

HrpS Is a Global Regulator on Type III Secretion System (T3SS) and Non-T3SS Genes in *Pseudomonas savastanoi* pv. *phaseolicola*

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The type III secretion system (T3SS) is the main machinery for *Pseudomonas savastanoi* and other gram-negative bacteria to invade plant cells. HrpR and HrpS form a hetero-hexamer, which activates the expression of HrpL, which induces all T3SS genes by binding to a ‘hrp box’ in promoters. However, the individual molecular mechanism of HrpR or HrpS has not been fully understood. Through chromatin immunoprecipitation coupled to high-throughput DNA sequencing, we found that HrpR, HrpS, and HrpL had four, 47, and 31 targets on the genome, respectively. HrpS directly bound to the promoter regions of a group of T3SS genes and non-T3SS genes. HrpS independently regulated these genes in a *hrpL* deletion strain. Additionally, a HrpS-binding motif (GTGCCAAA) was identified, which was verified by electrophoretic mobility shift assay and *lux*-reporter assay. HrpS also regulated motility and biofilm formation in *P. savastanoi*. The present study strongly suggests that HrpS alone can work as a global regulator on both T3SS and non-T3SS genes in *P. savastanoi*.

Pseudomonas savastanoi and other gram-negative pathogenic bacteria infect host organisms via the needle-like type III secretion system (T3SS) (Galán and Collmer 1999). T3SS in

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P. savastanoi includes a *hrp* (hypersensitive response and pathogenicity) pilus and inner and outer membrane rings (Kubori et al. 1998; Roine et al. 1997). In *P. savastanoi*, T3SS is encoded by the *hrp* genes, hypersensitive response conserved (*hrc*) genes, avirulence (*avr*) genes, and *hrp*-dependent outer protein (*hop*) genes (Alfano et al. 1997). The T3SS leads to disease development in host plants and hypersensitive response in nonhost (Collmer et al. 2000) and resistant plants. The expression of T3SS genes is repressed in nutrient-rich media (King’s B broth [KB]), while it is activated in minimal medium (MM) and plants (Xiao et al. 2007).

HrpL, an alternative sigma factor, is a key T3SS activator by recognizing a ‘hrp box’ (5′-GGAACC-N(15-16)-CCACNNA-3′) in promoters of T3SS genes (Lindeberg et al. 2005; Nissan et al. 2005). Under T3SS-inducing conditions, the transcription of *hrpL* is activated by HrpR and HrpS, which form a hetero-hexamer (Xiao et al. 1994). RpoN (σ^{54}) is also required for the induction of *hrpL* (Lindeberg et al. 2006; Nissan et al. 2005). HrpS independently induces *hrpL* transcription, which can be maximized by HrpRS (Hutcheson et al. 2001). HrpRS binds to a 47-bp region (101 to 147 bp upstream of ATG) in the *hrpL* promoter in *P. savastanoi* pv. *tomato* DC3000 (Jovanovic et al. 2011); however, the precise binding motif is unclear. HrpL is negatively autoregulated, which fine-tunes the expression of T3SS (Waite et al. 2017).

HrpR and HrpS are the bacterial enhancer-binding proteins and ATPases associated with various cellular activities (AAA+) (Morett and Segovia 1993; Schumacher et al. 2006). Previous studies have characterized their secondary structures, including the AAA+ domain (containing regions for ATP-binding, σ^{54} -binding, nucleotide hydrolysis, inter-subunit catalysis, and nucleotide-dependent oligomerization) and the DNA binding domain (helix-turn-helix structure in C-terminal) (Jovanovic et al. 2011; Lawton et al. 2014). Three residues in HrpR (D32, D34, E202, and K235) and three in HrpS (D32, G30, E200, and K233) are keys in the self-association process (Lawton et al. 2014). HrpR-D32 and HrpS-K233 residues are crucial to the formation of HrpRS hetero-hexamer (Lawton et al. 2014). In *Erwinia amylovora*, there is only one *hrpS* gene, which is evolved to homologous *hrpR* and *hrpS* via gene duplication in *P. savastanoi* (Vencato et al. 2006b).

HrpV acts as an inhibitor of T3SS by directly binding to HrpS (Preston et al. 1998). The inhibition of HrpS by HrpV can be released by a chaperone-like protein HrpG (Wei et al. 2005).

L108, A109, and G110 of HrpV are the critical sites for the interactions with HrpS and HrpG, while F6, L101, and L105 of HrpG are the keys for direct interaction with HrpV (Jovanovic et al. 2017). In addition, another T3SS inhibitor, Lon, an ATP-dependent protease, directly degrades HrpR to negatively regulate the repression of T3SS genes (Bretz et al. 2002). Our previous work shows that a two-component system response regulator,

RhpR, directly binds to the *hrpR* promoter by recognizing a putative IR motif (ATTTC-N₆-GATAC) (Deng et al. 2010, 2014). AefR, the key regulator of quorum sensing in *P. savastanoi* (Quiñones et al. 2004), also positively regulates *hrpR* (Deng et al. 2009).

Although the HrpRSL regulatory cascade has been characterized previously, the comprehensive profiling and individual

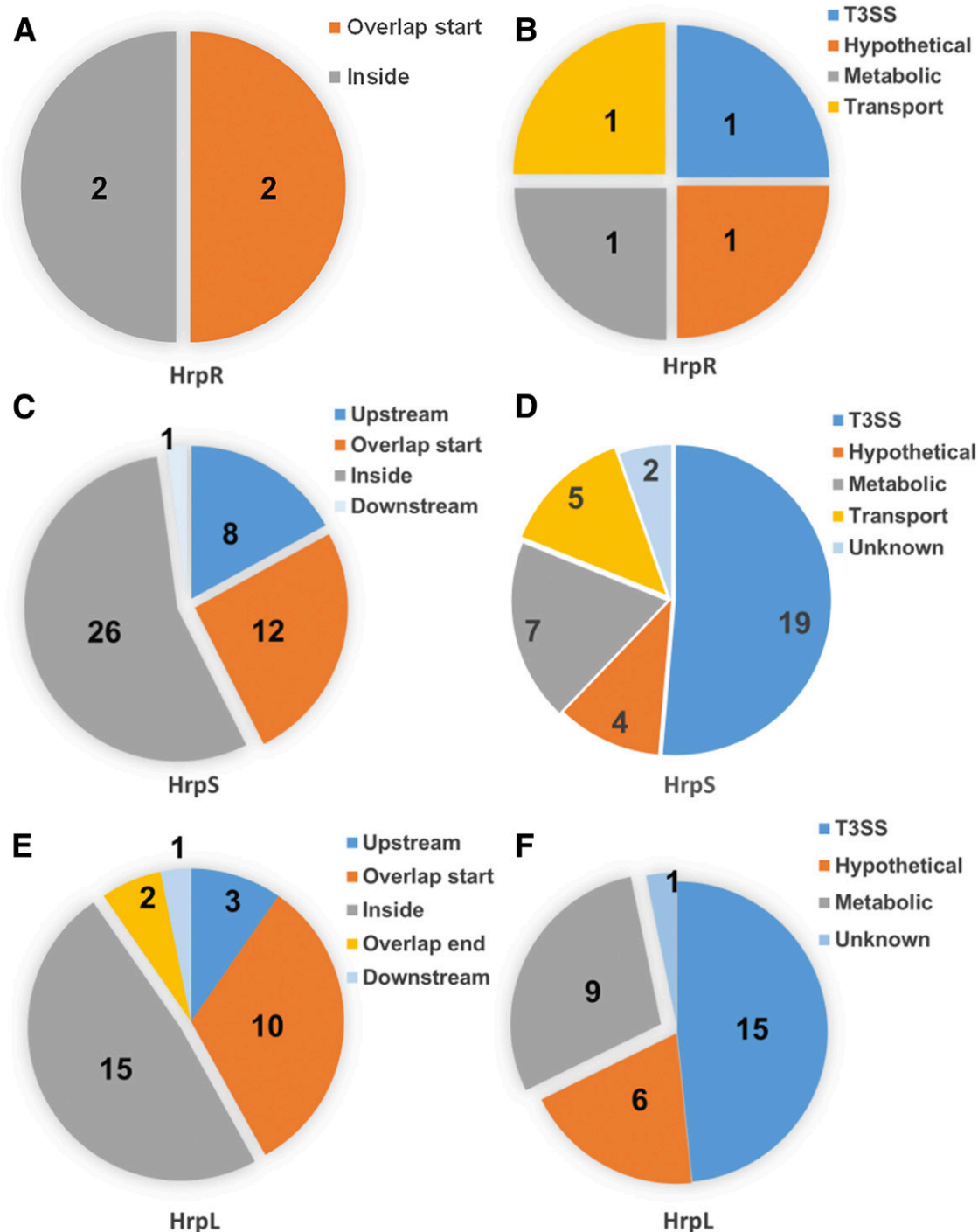


Fig. 1. Chromatin immunoprecipitation coupled to high-throughput DNA sequencing revealed in vivo binding sites of HrpS, HrpR, and HrpL in the *Pseudomonas savastanoi* genome. **A**, In HrpR-binding peaks, two loci were detected within coding regions and two in the overlap start region. **B**, The functions of HrpR-binding genes were diverse, including metabolism (1), type III secretion system (T3SS) (1), transport (1), and hypothetical proteins (1). **C**, In HrpS-binding peaks, 47 loci are distributed throughout the genome in intergenic regions (21) and coding regions (26). Notably, 20 peaks were located upstream of genes or overlapped with the promoter regions. **D**, The HrpS-bound genes encode proteins with diverse functions, including T3SS (19), metabolism (7), transport (5), hypothetical proteins (4), and unknown functions (2). **E**, Among 31 loci in the HrpL-binding peaks, 13 binding to the upstream or overlap starting region, 15 in inside region, two in overlap the end, and one in the downstream. **F**, The functions of HrpL-binding genes can be evenly classified into T3SS (15), metabolism (9), hypothetical proteins (6) and unknown (1).

characterization of HrpR or HrpS have yet to be carried out. In the present study, chromatin immunoprecipitation coupled to high-throughput DNA sequencing (ChIP-seq) was used to identify all direct binding loci of HrpR, HrpS, or HrpL. HrpS not only functions as an independent regulator to activate a group of T3SS but also control several non-T3SS genes in *P. savastanoi*. A specific HrpS-binding motif (GTGCCAAA) is identified in the *hrpL* promoter. The results show that HrpS directly regulates a group of downstream genes, which is independent on HrpL. The present study brings novel insights into the molecular regulatory mechanism of T3SS regulatory cascade.

