Functional Characterization of Key Residues in Regulatory Proteins HrpG and HrpV of *Pseudomonas syringae* pv. *tomato* DC3000

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The plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 uses a type III secretion system (T3SS) to transfer effector proteins into the host. The expression of T3SS proteins is controlled by the HrpL σ factor. Transcription of *hrpL* is σ^{54} -dependent and bacterial enhancer-binding proteins HrpR and HrpS coactivate the *hrpL* promoter. The HrpV protein imposes negative control upon HrpR and HrpS through direct interaction with HrpS. HrpG interacts with HrpV and relieves such negative control. The sequence alignments across Hrp group I-type plant pathogens revealed conserved HrpV and HrpG amino acids. To establish structure–function relationships in HrpV and HrpG, either truncated or alanine substitution mutants were constructed. Key functional residues in HrpV and HrpG are found within their C-terminal regions. In HrpG, L101 and L105 are indispensable for the ability of HrpG to directly interact with HrpV and suppress HrpV-dependent negative regulation of HrpR and HrpS. In HrpV, L108 and G110 are major determinants for interactions with HrpS and HrpG. We propose that mutually exclusive binding of HrpS and HrpG to the same binding site of HrpV governs a transition from negative control to activation of the HrpRS complex leading to HrpL expression and pathogenicity of *P. syringae*.

Pseudomonas syringae pv. *tomato* DC3000 (*P. syringae* DC3000) is a gram-negative bacterial pathogen of *Arabidopsis thaliana* and tomato (Whalen et al. 1991) that causes bacterial speck disease, characterized by necrotic lesions on plant leaves and fruit. *P. syringae*, like many other animal and plant pathogens, employs a type III secretion system (T3SS) to infect the host plant (Cornelis 2006). T3SS are highly conserved among gram-negative bacteria, which exploit this system to deliver effectors directly into the host cell cytosol. The T3SS consists of syringe-like export machinery, encoded by the *hrp/hrc* cluster that contains genes expressing proteins important for secretion, translocation, and regulation. The *hrp/hrc* genes are organized in seven operons located on a pathogenicity island. In Hrp group I-type plant pathogens such as *P. syringae*, *Erwinia* spp., *Pantoea* spp., and *Dickeya* spp., the expression of the

majority of *hrp*, *hrc*, *hop*, and *avr* genes, as well as other virulence factors, is dependent on an RNA polymerase (RNAP) containing the specialized extracytoplasmic σ factor HrpL (Alfano and Collmer 1997, Chatterjee et al. 2002, Hutcheson et al. 2001, Waite et al. 2017). The expression of *hrpL* is dependent on σ^{54} -RNAP, which requires activation by a specific group of transcriptional activators known as bacterial enhancer-binding proteins (bEBP) (Hutcheson et al. 2001). bEBP, via their interaction with the σ^{54} -RNAP closed promoter complex, catalyze the transition into the open promoter complex to initiate transcription. The bEBP binds DNA approximately 150 bp upstream of the σ^{54} promoter site and, via DNA looping, often facilitated by integration host factor (IHF) bound to the intervening regulatory region, comes into close proximity and interacts with the σ^{54} -RNAP complex. The orthodox bEBP consist of N-terminal regulatory, central catalytic AAA+, and C-terminal DNA binding domains (Dworkin et al. 1997; Joly et al. 2010, 2012; Ninfa et al. 1987; Wedel et al. 1990). Regulation of bEBP activity is mainly achieved through their N-terminal regulatory domain, which often negatively controls the self-assembly and action of the AAA+ catalytic domain. In *P. syringae* DC3000, *hrpL* expression is activated by two bEBP, HrpR and HrpS. These bEBP lack N-terminal regulatory domains and regulation of their activity is achieved in trans by an additional protein, HrpV. HrpV is a small protein (13,900 Da) encoded by *hrpV*, the last gene in the *hrcC* operon. HrpV acts as a negative regulator of the HrpRS complex formation through specific binding to HrpS (Jovanovic et al. 2011; Rufián et al. 2016; Wei et al. 2005). The AAA+ catalytic domain of HrpS is necessary for a direct interaction with HrpV, and HrpS I26 residue is a major determinant for this binding (Jovanovic et al. 2014).

The negative control imposed by HrpV is suppressed by the HrpG regulatory protein. HrpG (15,400 Da) is a small acidic, cytoplasmic, chaperone-like protein (Wei et al. 2005), encoded by *hrpG*, the second gene of the *hrcC* operon. Direct interaction between HrpG and HrpV relieves negative control upon HrpR and HrpS enabling activation of HrpL expression and, consequently, the synthesis of the T3SS machinery. Our previous in vivo studies on interactions between T3SS regulatory proteins showed that HrpG interacts only with HrpV but not with HrpR and HrpS and that neither HrpG nor HrpV self-associate (Jovanovic et al. 2011, 2014; Wei et al. 2005).

