kinase or more likely by certain small phosphorylated molecules such as acetyl-phosphate, phosphoramide and carbamyl phosphate that can phosphorylate many response regulator proteins (McCleary *et al*., 1993), and P-RohR represses the activation of TTSS genes. While the wild type RohS protein has the phosphatase activity that can remove the

phosphoryl group from P-RohR and thus relieves the repression, RohS(H147A) does not have this activity and therefore can not derepress P-RohR from inhibition of TTSS genes. According to these data, we propose that in the wild type bacteria under the the TTSS-inducing conditions, RohS acts mainly as the phosphatase and keeps the RohR in the non-phosphorylated form. The non-phosphorylated form of RohR is poor in DNA binding and therefore does not interfere significantly the induction of TTSS genes. It is possible that the P-RohR binding with DNA interferes with the function of a positive regulator of TTSS genes. The identity of the positive regulator that is interfered by P-RohR remains to be determined.

It is interesting to notice that *hrpR*$^-$ and *rohS*$^-$ mutants displayed similar levels of *avrPto-LUC* expression both in MM and *in planta*. However, the *hrpR*$^-$ mutant is nonpathogenic, whereas the *rohS*$^-$ mutants only lost partially the pathogenicity on host plants. This indicated that the pathogenic mechanisms represented by *avrPto-LUC* reporter contribute only partially to the overall bacterial pathogenicity. Consistent with this speculation, the *hrpR*$^-$ mutant still displayed a significant level of *avrPto-LUC* induction in the host bean plant, but the *hrpR*$^-$ mutant is nonpathogenic. This indicated that *hrpR* controls additional pathogenic mechanisms that are critical to bacterial pathogenicity but are not reflected by the *avrPto* promoter activity. The finding that the *hrpR*$^-$ mutants still displayed a significant induction of the *avrPto-LUC* activity in bean plants suggests additional factor(s) other than *hrpR* in regulation of *avrPto* and probably other type III genes as well. But the *hrpR*-independent mechanism does not contribute significantly to bacterial pathogenicity in the absence of the *hrpR*-dependent pathogenic mechanism. It is possible that part of the *hrpR*-controlled pathogenic mechanism is not

under the control of *RohS*, and therefore, even though mutation of *RohS* reduces the expression of the *hrpR* RNA, the remaining *hrpR* RNA is still able to direct the production of these virulence factors under the type III inducing conditions. However, the maximal expression of the *avrPto* or other *hrp* promoters requires a threshold of the HrpR protein and other unknown regulator(s).

EXPERIMENTAL PROCEDURES

Plant materials and bacterial strains

The *Arabidopsis att1* mutant plants (Xiao *et al.*, 2004) were used to screen NPS3121 mutants. Red Kidney bean (Lindgren *et al.*, 1986), Rio-Grande PtoS and tobacco W38 plants (Shan *et al.*, 2000) were used for pathogenicity and non-host HR assays. *Arabidopsis* plants were grown in growth chambers at 20°C during the night and 22°C during the day with a 10 h/day photoperiod. Tomato, tobacco, and bean plants were grown in a greenhouse.

Bacterial strains used in this study were *P. syringae* pv. *phaseolicola* NPS3121 (Lindgren *et al.*, 1986), *P. syringae* pv. *tomato* DC3000 strains (Buell *et al.*, 2003), and their derivatives. Bacteria were grown in King's B medium (King *et al.*, 1954) with appropriate antibiotics to $2x10^9$ CFU/mL before harvest for pathogenicity and *avrPto-LUC* expression assays.

Plasmids and construction

All plasmids used in this study are listed in Table III-2. Primers for PCR amplification of the promoters and open reading frames were designed based on the

genome sequences of *P. syringae* pv. *phaseolicola* 1448A (http://www.tigr.org) and *P. syringae* pv. *tomato* DC3000 (http://www.ncbi.nlm.nih.gov). All the primer sequences are listed in Table 3.

The pHM2::*avrPto-LUC* plasmid was constructed as described in chapter II. *hrpL-luc* reporter genes were previously constructed into the pPTE6 plasmid by Xiao *et al.* (2004). To generate the pLT::*hrpL-luc* reporter gene, the Kan^R gene in pPTE6::*hrpL-luc* plasmid was disrupted by EZ-Tn5<TET-1> transposon (Epicentre, Wisconsin, WI). The resulting plasmid confers tetracycline-resistance.

To construct the *hrpR-luc* reporter gene, the *hrpR* promoter was amplified from NSP3121 using primers phrpR-F and phrpR-R. The PCR product was digested with *Eco*RI and *Bam*HI, cloned into pBluscript-SK(+) plasmid and sequence confirmed. The promoter DNA was then released from the pBluscript-SK(+) plasmid with EcoR1/ *Bam*HI digestion and cloned upstream of the *luc* gene in the pPTE6::luc plasmid (Xiao *et al.*, 2004). To generate pLT::*hrpR-luc*, the Kan^R gene in pPTE6::*hrpR-luc* plasmid was knocked out by EZ-Tn5<TET-1> transposon insertion. pHM1::*pro2-LUC* was isolated from DC3000 genomic library that exhibited constitutive LUC activity.

pML123 plasmid (Labes *et al.*, 1990) was used to express the $rohS_{Psph}$ and $rohS_{Psto}$ genes in *P. syringae* strains. The $rohS\ (TTG)_{Psph}$ and $rohS(ATG)_{Psph}$ genes were amplified by PCR from NSP3121 strain using the following pairs of primers: *RohS*-F and *RohS*-R for $rohS\ (TTG)_{Psph}$; *RohS*(ATG)-F and *RohS*-R for $rohS\ (ATG)_{Psph}$, and the PCR products were cloned into pGEM-7Zf(+) and pBluescript-SK(+) plasmids, respectively. After sequence confirmation, the $rohS_{Psph}$ insert was released by *Bam*HI/*Xba*I digestion and cloned into pML123 plasmid predigested with the same enzymes. QuickChange®

Site-Directed Mutagenesis Kit (Stratagen, La Jolla, CA) and the primers *RohS*(TTC)-F and *RohS*(TTC)-R were used to change the start codon of $rohS_{Psph}$ in pBluescript-SK(+)::$rohS\ (TTG)_{Psph}$ from TTG to TTC. The mutant $rohS_{Psph}$ genes were confirmed by sequence and cloned into the pML123 plasmid. Similarly, the $rohS_{Psto}$ gene were amplified by PCR from DC3000 strain using the *RohS*-F and *RohS*-R primers, sequence confirmed, and cloned into the *Bam*HI and *Xba*I sites of pML123.

The coding regions of *hrpL* and *hrpR* were amplified from NSP3121 strain using the primer pairs of hrpL-F/hrpL-R and hrpRS-F/hrpR-R, respectively. The PCR products of *hrpL* and *hrpR* were digested with *Xba*I/*Hind*III, respectively, and cloned into pBluescript-SK(+) plasmid. After sequence confirmation, the inserts were released from the pBluescript-SK(+) plasmid with *Xba*I/*Hind*III and cloned into pML122 plasmid. The primer pair of hrpRS-F and hrpRS-R were used to amplified the *hrpR/S* locus from NSP3121, and the PCR product was cloned into pBluescript-SK(+) between the *Xba*I/*Bam*HI sites. To construct pML122::*hrpS*, the *Hind*III/*Bam*HI fragment containing the *hrpS* coding region was released from pBluescript-SK(+)::*hrpRS* construct and cloned into pML122.

Construction of transposon-insertion libraries in P. syringae pv. phaseolicola NPS3121 and P. syringae pv. tomato DC3000 strains

EZ-Tn5^{TM}<KAN-2> transposon and EZ-Tn5^{TM}<TET-1> transposon (Epicentre, Wisconsin, WI) were used to generate the transposon-insertion libraries of *P. syringae* pv. *phaseolicola* NPS3121 and *P. syringae* pv. *tomato DC3000*, respectively. Briefly, electrocompetent cells of NPS3121 carrying the pHM2::*avrPto-LUC* plasmid were mixed with EZ-Tn5^{TM}<KAN-2> transposon and transposase as instructed by manufacturer.

Following electroporation, the bacteria were plated on KB medium containing 10 mg/L kanamycin and 10 mg/L spectinomycin to selected for mutant bacteria carrying the EZ::TN^{TM}<KAN-2> transposon and the pHM2::*avrPto-LUC* reporter plasmid. The same procedures were also used to generate the DC3000 mutant library. The DC3000 mutant library was selected with KB plate containing 10mg/L tetracycline.

Screen of pin mutants and determination of transposon insertion sites

To screen for mutants with compromised *avrPto-LUC* expression *in planta*, the mutant bacterial colonies were first grown in liquid KB medium containing spectinomycin and kanamycin, washed twice with sterile water, resuspended in sterile water to OD_{600}=0.5, and injected into *att1* mutant plants. Six hours after inoculation, the inoculated leaves were sprayed with 1mM luciferin dissolved in 0.01% Tween-20, and the luciferase activity was determined by using a cooled CCD (Roper Scientific, Trenton, NJ). Putative bacterial mutants with <30% of the wild type luciferase activity were selected and confirmed. A total of 6, 000 colonies were screened.

The transposon insertion sites were determined by a two-stage semidegenerate PCR according to procedures described by Jacobs *et al.* (2003) using two transposon-specific primers (Kan2-SP1 and Kan2-SP2) and four degenerated primers (CEKG 2A, CEKG 2B, CEKG 2C, and CEKG). The PCR product was sequenced using the third transposon-specific primer Kan2-SP3. Blastn was used to search for the homologous sequence in NCBI database and the *P. syringae* pv. *phaseolicola* 1448A sequence in TIGR database.

To confirm the transposon insertion sites in *Min12* and *pin19* mutants, gene specific primer pin19-3'in and transposon specific primer Kan2-R were used to PCR-

amplify the transposon-flanking DNA. The PCR product was sequenced to determine the transposon insertion site.

Isolation of rohS$_{Psto}$- mutant from DC3000 transposon insertion library

DNA pooling and nested PCR were used for isolation of *rohS*$_{Psto}^{-}$ mutant from the transposon insertion library. A total of 19, 200 colonies from the DC3000 mutant library were grown in 200 96-well plates. Bacteria grown in each plate were pooled for DNA isolation. 10 pools of DNA representing 960 colonies were combined to form a superpool, and the superpool DNA was used for screening of *rohS*$_{Psto}^{-}$ mutant using nested PCR. Because the transposon can insert into the genome in two possible directions, we designed two sets of nest transposon-specific primers based on the sequence near the transposon ends (TN-Tet-L1 and TN-Tet-L2; TN-Tet-R1 and TN-Tet-R2). TN-Tet-L1 and TN-Tet-R1 in combination with the *rohS*$_{Psto}$-specific primer (DC*RohS*-R1) were used for the first round of PCR. The PCR product was then treated with Exo1 (USB, Cleveland, Ohio) and SAP (USB, Cleveland, Ohio) to clean the unused primers according to Jacob *et al.*, (2003). 0.5% of the first round PCR product was used as template for the second round PCR with the transposon-specific primer (TN-Tet-L2 or TN-Tet-R2) and *rohS*$_{Psto}$-specific primer (DC*RohS*-R2). The product of the second round PCR was separated with an agarose gel and the DNA was eluted from the gel and sequenced.

Once the superpool carrying the desired mutant was identified, the 10 corresponding subpools were PCR-screened to identify the plate carrying the mutant. The plate was then subjected to two dimensional pooling, forming 8 horizontal pools (each pool represents 12 colonies) and 12 vertical pools (each pool represents 8 colonies).

These pools were PCR-screened to identify the well of mutant. Bacteria from the well were streaked onto a KB plate, and individual colony was reconfirmed by PCR and sequence analysis.

Assay of avrPto-LUC induction in MM and in planta

P. s. pv. *phaseolicola* NPS3121 and the derived mutant strains carrying pHM2::*avrPto-LUC* were first grown in liquid KB medium containing 20 mg/L rifampcin,10 mg/L kanamycin, and 10 mg/L spectinomycin to OD_{600}=1. To determine the reporter gene induction in MM, the bacteria were harvested by centrifugation at 5, 000g, washed twice with sterile water, resuspended in MM to a concentration of OD_{600}=0.05, and cultured at 23°C with constant shaking at 250 rpm. One hundred micro liter of bacteria was mixed with 2 μl of 1 mM luciferin in 96-well plate, and the luciferase activity was determined using a cooled CCD. To determine the reporter gene induction in plant, bacteria at a concentration of OD_{600}=0.5 in sterile water were inoculated into plants. Leaves were detached 6hr after inoculation, sprayed with a solution containing 1 mM luciferin and 0.01% Tween-20, and kept in the dark for 5 min before imaging with a CCD. The relative LUC activity in MM and *in planta* was normalized to bacterial numbers.

Bacterial growth, disease symptom, and non-HR assays

Bacteria were cultured in liquid KB medium containing appropriate antibiotics overnight at room temperature with constant shaking. To prepare the inocula, the bacterial cells were centrifuged at 4, 000g, washed twice with sterile water, and resuspended in 10mM $MgCl_2$. NSP3121 at 2×10^5 CFU/ml and 2×10^4 CFU/ml was used for assays of disease symptoms and bacterial growth on bean, respectively. The inoculum

was infiltrated to the primary leaves of 10-day old bean plants by hand injection. DC3000 at 2×10^4 CFU/ml was used for bacterial growth and disease symptom assays on tomato. The bacteria in 10 mM $MgCl_2$ plus 30µl/L silwet L-77 were vacuum infiltrated into the Rio-Grande PtoS plants. Bacterial numbers were determined at the time indicated in the figures. Leaf disc of 1 cm^2 in size was ground in sterile water, and the diluted bacteria were plated onto TSA agar plate (10g/L Bacto tryptone, 10g/L sucrose, and 1g/L glutamic acid) containing appropriate antibiotics to determine the colony-forming units. Disease symptoms on bean leaves and tomato leaves were photographed 9 and 4 days after inoculation, respectively. For non-host HR assay, bacteria resuspended in 10mM $MgCl_2$ to $1x10^8$ CFU/ml were inoculated into fully expanded tobacco W38 leaves by hand-injection. The lesions were visually examined every 2 hrs after inoculation.

RNA extraction and Northern blotting

P. syringae strains were grown at room temperature in KB medium supplemented with appropriate antibiotics to OD_{600} =0.5–0.8. Bacteria then were pelleted and used for RNA isolation. For MM induction experiments, the pellets were washed twice with fructose MM and resuspended in the same medium to OD_{600}=0.3. Cultures were grown with moderate shaking at room temperature. After 6 hrs, the cells were collected by centrifugation at 8, 000g for 5 min.

RNA was extracted by using a modified hot phenol procedure (Aiba *et al.* 1981). Briefly, 15ml of bacterial culture was pelleted by centrifugation. The pellet was mixed with 2ml of extracting buffer (20 mM Sodium acetate at pH 5.5, 0.5% SDS, 1 mM EDTA) and an equal volume phenol pre-warmed to 60°C. After votex, the bacterial suspension was incubated at 60°C for 5-10 min, chilled on ice for 3-5 min, and then centrifuged. The

supernatant was extracted by acid phenol-chloroform twice and precipitated with 2.5 vol of 100% ethanol for overnight. RNA was pelleted by centrifugation at 12, 000g at 4°C for 15 min. The pellet was washed with 75% ethanol and re-suspended in RNase-free water. Contaminating DNA was digested with RQ1 DNase (Promega, Madison, WI). RNA was extracted with acid phenol-chloroform, precipitated with ethanol and sodium acetates, and re-suspended in RNase-free water. RNA concentration was measured with a spectrophotometer at 260nm. Ten micrograms of total RNA was used for Northern blot analysis following the procedures described by Tang *et al.* (1999). *rohS* and *hrpR* coding regions were radio-labeled with ^{32}P dCTP and used as probes.

REFERENCES

Aiba H., Adhya S., de Crombrugghe B., 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J. Biol. Chem.* 256: 11905–11910.