RESULTS

Genome-wide ChIP-seq analyses of HrpR, HrpS, and HrpL-binding regions.

HrpR and HrpS share the same upstream promoter and work as hetero-hexamers to activate the expression of the *hrpL* gene (Hutcheson et al. 2001). To comprehensively elucidate the direct targets of HrpR, HrpS, and HrpL, we mapped their binding loci on the chromosome of *Pseudomonas savastanoi* pv. *phaseolicola* 1448A, using the ChIP-seq assays in MM (Blasco et al. 2012). The FLAG-tagged HrpR, HrpS, or HrpL full length were respectively expressed under their own promoters in a low-copy pHM2 vector in the wild-type strain in MM. Each sequence

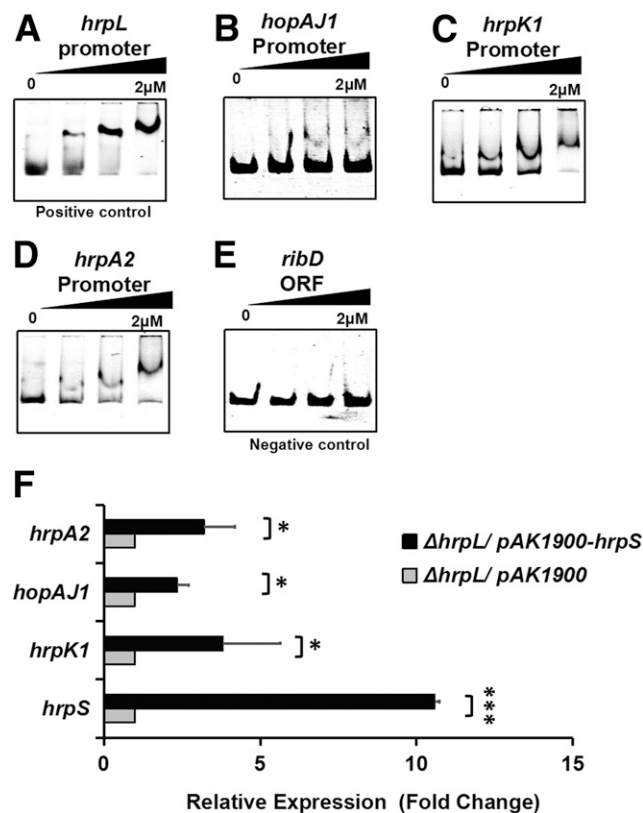


Fig. 2. HrpS directly bound to a group of type III secretion system genes. **A**, HrpS showed the maximal binding affinity with the *hrpL* promoter. Different concentrations of HrpS (0, 0.5, 1.0, and 2.0 μ M) were mixed with 40-ng DNA fragments. **B to D**, HrpS bound to the promoters of *hrpK1*, *hrpA2*, and *hopAJ1*. **E**, HrpS did not bind to negative control *ribD* at the same protein concentrations. **F**, Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) revealed that HrpS independently activated *hrpK1*, *hrpA2*, and *hopAJ1*. pAK1900-hrpS or pAK1900 empty vector were transformed into the *P. savastanoi* pv. *phaseolicola* $\Delta hrpL$ strain. RT-qPCR was performed to measure the transcription level of *hrpL*, *hrpK1*, *hrpA2*, and *hopAJ1* in both strains. One asterisk (*) denotes $P < 0.05$ and three (***) denote $P < 0.001$ compared with *hrpL*, by Student's *t* test. Each experiment has been performed three times.

data was obtained from two independent ChIP-seq assays using FLAG-specific antibody and were subsequently mapped to the *P. savastanoi* pv. *phaseolicola* genome. Using the MACS software (Zhang et al. 2008), we identified four, 47, and 31 enriched loci (P value = E-5) harboring HrpR, HrpS, or HrpL-binding peaks, respectively, which were enriched but were absent in control samples using wild-type *P. savastanoi* pv. *phaseolicola* without a FLAG tag (Supplementary Table S1).

Only four loci were identified in the HrpR-binding peaks, including two that were located in intergenic regions; the other two were in coding regions (Fig. 1A). They encode proteins associated with metabolism, T3SS, and transposition (Fig. 1B). In the HrpS-binding peaks, 47 loci were distributed throughout the genome in both intergenic regions (21 loci, 43%) and coding regions (26 loci) (Fig. 1C). Like HrpR, the HrpS-binding genes encode proteins associated with T3SS (19 genes), metabolism (seven genes), and transposition (five genes) (Fig. 1D). Among 31 loci in the HrpL-binding peaks, 13 were located in intergenic regions (Fig. 1E).

HrpR bound to a group of non-T3SS genes (PSPPH_2426, PSPPH_2767, *ribD*), which encode proteins associated with riboflavin biosynthesis, regulation, and transport. Interestingly, two non-T3SS genes (PSPPH_1525 and PSPPH_1855) and a group of T3SS genes, such as the *hrpA2-hrpZ1-hrpB-hrcJ-hrpD-hrpE-hrpF* operon, the *hrcV-hrpQ-hrcN-hrpO-hrpP-hrcQa-hrcQb-hrcR-hrcS-hrcT-hrcU* operon, *hopI1*, *hrpW1*, *hrpJ*, *hrpL*, *hrpK1*, and *hopX1*, were shared between HrpS-binding loci and HrpL-binding loci (Supplementary Fig. S1).

HrpS directly binds to a group of T3SS genes.

Since HrpS had more binding loci than HrpL, we hypothesized that HrpS alone has profound regulatory functions on T3SS and non-T3SS genes, which is tested in the following experiments. First, to verify the direct interaction between HrpS and its binding loci, we purified the full-length HrpS in *Escherichia coli* and then performed an electrophoretic mobility shift assay (EMSA). The *hrpL* promoter was used as a positive control (Hutcheson et al. 2001; Jovanovic et al. 2011). As a result, the full-length HrpS protein had low yield and low binding affinity with the promoter regions of *hrpL*, *hrpK1*, *hrpA2*, and *hopAJ1*. However, a recent study in *Erwinia amylovora* obtained optimal DNA-binding affinity of HrpS by expressing the C-terminal DNA-binding domain (Lee et al. 2016). Inspired by this work, a truncated HrpS containing the DNA-binding domain (amino acids 235 to 302, designated as HrpS^C) was cloned into pET28a. We successfully purified HrpS^C protein (Supplementary Fig. S2), which showed significantly improved binding affinity with the *hrpL* promoter (Fig. 2A). HrpS^C bound to the promoters of *hrpK1*, *hrpA2*, and *hopAJ1* (Fig. 2B to D), compared with the negative control *ribD* at the same protein concentration (Fig. 2E). These results suggested that HrpS directly regulates the transcription of *hrpK1*, *hrpA2* and *hopAJ1*.

HrpS activates a group of T3SS genes that are independent of HrpL.

Since HrpS^C directly bound to *hrpK1*, *hrpA2*, and *hopAJ1*, we then attempted to test if HrpS alone can activate these three genes in vivo, via quantitative reverse transcription-polymerase chain reaction (RT-qPCR). To avoid the induction of T3SS genes via HrpL, we compared the transcription level of these three T3SS genes between a $\Delta hrpL$ mutant containing pAK1900-*hrpS* overexpressing (10-fold) the full-length HrpS protein and a $\Delta hrpL$ strain containing the empty vector pAK1900 (Fig. 2F). The levels of *hrpK1*, *hrpA2*, and *hopAJ1* were induced fourfold by HrpS (Fig. 2F), suggesting that HrpS positively regulates them.

To further verify this result *in vivo*, a *lux*-reporter assay in MM was used to measure the activity of these three promoters by cloning their promoter regions into pMS402 plasmid. The promoter-*lux* reporters and pAK1900-*hrpS* were cotransformed into the *P. savastanoi* pv. *phaseolicola* Δ *hrpL* strain, while the empty pAK1900 was cotransformed with the promoter-*lux* reporters into the Δ *hrpL* mutant as a control. As expected, the activities of all tested promoters were significantly induced by overexpression of HrpS (Fig. 3A to C). As a positive control, *hrpL-lux* was induced dramatically by HrpS in the Δ *hrpL* mutant (Fig. 3D). These results indicate that HrpS directly activates the transcription of *hrpK1*, *hrpA2*, and *hopAJ1*, which are not dependent on HrpL.

A specific 8-bp motif (GTGCCAAA) in the *hrpL* promoter is essential for HrpS activation.

Although the interaction between HrpR/HrpS and the *hrpL* promoter has been well documented, the exact binding motif is still elusive. We attempted to use HrpS^C to pinpoint the HrpS-binding motif on the *hrpL* promoter. First, we truncated the *hrpL* promoter into different lengths (full-length, 242, 200, 191, 185, 175, 165, 158, 150, 143, and 128 bp from ATG) with the same 3' end (Supplementary Table S2). Subsequently, EMSA were performed to detect the binding regions of HrpS^C in these truncated *hrpL* promoters. HrpS^C showed obvious binding shift on the *hrpL* promoter truncations longer than 165 bp (Fig. 4A to F), while there was no binding shift when the truncations were shorter than 158 bp (Fig. 4G to J). We used the promoter of the gene *pepA* encoding leucyl aminopeptidase as a negative control, as it was absent in our ChIP-seq results (Fig. 4K). Therefore, we speculated that the HrpS-binding motif in the *hrpL* promoter is located in a specific sequence from -165 to -150 bp (Fig. 4L).

Previous studies show that the HrpS/HrpR hetero-hexamers binds to an 8-bp region in the *hrpL* promoter (from -146 to -139 bp) in *P. savastanoi* pv. *tomato* DC3000 (Jovanovic et al. 2011; Waite et al. 2017). A similar sequence was found from -161 to -153 bp (GTGCCAAA) in the *hrpL* promoter in *P. savastanoi* pv. *phaseolicola* (Supplementary Fig. S3), which suggests HrpS specifically binds to this motif. In order to verify and characterize this putative motif, we made 18-point mutants carrying single substitutions (A169C, G165A, T164C, T163C, T162C, G161A, T160C, G159T, C158T, C157A, A156C, A155C, A154C, or A153C), deletions (160D or 158D), or insertions (159I1 or 159I2) in the 171-bp *hrpL* promoter. EMSA were performed using the wild-type 165-bp *hrpL* promoter as the positive control and the *pepA* promoter as the negative control (Fig. 5A and B). Intriguingly, HrpS^C bound to probes carrying mutants between -165 to -162 bp (G165A, T164C, T163C, and T162C) (Supplementary Fig. S4) but did not bind to the probes carrying mutants ranged from -161 to -154 bp (G161A, T160C, G159T, C158T, C157A, A156C, A155C, and A154C) (Fig. 5C to G). In addition, the probes carrying deletions or insertions (deletion 160D or 158D, insertion 159I1 or 159I2) also compromised the binding affinity with HrpS^C (Fig. 5L to O). The promoter of *hrpL* with point mutations upstream of the -161 bp or downstream of -154 bp (A169C and A153C) retained binding shifts with HrpS^C (Fig. 5K). Taken together, the EMSA demonstrated that the 8-bp sequence (GTGCCAAA, from -161 to -153 bp) is essential for HrpS binding.