The effects of HrpV and HrpG in pathogenicity are well documented in many *P. syringae* strains (Deng et al. 1998; Ortiz-Matin et al. 2010; Preston et al. 1998; Wei et al. 2005). Because HrpV can bind either HrpS or HrpG but not both

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simultaneously (Hutcheson et al. 2001; Jovanovic et al. 2011, 2014), the important question is which determinants are responsible for the alternate HrpV interactions with HrpS and HrpG leading to the opposing outcomes of either negative control or activation of the HrpL expression. Although HrpV and HrpG lack domains or motifs common to other known or hypothetical proteins, multiple sequence alignments across group I plant pathogens revealed conserved HrpV and HrpG amino acids that may aid homolog definition in other organisms. To establish structure–function relationships in HrpV and HrpG, a range of single alanine substitutions of conserved residues in HrpV and HrpG were constructed and HrpV and HrpG variants were analyzed in vivo and in vitro assays for their ability to interact with each other and for functionality and ability to induce disease symptoms on *A. thaliana*. The results presented here reveal that L108, A109, and G110 are the major HrpV determinants responsible for both interaction with HrpS and, consequently, negative control of the HrpRS complex as well as for the interaction with HrpG and relief of negative control. The residues found within the HrpG C terminus (L101 and L105) are crucial for the ability of HrpG to interact with HrpV and, consequently, suppress the HrpV negative regulation over HrpR and HrpS.

RESULTS

Control of *hrpL* promoter activity in *P. syringae* DC3000 by HrpV and HrpG.

The role of HrpV and HrpG in pathogenicity has been reported previously for *P. syringae* pvs. *phaseolicola* and *syringae* 61 (Deng et al. 1998; Ortiz-Martín et al. 2010; Preston et al. 1998; Wei et al. 2005). Here, we report on the behavior of plasmid-borne *hrpL* promoter-*lacZ* (*PhrL-lacZ*) and *hrpL* promoter-*gfp* (*PhrL-gfp*) transcriptional fusions (Fig. 1) used as reporters in *P. syringae* DC3000 *hrpV* or *hrpG* knockout mutants. These fusions were constructed to directly assess the effect of HrpV and HrpG on HrpR and HrpS activity. The strains were grown at 25°C for 8 h in *hrp*-inducing medium (HIM), which mimics the conditions found in planta (Huynh et al. 1989), and the fluorescence per cell growth was measured. We showed that, in comparison with the wild type (WT), in the absence of HrpV, the HrpR and HrpS activity measured via *PhrL-gfp* increases whereas, in the absence of HrpG, the HrpR and HrpS activity decreases (Fig. 1A). In support of this, the activity of *PhrL-lacZ* fusion in Δ *hrpV* mutants was two

orders of magnitude greater ($P < 0.0001$) than in Δ *hrpG* mutants (Fig. 1B). These results support our experimental system as valid to follow the roles of HrpV or HrpG as regulators of HrpR and HrpS activity on *hrpL* transcription.

Effects of HrpV L108 and G110 residues on HrpV activity in vivo in *Escherichia coli*.

HrpV does not contain a recognizable functional domain. However, analysis of an HrpV multiple sequence alignment among 20 organisms that contain an *hrcC* operon showed that almost 100% of the examined HrpV sequences have one conserved primary sequence motif consisting of L108/A109/G110 residues (LAG motif), and the only exception (1 of 20) is *P. cichorii*, with an LTG amino acid sequence (Supplementary Fig. S1). In *P. syringae* DC3000, the LAG motif is situated within the last putative α helix (Fig. 2A). To analyze the role of the LAG motif and determine its potential effect on the functionality of HrpV, we introduced L108A and G110A alanine substitutions in HrpV and compared the function of this variant to the WT HrpV. The in vivo transcription assay revealed that, in comparison with HrpV, HrpV L108A/G110A was unable to efficiently repress the activity of HrpR and HrpS ($P < 0.0001$) and transcription of the *hrpL-lacZ* reporter in *Escherichia coli* (Fig. 2B).