Alarcon-Chaidez F.J., Keith L., Zhao Y., Bender C.L., 2003. RpoN (σ^{54}) is required for plasmid-encoded coronatine biosynthesis in *Pseudomonas syringae*. *Plasmid* 49:106-117.

Aldon D., Brito B., Boucher C., Genin S., 2000. A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. *EMBO J.* 19: 2304-2314.

Alfano J. R., Collmer A., 2004. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* 42: 385-414.

Boyd J. M., Lory S., 1996. Dual function of PilS during transcriptional activation of the *Pseudomonas aeruginosa* pilin subunit gene. *J. Bacteriol*. 178(3): 831-839.

Bretz J., Losada L., Lisboa K., Hutcheson S. W., 2002. Lon protease functions as a negative regulator of type III protein secretion in *Pseudomonas syringae*. *Mol. Microbiol.* 45: 397-409.

Buell C. R., Joardar V., Lindeberg M., Selengut J., Paulsen I. T., Gwinn M. L., *et al.*, 2003. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. USA* 100: 10181-10186.

Chatterjee A, Cui Y, Yang H, Collmer A, Alfano JR, Chatterjee AK. 2003. GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol. Plant Microbe. Interact*. 16:1106-1117.

Collmer A., Badel J. L., Charkowski A. O., Deng W. L., Fouts D. E., Ramos A. R., *et al.*, 2000. *Pseudomonas syringae* Hrp type III secretion system and effector proteins. *Proc. Natl. Acad. Sci. USA* 97: 8770-8777.

Dutta R., Qin L., Inouye M., 1999. Histidine kinases: diversity of domain organization. *Mol. Microbiol.* 34: 633-640.

Fouts D. E., Abramovitch R. B., Alfano J. R., Baldo A. M., Buell C. R., Cartinhour S., *et al.*, 2002. Genomewide identification of *Pseudomonas syringae* pv. tomato DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc. Natl. Acad. Sci. USA* 99: 2275-2280.

Francis M. S., Wolf-Watz H., Forsberg A., 2002. Regulation of type III secretion systems. *Curr. Opin. Microbiol.* 5: 166-172.

Galan J. E. and Collmer A., 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* 284: 1322-1328.

Graham M. R., Virtaneva K., Porcella S. F., Barry W. T., Gowen B. B., Johnson C. R., Wright F. A., Musser J. M., 2005. Group A *Streptococcus* transcriptome dynamics during growth in human blood reveals bacterial adaptive and survival strategies. *Am. J. Pathol.* 166: 455-465.

Grimm C., Aufsatz W., Panopoulos N. J., 1995. The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. *Mol. Microbiol.* 15: 155-165.

Hendrickson E. L., Guevera P., Ausubel F. M., 2000a. The alternative sigma factor RpoN is required for *hrp* activity in *Pseudomonas syringae* pv. *maculicola* and acts at the level of *hrpL* transcription. *J. Bacteriol.* 182: 3508-3516.

Hendrickson E. L., Guevera P., Penaloza-Vazquez A., Shao J., Bender C., Ausubel F. M., 2000b. Virulence of the phytopathogen *Pseudomonas syringae* pv. *maculicola* is *rpoN* dependent. *J. Bacteriol.* 182: 3498-3507.

Hirano S. S., Upper C. D., 2000. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*-a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. Rev.* 64: 624-653.

Hueck C. J., 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* 62: 379-433.

Hutcheson S. W., Bretz J., Sussan T., Jin S., Pak K., 2001. Enhancer-binding proteins HrpR and HrpS interact to regulate *hrp*-encoded Type III protein secretion in *Pseudomonas syringae* Strains. *J. Bacteriol.* 183: 5589–5598.

Huynh T. V., Dahlbeck D., Staskawicz B. J., 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* 245: 1374-1377.

Jacobs M. A., Alwood A., Thaipisuttikul I., Spencer D., Haugen E., Ernst S., Will O., Kaul R., Raymond C., Levy R., Chun-Rong L., Guenthner D., Bovee D., Olson M. V., Manoil C., 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 100: 14339-14344.

Jin Q., Hu W., Brown I., McGhee G., Hart P., Jones A. L., He S.Y., 2001. Visualization of secreted Hrp and Avr proteins along the Hrp pilus during type III secretion in Erwinia amylovora and *Pseudomonas syringae*. *Mol. Microbiol.* 40: 1129-1139.

Jin Q., Thilmony R., Zwiesler-Vollick J., He S. Y., 2003. Type III protein secretion in *Pseudomonas syringae*. *Microbes Infect.* 5: 301-310.

Jin Q., He S.Y., 2001. Role of the Hrp pilus in type III protein secretion in *Pseudomonas syringae*. *Science* 294: 2556-2558.

Kenney L.J., 2002. Structure/function relationships in OmpR and other winged-helix transcription factors. *Curr. Opin. Microbiol.* 5: 135-141.

King E. O., Ward M. K., Raney D. E., 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Med.* 22: 301-307.

Kubori T., Matsushima Y., Nakamura D., Uralil J., Lara-Tejero M., Sukhan A., GalanJ. E., Aizawa S. I., 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* 280: 602-605.

Labes M., Puhler A., Simon R., 1990. A new family of RSF1010-derived expression and lac-fusion broad-host-range vectors for gram-negative bacteria. *Gene* 89:37-46.

Leach J.E., White F.F., 1996. Bacterial avirulence genes. *Annu. Rev. Phytophathol.* 34: 153-179.

Lindgren P. B., Peet R. C., Panopoulos N. J., 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity of nonhost plants. *J. Bacteriol.* 168: 512-522.

Marenda M., Brito B., Callard D., Genin S., Barberis P., Boucher C., Arlat M., 1998. PrhA controls a novel regulatory pathway required for the specific induction of *Ralstonia solanacearum hrp* genes in the presence of plant cells. *Mol. Microbiol.* 27: 437–453.

Marlovits T.C., Kubori T., Sukhan A., Thomas D.R., Galan J.E., Unger V.M., 2004. Structural insights into the assembly of the type III secretion needle complex. *Science* 306: 1040-1042.

Martinez-Hackert E., Stock A. M., 1997. The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. *Structure* 5: 109-124.

McCleary W. R., Stock J. B., Ninfa A. J., 1993. Is acetyl phosphate a global signal in *Escherichia coli*? *J. Bacteriol*. 175: 2793-2798.

Park H., Saha S. K., Inouye M., 1998. Two-domain reconstitution of a functional protein histidine kinase. *Proc. Natl. Acad. Sci. USA* 95: 6728-6732.

Preston G., Deng W. L., Huang H. C., Collmer A., 1998. Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J. Bacteriol.* 180: 4532-4537.

Rahme L. G., Mindrinos M. N., Panopoulos N. J., 1992. Plant and environmental sensory signals control the expression of hrp genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 74: 3499-3507.

Salmeron J. M., Staskawicz B. J., 1993. Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato*. *Mol. Gen. Genet.* 239: 6–16.

Shan L., Thara V.K., Martin G.B., Zhou J.M., Tang X., 2000. The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. *Plant Cell* 12: 2323-2338.

Stock A. M., Robinson V. L., Goudreau P. N., 2000. Two-component signal transduction. *Annu. Rev. Biochem.* 69: 183-215.

Tang X., Xie M., Kim Y.J., Zhou J., Klessig D.F., Martin G.B., 1999. Overexpression of Pto activates defense responses and confers broad resistance. *Plant Cell* 11: 15-29.

van Dijk K., Fouts D. E., Rehm A. H., Hill A. R., Collmer A., Alfano J. R., 1999. The Avr (effector) proteins HrmA (HopPsyA) and AvrPto are secreted in culture from *Pseudomonas syringae* pathovars via the Hrp (type III) protein secretion system in a temperature- and pH-sensitive manner. *J. Bacteriol.* 181: 4790-4797.

Volz K.,1993. Structural conservation in the CheY superfamily. *Biochemistry* 32: 11741-11753.

Wei CF, Deng WL, Huang HC, 2005. A chaperone-like HrpG protein acts as a suppressor of HrpV in regulation of the *Pseudomonas syringae* pv. *syringae* type III secretion system. *Mol. Microbiol*. 57: 520-536

Wei W., Plovanich-Jones A., Deng W.L., Jin Q.L., Collmer A., Huang H.C., He S.Y., 2000. The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. tomato. *Proc. Natl. Acad. Sci. USA* 97: 2247-2252.

Xiao F., Mark Goodwin S., Xiao Y., Sun Z., Baker D., Tang X., Jenks M. A., Zhou J. M., 2004. *Arabidopsis* CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development. *EMBO J.* 23: 2903-2913.

Xiao Y., Heu S., Yi J., Lu Y., Hutcheson S. W., 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* 176: 1025-1036.

Xiao Y., Hutcheson S. W., 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. *J. Bacteriol.* 176: 3089-3091.

Xiao Y., Lu Y., Heu S., Hutcheson S. W., 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 hrp cluster. *J. Bacteriol.* 174: 1734-1741.

Zhu Y., Inouye M., 2004. The HAMP linker in histidine kinase dimeric receptors is critical for symmetric transmembrane signal transduction. *J. Biol. Chem.* 279: 48152-48158.

Zwiesler-Vollick J., Plovanich-Jones A. E., Nomura K., Bandyopadhyay S., Joardar V., Kunkel B. N., He S. Y., 2002. Identification of novel hrp-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. tomato DC3000 genome. *Mol. Microbiol.* 45: 1207-1218.

Table III-1. Plasmids used in this study

Plasmids	Description	Reference
pBluscript-SK(+)	Cloning and sequencing	Stratagen, La Jolla, CA
pGEM-7Zf(+)	Cloning and sequencing	Promega, Madison, WI
pGET-T	Cloning and sequencing	Promega, Madison, WI
pPTE6	Broad host plasmid	Ronald et al., 1993
pPTE6::*LUC*	Broad host plasmid carrying a promoter-less luc gene	Xiao et al., 2004
pHM1	Broad host plasmid	Zhu et al., 1999
pHM2	Broad host plasmid	This study
pPTE6::*avrPto-LUC*	*avrPto-LUC* reporter in pPTE6	Xiao et al., 2004
pHM2::*avrPto-LUC*	*avrPto-LUC* reporter in pHM2	This study
pPTE6::*hrpL--LUC*	hrpL-luc reporter in pPTE6	Xiao et al., 2004
pLT::*hrpL-LUC*	Derived from pPTE6::hrpL-luc by EZ-Tn5<TET-1> transposon insertion mutagenesis	This study
pPTE6::*hrpR-LUC*	hrpR-luc reporter in pPTE6	This study
pLT::*hrpR-LUC*	Derived from pPTE6::hrpR-luc by EZ-Tn5<TET-1> transposon insertion mutagenesis	This study
pML123	Broad host plasmid	Labes et al., 1990
pML122	Broad host plasmid	Labes et al., 1990
pML123::*rohS*$_{Psph}$(TTG)	*RohS*$_{Psph}$(TTG) in pML123 plasmid, under pNm promoter	This study
pML123::*rohS*$_{Psph}$(ATG)	*RohS*$_{Psph}$(ATG) in pML123 plasmid, under pNm promoter	This study
pML123::*rohS*$_{Psph}$(TTC)	*RohS*$_{Psph}$(TTC) in pML123 plasmid, under pNm promoter	This study
pML123::*rohS*$_{Psto}$	*RohS*$_{Psto}$ in pML123 plasmid, under pNm promoter	This study
PML123::*rohR/rohS*$_{Psph}$	*rohR/rohS*$_{Psph}$ in pML123 plasmid, under pNm promoter	This study
PML123::*rohR/rohS*$_{Psto}$	*rohR/rohS*$_{Psto}$ in pML123 plasmid, under pNm promoter	This study
PML123::*rohR*$_{Psph}$	*rohR*$_{Psph}$ in pML123 plasmid, under pNm promoter	This study
PML123::*rohS*$_{Psph}$ H147A	*rohS*$_{Psph}$ with H147A mutation in pML123 plasmid, under pNm promoter	This study
PML123::*rohR/rohS*$_{Psph}$H147A	*rohR/rohS*$_{Psph}$ with H147A mutation on *rohS* in pML123 plasmid, under pNm promoter	This study
pML122::*hrpR*	*hrpR* in pML122 plasmid, under pNm promoter	This study
pML122::*hrpS*	*hrpS* in pML122 plasmid, under pNm promoter	This study
pML122::*hrpL*	*hrpL* in pML122 plasmid, under pNm promoter	This study

Table III-2. Primers used in this study

Primer's name	5'- sequence -3'	Decription
Adaptor F	tccgaattcaagcttctcgagggtacctctaga	Modify pHM1
Adaptor R	tctctagaggtaccctcgagaagcttgaattcg	Modify pHM1
T7	taatacgactcactattggg	To sequence the inserts on pBluescript-SK(+) and pGEM-7Zf(+)
T3	attaaccctcactaaaggga	To sequence the inserts on pBluescript-SK(+)
SP6	tatttaggtgacactatag	To sequence the inserts on pGEM-7Zf(+)
LUC-FP	agaattcggatccgaagacgccaaaaacataaag	To amplify *luc* ORF
P-hrpR-F	ttgaattccccggttttactcgattg	To amplify the EcoRI/BamHI fragment of HrpR promoter
P-hrpR-R	ttggatcccatcgttcactctcatggt	To amplify the EcoRI/BamHI fragment of HrpR promoter
Kan2-SP1	gatagattgtcgcacctgattg	For *Psph* two-stage semidegenerate PCR
Kan2-SP2	aagacgtttcccgttgaatatg	For *Psph* two-stage semidegenerate PCR
Kan2-SP3	gcaatgtaacatcagagattttgag	For sequencing the PCR products of Tz flanking DNA
CEKG 2A	ggccacgcgtcgactagtacnnnnnnnnnnagag	for *Psph* two-stage semidegenerate PCR
CEKG 2B	ggccacgcgtcgactagtacnnnnnnnnnnacgcc	For *Psph* two-stage semidegenerate PCR
CEKG 2C	ggccacgcgtcgactagtacnnnnnnnnnngatat	For *Psph* s two-stage semidegenerate PCR
CEKG 4	ggccacgcgtcgactagtac	For *Psph* two-stage semidegenerate PCR
Kan2-RP	ctacctttgccatgtttcag	Regular PCR to confirm the results of the Tz-mapping
Pin19-3'in	gagaatgcgcaggacaatgg	*Psph rohR/S*$^-$ mutant confirmation
TN-Tet-L1	taccggcataaccaagcctatgcctacag	For *P.s.tomato* DC3000 ::Tz mutant library screening
TN-Tet-L2	aggatgacgatgagcgcattgttagatttc	For *P.s.tomato* DC3000 ::Tz mutant library screening
TN-Tet-R1	cacatggaacgggttggcatggattgtag	For *P.s.tomato* DC3000 ::Tz mutant library screening
TN-Tet-R2	actccaagaattggagccaatcaattcttg	For *P.s.tomato* DC3000 ::Tz mutant library screening
hrpS-G2	aggttaatcctgcaaatgcgaccatgatt	Mutant screening DC3000 lib. for *hrpS*$^-$
hrpS-G1	gcacgtccatggtctgcttcttcattgtt	Mutant screening DC3000 lib. for *hrpS*$^-$
hrpL-G2	aactggatatacgcatggtgagttgccat	Mutant screening DC3000 lib. for *hrpL*$^-$
hrpL-G1	ggctggacctgattattcacattggcatt	Mutant screening DC3000 lib. for *hrpL*$^-$
DC-*rohS*R1	gcatcggtacgatagtcacacacgtctaa	Mutant screening DC3000 lib. for *rohR/S*$^-$
DC-*rohS*R2	atggacaatcgctttcgtgagcacgctca	Mutant screening DC3000 lib. for *rohR/S*$^-$
rohR/S-FP	ttgaattccagggagctgggttga	To amplify EcoRI/XbaI fragment of *rohS*ORF or *rohR/S* ORF
rohR/S-RP	tttctagagtcaaaggcgcggcag	To amplify EcoRI/XbaI fragment of *rohS*ORF
rohR-FP	ttaattcacggcggcacacgcag	To amplify EcoRI/XbaI fragment of *rohR* ORF
rohR-RP	tttctaga tcaacccagctccctg	To amplify EcoRI/XbaI fragment of *rohS*ORF or *rohR/S* ORF
Psph-hrpRS-FP	atgctctagacgggtgttgctc	To amplify the XbaI/HindIII hrpR ORF or XbaI/BamHI *hrpR/S* ORF
Psph-hrpR-RP	gagacataagctttttggactcc	To amplify the XbaI/HindIII fragment of *hrpR* ORF
Psph-hrpRS-RP	aaagtggatccttggtacagataggtgg	To amplify the XbaI/BamHI fragment of *hrpR/S* ORF
PhrpR-FP	gaattcgttttaaagccggatgtatag	To amplify the EcoRI/BamHI fragment of *hrpR* promoter
PhrpR-RP	ttggatccgtccatatccagaaacgc	To amplify the EcoRI/BamHI fragment of *hrpR* promoter
Psph-hrpL-FP	atcgctctagagcttgcacacc	To amplify the XbaI/HindIII fragment of *hrpL* ORF
Psph-hrpL-RP	aatcaagcttccagacagatatccactcagg	To amplify the XbaI/HindIII fragment of *hrpL* ORF
PSPH-R2	gaccgttccgtggcaaagcaaaagttcaa	For *P.s.phaseolicola* ::Tz mutant library screening
PSPH-R1	ttgacgggacggcggctttgttgaataaat	For *P.s.phaseolicola* ::Tz mutant library screening
PSPH-L2	gaatatggctcataacacccttgtattac	For *P.s.phaseolicola* ::Tz mutant library screening
PSPH-L1	gggcttcccatacaatcgatagattgtc	For *P.s.phaseolicola* ::Tz mutant library screening
PSPH2223-G1	agcatgctgatcatgtcgttgag	For *P.s.phaseolicola* ::Tz mutant library screening
rohR/S-TTC-FP:	ggagctgggttcatccgccggttcgac	Mutagenesis, to replace start codon of *rohS* with TTC
rohR/S-TTC-RP:	gtcgaaccggcggatgaacccagctcc	Mutagenesis, to replace start codon of *rohS* with TTC
rohR/S-H147A-FP	ctggctgcggtatccgctgacctgcgcacg	Mutagenesis, introduce H147A to *rohR/S*
rohR/S-H147A-RP	cgtgcgcaggtcagcggataccgcagccag	Mutagenesis, introduce H147A to *rohR/S*