161G, 159G, and 157C are crucial sites in HrpS-binding motif.

To provide further evidence to evaluate the importance of HrpS-binding motif *in vivo*, we measured the activities of a

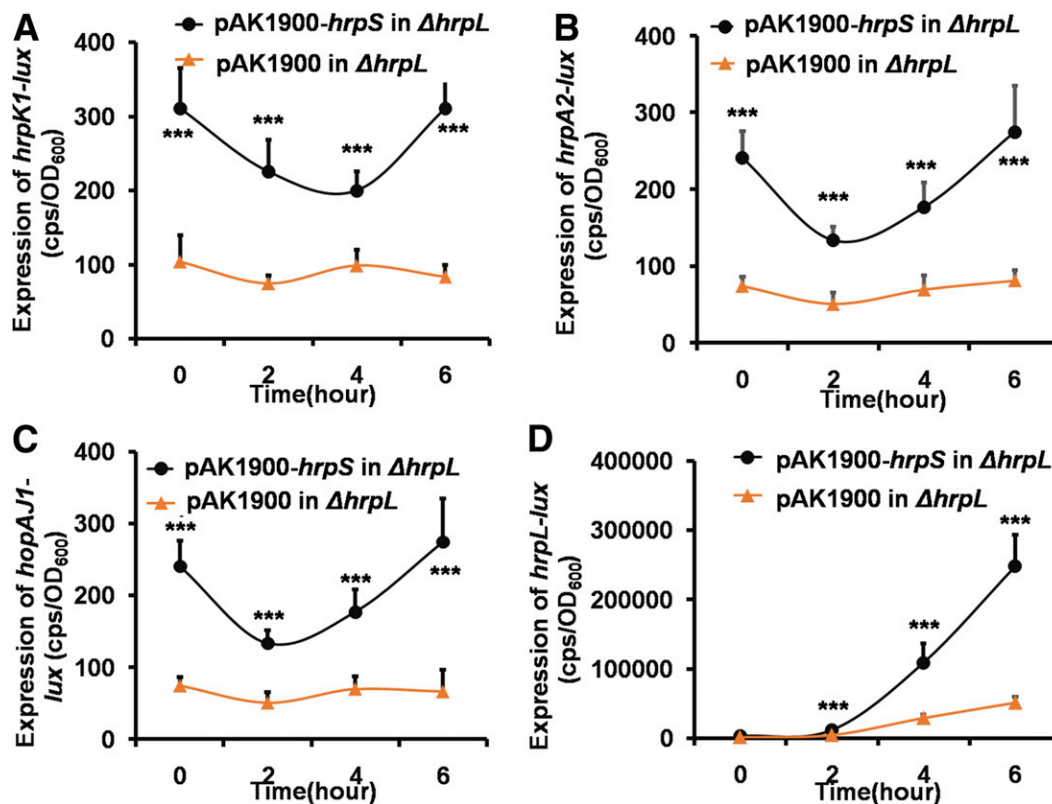


Fig. 3. HrpS directly activated the expression of *hrpA2*, *hrpK1*, and *hopAJ1* in vivo. pAK1900-*hrpS*/pAK1900 and promoter-*lux* reporters (*hrpL*, *hrpK1*, *hrpA2*, and *hopAJ1*) were cotransformed into *P. savastanoi* pv. *phaseolicola* Δ *hrpL* strain. **A**, Relative activities for *hrpK1-lux*, **B**, *hrpA2-lux*, **C**, *hopAJ1-lux*, and **D**, *hrpL-lux*. A single colony was cultured in King's B broth until optical density at 600 nm = 0.6 and was then transferred into minimal medium. Lux activities were measured over 6 h. Three asterisks (***) denote $P < 0.001$ compared with empty vector pAK1900, by Student's *t* test. Each experiment has been performed three times.

series of *hrpL* promoter-*lux* reporters carrying point mutations, insertions, or deletions (G161A, T160C, G159T, C158T, C157A, A156C, A155C, A154C, A153C, 160D, 158D, 159II, and 159I2) in the *P. savastanoi* pv. *phaseolicola* wild-type strain. The activities of *lux*-reporters with G161A, G159T, and C157A were significantly lower than that of the 171-bp wild-type control (20-, 16-, and eightfold, respectively), which demonstrated that they were crucial sites for HrpS binding (Fig. 5P). The *hrpL-lux* reporters containing insertions or deletions also had significantly lower activities compared with the reporter with wild-type *hrpL* promoter (fourfold for 160D, 4.3-fold for 159II, and sixfold for 159I2). In addition, we searched for the newly found HrpS motif in the promoters of *hrpK1*, *hrpA2*,

hopA11, and *hrpS* and found similar sequences (Supplementary Fig. S5). In sum, we showed that 161G, 159G, and 157C are crucial sites in the 8-bp motif of HrpS binding.

HrpS directly binds to a group of non-T3SS genes.

Besides T3SS genes, our ChIP-seq data suggested that HrpS also bound to a group of non-T3SS genes, including the upstream region of PSPPH_1496 (encoding a hypothetical protein), the promoter of PSPPH_3494 (encoding a hypothetical protein), the coding region of PSPPH_3495 (encoding a diguanylate cyclase), the coding region of *alaS* (encoding alanyl-tRNA synthetase), and the upstream as well as the coding region of PSPPH_1525 (encoding a protein homologous

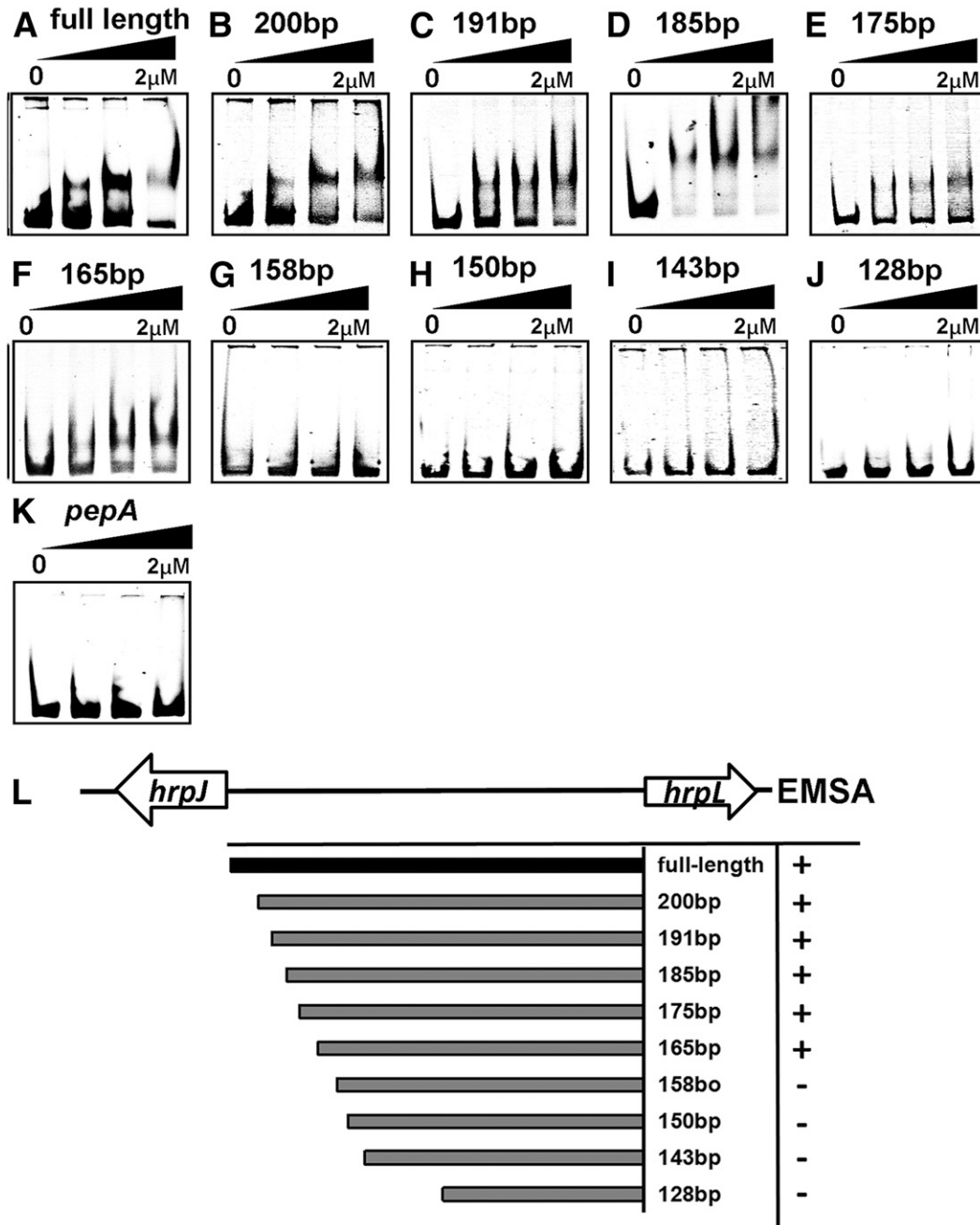


Fig. 4. HrpSC bound to a specific region (–165 to –150 bp) in the *hrpL* promoter. The *hrpL* promoter was truncated into different lengths (full-length, 242, 200, 191, 185, 175, 165, 158, 150, 143, and 128 bp), with the same 3' end, before being subjected to electrophoretic mobility shift assay (EMSA) with HrpSC (0, 0.5, 1.0, and 2.0 μ M). **A to F**, HrpSC showed an obvious binding shift on *hrpL* promoter truncations longer than 165 bp. **G to J**, No binding shift when the truncations were shorter than 150 bp. **K**, As the negative control, the *pepA* promoter had no binding shift with HrpSC. **L**, Summary of all EMSA (A to J). “+” represents obvious binding shift. “-” represents no binding shift.

to XopAD). We located potential HrpS-binding motifs in 12 of 25 regions. Subsequently, EMSA showed that HrpS^C directly bound efficiently to PSPPH_1496 (promoter), PSPPH_3494 (promoter), PSPPH_3495 (open reading frame [ORF]), *alaS* (ORF), and PSPPH_1525 (promoter) (Figs. 6A-E), compared with the negative controls *ribD* (Fig. 2B) and *pepA* (Fig. 4K). In sum, our result demonstrated that HrpS specifically binds to a

group of non-T3SS genes, strongly indicating that HrpS alone regulates these genes.