To assess the interactions between the HrpV variant and HrpG or HrpS, we used the bacterial adenylate cyclase two-hybrid (BACTH) system. The results revealed that, in contrast to HrpV, the HrpV variant was unable to interact with either HrpS or HrpG ($P < 0.0001$) (Fig. 2C). In support of this, in a three-component BACTH system where we studied the effect of coexpressed HrpV or HrpV L108A/G110A variant on the interaction between the HrpG and HrpV, we showed that an interaction can be detected only when HrpG is coexpressed with HrpV L108A/G110A but not with the HrpV WT ($P < 0.0001$; 18G(VLAG)-25V versus 18G(V)-25V) (Fig. 2C). Apparently, the HrpV WT and variant proteins are unstable when expressed alone in a BACTH assay and cannot be detected unless they interact with their counterpart. In separate experiments, we showed that HrpV instability cannot be attributed to the activity of Lon protease in *E. coli* studies.

Negative control function of HrpV L108A/G110A variant in *P. syringae* DC3000.

In order to characterize the role of the highly conserved HrpV LAG motif in the native *P. syringae* DC3000 strain, we

Fig. 1. Activities of the *hrpL* promoter in different *Pseudomonas syringae* DC3000 backgrounds. **A**, Activity of the pBBR1MCS*PhrL-gfp* reporter in *P. syringae* DC3000 Δ *hrpV* and Δ *hrpG*. The *hrpL* promoter activity (fluorescence at 520 nm and optical density at 600 nm [OD₆₀₀]) in wild-type (WT) DC3000 (black), Δ *hrpS* (gray), Δ *hrpV* (black squares), and Δ *hrpG* (black circles) strains carrying pBBR1MCS *PhrL-gfp* over 8 h in *hrp*-inducing medium. The *hrp* induction was simulated with growth at 25°C and 10 mM fructose. Results are presented as mean values for three biological replicates with standard error bars. **B**, β -Galactosidase (β -gal) activity of a pBBR1MCS *PhrL-lacZ* reporter in *P. syringae* DC3000 WT, Δ *hrpV*, and Δ *hrpG* strains. β -Gal activity was measured in the presence (black) and absence (light gray) of the *PhrL-lacZ* reporter plasmid. Light gray bars consistently score activity as less than 10 Miller Units. Results from three biological replicates with standard error bars are shown.

analyzed activity of a *PhrpL-gfp* transcriptional fusion in an HrpV knock-out (ΔV) *P. syringae* DC 3000 reporter strain in the presence of HrpV or HrpV L108/G110 variant. In vivo expression of green fluorescent protein (GFP) and cell density was measured in HIM media (Fig. 2D). The HrpV efficiently complemented the lack of chromosomal *hrpV*, significantly decreasing the *hrpL* promoter activity; whereas, in the presence of the HrpV L108/G110 variant, the promoter activity was not affected. This suggests that alanine substitutions of the residues L108/G110 affect the HrpV negative control function in *P. syringae* DC3000 in vivo. Notably, the negative control of plasmid-borne HrpV is somewhat stronger than the one observed by chromosomally expressed native HrpV (Fig. 2D).

Disease symptoms caused by the HrpV L108A/G110A variant.

Results in line with those of the transcription assays in vivo were obtained in a pathogenicity assay using *A. thaliana* leaves infected by *P. syringae* DC3000. The virulence assays (Fig. 2E)

were carried out using the same cultures as in the *gfp* expression assay (Fig. 2D). Bacterial cultures of *P. syringae* DC3000 ΔV expressing either HrpV or HrpV L108/G110 variant were diluted at an optical density at 600 nm (OD_{600}) of 0.01 and injected through the bottom of the *Arabidopsis* leaf with a blunt syringe. The leaf was placed onto a wet paper towel in a sealed Petri dish and incubated at 22°C, and infection data were collected after 24 and 72 h (Fig. 2E). Although the lack of HrpV causes severe disease symptoms after 72 h of incubation, the complementation by HrpV reduced the symptoms by impairing the T3SS synthesis and, thus, infectivity through negative control of HrpR and HrpS. As expected, the HrpV L108/G110 variant restored disease, indicating failure of this HrpV variant in repressing HrpR and HrpS activity.

Effects of the WT HrpG and HrpG variants on HrpR and HrpS activity in vivo.

