Expression of *Ralstonia solanacearum* type III secretion system is dependent on a novel type 4 pili (T4P) assembly protein (TapV) but is T4P independent

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**Abstract**

Type IV pili (T4P) are virulence factors in various pathogenic bacteria of animals and plants that play important roles in twitching motility, swimming motility, biofilm formation, and adhesion to host cells. Here, we genetically characterized functional roles of a putative T4P assembly protein TapV (*Rsc1986* in reference strain GM1000) and its homologue *Rsp0189* in *Ralstonia solanacearum*. Deletion of *tapV*, but not *rsp0189*, resulted in significantly impaired twitching motility, swimming motility, and adhesion to tomato roots, which are consistent as phenotypes of the *pilA* mutant (a known *R. solanacearum* T4P-deficient mutant). However, unlike the *pilA* mutant, the *tapV* mutant produced more biofilm than the wild-type strain. Our gene expression studies revealed that TapV, but not *Rsp0189*, is important for expression of a type III secretion system (T3SS, a pathogenicity determinant of *R. solanacearum*) both in vitro and in planta, but it is T4P independent. We further revealed that TapV affected the T3SS expression via the PhcA–TapV–PrhG–HrpB pathway, consistent with previous reports that PhcA positively regulates expression of *pilA* and *prhG*. Moreover, deletion of *tapV*, but not *Rsp0189*, significantly impaired the ability to migrate into and colonize xylem vessels of host plants, but there was no alteration in intercellular proliferation of *R. solanacearum* in tobacco leaves, which is similar to the *pilA* mutant. The *tapV* mutant showed significantly impaired virulence in host plants. This is the first report on the impact of T4P components on the T3SS, providing novel insights into our understanding of various biological functions of T4P and the complex regulatory pathway of T3SS in *R. solanacearum*.

**KEYWORDS**

pathogenesis, *Ralstonia solanacearum*, TapV, type 4 pili, type III secretion system

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1 INTRODUCTION

Pili or fimbriae are hair-like appendages found on the surface of a wide range of bacteria (Burdman et al., 2011; Dunger et al., 2016). There are several types of pili that differ in their mechanisms of assembly, structure, and function. Type IV pili (T4P) are the most abundant pili and the best studied thus far (Mattick, 2002; Gibiansky et al., 2010). T4P are proteinaceous, flexible filaments with a diameter of 5–8 nm and a length of several micrometres that are generally located at one or both poles of a cell (Strom et al., 1993; Fernandez and Berenguer, 2000). T4P are mainly composed of thousands of copies of a small (13–23 kDa) subunit named pilin (PilA in most cases) that are synthesized as prepilin and cleaved by the action of PilD to make the mature pilin. PilA units are assembled into pilins by the cytoplasmic membrane protein PilIc to form an extracellular helical polymer via the outer membrane secretin PilQ (Craig et al., 2004, 2019; Craig and Li, 2008). Several components of Pilc, PilM, PilN, PilO, and PilP form the inner membrane platform that interacts with pilins and two specialized hexameric ATPases of Pilb and PilT on the cytosolic face of the inner membrane (Craig et al., 2004; Craig and Li, 2008; Burdman et al., 2011).

To date, functions of T4P and several T4P-dependent specific phenotypes have been well characterized in various bacteria such as Pseudomonas, Neisseria, Escherichia, Vibrio, Xylella, and Xanthomonas spp. (Gibiansky et al., 2010; Burdman et al., 2011; Dunger et al., 2016). Adherence to eukaryotic cells, an important early step during infection of host cells by many pathogenic bacteria, is one of the original functions of T4P (Henderson et al., 1999; Finlay and Caparon, 2000; Burdman et al., 2011). Twitching motility, the most representative T4P-dependent phenotype, is a form of bacterial translocation over moist organic and inorganic surfaces. It is an efficient and versatile flagellar-independent form of bacterial surface motility that is promoted by extension, attachment, and subsequent retraction of T4P in many different bacteria (Wall et al., 1999; Merz et al., 2000; Skerker et al., 2001; Maier and Wong, 2015). A remarkable feature of twitching motility is the edge of the expanding colonies, termed the twitching zone, which is made up of small groups or individual cells with poorly defined and irregular boundaries. T4P mutants usually present a more uniform and well-defined boundary with tightly packed cells (Bradley, 1980; Wall and Kaiser, 2010). Besides adherence to host cells and twitching motility, T4P also play important roles in surface attachment, biofilm formation, genetic material uptake, and bacteriophage infection (Kang et al., 2002; Gibiansky et al., 2010; Dunger et al., 2016).