Table III-3. Fold of *avrPto-luc* induction in minimal medium and plants in 6 hours (6 hr/0 hr)

Bacterial strain	In minimal medium	In *att1* plants	In bean plants
WT NPS3121	171.2 ± 15.7	76.7 ±10.0	310.0 ±63.4
$hrpR_{Pph}^{-}$ mutant	9.3 ± 0.6	4.6 ±0.9	32.8 ±8.4
pin19	11.00 ± 1.2	8.5 ±1.1	53.0 ±14.9
pin19 + pML123::$rohS\ (ATG)_{pph}$	167.1 ± 24.8	74.6 ±21.3	318.0 ±84.8
pin19 + pML123::$rohS\ (TTC)_{pph}$	12.5± N.D.	N.D.	N.D.
pin19 + pML123::$rohS\ (TTG)_{pph}$	10.3 ± 1.6	5.2 ±2.2	N.D.
pin19 + pML123::$rohS_{psto}$	174.6 ±1 6.8	23.5 ±11.6	148.0 ±62.3
Pin19+ pML123::$rohS/rohR_{pph}$	36.7±16.4	15.5±8.6	92.2±20.5
Pin19+pML123::$rohS/rohR_{psto}$	48.2±18.9	18.6±7.9	55.6±13.5
Pin19+pML123::$rohR/rohS_{pph}$H147A	36.7±11.6	10.7±6.2	14.4±7.5
Pin19+ pML123::*rohRpsph*	6.8± N.D.	6.7± N.D.	6.6± N.D.
Pin19+pML123::$rohS_{pph}$H147A	8.6± N.D.	N.D.	N.D.
WT NPS3121 +pML123::$rohR/rohS_{pph}$H147A	26.98±15.1	8.04±4.8	67.7±19.4
WT *P. s. ph* NPS3121 +pML123::$rohS_{pph}$	171.0±25.2	78.9±15.6	296.2±78.5
WT *P. s. ph* NPS3121 +pML123::$rohR/rohS_{pph}$	47.4±18.3	12.57±9.2	86.49±30.1
WT *P. s. ph* NPS3121 + pML123::$rohR_{pph}$	14.1± N.D.	4.0± N.D.	16.8± N.D.

* *Pin9* showed the similar results

** ± N.D.: Experiment not repeated

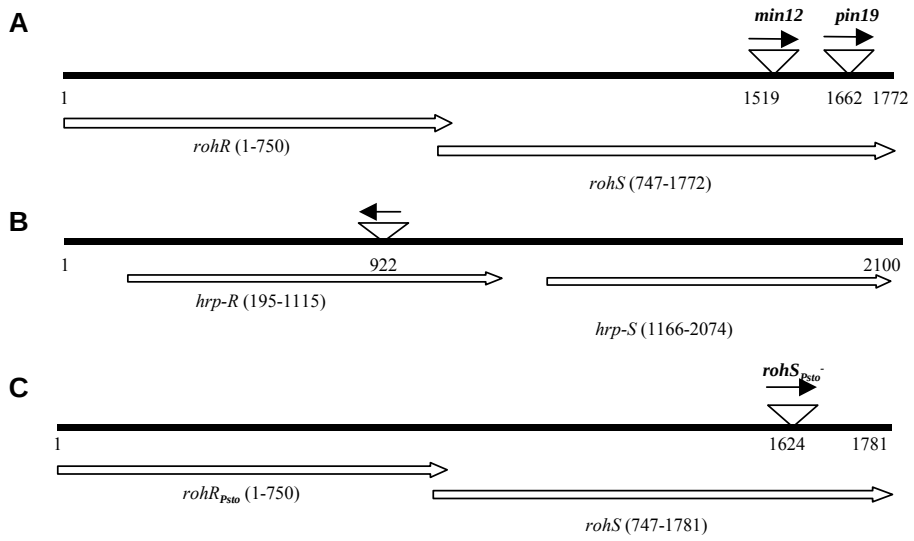


Figure III-1. Gene organization and transposon insertion sites in *RohS*$_{Psph}^{-}$, *hrpR*$_{Psph}^{-}$, and *RohS*$_{Psto}^{-}$ mutants.

A. Transposon insertions in the *P. syringae* pv. *phaseolicola* NSP3121 *rohR/rohS* locus. The beginning of *rohR* coding region (1) and the end of *rohS* coding region (1772) are designated for the NSP3121 strain. Transposons are inserted next to nucleotides 1519 and 1662 in *min12* and *pin19* mutants, respectively. Open arrows denote the two open reading frames in this locus. Arrows above the triangles indicate the orientation of transposons.

B. Transposon insertion in the NSP3121 *hrpR/hrpS* locus. The beginning (1) and end (2100) of published *hrpR/hrpS* locus sequence (accession number, X77638; Grimm et al., 1995) are indicated. The transposon is inserted next to the nucleotide 922 in the *hrpR* gene.

C. Transposon insertion in the DC3000 *rohR/rohS* locus. The beginning of *rohR* (*PSPTO2223*) coding region (1) and the end of *rohS* (*PSPTO2222*) coding region (1781) are designated for the *P. syringae* pv. *tomato* DC3000 strain (Buell et al., 2003). Transposon is inserted next to nucleotide 1624 in the *rohS* gene.

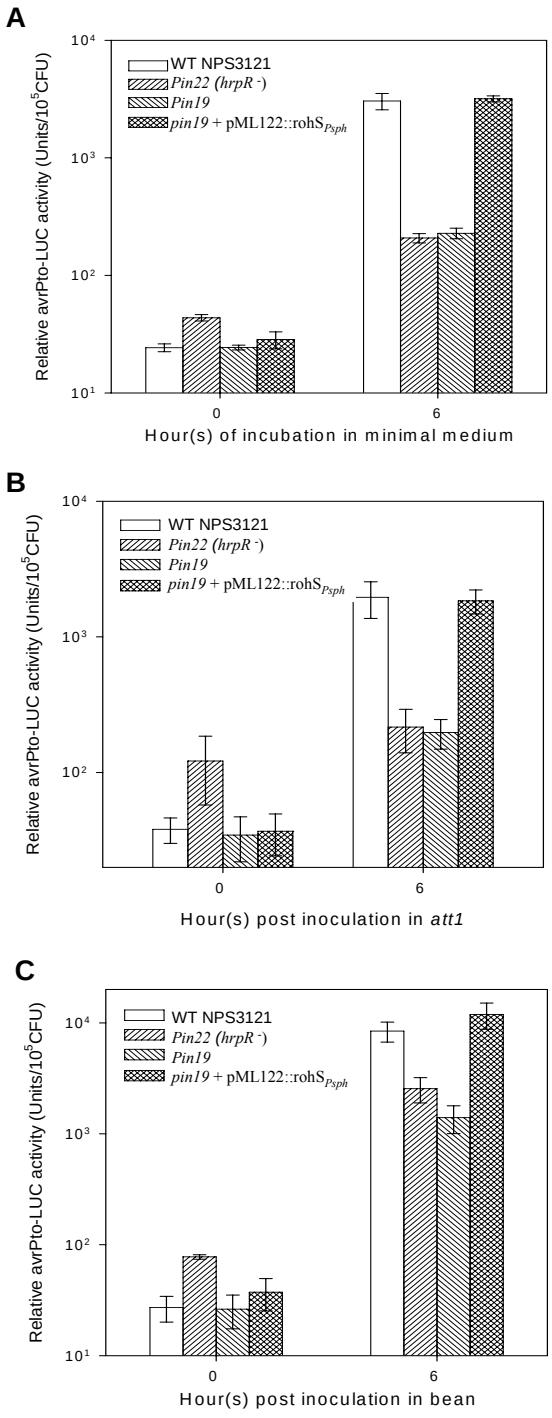


Figure III-2. Mutation of *rohS* reduces the *avrPto-LUC* induction in minimal medium and *in planta*.

Overnight cultures of the wild type (WT) NSP3121, *pin19*, *hrpR*$^-$, and the *rohS*$_{Psph}$*(ATG)*-complemented *pin19* strains carrying pHM2::*avrPto-LUC* reporter plasmid in KB medium were collected by centrifugation and washed twice with sterile water. The bacteria were diluted in minimal medium to OD_{600}=0.02 for induction assay of *avrPto-LUC* by minimal medium (A). The bacteria were diluted in 10mM $MgCl_2$ to OD_{600}=0.5 and injected into the leaves of 5-6 week old *att1* plant (B) or primary leaves of 10 day old bean (C) for measurement of *avrPto-LUC* induction *in planta*. The LUC activity was measured at 0 and 6 hrs after induction using a cooled CCD and normalized by the bacterial number. Error bars indicated standard error. The experiments were repeated numerous times with similar results.

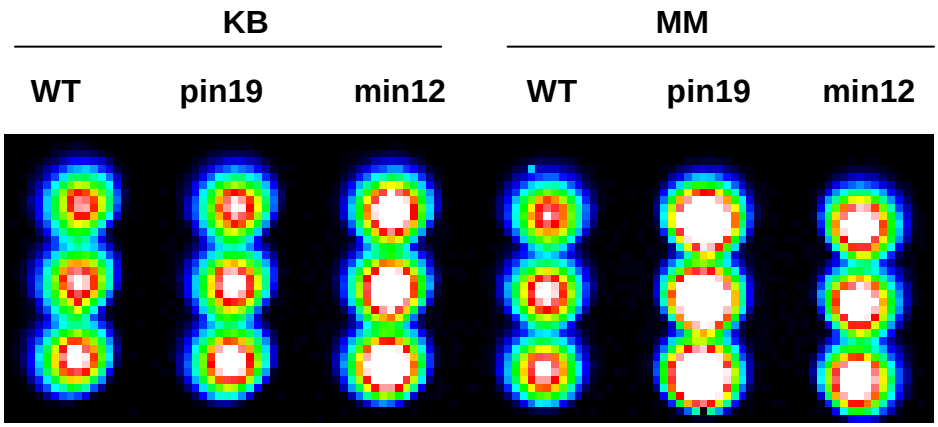


Figure III-3. *rohS* mutation did not affect the expression of the promoter activity of constitutive gene *pro2*

LUC reporter driven by the promoter of constitutive gene *pro2* was introduced to the wild type NSP3121 strain and *rohS*⁻ mutants (*Pin19* and *Min12*) cured of *avrPto-LUC* reporter. The resulting strains were cultured in KB media supplemented with appropriate antibiotics overnight at room temperature. Bacterial cells were collected by centrifugation and washed twice with sterile water and diluted in MM to OD_{600}=0.02 for *LUC* activity assay. The images were captured at 0 hr (indicating the *pro2-LUC* activity in KB medium) and after being cultured in MM at room temperature for 6 hrs (indicating the *pro2-LUC* activity in MM) using the cooled CCD.

Each column represents a strain, and each cell in a column represents a replication

(Contributed by Dr. Xiaoyan Tang)

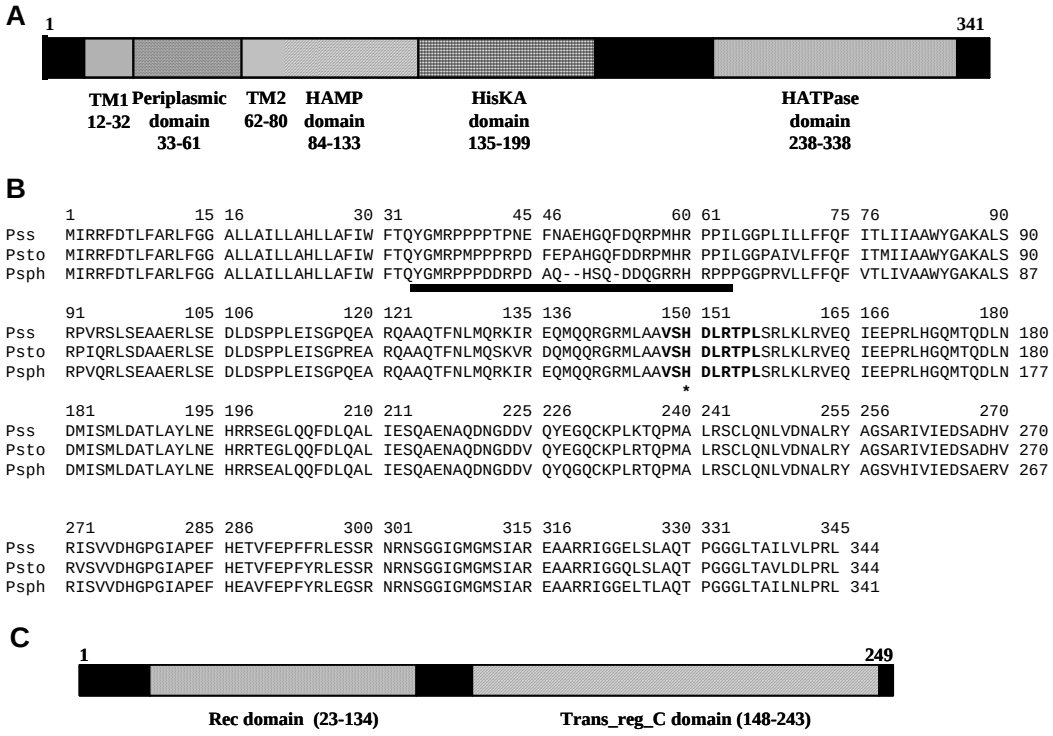


Figure III-4. Structural characteristics of the RohS and RohR proteins.