Studies by Gazi and associates (2015) and Lilic and associates (2006) showed that specific residues of the class I chaperones HrpG and HrpG-like proteins of *Erwinia* and *Salmonella*

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Fig. 3. Effect of the HrpG wild type (WT) and HrpG variants on HrpV-negative regulation of HrpR and HrpS activity. **A**, Secondary structure prediction of HrpG from *Pseudomonas syringae* DC3000; H = helix, E = β -strand, and – = coil or loop. **B**, Transcription activity in vivo of HrpR and HrpS proteins in the presence of HrpV and HrpG or HrpG variants in *Escherichia coli* *hrpL-lacZ* (*PhrL-lacZ*) reporter strain. HrpR and HrpS proteins were coexpressed from pAPT110 vector while HrpV was expressed alone or coexpressed with HrpG or HrpG variants from pBAD18-based vector. Activity of *hrpL-lacZ* promoter fusion was measured using the β -galactosidase (β -gal) assay. Mean values with standard error bars from three independent biological samples are shown. MU = Miller Units. **C**, Binding interactions between the HrpG protein or HrpG variants and HrpV detected by the bacterial adenylate cyclase two-hybrid (BACTH) system. Numbers 18 and 25 stand for T18 and T25 adenylate cyclase fragments on pUT18C and pKT25 vectors, respectively. The WT HrpG and HrpG variants fused to T18 were labeled as 18G, 18G(F6A), 18G(L101A), 18G(L105A), and 18G(L101A/L105A). The pKT25-*hrpV* expressing T25-HrpV was labeled as 25V. **D**, Effects of HrpG WT or HrpG variants (amino acid substitutions given in parentheses) on interaction between HrpS and HrpV analyzed by BACTH three-component system. For the BACTH three-component system, pUT18C-based constructs expressing either HrpS and HrpG or HrpS and HrpG variants (F6A, L101A, L105A, and L101A/L105A) were labeled as 18S(G), 18S(GF6A), 18S(GL101A), 18S(GL105A), and 18S(GL101A/L105A), respectively (see inset). For C and D, the interaction between proteins was measured using the β -gal assay and results are presented as mean values of three independent biological samples with standard error bars. Inset: schematic drawing of pUT18C derivative with coexpressed T18-HrpS and HrpG WT or HrpG variant: Pr *lac* = promoter, SD = Shine-Dalgarno sequence, and *bla* = ampicillin resistance. **E**, Activity of the *PhrL-gfp* reporter (on plasmid pBBR1MCSP*hrpL-gfp*) in *P. syringae* WT DC3000 (WT) and DC3000 Δ *hrpG* (Δ G) cells in the presence of HrpG or HrpG variants expressed from the pSEVA-based constructs: red = WT cells carrying pSEVA empty vector (WT+pSEVA), dark blue = Δ G cells carrying pSEVA empty vector (Δ G+pSEVA), light green = Δ G carrying pSEVA-*hrpG* (Δ G+G WT), light blue = Δ G carrying pSEVA-*hrpG*(F6A) (Δ G+G6), orange = Δ G carrying pSEVA-*hrpG*(L101A) (Δ G+G101), dark green = Δ G carrying pSEVA-*hrpG*(L105A) (Δ G+G105), purple = Δ G carrying pSEVA-*hrpG*(L101A/L105A) (Δ G+G101/105), and black = *P. syringae* WT DC3000 without *PhrL-gfp* reporter plasmid and pSEVA as intrinsic cell fluorescence control. Presented results show mean values from three independent biological samples with standard error bars. **F**, Disease symptoms developed on the leaves of *Arabidopsis thaliana* 24 and 72 h after infiltration with *P. syringae* DC3000 Δ *hrpG* carrying pBBR1MCSP*hrpL-gfp* and pSEVA empty vector (Δ G+pSEVA), pSEVA-*hrpG* (Δ G+WT HrpG), pSEVA-*hrpG*(F6A) (Δ G+HrpGF6A), pSEVA-*hrpG*(L101A) (Δ G+HrpGL101A), pSEVA-*hrpG*(L105A) (Δ G+HrpGL105A), or pSEVA-*hrpG*(L101A/L105A) (Δ G+HrpGL101A/L105A). WT DC3000 with *PhrL-gfp* reporter plasmid and pSEVA (WT+pSEVA) was used as a control. Mock = leaves treated with 10 mM $MgCl_2$. At least 10 leaves were treated with each strain and three typical leaf samples were shown for each experiment.

spp., respectively, are part of a structural motif important for the stability of the chaperone-substrate complex and that their disruption leads to destabilization of this complex. To determine the role of equivalent residues on the function of HrpG in *P. syringae* DC3000, we introduced the alanine substitutions in F6, situated in the first putative α -helix, and in L101 and L105, situated in the last putative α -helix of HrpG (Fig. 3A; Supplementary Figs. S2 and S3). These HrpG variants were analyzed in vivo in *E. coli* carrying the *PhrpL-lacZ* transcriptional fusion reporter (Fig. 3B). Our results demonstrated

that any single substitution does not significantly affect the functionality of HrpG ($P > 0.05$). However, the HrpG variants carrying either double L101A/L105A or triple F6A/L101A/L105A alanine substitutions reduced the ability of HrpG to suppress the HrpV negative control of HrpR and HrpS at $P < 0.05$ and $P < 0.0001$, respectively.