HrpS alone regulates a group of non-T3SS genes.

To investigate if HrpS regulates PSPPH_1496, PSPPH_3494, and PSPPH_1525, the promoter-*lux* or promoter-ORF-*lux* reporters were constructed to measure their transcriptional and

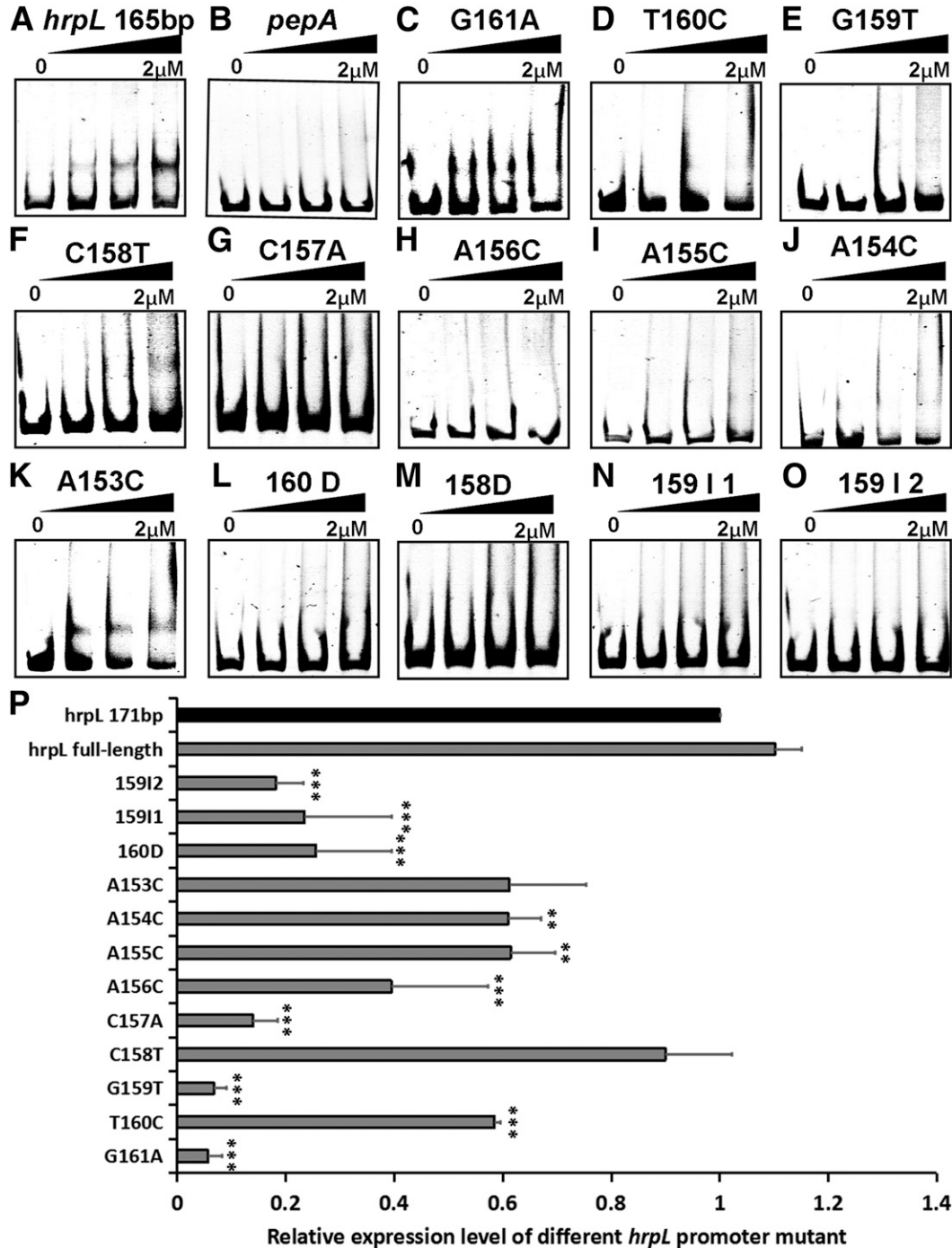


Fig. 5. Electrophoretic mobility shift assay revealed a specific 8-bp HrpS-motif (GTGCCAAA) in the *hrpL* promoter. HrpSC (0, 0.5, 1.0, and 2.0 μ M) was mixed with **A**, the 165-bp *hrpL* promoter as the positive control and **B**, the *pepA* promoter as the negative control. **C** to **J**, mutants ranging from -161 to -154 bp (G161A, T160C, G159T, C158T, C157A, A156C, A155C, and A154C). **K**, A mutant out of the -161 to 154 bp region in the *hrpL* promoter (A153C). **L** to **O**, Mutants carrying deletion or insertion (deletion 160D and 158D, insertion 159I1 and 159I2). Each reaction was performed three times. **P**, Mutagenesis in the HrpS-binding motif via a *hrpL*-*lux* reporter. Activities of *hrpL*-*lux* reporters carrying point mutations, insertions, or deletions (G161A, T160C, G159T, C158T, C157A, A156C, A155C, A154C, A153C, 160D, 158D, 159I1, and 159I2) in the *P. savastanoi* pv. *phaseolicola* wild-type strain in minimal medium for 6 h. An asterisk (*) denotes statistical significance among these mutants, two asterisks (**) denote $P < 0.01$, and three (***) $P < 0.001$ compared with the empty vector pAK1900, HrpR, and RphR, by Student's *t* test, respectively. Each experiment has been performed three times.

translational levels. First, we cotransformed pAK1900-*hrpS* and different *lux*-reporters into the *P. savastanoi* pv. *phaseolicola* Δ *hrpL* strain. The cotransformation of pAK1900 empty vector and the *lux*-reporters was used as the negative control. The overexpression of HrpS induced the level of PSPPH_1496-*lux* and PSPPH_3494-*lux* eight- and fourfold in MM, respectively (Fig. 7A and B). On the contrary, the level of PSPPH_1525-*lux* displayed a fivefold decrease by HrpS in MM (Fig. 7C). In addition, these reporter strains were also cultured and were tested in KB medium. After 20 h, all these reporters were significantly induced by HrpS in KB (Supplementary Fig. S6). We cotransformed a negative control *gntR-lux* (*gntR* encoding the repressor for gluconate utilization system GNT-I) and pAK1900-*hrpS*/pAK1900 into Δ *hrpL*. There was no change in the *gntR-lux* levels between the Δ *hrpL*/pAK1900-*hrpS* and Δ *hrpL*/pAK1900 in MM or KB. We concluded that, in MM, HrpS positively regulated PSPPH_1496 and PSPPH_3494 but negatively regulated PSPPH_1525. However, in KB, HrpS positively regulates all three genes. These results demonstrate that HrpS specifically regulates a group of non-T3SS in *P. savastanoi* pv. *phaseolicola*, which expands its original regulatory pathway in T3SS. Taken together, our results reveal that HrpS directly regulates PSPPH_1496, PSPPH_3494, and PSPPH_1525. HrpS not only regulates T3SS genes but also a group of non-T3SS genes.

HrpS positively regulates motility and negatively regulates biofilm formation.

A recent study showed that Chp8 (PSPTO_2907), which can respond to c-di-GMP and HrpRS, is related to biofilm formation and motility in *P. savastanoi* pv. *tomato* DC3000 (Engl et al. 2014). Therefore, we tested whether HrpS plays a role in biofilm formation and motility. We detected the motility for wild-type *P. savastanoi* pv. *phaseolicola*, a Δ *hrpS* strain, and its complemented strain (Δ *hrpS*/pAK1900-*hrpS*) on KB medium (0.4% agar) at 28°C. After 2 days, the Δ *hrpS* strain showed a compromised motility compared with the wild-type *P. savastanoi* pv. *phaseolicola* (Fig. 8A). Biofilm formation was also measured for these three strains, which were incubated in a 96-well plate at an optical density at 600 nm (OD₆₀₀) = 0.1. The Δ *hrpS* mutant strain produced more biofilm than did wild type and the complemented strain (Fig. 8B), indicating that HrpS has a negative effect on biofilm formation. As a control, the loss of the *hrpS* gene

did not affect the growth rate in KB, compared with the wild-type *P. savastanoi* pv. *phaseolicola* (Supplementary Fig S9).

HrpR alone didn't bind to promoters of *hrpK1*, *hrpA2*, and *hopAJ1*.

Given that HrpR and HrpS form a hetero-hexamers in the previous report (Lawton et al. 2014), we investigated if HrpR alone can bind to the promoters of three tested T3SS genes. First, the C-terminal DNA binding domain HrpR^C (amino acids 241 to 306, homologous to the counterpart in HrpS^C) was successfully expressed and was purified by His6x-tag in *E. coli* BL21 star (DE3). Subsequent EMSA showed that HrpR^C bound to the *hrpL* promoter and *ribD* (ORF) (Supplementary Fig. S8) but did not bind to the promoter of *hrpK1*, *hrpA2*, and *hopAJ1*, because they were absent in the ChIP-seq result of HrpR. In sum, although HrpR directly binds to the *hrpL* promoter, it cannot bind to the T3SS genes (*hrpK1*, *hrpA2*, and *hopAJ1*), which suggests the global regulation of HrpS in the *P. savastanoi* T3SS.