A. Predicted domains of *RohS*$_{Psph}$. The *RohS*$_{Psph}$ protein is predicted to have 341 amino acids. A periplasmic domain (aa 33-62) is flanked by two transmembrane domains (aa 12-32 and aa 63-83). Other conserved domains are HAMP domain (aa 84-133), HisKA domain (aa 135-199), and HATPase domain (aa 238-338).

B. Alignment of the orthologs of *RohS* from *P. syringae* pv. *tomato* DC3000 (Psto), *P. syringae* pv. *phaseolicola* 1448A (Psph, identical to the NPS3121 *RohS*), and *P. syringae* pv. *syringae* B728a (Pss). This alignment was performed using ClustalW 1.8. The periplasmic domain is indicted by the bar. The H147 residue denoted by * is predicted to be the autophosphorylation site of *RohS*$_{Psph}$.

C. Structural domains of *RohR*$_{Psph}$. Indicated are the conserved signal receiver domain (Rec domain) and winged-helix DNA-binding domain (Trans_reg_C domain).

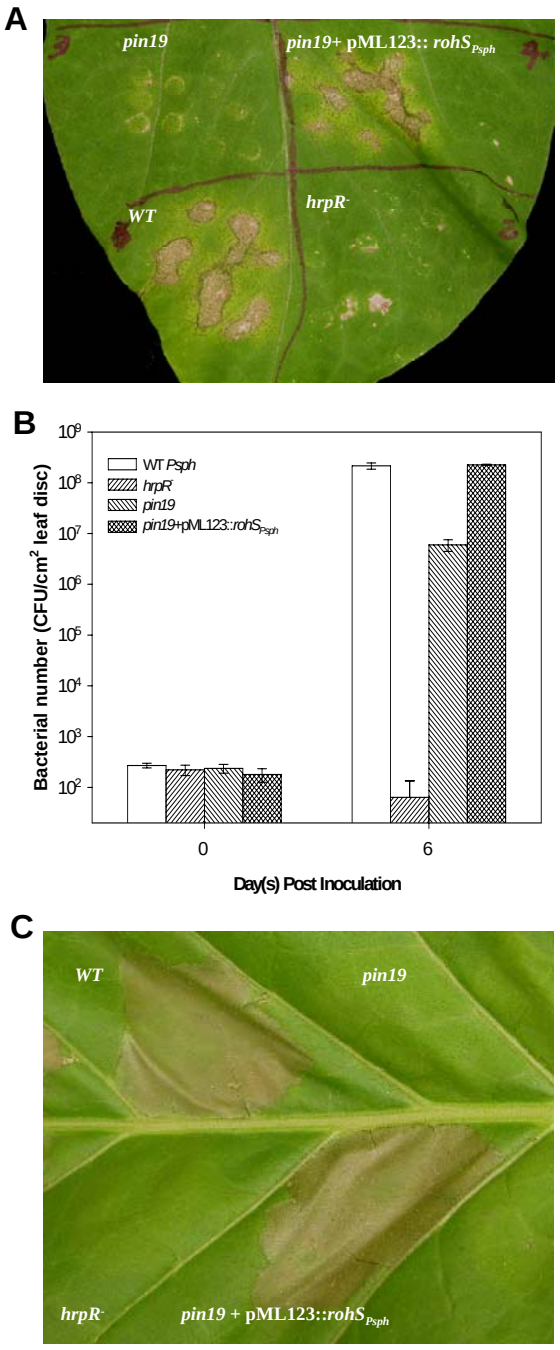


Figure III-5. Mutation of *rohS*$_{Psph}$ compromised bacterial pathogenicity and elicitation of HR.

A. Disease symptoms on Red Kidney bean plant. Primary leaves were inoculated with 10^5 CFU/ml bacteria and photographed 9 days after inoculation.

B. Bacterial growth on Red Kidney bean plants. Primary leaves were inoculated with 10^4 CFU/ml bacteria. Bacterial numbers were measured at 0 and 6 days after inoculation. The numbers represent average of bacteria in four leaf discs. Error bars indicate standard error.

C. Non-host HR on tobacco W38 plants. W38 plants were inoculated with 10^8 CFU/ml bacteria. The non-host HR was photographed at 16 hrs after inoculation. The experiments were repeated at least three times with similar results.

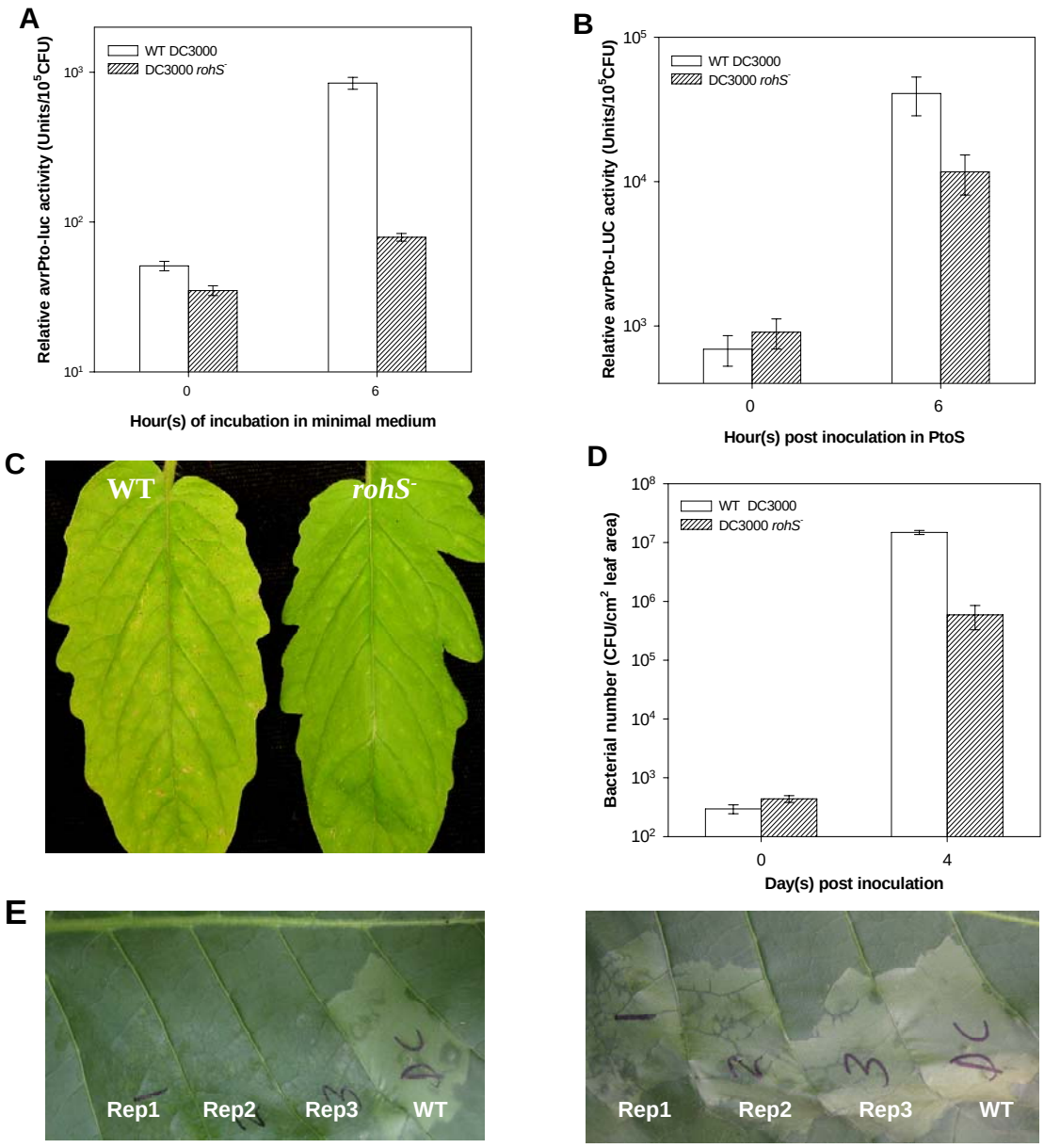


Figure III-6. Mutation of *rohS*$_{Psto}$ in *P. syringae* pv. *tomato* DC3000 compromised *avrPto-LUC* induction, bacterial pathogenicity and elicitation of HR.

A. Induction of the *avrPto-LUC* gene in minimal medium.

B. Induction of the *avrPto-LUC* gene in tomato PtoS plants.

C. Disease symptom on tomato PtoS plants inoculated with 2×10^4 CFU/ml bacteria. The inoculated leaves were photographed 6 days after inoculation.

D. Bacterial growth on PtoS plants inoculated with 2×10^4 CFU/ml bacteria. Bacterial numbers were measured at 0 and 4 days after inoculation.

E. Non-host HR on tobacco W38 plants. Rep1, Rep2 and Rep3 are three independent colonies of the *rohS*$_{Psto}^-$ mutant. WT is the wild type DC3000. The non-host HR was photographed at 10 (left) and 16 (right) hrs after inoculation with 1×10^8 CFU/ml bacteria. The wild type DC3000 and *rohS*$_{Psto}^-$ mutant carrying the pPTE6::*avrPto-LUC* plasmid were used for the experiments. The growth of bacteria in KB medium, preparation of inocula, reporter gene assay, bacterial inoculation and growth assays were as described in Experimental Procedures (Contributed by Doug Baker).

Each experiment was repeated at least twice with similar results. Error bars indicated standard error.

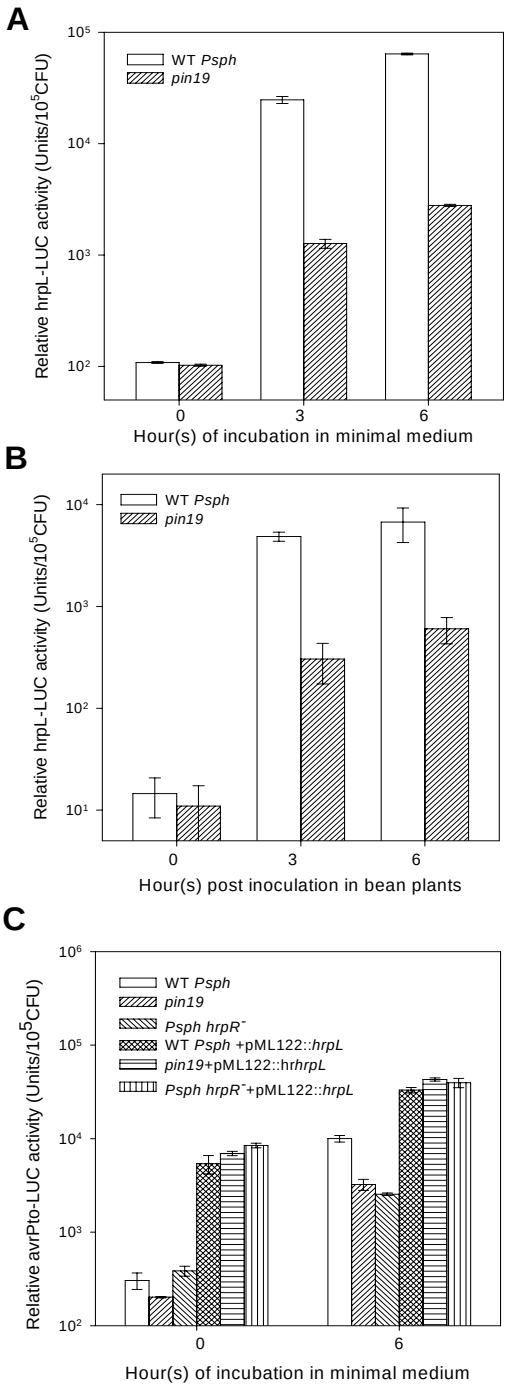


Figure III-7. Epistatic analysis between *rohS* and *hrpL*.

The wild type *P. syringae* pv. *phaseolicola* NPS3121 and $rohS_{Psph}^{-}$ mutant carrying pLT:: *hrpL-luc* was grown in KB medium carrying 10 mg/L tetracycline and induced in minimal medium (A) and in bean plants (B). The LUC activities were measured at 0 hr and 6 hrs after induction. pML122::*hrpL* was introduced into the wild type, $hrpR^{-}$ and $rohS^{-}$ strains of *P. syringae* pv. *phaseolicola* NPS3121 to test the effect of HrpL overexpression on transcription of *avrPto-LUC*(C). The LUC activity was measured before induction (0 hr) and 6 hrs after induction in minimal medium. Error bars indicated standard error.

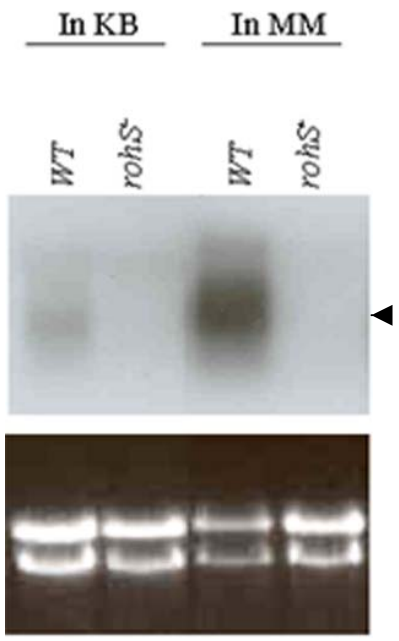


Figure III-8. *rohS* mutation reduces the *hrpR* RNA expression.

The wild type DC3000 and $rohS_{Psto}^{-}$ mutant were grown in KB liquid medium containing 50 mg/L rifampicin and induced in minimal medium for 6 hrs. Ten microgram of total RNA per sample was used for RNA blot analysis. The blot was probed with radiolabeled *rohS* probes. The two weak bands presented in all samples on the blot (top) are from rRNA. Arrows indicate the *rohS* signal from the wild type DC3000 strain and the $rohS_{Psto}^{-}$ mutant. The signal displayed by the $rohS_{Psto}^{-}$ mutant is likely from the truncated gene caused by transposon insertion. The bottom RNA gel picture indicates equal loading of RNA in lanes.

(Contributed by Dr. Lefu Lan)

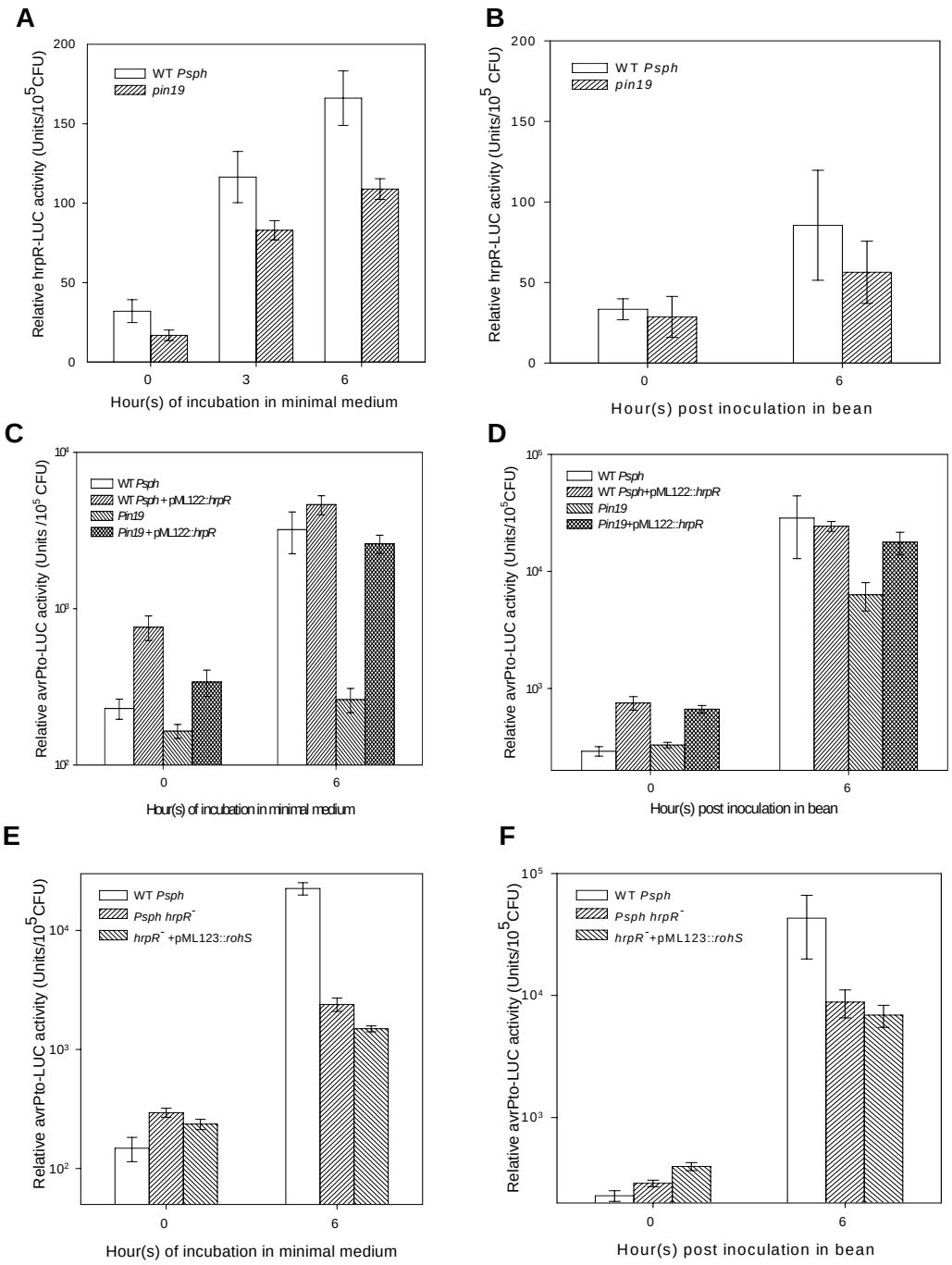


Figure III-9. Epistasis analysis between *rohS* and *hrpR*.