To analyze the potential role of F6/L101/L105 in interactions with HrpV, we performed BACTH assays (Fig. 3C). The results showed that L101A substitution decreased the interaction between HrpG and HrpV ($P < 0.0001$) whereas a double L101A/L105A

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substitution in HrpG showed no interaction ($P < 0.0001$), indicating that these two residues are involved and important for formation of the HrpG-HrpV regulatory complex. In addition, truncation analyses of HrpG showed that deletion of the last 20 amino acids at the C terminus, leaving the last α -helix and L101 and L105 residues intact, does not affect the HrpG stability and ability to interact with HrpV.

It has been shown that the presence of HrpG can at least partially relieve the repression of HrpS caused by HrpV (Jovanovic et al. 2011; Wei et al. 2005). Also, we showed previously that HrpV interacts with HrpS and HrpG but cannot interact simultaneously with both proteins, indicating that HrpS and HrpG could compete and bind for the same HrpV binding site (Jovanovic et al. 2014). Here, we performed a BACTH three-component assay where an HrpG WT (HrpG) or HrpG variant is coexpressed with HrpS-T18 (see Materials and Methods) and showed that HrpG can, indeed, outcompete HrpS for binding with HrpV ($P < 0.0001$; 18S-25V versus 18S(G)-25V) (Fig. 3D). The introduction of single L101A or L105A mutations or double L101A/L105A mutations in HrpG affected the efficiency of interaction between HrpG and HrpV ($P < 0.001$ for L101A or L105A and $P < 0.0001$ for L101A/L105A) and revealed that HrpG L101 and L105 can act as major determinants for interaction with HrpV (Fig. 3D). Because the effects of F6A were nonsignificant ($P > 0.05$), (Fig. 3B), this mutant was not further studied.

Activity of the HrpG L101A/L105A variant in *P. syringae* pv. *tomato* DC3000.

The regulatory functions of HrpG and HrpG variants carrying single or double alanine substitutions of F6/L101/L105 were tested by measuring the *hrpL* promoter activity in an *hrpG* knock-out (Δ G) *P. syringae* DC3000 reporter strain (Fig. 3E). The WT HrpG efficiently suppressed the HrpV negative control of HrpR and HrpS, leading to increased activity of the *hrpL* promoter. The single substitutions F6 and L101 significantly decreased the *hrpL* activity, whereas the L105 and double L101A/L105A mutations showed an even more pronounced effect, leading to inefficient suppression of the HrpV negative control.

Disease symptoms caused by HrpG F6A/L101A/L105A variants.

Results from the previous experiments indicate that the double L101A/L105A substitution in HrpG reduces *P. syringae* DC3000 virulence by reducing the HrpG-HrpV interactions and, consequently, the *hrpL* promoter activity. Subsequently, *A. thaliana* was infected with *P. syringae* DC3000 Δ G expressing the HrpG or HrpG variants. These virulence assays (Fig. 3F) were performed using the same cultures used in the *gfp* expression assay (Fig. 3E). In addition, the same control (WT/pSEVA) was used for assays presented in Figures 2E and 3F). A strain lacking HrpG (Δ G) displayed reduced disease symptoms (Fig. 3F). Complementation of Δ G with either HrpG WT, HrpG F6A, or HrpG L101A restored the disease severity to a near WT level, whereas the HrpG L101A/L105A variant reduced the disease symptoms to the level seen in the Δ G strain, supporting the *hrpL* promoter gene regulation data.

HrpG L101A/L105A substitutions affect HrpG activity in vitro.

In order to analyze the organization of HrpV and HrpG proteins and their effect on HrpR- and HrpS-dependent *hrpL* transcription in vitro, we purified HrpG and HrpV (Supplementary Fig. S4). We used a short-primed RNA (spRNA) assay to determine the effect of HrpG and the HrpG L101A/L105A variant on the HrpV negative control of HrpR and HrpS transcription activation (Fig. 4). The results showed a significant difference between the WT HrpG and its L101A/L105A variant