Over the past few decades, the function, structure, and regulation of T4P have been well studied in genera of Pseudomonas, Neisseria, Escherichia, and Vibrio, where they are essential virulence factors of many human pathogenic bacteria (Burdman et al., 2011; Dunger et al., 2016), while they are less studied in plant pathogenic bacteria except for Xylella and Xanthomonas species, belonging to the Xanthomonadaceae family (Hu et al., 1995; Yang et al., 2004; Meng et al., 2005; Li et al., 2007). Although pioneering work has provided several links between T4P and Xanthomonas physiology and virulence, the role of T4P in the pathogenicity of plant pathogenic bacteria is poorly understood (Burdman et al., 2011; Dunger et al., 2016). In plant pathogenic bacteria, the contribution of T4P to virulence has mainly been investigated in a few vascular pathogens, including Ralstonia solanacearum, where it may contribute to colonization and dispersal in xylem vessels through cell attachment, biofilm formation, and twitching motility (Burdman et al., 2011; Dunger et al., 2016). R. solanacearum, the causal agent of bacterial wilt disease in many plant species worldwide, has been employed as a model system to decipher molecular interactions between plant and pathogenic bacterium (Genin and Denny, 2012; Jiang et al., 2017), although there are only a few studies to date that demonstrate the contribution of T4P to its virulence (Liu et al., 2001; Kang et al., 2002; Wairuri et al., 2012).

As a soilborne vascular bacterium, R. solanacearum generally invades host plants through natural root openings or root wounds (Vasse et al., 1995; Janse et al., 2004). Once it has invaded xylem vessels, it proliferates extensively and produces a huge number of exopolysaccharides (EPS) to block sap flow, resulting in quick stunning and wilting (Roberts et al., 1988; Denny, 1995). A syringe-like type III secretion system (T3SS) is another essential pathogenicity determinant in R. solanacearum, which many pathogenic bacteria use to inject virulence factors (type III effectors, T3Es) into host cytoplasm to subvert host defence (Cunnac et al., 2004; Angot et al., 2006; Jones and Dangl, 2006). The T3SS in R. solanacearum is encoded by 22 genes forming an hrp regulon and is globally regulated by a complex network (Arlat et al., 1992; Hikichi et al., 2017; Genin and Denny, 2012). In general, a master regulator HrpB of the AraC family of transcriptional regulators directly controls the T3SS and T3Es (Mukaihara et al., 2010; Coll and Valls, 2013). Expression of the T3SS and hrpB is not activated until the bacterium comes into contact with host signals or some mimic signals, such as those in nutrient-limited medium that mimics plant apoplastic fluids (Marenda et al., 1998; Yoshimochi et al., 2009; Zhang et al., 2013). Expression of hrpB is positively regulated by HrpG and PrhG in a parallel way. These are two close paralogs of two-component system response regulators and can respond to host signals by phosphorylation (Plener et al., 2010; Zhang et al., 2013). Host signals or some mimic signals are presumed to be recognized by an outer membrane PrhA and transferred to HrpG via a signalling cascade of PrhA-PrhR/I-PrhJ or some novel cascades (Valls et al., 2006; Genin and Denny, 2012; Hikichi et al., 2017; Zhang et al., 2018). Besides HrpG and PrhG, the global regulation network also includes numerous well-studied regulators such as PhcA, PrhN, PrhO, and XpsR cascades (Valls et al., 2006; Genin and Denny, 2012; Hikichi et al., 2017; Zhang et al., 2018).

To further elucidate the global regulation of T3SS in R. solanacearum, we previously screened several T3SS-regulating candidates with transposon mutagenesis, in which expression profiles of the T3SS were monitored with a popA-lacZYA fusion (Zhang et al., 2013). Among these was Rsc1986 (962 amino acids in the reference strain GMI1000), (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi), which is annotated as a putative T4P-assembly protein TapV, sharing 30% amino acid identity to known Tap proteins. Tap
proteins were originally identified in Aeromonas species and abbreviated as type IV Aeromonas pilus (Tap), in which T4P is encoded in part by the tapABCD operon (Barnett and Kirov, 1999; Boyd et al., 2008). Rsp0189 (673 amino acids) was identified as a homolog of TapV, sharing 58% amino acid identity. Expression of T3SS was substantially reduced in tapV transposon mutants, and is thus the first report linking the T3SS and T4P components. We therefore focused on TapV and Rsp0189 to investigate their roles in T4P properties, T3SS regulation, and contribution to pathogenicity in R. solanacearum.