To determine how *rohS* mutation affects the hrpR promoter activity, pLT::*hrpR-luc* was introduced into the wild type (WT) *P. syringae* pv. *phaseolicola* NPS3121 and $rohS_{Psph}^{-}$

mutant, and the LUC activity was measured in minimal medium (A) and in bean plants (B) 0 and 6 hrs after induction. To study if *hrpR* overexpression could complement the *avrPto-LUC* expression in $rohS_{Psph}^{-}$ mutant, pML122::*hrpR* was introduced to the wild type NPS3121 and $rohS_{Psph}^{-}$ mutant strains carrying pHM2::*avrPto-LUC*. The LUC activity was measured in minimal medium (C) and bean plants (D) 0 and 6 hrs after induction. To test if overexpression of *RohS* could complement the *avrPto-LUC* expression in $hrpR^{-}$ mutant, pML123::$RohS_{Psph}$*(ATG)* was introduced into the wild type NPS3121 and $hrpR^{-}$ mutant strains carrying pHM2::*avrPto-LUC*. The LUC activity was measured in minimal medium (E) and bean plants (F) 0 and 6 hrs after induction.

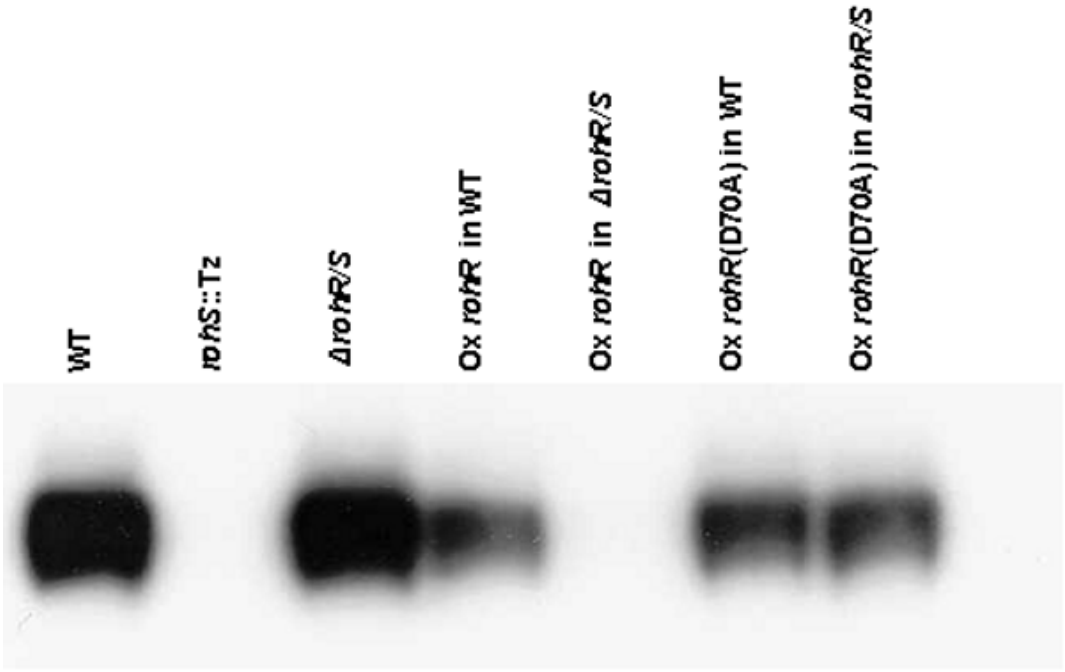


Figure III-10. Northern blot analysis of *avrPto* transcripts

Bacteria were grown in KB liquid medium containing 50 mg/L rifampcin and induced in minimal medium for 6 hrs before RNA extraction. Equal amount of total RNA per sample was analyzed by RNA blotting with radiolabeled *avrPto* probes.

Abbreviation: WT = Wild type strain; *rohS*::*Tz* = *rohS* Transposon insertion mutant; OX = Overexpression; Δ*rohR/S* = *rohR/S* deletion mutant; *rohR*(D70A) = *rohR* mutant gene with aspartate 70 substituted with alanine

(Contributed by Dr. Chutao Yin)

CHAPTER IV

Fructose Acts as Carbon Nutrient for Induction of *Hrp* Box Promoter in *Pseudomonas syringae*

SUMMARY

Type III secretion system (TTSS) is a specialized bacterial protein secretion system that delivers effector proteins into the host cells. Many plant bacterial pathogens rely on the TTSS for pathogenicity on host plants and induction of hypersensitive response (HR) on non-host plants. *Pseudomonas syringae* genes encoding TTSS and effectors have a conserved "*hrp* box" motif in the promoter that can be induced *in planta* and in minimal medium (MM), and the induction of the "*hrp* box" promoter in MM is modulated by fructose and other sugars. However, it is unclear if sugars serve as extracellular signals or as essential nutrients. We have isolated four transposon insertion mutants of *P. s.* pv. *phaseolicola* defective in "*hrp* box" promoter induction in MM containing fructose. These mutants carry transposons in an operon for fructose uptake. Replacement of fructose with several catabolic carbons in MM enabled normal "*hrp* box" promoter induction in these mutants, suggesting that fructose acts as a nutrient for the activation of type III genes. The fructose-uptake mutants displayed a slightly lower induction of the "*hrp* box" promoter in *Arabidopsis*, normal induction of HR on non-host plant tobacco, and reduced pathogenicity on the host bean plants. The reduced pathogenicity suggested that exploitation of fructose from host tissue is an important means for pathogenesis of *P. s. phaseolicola*.

INTRODUCTION

Many Gram-negative bacterial pathogens have a specialized protein secretion system, termed TTSS that delivers effector proteins into the host cells (Galan and Collmer, 1999). TTSS of plant bacterial pathogens is encoded by hypersensitive response

(HR) and pathogenicity (*hrp*) genes because mutation of the *hrp* genes eliminates HR on nonhost plants and pathogenicity on host plants (Lindgren *et al.,* 1986). The function of *hrp* genes in HR and pathogenesis is realized by effector proteins that are secreted by the TTSS (Buttner and Bonas, 2002). Certain effector proteins can be recognized by the disease resistance (R) proteins in the plant cell, and this recognition elicits HR and disease resistance (Lahaye and Bonas, 2001). A large number of effectors have been shown to confer a virulence function in plants lacking the *R* gene (Innes, 2003). It is generally believed that type III effectors of a bacterial pathogen act collectively to determine the pathogenicity (Alfano and Collmer, 2004).

Genes encoding TTSS and effectors (both of which are referred to as type III genes hereafter) are induced during the bacterial encounter with plant cells (Arlat *et al.,* 1992; Rahme *et al.,* 1992; Schulte and Bonas, 1992; Wei *et al.,* 1992). The host signal for type III gene activation is not known. But certain media that are believed to mimic the host environments also induce the type III genes (Arlat *et al.,* 1992; Rahme *et al.,* 1992; Wei *et al.,* 1992; Wengelnik *et al.,* 1996). Recent studies in *Ralstonia solanacearum* suggested distinct signals responsible for type III gene induction in plant and in media (Marenda *et al.,* 1998; Brito *et al.,* 1999, 2002; Aldon *et al.,* 2000). The plant signal is likely a non-diffusible wall component that is perceived by PrhA, a membrane protein with significant similarities to siderophore receptors (Marenda *et al.,* 1998; Aldon *et al.,* 2000). Mutation of PrhA distinguishes the induction of *R. solanacearum* type III genes in plant and medium.

In *P. syringae* bacteria, the activation of type III genes in plant and MM is mediated by a conserved *cis* element in the promoter, termed "*hrp* box" (Xiao *et al.,*

1994). The "*hrp* box" motif is recognized by HrpL, a transcriptional activator belonging to the ECF family alternate sigma factors (Xiao *et al.,* 1994). The expression of *hrpL* is controlled by HrpR and HrpS, two highly homologous proteins that are similar to the NtrC family transcription regulators (Xiao *et al.,* 1994). HrpS and HrpR form a heterodimmer that binds the *hrpL* promoter to activate *hrpL* transcription (Hutcheson *et al.,* 2001). Recently the Lon proteinase was found to be a negative regulator of type III genes in *P. s.* pv. *tomato* (Bretz *et al.,* 2002). The Lon proteinase appears to regulate the type III gene expression by destabilizing the HrpR protein.

In MM, the expression of *P. syringae* type III genes is modulated by different catabolites, pH, and osmolarity (Huynh *et al.,* 1989; Rahme *et al.,* 1992; Xiao *et al.,* 1992). These conditions were thought to mimic the environment of plant intercellular spaces where bacteria proliferate. Among the various carbon sources tested, fructose and sucrose are the best inducers of type III genes in MM (Huynh *et al.,* 1989). Consistent with this observation, sucrose is the most abundant apoplast sugar and can be converted to fructose and glucose. However, whether these sugars act as extracellular signal or essential carbon sources for the induction of type III genes is not known. Sugars are known chemotaxis signals to many bacteria (Postma *et al.,* 1993). In Agrobacterium, sugars serve as extracellular signal and act synergistically with the phenolic compounds to activate the expression of *vir* genes (Ankenbauer and Nester, 1990; Cangelosi *et al.,* 1990; Shimoda *et al.,* 1990). This action is mediated by ChvE, a periplasmic sugar binding protein, in cooperation with VirA, the two component sensor protein for phenolics, that signals the activation of *vir* genes (Cangelosi *et al.,* 1990; Huang *et al.,* 1990; Shimoda *et al.,* 1993).

In an effort to identify *P. syringae* genes required for type III gene induction, we have isolated four *P. s.* pv. *phaseolicola* mutants that displayed no induction of a "*hrp* box" promoter in the MM supplemented with fructose. The four mutants carry transposon inserted in a locus encoding phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) specific for fructose. PTS catalyzes the transfer of the phosphate group from PEP to carbohydrate during the translocation of carbohydrate across the plasma membrane. This system is also known for the role in chemotaxis signaling and regulation of a number of other metabolic pathways (Postma *et al.,* 1993). The mutants were used to test whether fructose serves as an extracellular signal or as carbon nutrient for the induction of *Pseudomonas* type III genes.

RESULTS

Construction of the *avrPto-luc* reporter gene

Most of *Pseudomonas syringae* type III genes carry a "*hrp* box" promoter responsible for induction of the gene in MM and in plants (Fouts *et al.,* 2002; Zwiesler-Vollick *et al.,* 2002). The *avrPto* gene of *P. s.* pv. *tomato* contains a typical "*hrp* box" in the promoter (Salmeron *et al.,*1993). To non-intrusively monitor the activation of a "*hrp* box" promoter, we constructed an *avrPto-luc* reporter gene by placing the firefly luciferase (*luc*) gene under the control of the *avrPto* gene promoter and cloned this reporter gene into the broad host vector pHM2. The reporter construct was transformed into *P. s.* pv. *phaseolicola* NPS3121 strain, and the activation of the reporter gene was tested in MM and in plants. The bacterium displayed no luciferase activity in the rich KB medium but strong induction of luciferase activity after culture in MM (Fig. IV-1A) and being infiltrated into *Arabidopsis* plants (Fig. IV-1B). The *avrPto-luc* gene was induced to a

high level 6 hrs after culture in MM, but at this time point less than two fold of bacterial multiplication was detected. Therefore, this time point was used for measurement of *avrPto-luc* induction in the rest of experiments unless otherwise specified.

Fructose stimulates *avrPto-luc* induction in MM

Fructose can stimulate the *P. syringae* type III gene expression in MM (Huynh *et al.,* 1989), but whether fructose serves as an extracellular signal or as an essential carbon nutrient for the induction of type III genes remains unknown. To better understand the role of fructose in type III gene induction, a study of the dosage-dependent induction of *avrPto-luc* in MM by fructose was performed (Fig. IV-2). *avrPto-luc* showed little induction in MM without fructose or with a very low concentration of fructose. However, ~60 fold induction was detected when fructose was added to 100μM-10mM, and an increase of fructose dosage up to 100mM further stimulated the *avrPto-Luc* induction. Fructose at 500mM almost completely suppressed the activity of *avrPto* promoter.

Fructose-uptake mutants showed no *avrPto-luc* induction in MM

In an effort to identify *P. syringae* genes required for type III gene activation in MM, we screened a *P. s.* pv. *phaseolicola* NPS3121 mutant library (created by random EZ::TN^{TM}<KAN-2> transposon insertion) for mutants that were unable to activate *avrPto-luc* in MM. Mapping of the transposon-insertion site revealed four mutants, termed MM-insensitive (*min*) carrying transposons inserted into an operon encoding the PEP phosphotransferase system specific for fructose (Fig. IV-3). Transposons in *min65* and *min98* mutants were inserted into the first ORF of the operon encoding PEP-protein phosphotransferase containing EI/HPr/EIIA components (ORF1). *min18* and *min41*

carried transposons in the third ORF encoding fructose-specific IIBC components (ORF3). *ORF1* and *ORF3* are separated by *ORF2* encoding 1-phosphofructokinase. All the mutants displayed a constitutive low level of the luciferase activity during growth in MM (Fig. IV-4A) and insensitivity to the changes of fructose concentration in MM (Fig. IV-4B). None of the mutations affected bacterial growth in the rich KB medium (data not shown).