in suppressing the HrpV negative control. The 1.5- μ M HrpG L101A/L105A showed more than twofold lower efficiency than HrpG WT. To analyze the organization of HrpV and HrpG proteins, we performed gel filtration chromatography using a Superdex 200 column (Supplementary Fig. S5). Even though both proteins are mainly found in the insoluble fraction upon overexpression, the soluble fraction of HrpV is an apparent monomer whereas HrpG forms an apparent dimer. Gazi and associates (2015) showed that, when HrpG and HrpV from *Erwinia amylovora* were copurified, they formed a very stable complex. We copurified *P. syringae* DC3000 HrpG and HrpV and obtained a stable and soluble HrpG-HrpV complex. Gel filtration of copurified HrpG-HrpV resolved one distinct peak with a molecular mass corresponding to 31,000 Da. The relative HrpG-HrpV staining intensities imply that the HrpG-HrpV complex is a hetero-dimer comprising one HrpG and one HrpV subunit. The HrpG-HrpV interaction is specific and relies on HrpV L101 and L105 residues. Namely, when we copurified the HrpG with the HrpV L101A/L105A variant, the yield of the HrpG-HrpV complex was strongly reduced.

We showed previously that the HrpRS complex binds the *hrpL* promoter DNA UAS sequence, and that the *hrpL* regulatory region also contains an IHF binding site situated between the UAS and the σ^{54} binding site (Supplementary Fig. S6). IHF strongly stimulates HrpR- and HrpS-dependent transcription of *hrpL* (Jovanovic et al. 2011). Here, we extended this observation and, using the gel mobility binding assay, showed that, in addition to strong binding of IHF to the *hrpL* promoter region, the IHF and HrpRS complex together bind this region and may reorganize the DNA conformation, seen as a supershift of the probe. We also showed that the HrpRS complex alone strongly binds the *hrpL* promoter region and, hence, we used this simple setup to test the influence of HrpV and HrpG on HrpRS-DNA interactions. We showed that neither HrpG nor HrpV alone bind the DNA promoter region or affect the HrpRS-DNA interactions, implying that HrpV may act following UAS occupancy by HrpR and HrpS.

DISCUSSION

The T3SS expression in *P. syringae* is activated by RNA polymerase containing the HrpL σ factor, expression of which is tightly controlled by a complex network of regulatory factors. The main regulatory elements include the HrpRS transcriptional activator complex, the negative regulator HrpV, and HrpG, the protein that suppresses HrpV negative regulation. HrpV and HrpG play important roles in this regulatory network and very little is known about their structure, modes, and preferences of interactions, or the course of their interaction events during plant infection. Our findings help elaborate a structure-function model for the binding interactions made by HrpV and HrpG to control HrpR- and HrpS-dependent expression of HrpL. We found that HrpV residues L108 and G110 within the LAG motif placed in the C-terminal helix are important for the HrpV direct interaction with HrpS, consequent negative control function, and repression of disease symptom development. Moreover, HrpV L108 and G110 are implicated in establishing a stable binding interaction with HrpG, which then suppresses the negative HrpV control over HrpS, leading to infectivity and appearance of disease symptoms. These outcomes strongly suggest that HrpG and HrpS recognize and bind the same determinants in HrpV and, through use of a common binding site, regulate the HrpR and HrpS activity. This mechanism of alternate and mutually exclusive binding of HrpV to HrpS or HrpG determines the expression level of HrpL, leading to basal standby mode or synthesis of the T3SS proteins and pathogenicity of *P. syringae*, respectively.

Our results and the current model of the Hrp regulatory circuit are supported by a range of observations (Jovanovic et al. 2011; Wei et al. 2005), in which a strong interaction was observed between HrpV and HrpS in the absence of HrpG, whereas no such interaction was seen when HrpG was present. In line with this, a strong interaction was observed between HrpV and HrpS in the presence of the HrpG L101A/L105A variant, suggesting that this HrpG double-leucine substitution abolishes HrpG-HrpV binding and HrpG function, therefore allowing a constitutive and strong binding between HrpV and HrpS and consequential negative control of the HrpL expression. Clearly, HrpG L101 and L105 are key determinants for interaction with HrpV and suppression of negative control mode imposed by HrpV. In addition, the HrpG F6A mutation further enhances the effect of the HrpG L101A/L105A double mutant, allowing HrpV-HrpS binding and the negative control effect upon HrpR and HrpS activity. Apparently, single-leucine substitutions (L101A or L105A) in HrpG had intermediate effects on the HrpV-HrpS interaction. One major difference between data obtained in the BACTH interactions and functional assays in vivo and in vitro is that the HrpG L101A/L105A double mutant does not interact with HrpV but still suppresses the HrpV negative control. The possible explanation for this may be that the HrpG fusion protein used in BACTH somehow has the F6 residue masked and, thus, behaves as a triple mutant, abolishing the interaction between HrpG and HrpV. The triple HrpG F6A/L101A/L105A mutant in the in vivo transcription assay showed a more pronounced effect than the double L101A/L105A mutant, supporting this hypothesis.