DISCUSSION

In this study, we provided biochemical and genetic evidence illustrating the molecular mechanism of HrpS in regulating a group of T3SS genes and non-T3SS genes. Our ChIP-seq assays for HrpR, HrpS, and HrpL found that two, 20, and 13 binding peaks were located in the intergenic region, respectively (Fig. 1A, C, and E). These results led us to test if HrpS independently regulates both T3SS and non-T3SS genes. We noticed that a majority of HrpS-binding peaks were located in coding regions of the genome, which is different from the notion that transcription factors mostly bind to promoter regions. We have shown direct binding between HrpS and two peaks in coding regions (PSPPH_3495 and *alaS*) (Fig. 6C and E), suggesting that HrpS has more and complicated regulatory functions in recognizing coding regions. We propose that HrpS directly regulates non-T3SS genes but still relies on HrpR and HrpL to regulate other T3SS genes. Given the limited peaks and low DNA-binding affinity by HrpR, we could not specify the binding site of HrpR in this study. We speculate that HrpR has a different and weak binding site, which might be close to the HrpS motif in the promoter of *hrpL*. HrpS may form a homo-hexamers to regulate gene expression. We propose that the formation of a HrpRS complex facilitates the binding between HrpS and DNA.

HrpL-binding loci have been previously characterized by ChIP-seq assays in *P. savastanoi* pv. *tomato* DC3000 (Lam et al. 2014). Here, our ChIP-seq revealed 31 HrpL-binding peaks, including 15 T3SS genes in *P. savastanoi* pv. *phaseolicola*. Among these T3SS genes, 12 were found in *P. savastanoi* pv. *tomato* DC3000 (Lam et al. 2014). One non-T3SS gene (PSPPH_1855) was shared in ChIP-seq results for HrpS and HrpL. In a previous study, PSPPH_1525 is regulated by HrpL in *P. savastanoi* pv. *phaseolicola* (Vencato et al. 2006a). Thirteen new HrpL targets were identified in this study: *cysS*, PSPPH_2242, PSPPH_2291, PSPPH_2408, PSPPH_2649, *nirB*, *garK*, PSPPH_3733, PSPPH_2520, *baeS1*, *dnaE2*, PSPPH_2789, and PSPPH_2897 (Supplementary Table S3). Further study needs to be done to test whether HrpL regulates these non-T3SS genes, which may find novel and important roles of HrpL in other cellular processes. In sum, our HrpL ChIP-seq data not only agreed with the previous publication but also revealed new potential HrpL targets with novel functions.

The DNA-binding motif of HrpS in *Erwinia amylovora* is a dyad symmetry sequence (TGCAA-N4-TTGCA) (Lee et al. 2016), which is absent in the promoter of *hrpL* in *P. savastanoi*. In the present study, we pinpointed the HrpS-binding motif as an 8-bp crucial sequence (GTGCCAAA) from -161 to -154 bp in the *hrpL* promoter (Fig. 4). Among this motif, 161G, 159G,

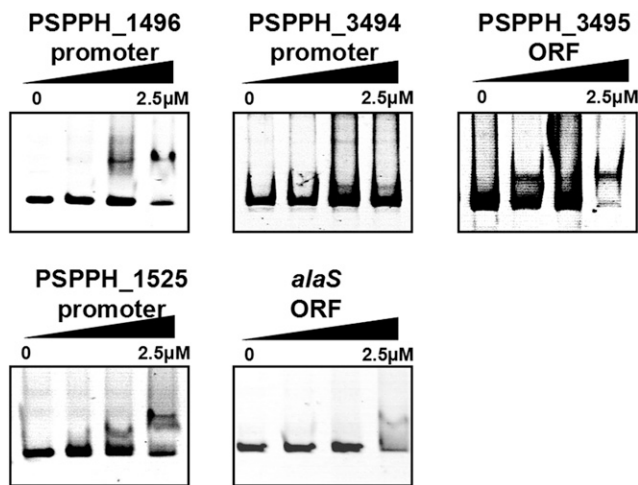


Fig. 6. HrpS directly bound to a group of non-type III secretion system genes. HrpS (0, 0.5, 1.0, and 2.0 μ M) was mixed with 40-ng probes PSPPH_1496 (promoter), PSPPH_3494 (promoter), PSPPH_3495 (ORF), PSPPH_1525 (promoter), and *alaS* (ORF [open reading frame]).

and 157C were crucial sites for HrpS binding (Fig. 5). The motif can also be found in the promoters of three new HrpS targets (*hrpK1*, *hrpA2*, and *hopAJ1*).

In our previous study, microarray analyses were conducted to identify the gene expression profiles in a *hrpR* mutant, a *hrpS* mutant, a *hrpL* mutant, and the wild-type strain under T3SS-

inducing conditions in *P. savastanoi* pv. *tomato* DC3000 (Lan et al. 2006). Our previous microarray showed that HrpR regulates itself and a group of non-T3SS genes. HrpS controls a large group of T3SS as well as non-T3SS genes. HrpL mainly regulates T3SS genes that largely overlapped with the HrpS regulon. Given that two different strains were used for ChIP-seq

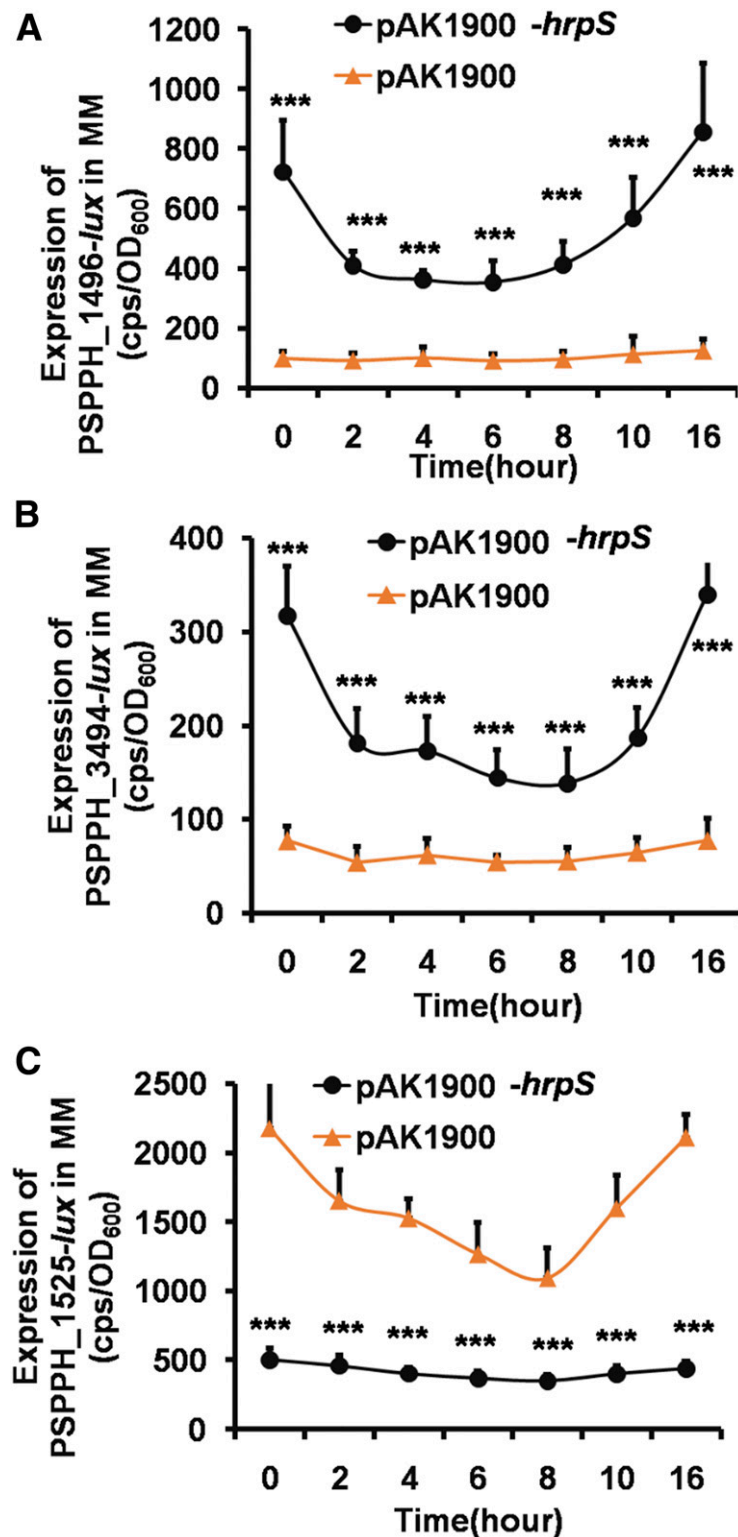


Fig. 7. HrpS regulated a group of non-type III secretion system genes. **A**, Activities of P_{SPPH_1496}-lux, **B**, P_{SPPH_3494}-lux, and **C**, P_{SPPH_1525}-lux in the *P. savastanoi* pv. *phaseolicola* Δ *hrpL* strain carrying pAK1900-*hrpS* or empty pAK1900. Single colonies were cultured in King's B broth until optical density at 600 nm = 0.6 and were then transferred into minimal medium. One asterisk (*) denotes $P < 0.05$, two (**) denote $P < 0.01$, and three (***) $P < 0.001$ compared with the empty vector pAK1900, HrpR, and RphR, by Student's *t* test, respectively. Each experiment has been performed three times.

(*P. savastanoi* pv. *phaseolicola*) and microarray (*P. savastanoi* pv. *tomato* DC3000), we first tried to find the corresponding genes between two strains. In microarray data, we found three homologous genes that appeared in ChIP-seq of HrpR, and one of them showed a more than twofold difference. The *hrpR* microarray data showed that HrpR had a positive regulation on itself. Among 28 genes shared between our ChIP-seq of HrpS and microarray for the *hrpS* mutant, 15 of the genes showed significant differences compared with the wild-type, including non-T3SS genes PSPTO_0834 (homologous to PSPPH_3759), PSPTO_3087 (homologous to PSPPH_2294), and T3SS genes *hopI1*, *hopAJ1*, *hrpK1*, *hrpL*, *hrpA2* operon, *hrcV* operon, *hrpW1*, and *hopAA1*. Eighty-seven percent of the HrpL ChIP-seq binding genes have similar genes in *P. savastanoi* pv. *tomato* DC3000. Half of these genes were differently expressed in the *hrpL* mutant compared with the wild type in the microarray, including *hrpW1*, *hrpA1-hrpZ1-hrpB-hrcJ-hrpD-hrpE-HrpF* operon (*hrpA1* operon), and *hrpJ-hrcV-hrpQ-hrcN-hrpO-hrpP-hrcQa-hrcQb-hrcR-hrcS-hrcT-hrcU* operon (*hrpJ* operon), *hrpK1*, PSPTO_2105 (homologous to PSPPH_1855, encoding thiamine biosynthesis lipoprotein), *shcV*, *hopR1*, and *hopI1*.