Fructose-uptake mutants displayed *avrPto-luc* induction in response to other carbons

The isolation of the fructose uptake mutants allowed us to test if fructose acts as a signal or an essential carbon nutrient for the induction of the type III gene. If fructose serves as a carbon nutrient, the *avrPto-luc* induction in the fructose uptake mutants is expected to be restored by other catabolic carbons but not by non-catabolic carbons. The fructose uptake mutants were cultured in MM with the fructose replaced by 1mM glycerol, sucrose, glucose, glutamate, citrate, and γ-aminobutyric acid (GABA), respectively, and the luciferase activity was measured 6 hrs after induction. All the mutants exhibited induction of *avrPto-luc*, although different carbons induced the *avrPto-luc* gene to different levels (Fig. IV-5, A and B). Glycerol, glutamate, and citrate caused 60-100 fold induction, whereas GABA induced the reporter gene only to 6-8 fold. With sucrose, approximately 3-fold higher induction of *avrPto-luc* was detected in the wild type strain than in the fructose uptake mutant strains, implying hydrolysis of sucrose to fructose and glucose contributes at least partly to the sucrose induction. To our surprise, replacement of fructose with mannitol did not support any induction of *avrPto-luc* in both the wild type and the mutant strains (Fig. IV-5A). To determine if mannitol can be used

by NPS3121 strain as a carbon source, we measured the bacterial growth in minimal medium supplemented with 10 mM mannitol. Twenty four hours after culture, the bacterial numbers remained the same in minimal medium with or without mannitol but increased ~8 fold in minimal medium with 10 mM fructose, suggesting that mannitol can not be used as a carbon source for the NPS3121 strain. These results indicated that deprivation of carbon nutrition is the cause of *avrPto-Luc* repression in the fructose uptake mutants.

The inability of mannitol to induce *avrPto-luc* in NPS3121 strain was different from what was reported using *P. s.* pv. *glycinea* race 0 strain (Huynh *et al.,* 1989). In this strain, MM containing mannitol induced the *avrB* gene almost as well as MM containing fructose. The difference between the two strains may result from different ability to use mannitol as a carbon source or different requirement of a carbon source for the induction of type III genes in MM. We therefore tested four different *P. syringae* strains for the induction of *avrPto-luc* gene in MM with 10 mM mannitol or without any carbon source (Fig. IV-6). Interestingly, these bacterial strains displayed dramatic difference on *avrPto-luc* induction in response to the changes of carbons in MM. *P. s.* pv. *tomato* DC3000 and *P. s*. pv. *maculicola* ES4326 both showed strong *avrPto-luc* induction 6 hrs after culture in MM without any carbons, whereas *P. s.* pv. *phaseolicola* NPS3121 and *P. s.* pv. *tabaci* 11528 strains showed no induction in the absence of carbon. By including mannitol in the MM, a stronger and more extended induction of the reporter gene was detected in *P. s.* pv. *tomato* DC3000, *P. s*. pv. *maculicola* ES4326, and *P. s.* pv. *tabaci* 11528 as well. Different from the *P. s.* pv. *phaseolicola* NPS3121 strain, *P. s.* pv. *tomato* DC3000, *P. s*. pv. *maculicola* ES4326, *P. s.* pv. *tabaci* 11528 strains all multiplied in MM with mannitol,

and the multiplication rate of DC3000 and ES4326 was 8-10 fold higher than that of the 11528 strain. But none of the bacteria multiplied in MM without any carbon. These results demonstrated the different metabolic activities among various *P. syringae* strains towards certain carbons. The data also supported the role of fructose as nutrient instead of signal for the induction of type III genes in *P. syringae* strains in MM.

Fructose uptake mutants displayed a slightly lower expression of *avrPto-luc* in *Arabidopsis* plants

To test how the fructose uptake mutations affect the *avrPto-luc* expression in plant, the mutant bacteria were inoculated into the *Arabidopsis att1* mutant plant. *att1* mutant carries a dysfunctional cytochrome P450 monooxygenase gene that inhibits the production of a negative signal for the induction of *P. syringae* type III genes (Xiao *et al.,* 2004). As a result, *att1* mutant plants super-induces the *avrPto-luc* gene and provides a sensitive assay system for the activation of type III genes in plant. Compared with the wild type strain, all the fructose uptake mutants showed weaker induction of *avrPto-luc* in *att1* plants, and the reduction of the luciferase activity of the mutant strains was statistically significant (Fig. IV-7). The result indicated that fructose availability affects partially the type III gene induction in plant.

Fructose uptake mutations did not affect the non-host HR on tobacco plants

TTSS is required by many bacterial pathogens to induce an HR on non-host plants. *P. s. phaseolicola* NSP3121 strain can induce a non-host HR on tobacco plants, although the responsible *avr* and *R* genes have not been defined. To determine how the fructose uptake mutations affect the HR-inducing activity, the mutant bacteria were infiltrated into

the tobacco W38 plants. All the mutant bacteria exhibited the same HR-inducing activity, as did the wild type strain (Fig IV-8A). This result indicated that the reduced *in planta* type III gene induction in the fructose-uptake mutants did not impact significantly to the HR induction.

Fructose uptake mutants compromised the bacterial pathogenicity on host plants.

To determine whether defect on fructose-uptake affects the bacterial pathogenicity in host plants, we inoculated the wild type and the mutant strains at 10^5cfu/mL into the Red Kidney bean plants and monitored the growth and symptom development. Compared with the wild type NSP3121 strain, all the mutant bacteria showed ~5-8 fold lower bacterial growth 4 days after inoculation into the bean plants (Fig. IV-8B). The mutant strains also caused less severe disease symptoms than did the wild type strain on bean leaves (Fig. IV-8C). Six days after inoculation, leaves inoculated with the wild type strain turned almost completely yellow while those inoculated with the mutant strains remained green.

DISCUSSION

In an effort to identify *P. syringae* genes responsible for the induction of type III genes in MM, we isolated four mutants of the *P. s.* pv. *phaseolicola* NPS3121 strain carrying transposon in the fructose-uptake genes. Using these mutants, we investigated whether fructose, a major modulator of type III genes in MM, acts as an extracellular signal or as an essential carbon nutrient. We found that replacement of fructose with various catabolic carbons induced the type III gene *avrPto* in the mutants, indicating the role of fructose as an essential carbon nutrient rather than an extracellular signal in type

III gene induction. We also found that some *P. syringae* strains can induce type III genes in the absence of a carbon source in minimal medium. This result also supports that fructose, and probably other carbons as well, are not the inducing signal for *P. syringae* type III genes.

Our result showed that, in *P. s.* pv. *phaseolicola* NPS3121 strain, uptake of fructose is mediated by the fructose-specific PTS, because mutation of the PTS genes renders the bacterium completely null to the presence of fructose in MM. PTS is well known for its function in carbohydrate translocation and sugar chemotaxis. Most bacterial PTSs have the following catalytic components, including Enzyme I (EI), Histidine protein (Hpr), Enzyme IIA (EIIA), Enzyme IIB (EIIB), and Enzyme IIC (EIIC), that catalyze the sequential transfer of phosphate group from PEP to EI, Hpr, EIIA, EIIB, and finally carbohydrate (Postma *et al.,* 1993). These catalytic components exist either as individual peptide or as modules of a fusion protein. The carbohydrate specificity is determined by the EII components (Postma *et al.,* 1993). Sequence analysis of the fructose-specific PTS genes in *P. s.* pv. *phaseolicola* 1448A strain indicated that EI, HPr, and EIIA domains are fused in one protein (PSPPH0847), while EIIB and EIIC are fused in another protein (PSPPH0849; Joardar *et al.,* 2005). Through the function of these two proteins, fructose is translocated across the bacterial inner membrane into the cell and phosphorylated to fructose 1-P. PSPPH0847 and PSPPH0849 genes are separated by another ORF encoding FruK (PSPPH0848) that catalyzes the phosphorylation of fructose 1-P to fructose1,6 bis-P which can be used as substrate for glycolysis. Similar gene organization was also found in the *P. s.* pv. *tomato* DC3000 strain (Buell *et al.,* 2003).

The strong induction of *avrPto-luc* in the mutant strains *in planta* is consistent with the fact that plant apoplast fluid contains a mixture of carbohydrates and amino acids that can complement the fructose deficiency. It was reported that sucrose accumulates in the apoplast of the legume plant *V. faba* to a concentration of 3-4 mM (reference in Ritte *et al.,* 1999). Glucose and fructose also accumulate to >0.3 mM in *V. faba* apoplast fluid (reference in Ritte *et al.,* 1999). In tomato apoplasts, glutamic acid, glutamine, serine, and GABA each accumulate to 0.6-0.8 mM (Solomon and Oliver, 2001). The levels of carbohydrates and amino acids in *Arabidopsis* apoplast are not clear. But the strong induction of *avrPto-luc* in *Arabidopsis* indicated that the mixture of the apoplast carbon nutrients is above the threshold for induction of the *avrPto* gene. The lower *avrPto-luc* induction in the fructose mutants in *Arabidopsis* indicates that fructose is a necessary carbon in plants to support the optimal expression of type III genes. This result also indicates that fructose is not the plant signal for induction of type III genes.

Although mutation on fructose uptake reduced the induction of *hrp* promoter in *Arabidopsis*, it did not affect the HR development on tobacco plants. One explanation of this result is that fructose uptake mutation does not affect type III gene expression in tobacco. Alternatively, it implies that the full induction of HR can be achieved with suboptimal induction of TTSS. However, on the host bean plants, the fructose mutants did show reduced growth and weaker disease symptoms compared to the wild type strain, suggesting the importance of fructose for bacterial pathogenesis. This result is somewhat unexpected because sucrose is much more abundant than fructose in the plant apoplast (Ritte *et al.,* 1999), and in minimal medium, sucrose can stimulate the *avrPto-luc* gene expression in NSP3121 strain. As the abundant sucrose can serve as a carbon source for

the bacterium, why does the bacterium still rely on the much less abundant fructose for full pathogenicity? A plausible explanation is that conversion of sucrose to fructose and glucose is probably an important means for sucrose uptake by the NSP3121 strain. Fig. IV-5 shows that, in MM, sucrose induces the *avrPto-luc* gene better in the wild type strain than in the fructose uptake mutant strains, implying hydrolysis of sucrose to fructose and glucose before uptake. We examined the DC3000 genomic sequence and found a glucose uptake system encoded by the PSPTO1292, PSPTO1293, PSPTO1294, and PSPTO1295 genes but not a typical sucrose uptake system in this bacterium (Buell *et al.,* 2003). Genes encoding the sucrose uptake proteins were not found in the *P. s. phaseolicola* 1448A genome (TIGR, unfinished microbial genome). If hydrolysis of sucrose is the sucrose-uptake mechanism in the *P. s.* pv. *phaseolicola* NPS3121 strain, it would readily explain the weaker pathogenicity of the fructose uptake mutant strains, because these mutants can not absorb fructose derived from sucrose.

The inability of mannitol to induce the *avrPto-luc* gene in the *P. s. phaseolicola* NSP3121 strain in MM suggests that mannitol can not be used as a carbon source by this bacterium. To support this possibility, we found that NSP3121 was unable to multiply in MM supplemented with mannitol. However, mannitol did support an extended *avrPto-luc* expression in other tested *P. syringae* strains, and in these strains, mannitol also supported the growth of these bacteria. These results strongly support the nutritional role of various carbons in *Pseudomonas* type III gene induction. It is unclear if the difference in mannitol utilization among the various *P. syringae* strains is related to the mannitol availability in their corresponding host plants. It remains to be determined if *P. s. phaseolicola* NSP3121 strain is defective in mannitol uptake or catabolism.

MATERIALS AND METHODS

Bacterial strains and plants

Bacterial strains used in this study were *P. s.* pv. *phaseolicola* NPS3121 (Lindgren *et al.,* 1986), P. s. pv. tomato DC3000 (Davis *et al.,* 1991), P. s. pv. tabaci 11528 (Tang *et al.,* 1996), and P. s. pv. maculicola ES4326 strains (Davis *et al.,* 1991). Bacteria were grown in King's B medium (King *et al.,* 1954) with appropriate antibiotics and induced in minimal medium (Huynh *et al.,* 1989) at a concentration of $1x10^7$ CFU/mL (Huynh *et al.,* 1989). Plants used for assays of avrPto-luc induction, non-host HR, and pathogenicity were the *Arabidopsis* att1 mutant (Xiao *et al.,* 2004), tobacco W38, and the Red Kidney bean (Lindgren *et al.,* 1986), respectively. *Arabidopsis* plants were grown in growth chambers at 20°C during the night and 22°C during the day with a 10 h/day photoperiod. Tobacco W38 and Red Kidney bean plants were grown in a greenhouse.

Construction of the avrPto-luc reporter gene into the pHM2 vector

Two broad host plasmids pHM2 was used to express the *avrPto-luc* reporter gene in *P. syringae* strains. The *avrPto-luc* reporter gene was previously constructed into the pPTE6 plasmid by Xiao *et al.,* (2004). pHM2 plasmid was derived from the pHM1 plasmid (Zhu *et al.,* 1999) through the following modifications. First, the pHM1 plasmid was digested with *Bam*HI to remove the *lacZ* promoter and multiple cloning sites. The resulting vector was partially filled in with dATP and dGTP and Klenow, and subsequently ligated with an adaptor DNA formed by association of the following two oligo nucleotides: 5'-TCCGAATTCAAGCTTCTCGAGGGTACCTCTAGA-3' and 5'-TCTCTAGAGGTACCCTCGAGAAGCTTGAATTCG-3'. To clone the *avrPto-luc* reporter gene into pHM2, pPTE6 plasmid containing the *avrPto-luc* reporter gene was

digested with *Eco*RI and *Xba*I, and the released *avrPto-luc* expression cassette was subsequently cloned into the pHM2 plasmid predigested with *Eco*RI and *Xba*I, resulting in pHM2::*avrPto-luc* plasmid.

Construction and screen of P. s. pv. phaseolicola NPS3121 mutant library

EZ::TN^{TM}<KAN-2> Tn transposome^{TM} Kit was used to generate the *P. s.* pv. *phaseolicola* NPS3121 mutant library. Briefly, electrocompetent cells of *P. s.* pv. *phaseolicola* NPS3121 carrying the pHM2::*avrPto-luc* plasmid were mixed with EZ::TN^{TM}<KAN-2> transposon and transposase as instructed by manufacturer (Epicentre, Wisconsin, WI). Following electroperation, the bacteria were plated on KB medium containing kanamycin and spectinomycin to select for mutant bacteria carrying the EZ::TN^{TM}<KAN-2> transposon and pHM2::*avrPto-luc* plasmid. To screen for mutants with compromised *avrPto-luc* expression, the mutant bacterial colonies were first grown in liquid KB medium containing spectinomycin and kanamycin in 96-well plate, washed twice with sterile water, resuspended in MM (50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, and 10 mM fructose, pH 5.7) and incubated at 23^{o}C for 6 hrs. To measure the luciferase activity, luciferin was added to the bacterial culture to a final concentration of 50 μM, and the activity was determined by using a cooled CCD (Roper Scientific, Trenton, NJ). Colonies with luciferase activity ~5 fold lower than that in the wild type strain was selected as putative mutants. A total of 12, 000 colonies were screened.

Transposon insertion location

The transposon insertion sites were determined by a two-stage semidegenerate PCR according to procedures described by Jacobs *et al.* (2003) using two transposon-specific primers (5'-GATAGATTGTCGCACCTGATTG-3' and 5'-AAGACGTTTCCCGTTGAATATG-3') and four degenerated primers (5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG-3', 5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC-3', 5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT-3', and 5'-GGCCACGCGTCGACTAGTAC-3'). The PCR product was sequenced using a transposon-specific primer (5'-GCAATGTAACATCAGAGATTTTGAG-3'). Blastn was used to search the homologous sequence in database and the *P. s. phaseolicola* 1448A sequence in TIGR database. To further confirm the transposon insertion site, we designed gene specific primers according to the *P. s. phaseolicola* 1448A gene sequences and PCR-amplified the DNA at transposon insertion site using gene specific primer and transposon specific primer. The PCR product was sequenced to determine the exact transposon insertion site.

avrPto-luc expression assay in minimal medium and in plant

P. s. pv. *phaseolicola* NPS3121 and the derived mutant strains carrying pHM2::*avrPto-luc* were first grown in liquid KB medium containing 20 mg/L rifampcin,10 mg/L kanamycin, and 10 mg/L spectinomycin to ~OD_{600}=1. To determine the *avrPto-luc* induction in minimal medium, the bacteria were harvested by centrifugation at 4000g, washed twice with sterile water, resuspended in minimal medium to a concentration of OD_{600}=0.05, and cultured at 23^{o}C with constant shaking at

200rpm. The luciferase activity was determined as described above. To determine the *avrPto-luc* induction in plant, bacteria at $5x10^8$ CFU/ml in sterile water were inoculated into the *Arabidopsis* plants. Leaves were detached 6hr after inoculation, sprayed with a solution containing 1 mM luciferin and 0.01% Tween-20, and kept in the dark for 5 min before imaging with a CCD. The relative LUC activity was normalized to leaf bacterial numbers.