Taken together with the previous observation that HrpS I26 residue is important for HrpV-HrpS association, our results establish that amino acid residues implicated in Hrp regulatory protein interactions and functions all have hydrophobic side chains (HrpV L108/A109/G110 and HrpG F6/L101/L105). This provides strong evidence that hydrophobic interactions likely dominate the recognition of major determinants involved in protein-protein binding within the Hrp regulatory network (as already observed in interactions between bEBP PspF and its in trans negative regulator, PspA) (McDonald et al. 2017). These are of importance for the effective and reversible modes of control and efficient signal transduction when sensing environmental conditions; in particular, during the in planta interaction. Moreover, knowing major functional determinants of the Hrp regulatory proteins raises the prospect of using them as viable antimicrobial targets.

The conservation of L108/A109/G110 residues in HrpV across the 20 species in the amino acid alignment suggests that these residues could be similarly important in all group I-type plant pathogens. Also, in HrpG at position L101 and L105, all 20 species have either isoleucine, leucine, or valine, suggesting a conserved role for these residues. HrpG F6 is only conserved in organisms containing HrpR and HrpS rather than HrpS alone.

Although the HIM can be used empirically to mimic *P. syringae*-plant interaction conditions, rather little is known about the molecular signals and the subsequent regulatory controls that lead to activation of the Hrp system and infection in planta. Considering that *hrpG* and *hrpV* are the second and last gene in the *hrpC* operon, respectively, HrpG and HrpV are most likely both present in the cytoplasm following exposure to Hrp-activating conditions. How HrpG and HrpV are organized in preparation for their regulatory roles is still unknown. Our in vivo and in vitro data show that HrpG and HrpV strongly interact and form a stable dimer when copurified. The fact that the same residues on HrpV are important for interaction with both HrpG and HrpS, and that HrpG outcompetes HrpS (interactions with HrpV), suggests that relief of the negative control function of HrpV by HrpG is the dominant state of the

Hrp regulatory network during Hrp-activating conditions. Sequestration of regulatory factors can cause ultrasensitive responses and, potentially, the interplay between HrpV, HrpG, and the HrpRS complex causes such behavior (Bradley et al. 2016).

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. Antibiotics were used at the following concentrations: ampicillin (Amp) at 100 µg/ml, kanamycin (Kan) at 50 µg/ml, chloramphenicol (Cm) at 30 µg/ml, and rifampicin (Rif) at 50 µg/ml. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 25°C. For analysis of *hrpL-gfp* activity in *P. syringae* DC3000, we used HIM: 50 mM potassium phosphate buffer (pH 7.0), 1.7 mM MgCl₂, 1.7 mM NaCl, 7.6 mM (NH₄)SO₄, and 10 mM fructose (Huynh et al. 1989).

Electroporation.

P. syringae DC3000 strains were prepared for electroporation according to a method previously developed for *P. aeruginosa* (Choi et al. 2006). Cells grown to exponential phase in LB medium were washed twice and resuspended in a 100 µl of sterile 300 mM sucrose and mixed with DNA. Electroporation was performed in a 1-mm Micropulser cuvette (Bio-Rad) at 1,800 V. Cells were recovered in LB at 28°C for 1 h before plating onto selective LB agar supplemented with Rif at 25 µg/ml, Amp at 50 µg/ml, and Kan at 25 µg/ml and incubated for 2 days at 25°C.

Fluorescent reporter assay.

P. syringae DC3000 transformants were grown overnight in LB (supplemented with appropriate antibiotics) at 25°C, washed twice in 10 mM MgCl₂, and diluted in HIM to an OD₆₀₀ of 0.25. Triplicate samples were incubated in a 96-well tissue culture plate in 200-µl volumes (Greiner). Fluorescence (excitation at 420 nm and emission at 520 nm) and cell density (OD₆₀₀) were measured simultaneously every 20 min for 20 h using a BMG FLUOstar fluorometer. Fluorescence per unit cell was calculated.

Arabidopsis inoculations and symptom development.

P. syringae DC3000 strains were grown overnight in LB (supplemented with appropriate antibiotics) at 25°C and washed twice with 10 mM MgCl₂ to an OD₆₀₀ of 0.01. Bacteria culture (200 µl) was carefully infiltrated into the bottom of *A. thaliana* leaves. Inoculated leaves were placed onto a wet paper towel in a sealed Petri dish and incubated at 22°C for 72 h.