In the present study, we found that HrpS directly regulated a group of T3SS and non-T3SS genes for the first time, which made HrpS a global regulator. By using EMSA and *lux*-reporter assays in *P. savastanoi*, we showed that HrpS directly regulated PSPPH_1496 operon, PSPPH_3494, and PSPPH_1525. In the Δ *hrpL* mutant cultured in MM, HrpS functioned as a negative regulator of PSPPH_1525 (Fig. 7D). However, in the Δ *hrpL* mutant cultured in KB, HrpS is a positive regulator of

PSPPH_1525 (Fig. 7H). In addition, HrpS positively regulates PSPPH_1496 in KB and PSPPH_1525 in MM, which indicates that HrpS may sense different environmental signals to exert opposite regulatory roles. We also observed that HrpS positively regulated motility and negatively tuned biofilm formation in KB but no significant differences occurred in MM. The highly activated T3SS may interfere with motility and biofilm formation. HrpS might have similar binding peaks in KB as in MM. Taken together, these findings expanded our original understanding of HrpS and suggests possibilities for the study of potential functions of HrpS on other pathways in the future. Previous study has demonstrated that HrpR alone bound to the *hrpL* promoter (Jovanovic et al. 2011). Our EMSA verified their binding (Supplementary Fig. S7), although the affinity (dissociation constant [Kd] = 7 μ M) was lower than that of HrpS (Kd = 0.5 μ M). These results might explain the absence of the *hrpL* promoter in our ChIP-seq data of HrpR. We propose that, although HrpRS mostly function as a hetero-hexamers, they have different individual targets and regulons. HrpS has more important roles than HrpR in regulating their own downstream genes.

Here, we present a new regulatory model of HrpR and HrpS in regulating various T3SS and non-T3SS genes. HrpS induces three T3SS genes (*hrpK1*, *hrpA2*, and *hopAJ1*) by directly binding to their promoters, which is independent of HrpL. HrpS also directly activates the expression of non-T3SS genes (PSPPH_1496, PSPPH_1525, and PSPPH_3494) (Fig. 9). In particular, HrpS directly regulates the downstream genes by recognizing a specific 8-bp motif (GTGCCAAA) in the promoters of *hrpL* and other downstream genes. HrpR alone has no regulatory role on the *hrpL* expression. In summary, our study demonstrates that HrpS plays a central role in the T3SS regulatory cascade and activates a group of T3SS as well as non-T3SS genes that bypass HrpL. This work decipheres the key individual roles of HrpS in the regulatory network of T3SS, providing novel insights for developing new strategies to control *P. savastanoi* infection in the future.

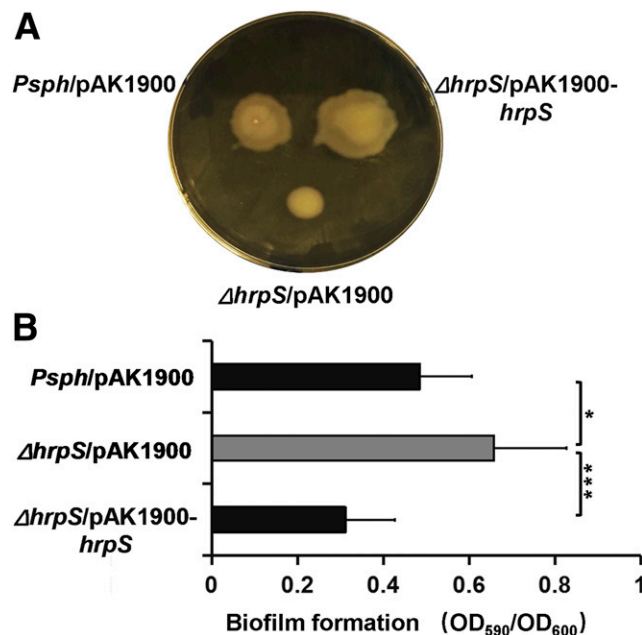


Fig. 8. The effects of HrpS on motility and biofilm formation. **A**, Motility was observed for the Δ *hrpS* complementation strain (pAK1900-*hrpS*), Δ *hrpS* (pAK1900), and *P. savastanoi* pv. *phaseolicola* wild type (pAK1900) grown in King's B medium (0.4% agar) at 28°C for 2 days. **B**, Biofilm formation was detected among Δ *hrpS* complementation strain (pAK1900-*hrpS*), Δ *hrpS* (pAK1900), and *P. savastanoi* pv. *phaseolicola* wild type (pAK1900). Strains were incubated to an optical density at 600 nm (OD₆₀₀) = 1.0 and were then diluted to OD₆₀₀ = 0.1 in the 96-well plate. After 3 days of static conditions, the biofilm was stained by 0.1% crystal violet and measurements were taken at 590 nm absorbance. The total result was expressed as OD₅₉₀/OD₆₀₀. One asterisk (*) denotes $P < 0.05$ and three asterisks (***) denote $P < 0.001$ compared with *P. savastanoi* pv. *phaseolicola* wild type (pAK1900) and Δ *hrpS* complementation strain (pAK1900-*hrpS*), by Student's *t* test.

MATERIALS AND METHODS

Bacterial strains and culture condition.

The bacterial strains, plasmids, and primers used are listed in Supplementary Table S4. The *P. savastanoi* pv. *phaseolicola* 1448A strains used in this study were the wild-type and the Δ *hrpL* mutant. The *P. savastanoi* pv. *phaseolicola* strains were grown in KB medium (King et al. 1954) at 28°C to OD₆₀₀ = 2.0 to 2.5. Then bacteria were centrifuged, were washed twice with MM (50 mM KH₂PO₄, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, 1.7 mM NaCl, and 10 mM fructose, pH 6.0) (Huynh et al. 1989; Schumacher et al. 2014), were cultured in MM for 6 h before further experimentation. Antibiotic concentrations used were: rifampicin, 25 μ g/ml; kanamycin, 100 μ g/ml; and carbenicillin, 200 μ g/ml. The *E. coli* BL21 (DE3) strain was grown in Luria Bertani (LB) medium at 37°C and antibiotics were used at the following concentrations: kanamycin, 50 μ g/ml and ampicillin, 50 μ g/ml.

ChIP-seq.

ChIP was performed in MM as previously described (Blasco et al. 2012), with minor modifications. An empty pHM2, pHM2-*hrpS*_{psph}-FLAG, pHM2-*hrpR*_{psph}-FLAG, or pHM2-*hrpL*_{psph}-FLAG plasmid was transformed into wild-type *P. savastanoi* pv. *phaseolicola* and were then cultured in KB medium until the OD₆₀₀ = 0.4 to 0.6 before transferring to MM liquid medium with spectinomycin (50 μ g/ml) for 6 h. The strains were treated with 1% formaldehyde for 10 min at 37°C. Subsequently, 125 mM glycine was added to stop the cross-linking. After centrifugation, bacterial pellets were washed

twice with Tris buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and were then resuspended in 500 μ l IP buffer (50 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, mini-protease inhibitor cocktail [Roche]). Next the DNA was sonicated into fragments ranged from 100 to 300 bp. The cellular debris was removed, and the supernatant was saved as the input sample in the IP experiments. Both the control and IP samples were washed by protein A beads (General Electric). Then in the IP buffer, the samples were mixed with 50 μ l of agarose-conjugated anti-FLAG antibodies (Sigma). The subsequent washing, crosslink reversal, and purification of ChIP-DNA was performed following previously reported protocols (Blasco et al. 2012). DNA fragments (150 to 250 bp) were selected for library construction and sequencing using the NEXTflex ChIP-Seq kit (Bio Scientific). The libraries were sequenced using the HiSeq 2000 system (Illumina). ChIP-seq results were mapped to the *P. savastanoi* pv. *phaseolicola* genome using TopHat (version 2.0.0) with two mismatches allowed (Trapnell et al. 2009). The binding peaks were detected by MACS software (version 2.0.0) (Zhang et al. 2008); nearly all of them were performed in two experiments and the reported peaks are found in both experiments. All the ChIP-seq data were submitted to NCBI (accession number GSE103921).

Construction of $\Delta hrpL$ and $\Delta hrpS$ mutants.

To construct the $\Delta hrpL$ and $\Delta hrpS$ mutant, a pAK18-mobsacB suicide plasmid was used (Kvitko and Collmer 2011).

hrpL-Up-F/R and *hrpS*-Up-F/R were used to amplify 1-kb fragments upstream of *hrpL* and *hrpS* genes, while *hrpL*-Down-F/R and *hrpS*-Down-F/R were used to amplify 1.5-kb fragments downstream of *hrpL* and *hrpS*, respectively. First, the purified upstream and downstream fragments of *hrpS* (using *Pst*I) or *hrpL* (using *Xba*I) were cut with *Pst*I and *Xba*I, respectively, before being linked by T4 ligase. Second, the linked fragments were cloned to pAK18mobsacB (*hrpL* using *Xba*I/*Hind*III and *hrpS* using *Bam*HI/*Pst*I). Next, the constructed vectors were transformed into *E. coli* S17. After 2 days of mating of the *P. savastanoi* pv. *phaseolicola* wild-type strain and the transformed *E. coli* S17 in LM medium (10.0 g of tryptone, 6.0 g of yeast extract, 0.6 g of NaCl, 0.4 g of MgSO₄·7H₂O, and 1.5 g of K₂HPO₄ in 1 liter), the conjugated strains survived in the KB plate with rifampin (25 μ g/ml) and kanamycin (100 μ g/ml). Next, colonies were transferred to KB plates with kanamycin, rifampin, and 10% sucrose, to screen for a single-crossover event. Colonies on the sucrose plate were then cultured in both KB with kanamycin and rifampin and KB with only rifampin. Loss of kanamycin resistance indicated a double crossover. Finally, the $\Delta hrpL$ and $\Delta hrpS$ mutants were verified by PCR, using primers *hrpL*-ORF-F/R and *hrpS*-ORF-F/R.

Protein expression and purification.