Non-host HR and pathogenicity assay

For HR assay, *P. s.* pv. *phaseolicola* NPS3121 and the fructose uptake mutant strains at $1x10^8$ CFU/ml were inoculated into young, fully expanded tobacco W38 leaves. The lesions caused by HR were photographed at 20 hrs after inoculation. To monitor the growth of *P. s.* pv. *phaseolicola* NPS3121 and the fructose uptake mutants in bean plants, bacteria at $2x10^4$ CFU/ml were inoculated using a needle-less syringe into the first two true leaves of Red Kidney bean plants two weeks after the seed germination. Leaf bacteria were determined by grinding two leaf discs in sterile water, plating diluted suspensions on KB agar plate, and counting colony-forming units. To document disease symptoms, the inoculated bean leaves were photographed 9 days after inoculation.

REFERENCES

Aldon, D., Brito, B., Boucher, C., and Genin, S. 2000. A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. EMBO J. 19: 2304–2314.

Alfano, J. R., and Collmer, A. 2004. TYPE III SECRETION SYSTEM EFFECTOR PROTEINS: Double Agents in Bacterial Disease and Plant Defense. Annu. Rev. Phytopathol. 42: 385-414.

Ankenbauer, R. G, and Nester, E. W. 1990. Sugar-mediated induction of *Agrobacterium tumefaciens virulence* genes: structural specificity and activities of monosaccharides. J. Bacteriol. 172: 6442-6446.

Arlat, M., Gough, C. L., Zischek, C., Barberis, P. A., Trigalet, A., and Boucher, C. A. 1992. Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. Mol. Plant Microbe Interact. 5:187–193.

Bretz, J., Losada, L., Lisboa, K., and Hutcheson, S. W. 2002. Lon protease functions as a negative regulator of type III protein secretion in *Pseudomonas syringae*. Mol. Microbiol. 45: 397-409.

Brito, B., Aldon, D., Barberis, P., Boucher, C., and Genin, S. 2002. A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum hrp* genes. Mol. Plant Microbe Interact. 15: 109–119.

Brito, B., Marenda, M., Barberis, P., Boucher, C., and Genin, S. 1999. *prhJ* and *hrpG*, two new components of the plant signal-dependent regulatory cascade controlled by PrhA in *Ralstonia solanacearum*. Mol. Microbiol 31: 237–251.

Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., Dodson, R. J., Deboy, R. T., Durkin, A. S., Kolonay, J. F., Madupu, R., Daugherty, S., Brinkac, L., Beanan, M. J., Haft, D. H., Nelson, W. C., Davidsen, T., Zafar, N., Zhou, L., Liu, J., Yuan, Q., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K.,

Utterback, T., Van Aken, S. E., Feldblyum, T. V., D'Ascenzo, M., Deng, W. L., Ramos, A. R., Alfano, J. R., Cartinhour, S., Chatterjee, A. K., Delaney, T. P., Lazarowitz, S. G., Martin, G. B., Schneider, D. J., Tang, X., Bender, C. L., White, O., Fraser, C. M., and Collmer, A. 2003. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Proc. Natl. Acad. Sci. U.S.A. 100: 10181-10186.

Buttner, D., and Bonas, U. 2002. Getting across--bacterial type III effector proteins on their way to the plant cell. EMBO J. 21: 5313-5322.

Cangelosi, G. A., Ankenbauer, R. G., and Nester, E. W. 1990. Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl. Acad. Sci. U.S.A. 87: 6708-6712.

Davis, K. R., Schott, E., and Ausubel, F. M. 1991. Virulence of selected phytopathogenic pseudomonads in *Arabidopsis thaliana*. Mol. Plant Microbe Interact. 4: 477–488.

Fouts, D. E., Abramovitch, R. B., Alfano, J. R., Baldo, A. M., Buell, C. R., Cartinhour, S., Chatterjee, A. K., D'Ascenzo, M., Gwinn, M. L., Lazarowitz, S. G., Lin, N. C., Martin, G. B., Rehm, A. H., Schneider, D. J., van Dijk, K., Tang, X., and Collmer, A. 2002. Genomewide identification of *Pseudomonas syringae* pv. *tomato* DC3000 promoters controlled by the HrpL alternative sigma factor. Proc. Natl. Acad. Sci. U.S.A. 99: 2275-2280

Galan, J. E., and Collmer, A.1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284: 1322–1328.

Huang, M. L., Cangelosi, G. A., Halperin, W., and Nester, E. W. 1990. A chromosomal *Agrobacterium tumefaciens* gene required for effective plant signal transduction. J. Bacteriol. 172: 1814-1822.

Hutcheson, S. W., Bretz, J., Sussan, T., Jin, S., and Pak, K. 2001. Enhancer-binding proteins HrpR and HrpS interact to regulate hrp-encoded type III protein secretion in *Pseudomonas syringae* strains. J. Bacteriol. 183: 5589-5598.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science 245:1374–1377.

Innes, R. 2003. New effects of type III effectors. Mol. Microbiol. 50: 363-365.

Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenthner, D., Bovee, D., Olson, M.V., and Manoil, C. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. 100:14339-14344.

Joardar,V., Lindeberg,M., Jackson,R., Selengut,J., Dodson,R., Brinkac,L.M., *et al.,* 2005. Whole-Genome Sequence Analysis of *Pseudomonas syringae* pv. *phaseolicola* 1448A reveals divergence among pathovars in genes involved in virulence and transposition. *J. Bacteriol.* 187: 6488-6498.

King, E.O., Ward, M.K., and Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab Med. 22: 301-307.

Lahaye, T., and Bonas, U. 2001. Molecular secrets of bacterial type III effector proteins. Trends Plant Sci. 6: 479-485.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. '*phaseolicola*' controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168: 512–522.

Marenda, M., Brito, B., Callard, D., Genin, S., Barberis, P., Boucher, C., and Arlat, M. 1998. PrhA controls a novel regulatory pathway required for the specific induction of *Ralstonia solanacearum hrp* genes in the presence of plant cells. Mol. Microbiol. 27: 437–453.

Postma, P. W., Lengeler, J. W., and Jacobson, G. R. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol. Rev*. 57: 543-594.

Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syrinagae* pv. *phaseolicola*. J. Bacteriol. 174: 3499–3507.

Ritte, G., Rosenfeld, J., Rohrig, K., and Raschke, K. 1999. Rates of sugar uptake by guard cell protoplasts of *pisum sativum* L. related to the solute requirement for stomatal opening. Plant Physiol. 121: 647-656.

Salmeron, J. M., and Staskawicz, B. J. 1993. Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato*. Mol. Gen. Genet. 239: 6–16.

Schulte, R., and Bonas, U. 1992. Expression of the *Xanthomonas campestris* pv. *vesicatoria hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. J. Bacteriol. 174: 815-823.

Shimoda, N., Toyoda-Yamamoto, A., Aoki, S., and Machida, Y. 1993. Genetic evidence for an interaction between the VirA sensor protein and the ChvE sugar-binding protein of Agrobacterium. J. Biol. Chem. 268: 26552-26558.

Shimoda, N., Toyoda-Yamamoto, A., Nagamine, J., Usami, S., Katayama, M., Sakagami, Y., and Machida, Y. 1990. Control of expression of Agrobacterium *vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. Proc. Natl. Acad. Sci. U.S.A. 87: 6684-6688.

Solomon, P. S., and Oliver, R. P. 2001. The nitrogen content of the tomato leaf apoplast increases during infection by *Cladosporium fulvum*. Planta 213: 241-249.

Solomon, P. S., and Oliver, R. P. 2002. Evidence that gamma-aminobutyric acid is a major nitrogen source during *Cladosporium fulvum* infection of tomato. Planta 214: 414-420.

Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. 1996. Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase. Science 274: 2060-2063.

Wei, Z. M., Sneath, B. J., and Beer, S. V. 1992. Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. J. Bacteriol. 174:1875–1882.

Wengelnik K and Bonas U. 1996. HrpXv, an AraC-type regulator, activates expression of five of the six loci in the *hrp* cluster of *Xanthomonas campestris* pv. *vesicatoria*. J. Bacteriol. 178: 3462-3469.

Xiao, F., Mark Goodwin, S., Xiao, Y., Sun, Z., Baker, D., Tang, X., Jenks, M. A., and Zhou, J. M. 2004. *Arabidopsis* CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development. EMBO J. 23:2903-2913.

Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176: 1025–1036.

Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. J. Bacteriol. 174:1734-1741.

Zhu, W., Yang, B., Wills, N., Johnson, L. B., and White, F. F. 1999. The C Terminus of AvrXa10 Can Be Replaced by the Transcriptional Activation Domain of VP16 from the Herpes Simplex Virus. Plant Cell 11:1665-1674.

Zwiesler-Vollick, J., Plovanich-Jones, A. E., Nomura, K., Brandyopadhyay, S., Joardar, V., Kunkel, B. N., and He, S, Y. 2002. Identification of novel *hrp*-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome. Mol. Microbiol. 45:1207-1218.

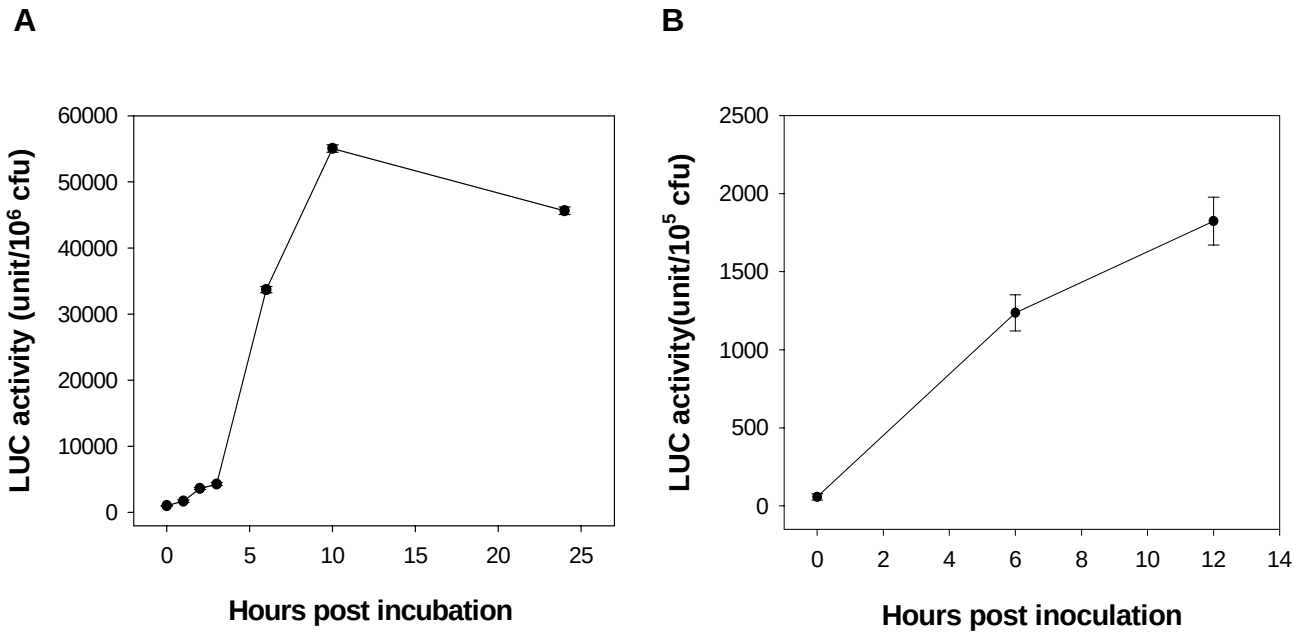


Figure IV-1. Kinetics of *avrPto-luc* gene expression in minimal medium and in plant.

P. s. pv. *phaseolicola* NPS3121 strain carrying *avrPto-luc* was cultured in minimal medium (A) or inoculated into the *Arabidopsis* leaves (B). Luciferase activity was measured at the indicated times. Error bars indicate standard errors.

(Contributed by Xiaoyan Tang)

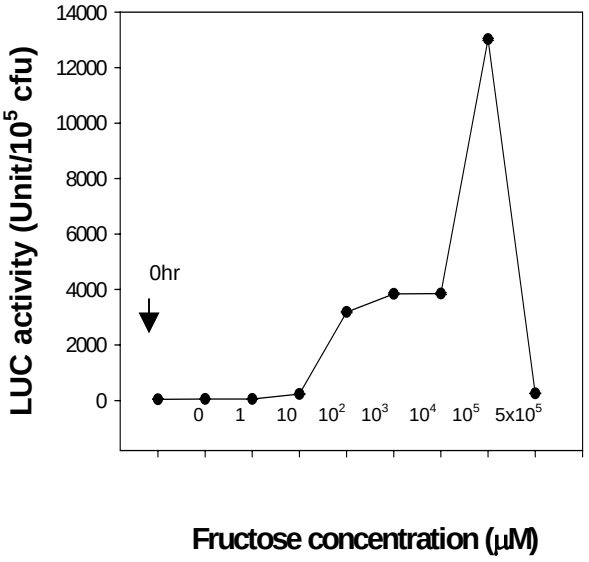


Figure IV-2. Induction of *avrPto-luc* in minimal medium by different fructose dosages.

P. s. pv. *phaseolicola* NPS3121 strain carrying *avrPto-luc* was cultured in minimal medium containing fructose at the indicated concentrations. Arrow indicates the luciferase activity at 0 hr. Other data points indicate luciferase activity at 6 hrs after culture in MM. Error bars indicate standard errors. Experiment was repeated three times with similar result.

(Contributed by Xiaoyan Tang)

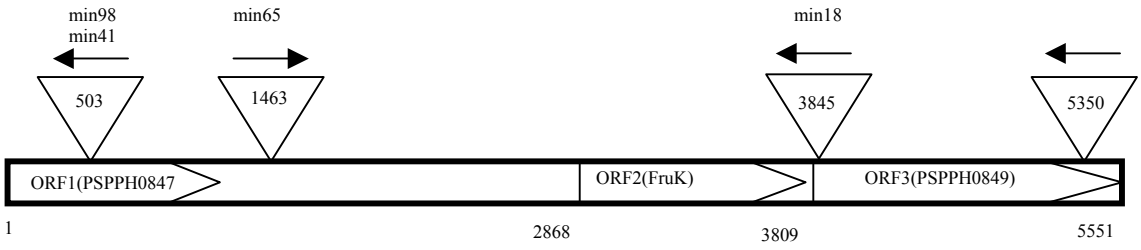


Figure IV-3. Transposon insertions in the fructose uptake mutants.