Fig. 4. Short primed RNA (spRNA) assay in the presence of HrpR, HrpS, HrpV, HrpG, and HrpG L101A/L105A variant. The quantitative analysis of spRNA (%) was performed using the ImageJ program.

β -Galactosidase assay.

The β -galactosidase (β -gal) assay was performed as described by Jovanovic and associates (2014).

BACTH assay.

The BACTH assay was performed as described by Jovanovic and associates (2014). Briefly, pKT25 and pUT18C vectors carrying Hrp regulatory proteins or their derivatives were used to cotransform BTH101 cells. After incubation at room temperature (RT) for 2 to 3 days, transformants were inoculated in LB media supplemented with Amp at 100 μ g/ml, Kan at 50 μ g/ml, and 0.5 mM isopropyl-thio-galactopyranoside (IPTG), and incubated overnight at 25°C; and protein interactions were quantified by β -gal assays. In BACTH, the three-component system protein of interest was coexpressed with another protein fused to a T18 adenylate cyclase subunit and interactions between two T18/T25-fused proteins were measured in the presence of that not-fused third component.

Statistical analysis of β -gal assays.

For statistical analysis of β -gal assays, one-way analysis of variance (ANOVA) and two-way ANOVA as implemented by GraphPad PRISM 6 were used, and *P* values smaller than 0.05 were considered statistically significant (Supplementary Table S2).

Protein purification.

Vectors carrying genes of interest pET28b⁺-*hrpG*, pET28b⁺-*hrpV*, or pET28b⁺-*hrpGV* were transformed into BL21 cells, inoculated into 0.5 liter of LB supplemented with Kan at 50 μ g/ml, and grown at 25°C to an OD₆₀₀ of approximately 0.5 before 0.3 mM IPTG induction. After 18 h of incubation at 16°C, cells were pelleted by centrifugation at 6,000 rpm for 30 min. Cell pellets were resuspended with buffer A (50 mM sodium phosphate buffer [pH 7.0], 50 mM NaCl, 0.1 mM EDTA, 5% [wt/vol] glycerol, and a protease inhibitor cocktail tablet) (Roche). Cells were sonicated on ice using Sonics Vibra Cell Model VCX 130 (40% amplitude for 2 $\times$ 10 min with an on/off pulse rate of 2 s) before centrifugation 30 min at 15,000 rpm. The soluble fraction was purified by Ni-affinity chromatography. Protein fractions were dialyzed overnight against storage buffer (20 mM Tris [pH 8.0], 50 mM NaCl, 0.1 mM EDTA, and 5% [wt/vol] glycerol). Other proteins used in this study are from lab collections: σ^{54} was purified as described (Cannon et al. 2000), IHF was purified as described (Nash et al. 1987), and RNAP core enzyme was purchased from Epicentre.

Gel-filtration assay.

Gel-filtration chromatography (Superdex 200 column, 10 by 300 mm, 24 ml; GE Healthcare) was carried out in a buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 15 mM MgCl₂ at RT and a 0.5 ml/min flow rate. The column was calibrated with the globular proteins thyroglobulin (669,000 Da), apoferritin (443,000 Da), β -amylase (200,000 Da), bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da) and lysozyme (14,307 Da).

spRNA assay.

In a 10- μ l reaction with 5 $\times$ TTH (10 mM Tris-HCl [pH 7.5], 70 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, 12.5% [wt/vol] glycerol, and 0.1% [wt/vol] Triton X-100), 100 nM holoenzyme (core: σ^{54} ratio of 1:5), 5 mM ATP, 20 mM IHF, and 20 nM DNA template were incubated for 10 min at RT. This was followed by the subsequent addition of HrpR, HrpS, HrpV, or HrpG and incubated for a further 40 min at RT. Transcription was initiated with an elongation mix containing

heparin at 100 μ g/ml, 0.3 μ l of UpA, and 1 μ Ci of [α -³²P] CTP and left for a further 20 min at RT. Reactions were quenched with loading dye and analyzed on 20% denaturing gels. Transcripts were visualized and quantified using a Fuji FLA-5000 Phosphorimager and AIDA image analyzer.

Native gel-shift assay.

In a 10- μ l reaction with 5 $\times$ TTH buffer, 20 nM DNA template Cy3 labeled and proteins of interest were incubated at RT for 40 min. The reactions were analyzed on a 4.5% native polyacrylamide gel and visualized using Fuji FLA-5000 Phosphorimager. Gels were quantified using AIDA Image Analyzer.

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