To express HrpR and HrpS, their coding regions were amplified by PCR (*hrpR*-ORF-F/R and *hrpS*-ORF-F/R), were then digested, and were ligated to pET28a (*Bam*HI/*Xho*I), which has

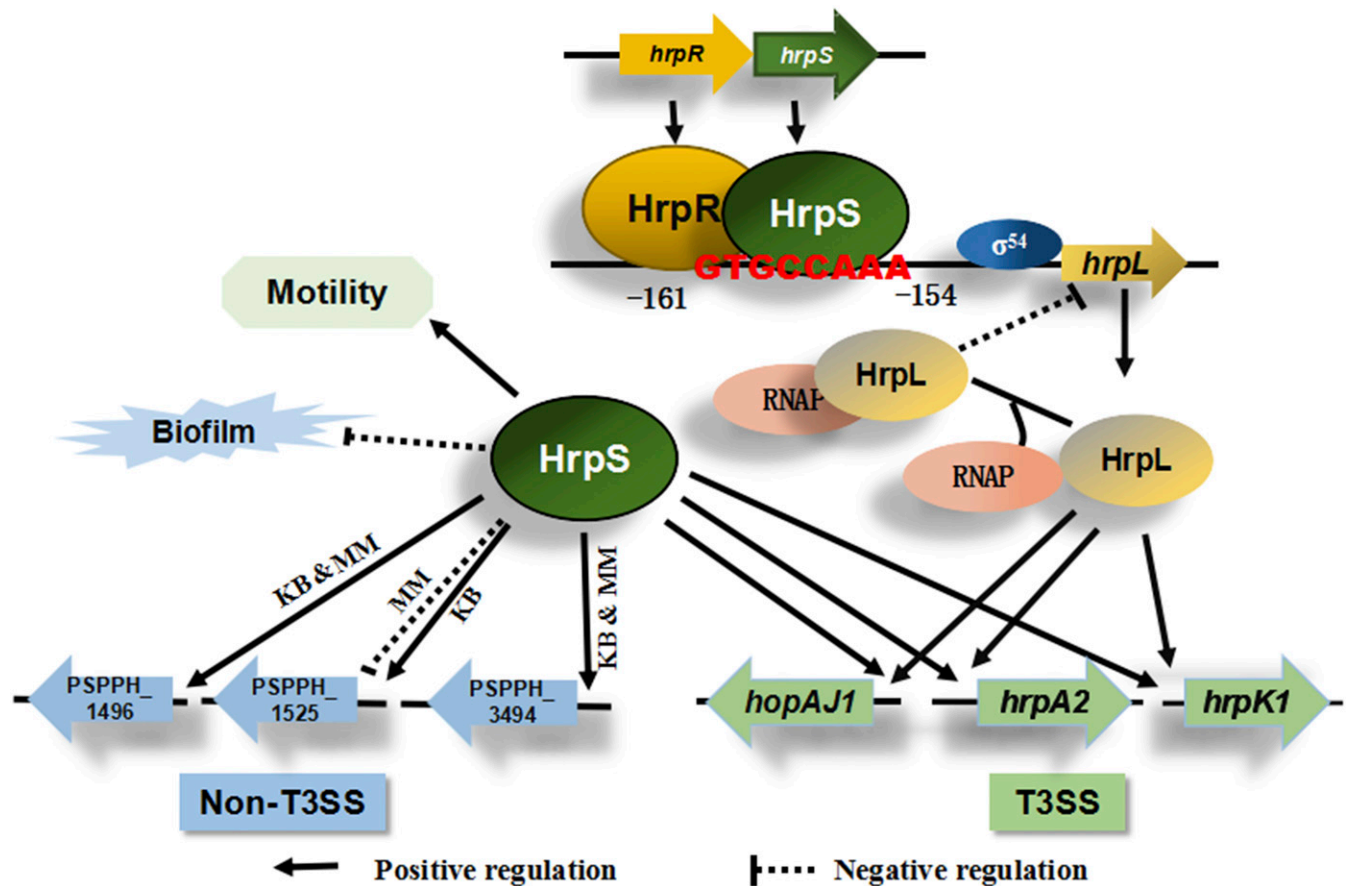


Fig. 9. Proposed models of HrpS-mediated regulation of type III secretion system (T3SS) and non-T3SS genes in *Pseudomonas savastanoi*. HrpR and HrpS are encoded by the *hrpRS* operon and form a hetero-hexamers. In T3SS-inducing conditions, HrpS not only activates *hrpL* but also *hrpK1*, *hrpA2*, and *hopAJ1*, which are also activated by HrpL. HrpL performs self-repression via the HrpL-RNAP complex occupying the HrpRS binding regions. An 8-bp sequence (GTGCCAAA) on the *hrpL* promoter is essential for HrpS binding. HrpR alone can bind to the *hrpL* promoter but cannot activate its transcription. HrpS is a global regulator on both T3SS and non-T3SS genes, including PSPPH_1496, PSPPH_1525, and PSPPH_3494. Further, HrpS positively regulates pathways involved in motility but negatively regulates pathways involved in biofilm formation.

a His6-tag at its N-terminus. To coexpress HrpRS, the *hrpRS* operon was amplified by PCR (HrpR-ORF-F and HrpS-ORF-R) and was then ligated to pET28a. Although HrpRS had higher activity, it was lower than expected. The C-terminal DNA binding domain of HrpR was truncated from amino acids 241 to 306 aa (nucleotide 721 to 921 bp), and HrpS was truncated from 235 to 303 aa (nucleotide 703 to 909 bp). *hrpR*₇₂₁₋₉₂₁ and *hrpS*₇₀₃₋₉₀₉ fragments were amplified by PCR, were then digested, and were linked to pET28a (*Bam*HI/*Xho*I). pET28a-*hrpR*₇₂₁₋₉₂₁ and pET28a-*hrpS*₇₀₃₋₉₀₉ were then transformed into *E. coli* BL21 star (DE3). Briefly, the selected single colony was cultured in LB liquid overnight, was then transferred to 500 ml of fresh LB at 37°C, with shaking. When the OD₆₀₀ = 0.4 to 0.6, 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) was added into the volume to overexpress protein at 16°C for 16 h. After centrifugation, strain pellets were resuspended in buffer A (500 mM NaCl, 25 mM Tris-HCl, pH7.5, 5% glycerol, 1 mM dithiothreitol [DTT], 1 mM phenyl-methanesulfonyl fluoride). Sonication disrupted cells, which were then centrifuged at 4°C, 12000 rpm for 30 min. The supernatant was filtered through a 0.45-μm filter and was mixed with a Ni-NTA column (Qiagen) that had been treated with buffer A. Then the column was washed twice with washing buffer (500 mM NaCl, 25 mM Tris-HCl, 60 mM imidazole, pH7.5, 5% glycerol, 1 mM DTT, 1 mM phenyl-methanesulfonyl fluoride) and was purified by gradients of 100 to 500 mM imidazole prepared in buffer A. Proteins were concentrated by centrifuge (Millipore) at 4°C and were then supplemented with 20% glycerol and stored at -80°C.

EMSA.

DNA fragments were amplified by PCR. Different concentrations of HrpS (0, 0.5, 1.0, and 2.0 μM) and HrpR (0, 1.6, 3.5, and 7.0 μM) were interacted with 40 ng of purified DNA fragments in a 20-μl volume with sample buffer (10 mM Tris-HCl, pH 7.5, 70 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1 mM EDTA, and 10% glycerol and 0.1% [wt/vol] Triton X-100) (Jovanovic et al. 2011) at room temperature for 20 min. Free DNA and protein-DNA was divided in 8% native polyacrylamide gel electrophoresis at 80 V for 2 h and the gel was stained by Gel stain (TransGen Biotech) at room temperature and was visualized by the image analysis program (Beijing Liuyi).

Truncation and site mutagenesis assay of *hrpL* promoter.

To pinpoint the precise HrpS^c binding region on the *hrpL* promoter, it was truncated into 128, 143, 150, 158, 165, 175, 185, 191, and 200 bp (same 3' end at the ATG). Then, an EMSA array was performed, using HrpS^c and these truncates to identify the binding region. To map the binding regions, 171 bp of *hrpL* promoter containing single-point mutation (A169C, G165A, T164C, T163C, T162C, G161A, T160C, G159T, C158T, C157A, A156C, A155C, A154C, A153C, deletion 160D, or 158D and insertion 159I1 or 159I2) were designed and linked to the pMS402 (*Xho*I/*Bam*HI). EMSA was performed to identify the key nucleotides. Using the 165-bp *hrpL* promoter as positive control, the fragments containing single-point mutation (A169C, G165A, T164C, T163C, T162C, G161A, T160C, G159T, C158T, C157A, A156C, A155C, A154C, A153C, deletion 160D or 158D, and insertion 159I1 or 159I2) were used as probes to bind with *hrpL* promoter. The corresponding *hrpL-lux* reporters containing point mutation were transformed into *P. savastanoi* pv. *phaseolicola* wild type, measuring the luminance using a plate reader in MM.

Luminescence screening assays.

For *lux*-reporter fusion, the promoters of *hrpL*, *hrpK1*, *hrpA2*, *hopAJ1*, *PSPPH_1525*, *PSPPH_1496*, or *PSPPH_1496* were cloned into the pMS402 plasmid (*Xho*I/*Bam*HI) (Duan et al.

2003). The *lux* reporters were transformed into *P. savastanoi* pv. *phaseolicola* strains. The resulting strains were first cultured to OD₆₀₀ = 0.6 in KB. Then the cultures were washed twice with MM medium and were incubated in MM with shaking to measure LUX activities. In addition, the LUX activities for *PSPPH_1525*, *PSPPH_1496*, or *PSPPH_1496* were also measured in KB, starting at OD₆₀₀ = 0.01. Bacterial growth was monitored at the same time by measuring the OD₆₀₀ in a Synergy 2 plate reader (BioTek). The LUX activity value was acquired by CPS (counts per second)/OD₆₀₀.

RT-qPCR.

P. savastanoi pv. *phaseolicola* strains were cultured at 28°C in KB and were then transformed to MM medium at OD₆₀₀ = 0.4 to 0.6. After inducing for 6 h, the bacteria were harvested by centrifugation at 6,000 rpm for 4 min. The RNA was purified by the Purification Kit (Sangon Biotech). The cDNA synthesis was performed by using a cDNA synthesis kit (Takara) with 1.2 μg of total RNA in each reaction; 100 ng of each sample was added into each well. Each reaction was performed in triplicate in 20-μl reaction volumes, with 16S rRNA as a control. For each reaction, 200 nM primers were used for RT-qPCR. The reactions were run at 37°C for 15 min, 85°C, for 5 s, and 4°C to save. Next, RT-qPCR was conducted under SYBR Green (Tiangen Biotech) instruction using the ABI 7500 Real-Time PCR System. The reaction includes 15 min at 95°C and 40 cycles of 10 s at 95°C and 32 s at 60°C. The fold change represents relative expression level of mRNA and can be calculated by the values of 2^{-ΔΔCt}.