The bar represents the fructose uptake locus in *P. s. phaseolicola* 1448A strain. Open arrows inside the bar indicate the direction of genes in this locus. Numbers under the bar indicate the stop codon of each *ORF* (i. e. *ORF1*, 1-2868; *ORF2*, 2868-3809; *ORF3*, 3822-5551). Triangles represent the transposon insertions, and the numbers inside the triangle designate the insertion sites that were determined by sequence alignment between the *P. s. phaseolicola* 1448A sequence and the transposon flanking sequences. Arrows above the triangles indicate the direction of kanamycin resistance gene carried by the Tn5 transposons

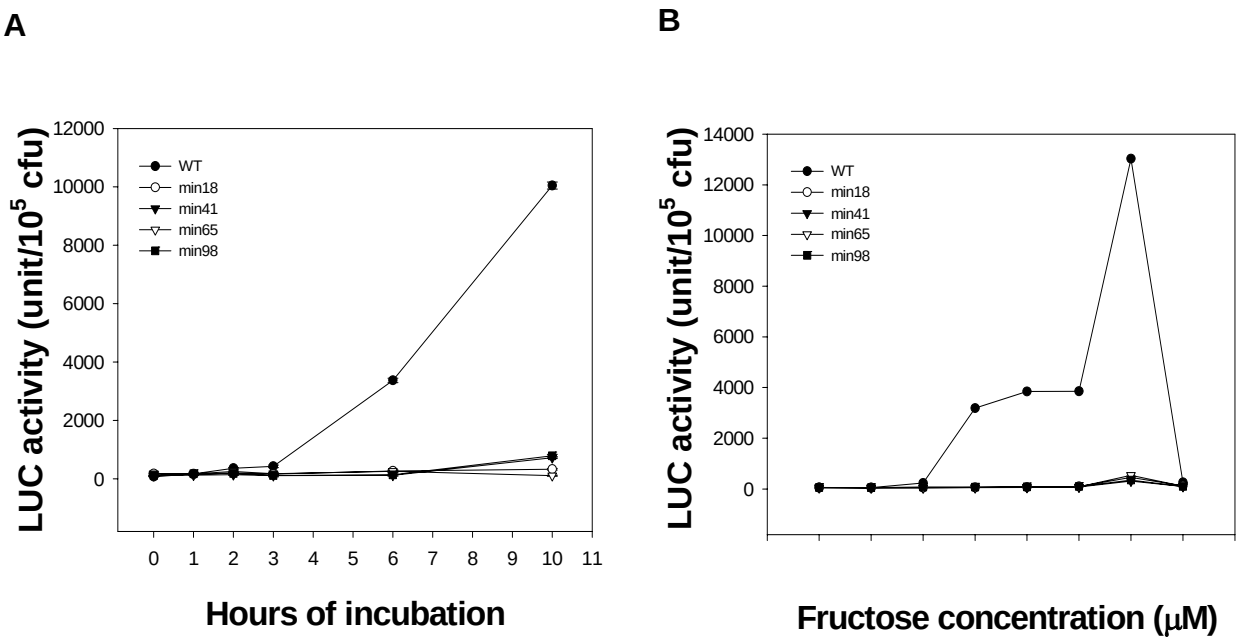


Figure IV-4 *avrPto-luc* expression in the fructose uptake mutants.

A, Kinetics of *avrPto-luc* gene expression in minimal medium containing 10 mM fructose. **B,** Reaction of fructose-uptake mutants to changes of fructose dosage in minimal medium. The wild type NPS3121 and the derived mutant strains carrying *avrPto-luc* were cultured in minimal media. Luciferase activities were measured at the indicated times in A and 6 hrs in B. Error bars indicate standard errors. The experiments were repeated three times with similar results.

(Contributed by Xiaoyan Tang)

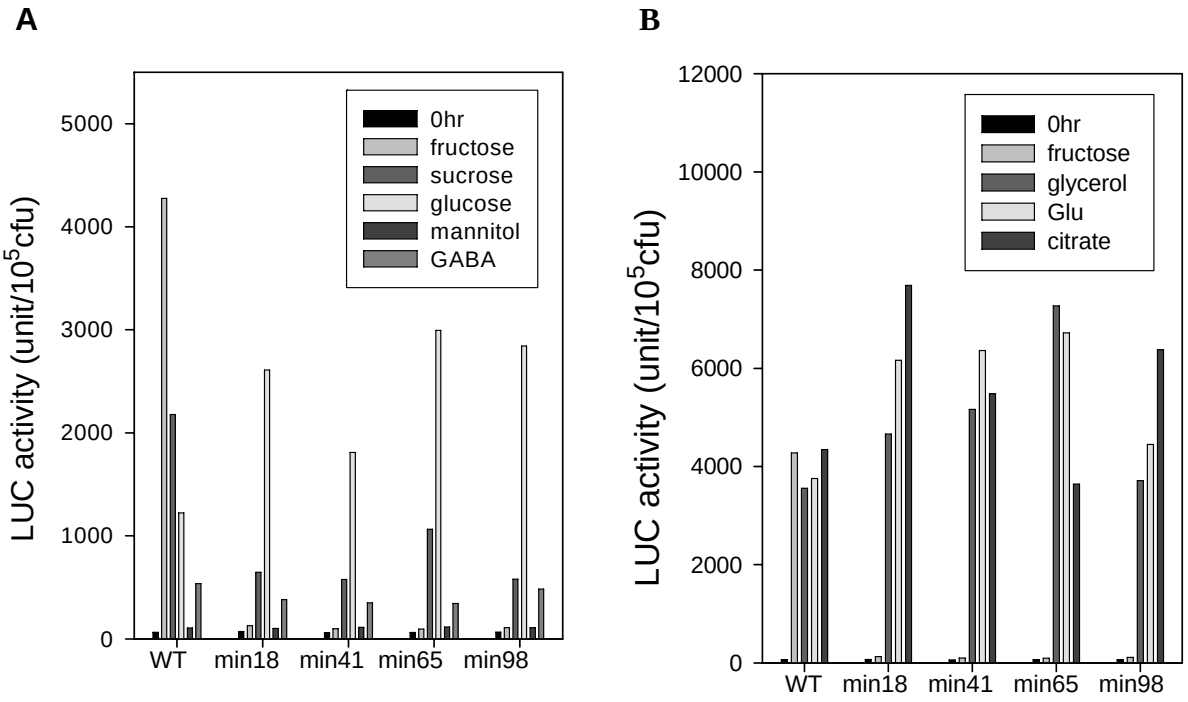


Figure IV-5. *avrPto-Luc* expression in the fructose uptake mutants in minimal media containing different carbons.

1 mM fructose, sucrose, glucose, mannitol, GABA, glycerol, glutamic acid, and citrate were added individually to the minimal medium. LUC activities were measured at 6 hrs after culture. The experiment was repeated three times with similar result.

(Contributed by Xiaoyan Tang)

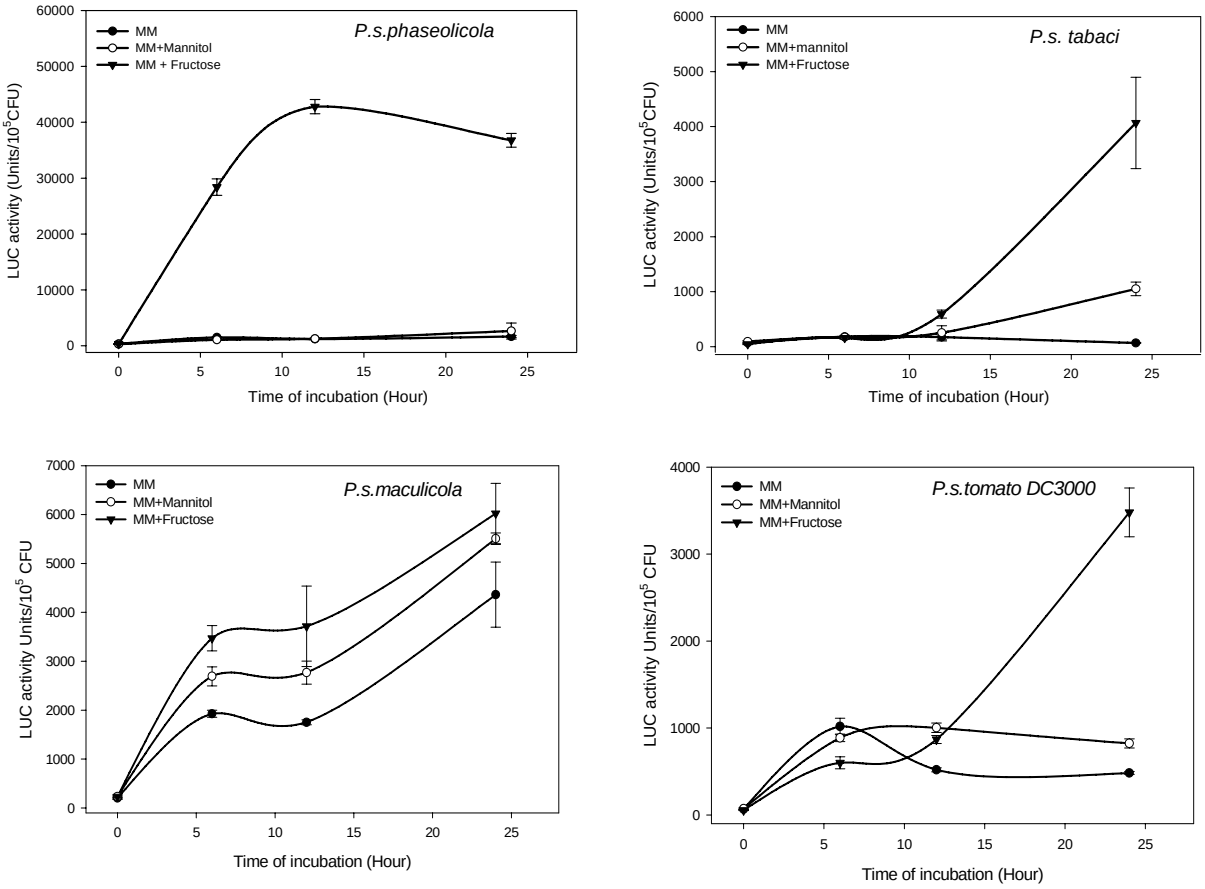


Figure IV-6. Differential requirement of mannitol for the induction *avrPto-luc* in various *P. syringae* strains.

Phaseolicola NPS3121, *P. s.* pv. *tomato* DC3000, *P. s*. pv. *maculicola* ES4326, and *P. s.* pv. *tabaci* 11528 strains carrying *avrPto-luc* gene were cultured in MM without carbon, MM with 10 mM fructose, and MM with 10 mM mannitol. Luciferase activities were measured at the indicated times. Error bars indicated standard error. The experiment was repeated twice with similar result.

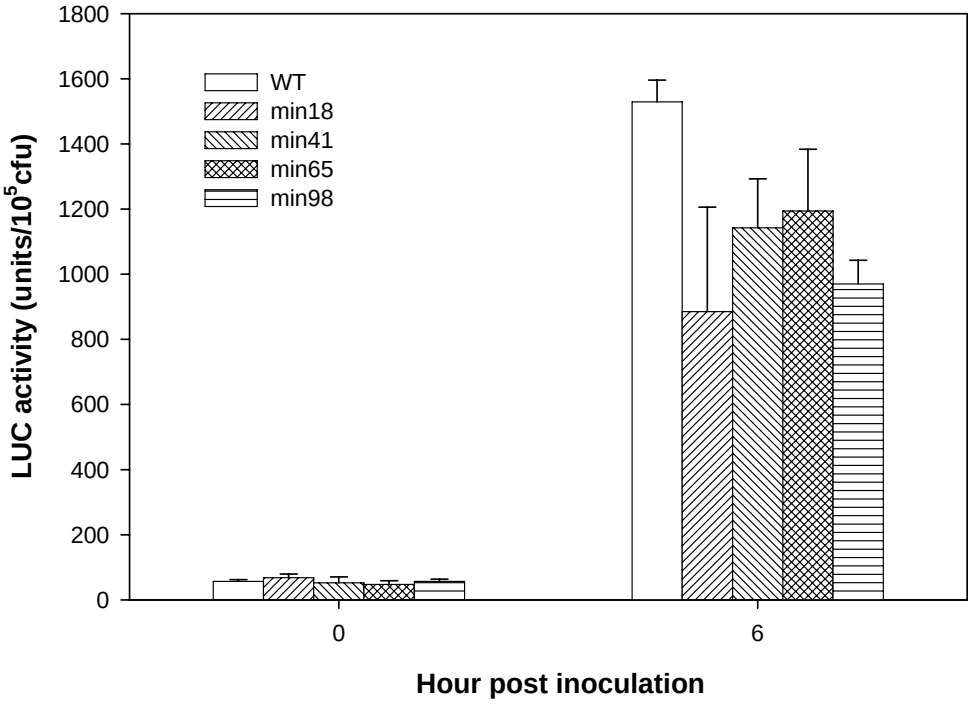


Figure IV-7. *In planta* expression of *avrPto-luc* in the fructose uptake mutants.

The fructose uptake mutants and the wild type strain at the concentration of $5x10^8$ cfu/mL were infiltrated into the *Arabidopsis att1* mutant plants. Luciferase activities were measured 6 hrs after inoculation. Error bars indicated standard error. The experiment was repeated three times with similar results.

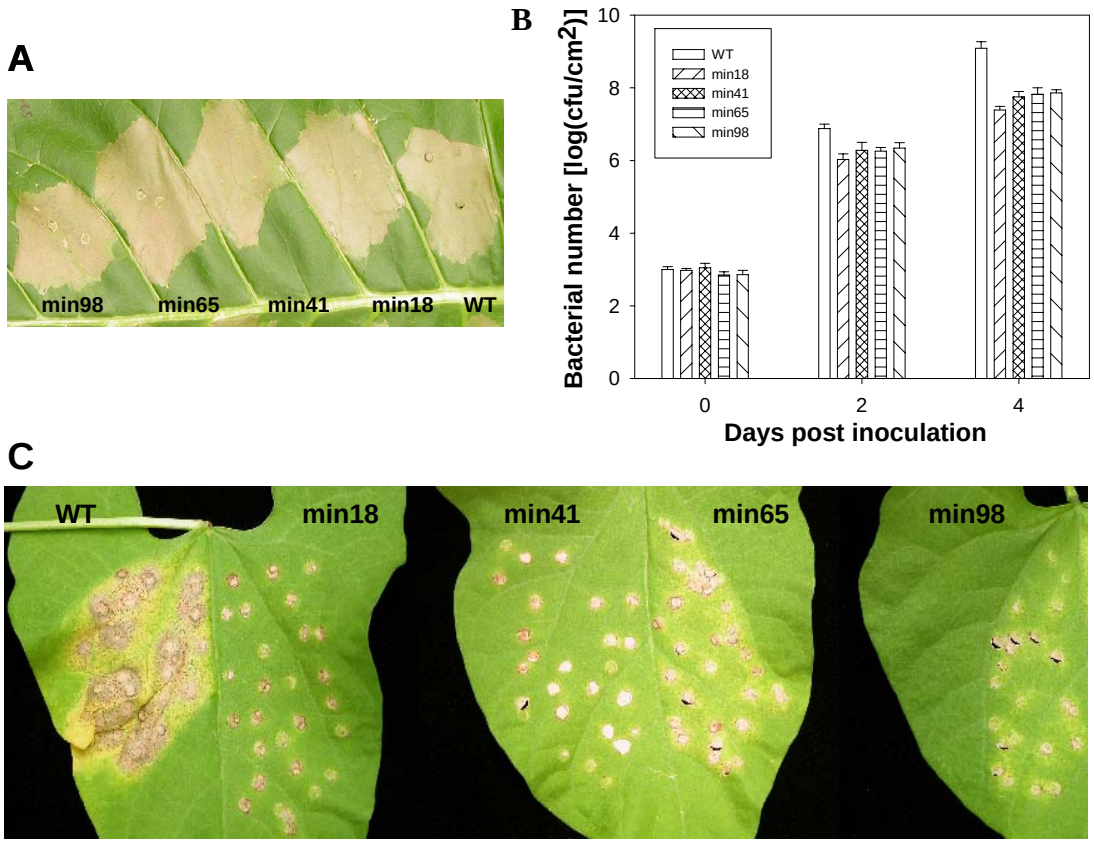


Figure IV-8. HR and pathogenicity assays of the fructose uptake mutants.

A, Fructose uptake mutants and the wild type strain at 10^8cfu/mL were injected into tobacco W38 plants. HR was photographed 20 hrs after inoculation. **B,** Growth of fructose uptake mutants in bean leaves. Wild type and mutant strains at 10^5cfu/mL were infiltrated into the Red Kidney bean leaves. Bacterial numbers were measured at the indicated times. Error bars indicated standard error. **C,** Disease symptoms caused by fructose uptake mutants in bean leaves. Wild type and mutant strains at 10^5cfu/mL were infiltrated into the Red Kidney bean leaves. Disease symptoms were documented 9 days after inoculation. The experiment was repeated three times with similar results.