Swarming motility assay.

A single colony was grown in KB liquid overnight and was then transferred at 1:100 to 2 ml of fresh KB medium and grown at 28°C until OD₆₀₀ = 0.6. Two microliters of the cultures were spotted onto soft KB or MM plates (0.4% agar). The plates were incubated at 28°C for 48 h and swarming across the plate was measured as the diameter of spread. All assays were performed in triplicate.

Biofilm formation assay.

Biofilm formation was measured as described previously (Engl et al. 2014). Briefly, the *ΔhrpS* mutant and wild-type strains were grown in KB or MM at 28°C to OD₆₀₀ = 1.0, were then diluted to OD₆₀₀ = 0.1 and 150 μl was added to 96-well plates, and all were incubated at 28°C for 72 h under static conditions. After 72 h of incubation the OD₆₀₀ was measured and medium was removed. Biofilm formation in each well was washed gently three times with sterile phosphate buffered saline and was then stained by 150 μl of 0.1% crystal violet and incubated for 20 min. The extra crystal violet was removed and wells were washed gently three times with distilled water. The stained biofilms were resuspended in 150 μl of 75% ethanol and the OD₅₉₀ was measured for each well. Biofilm formation was expressed as OD₅₉₀/OD₆₀₀. All assays were performed three times.

LITERATURE CITED

- Alfano, J. R., Klm, H. S., Delaney, T. P., and Collmer, A. 1997. Evidence that the *Pseudomonas syringae* pv. *syringae* *hrp*-linked *hrmA* gene encodes an Avr-like protein that acts in an *hrp*-dependent manner within tobacco cells. *Mol. Plant-Microbe Interact.* 10:580-588.
- Blasco, B., Chen, J. M., Hartkoorn, R., Sala, C., Uplekar, S., Rougemont, J., Pojer, F., and Cole, S. T. 2012. Virulence regulator EspR of *Mycobacterium tuberculosis* is a nucleoid-associated protein. *PLoS Pathog.* 8: e1002621.
- 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.

- 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., and Alfano, J. R. 2000. *Pseudomonas syringae* Hrp type III secretion system and effector proteins. *Proc. Natl. Acad. Sci. U.S.A.* 97:8770-8777.
- Deng, X., Lan, L., Xiao, Y., Kennelly, M., Zhou, J. M., and Tang, X. 2010. *Pseudomonas syringae* two-component response regulator RhpR regulates promoters carrying an inverted repeat element. *Mol. Plant-Microbe Interact.* 23:927-939.
- Deng, X., Liang, H., Chen, K., He, C., Lan, L., and Tang, X. 2014. Molecular mechanisms of two-component system RhpRS regulating type III secretion system in *Pseudomonas syringae*. *Nucleic Acids Res.* 42:11472-11486.
- Deng, X., Xiao, Y., Lan, L., Zhou, J. M., and Tang, X. 2009. *Pseudomonas syringae* pv. *phaseolicola* mutants compromised for type III secretion system gene induction. *Mol. Plant-Microbe Interact.* 22:964-976.
- Duan, K., Dammel, C., Stein, J., Rabin, H., and Surette, M. G. 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol. Microbiol.* 50:1477-1491.
- Engl, C., Waite, C. J., McKenna, J. F., Bennett, M. H., Hamann, T., and Buck, M. 2014. Chp8, a diguanylate cyclase from *Pseudomonas syringae* pv. *tomato* DC3000, suppresses the pathogen-associated molecular pattern flagellin, increases extracellular polysaccharides, and promotes plant immune evasion. *MBio* 5:e01168-14.
- Galán, J. E., and Collmer, A. 1999. Type III secretion machines: Bacterial devices for protein delivery into host cells. *Science* 284:1322-1328.
- 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.
- Jovanovic, M., James, E. H., Burrows, P. C., Rego, F. G., Buck, M., and Schumacher, J. 2011. Regulation of the co-evolved HrpR and HrpS AAA + proteins required for *Pseudomonas syringae* pathogenicity. *Nat. Commun.* 2:177.
- Jovanovic, M., Waite, C., James, E., Synn, N., Simpson, T., Kotta-Loizou, I., and Buck, M. 2017. Functional characterization of key residues in regulatory proteins HrpG and HrpV of *Pseudomonas syringae* pv. *tomato* DC3000. *Mol Plant Microbe Interact.* 30:656-665.
- King, E. E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
- Kubori, T., Matsushima, Y., Nakamura, D., and Uralil, J., Lara-Tejero, M., Sukhan, A., Galán, J. E., and Aizawa, S.-I. 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* 280:602.
- Kvitko, B. H., and Collmer, A. 2011. Construction of *Pseudomonas syringae* pv. *tomato* DC3000 Mutant and Polymutant Strains. In: *Plant Immunity. Methods in Molecular Biology (Methods and Protocols)*, Vol. 712. J. McDowell, ed. Humana Press, Totowa, NJ, U.S.A.
- Lam, H. N., Chakravarthy, S., Wei, H. L., BuiNguyen, H., Stodghill, P. V., Collmer, A., Swingle, B. M., and Cartinhour, S. W. 2014. Global analysis of the HrpL regulon in the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 reveals new regulon members with diverse functions. *PLoS One* 9:e106115.
- Lan, L., Deng, X., Zhou, J., and Tang, X. 2006. Genome-wide gene expression analysis of *Pseudomonas syringae* pv. *tomato* DC3000 reveals overlapping and distinct pathways regulated by *hrpL* and *hrpRS*. *Mol. Plant-Microbe Interact.* 19:976-987.
- Lawton, E., Jovanovic, M., Joly, N., Waite, C., Zhang, N., Wang, B., Burrows, P., and Buck, M. 2014. Determination of the self-association residues within a homomeric and a heteromeric AAA+ enhancer binding protein. *J. Mol. Biol.* 426:1692-1710.
- Lee, J. H., Sundin, G. W., and Zhao, Y. 2016. Identification of the HrpS binding site in the *hrpL* promoter and effect of the RpoN binding site of HrpS on the regulation of the type III secretion system in *Erwinia amylovora*. *Mol. Plant Pathol.* 17:691-702.
- Lindeberg, M., Cartinhour, S., Myers, C. R., Schechter, L. M., Schneider, D. J., and Collmer, A. 2006. Closing the circle on the discovery of genes encoding Hrp regulon members and type III secretion system effectors in the genomes of three model *Pseudomonas syringae* strains. *Mol. Plant-Microbe Interact.* 19:1151-1158.
- Lindeberg, M., Stavrinides, J., Chang, J. H., Alfano, J. R., Collmer, A., Dangel, J. L., Greenberg, J. T., Mansfield, J. W., and Guttman, D. S. 2005. Proposed guidelines for a unified nomenclature and phylogenetic analysis of type III Hop effector proteins in the plant pathogen *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 18:275-282.
- Morett, E., and Segovia, L. 1993. The sigma 54 bacterial enhancer-binding protein family: Mechanism of action and phylogenetic relationship of their functional domains. *J. Bacteriol.* 175:6067-6074.
- Nissan, G., Manulis, S., Weinthal, D. M., Sessa, G., and Barash, I. 2005. Analysis of promoters recognized by HrpL, an alternative sigma-factor protein from *Pantoea agglomerans* pv. *gypsophylae*. *Mol. Plant-Microbe Interact.* 18:634-643.
- Preston, G., Deng, W. L., Huang, H. C., and Collmer, A. 1998. Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J. Bacteriol.* 180:4532-4537.
- Quiñones, B., Pujol, C. J., and Lindow, S. E. 2004. Regulation of AHL production and its contribution to epiphytic fitness in *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 17:521-531.
- Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E. L., Kalkkinen, N., Romantschuk, M., and He, S. Y. 1997. Hrp pilus: An *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. U.S.A.* 94:3459-3464.
- Schumacher, J., Joly, N., Rappas, M., Zhang, X., and Buck, M. 2006. Structures and organisation of AAA+ enhancer binding proteins in transcriptional activation. *J. Struct. Biol.* 156:190-199.
- Schumacher, J., Waite, C. J., Bennett, M. H., Perez, M. F., Shethi, K., and Buck, M. 2014. Differential secretome analysis of *Pseudomonas syringae* pv. *tomato* using gel-free MS proteomics. *Front. Plant Sci.* 5:242.
- Trapnell, C., Pachter, L., and Salzberg, S. L. 2009. TopHat: Discovering splice junctions with RNA-Seq. *Bioinformatics* 25:1105-1111.
- Vencato, M., Tian, F., Alfano, J. R., Buell, C. R., Cartinhour, S., DeClerck, G. A., Guttman, D. S., Stavrinides, J., Joardar, V., Lindeberg, M., Bronstein, P. A., Mansfield, J. W., Myers, C. R., Collmer, A., and Schneider, D. J. 2006a. Bioinformatics-enabled identification of the HrpL regulon and type III secretion system effector proteins of *Pseudomonas syringae* pv. *phaseolicola* 1448A. *Mol. Plant-Microbe Interact.* 19:1193-1206.
- Vencato, M., Tian, F., Alfano, J. R., Buell, C. R., Cartinhour, S., DeClerck, G. A., Guttman, D. S., Stavrinides, J., Joardar, V., Lindeberg, M., Bronstein, P. A., Mansfield, J. W., Myers, C. R., Collmer, A., and Schneider, D. J. 2006b. Bioinformatics-enabled identification of the HrpL regulon and type III secretion system effector proteins of *Pseudomonas syringae* pv. *phaseolicola* 1448A. *Mol. Plant-Microbe Interact.* 19:1193-1206.
- Waite, C., Schumacher, J., Jovanovic, M., Bennett, M., and Buck, M. 2017. Negative autogenous control of the master type III secretion system regulator HrpL in *Pseudomonas syringae*. *MBio* 8:e02273-16.
- Wei, C. F., Deng, W. L., and 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.
- 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., Lan, L., Yin, C., Deng, X., Baker, D., Zhou, J. M., and Tang, X. 2007. Two-component sensor RhpS promotes induction of *Pseudomonas syringae* type III secretion system by repressing negative regulator RhpR. *Mol. Plant-Microbe Interact.* 20:223-234.
- Zhang, Y., Liu, T., Meyer, C. A., Eeckhoutte, J., Johnson, D. S., Bernstein, B. E., Nusbaum, C., Myers, R. M., Brown, M., Li, W., and Liu, X. S. 2008. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 9:R137.