2 | RESULTS

2.1 | Structural features of TapV and Rsp0189

Genomes of R. solanacearum strains possess a unique feature of two replicons: one chromosome (3.7 Mb in GMI1000) and one megaplasmid (2.1 Mb in GMI1000) (Salanoubat et al., 2002). TapV (Rsc1986 in GMI1000) is located in the chromosome of GMI1000, but Rsp0189 in the megaplasmid. R. solanacearum strains are extremely heterogeneous, while TapV and Rsp0189 are greatly conserved, exhibiting more than 95% amino acid identity among different R. solanacearum strains. TapV and Rsp0189 appear to be orphans because they do not transcriptionally link with other genes and no transposase or integrase have been identified nearby, indicating that these two genes are not integrated by simple transposition or horizontal gene transfer (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi). For instance, TapV is located between two genes encoding aspartate-semialdehyde dehydrogenase (Rsc1987) and tRNA pseudouridine synthase A (Rsc1985), and clear intergenic spacers can be observed between these genes. Moreover, TapV and Rsp0189 exhibit quite high guanine and cytosine (GC) contents of 70.8% and 71.2%, respectively, which is consistent with about 69% of GC content in the genome of R. solanacearum GMI1000 (Salanoubat et al., 2002).

Blast search analysis at National Center for Biotechnology Information suggests that R. solanacearum TapV is a member of the FimV super family that contains a putative peptidoglycan (PG)-binding LysM domain (residues 214–259), a putative tetra-tricopeptide repeat (TPR) domain (residues 602–669), and a putative gametogenetin (GGN) domain (residues 745–823) (Figure 1a). The TPR domain is typically involved in protein–protein interactions (Dandrea and Regan, 2003; Robinson et al., 2014). The TPR domain is typically involved in protein–protein interactions (Dandrea and Regan, 2003; Robinson et al., 2005), and the GGN domain has been found to be associated with the intracellular membrane and is involved in vesicular trafficking (Strong and Schimienti, 2010). FimV has been validated to be important for T4P assembly and T4P-dependent twitching motility in Pseudomonas aeruginosa (Bateman and Bycroft, 2000; Webhi et al., 2011; Sieweringa et al., 2014). The TPR domain is typically involved in protein–protein interactions (Dandrea and Regan, 2003; Robinson et al., 2005), and the GGN domain has been found to be associated with the intracellular membrane and is involved in vesicular trafficking (Strong and Schimienti, 2010). FimV has been validated to be important for T4P assembly and T4P-dependent twitching motility in Pseudomonas aeruginosa (Bateman and Bycroft, 2000; Semmler et al., 2000). TapV is thus assumed to be one of T4P assembly proteins that may span the membrane and interact with components of T4P. Rsp0189 (673 amino acids) has been identified as a homolog to TapV, sharing 54% of identity at N-termini of about 300 amino acids containing the typical LysM domain, and 82% of identity in the central region of 150 amino acids containing the TPR domain (Figure 1a). It is worthwhile noting that LysM domains of TapV and Rsp0189 share 85% amino acid identity.

2.2 | TapV but not Rsp0189 is essential for T4p-dependent twitching motility in R. solanacearum

To determine whether TapV and Rsp0189 are involved in functional T4P of R. solanacearum, we first evaluated their requirement for twitching motility, one of the most representative T4P-dependent features in many bacteria. Colonies of the wild-type strain (RK5050) growing on the surface of broth agar plates exhibited typical twitching zones that are made up of small groups or individual cells with a poorly defined and irregular boundary, while colonies of the tapV mutant (RQ5703) had a more uniform and well-defined boundary with tightly packed cells, which was similar to phenotypes of the pilA mutant (RQ6055), a well-known T4P-deficient mutant in many bacteria (Figure 1b). It was noteworthy that these typical twitching zones could be observed on all colonies from the wild-type strain, but not on any colonies from tapV or pilA mutants. Different from those of the tapV and pilA mutants, colonies of the rsp0189 mutant (RQ6059) exhibited similar twitching zones to those in the wild-type strain (Figure 1b). Expression of complemented tapV or pilA with their native promoters in the respective mutants could fully restore the twitching motility phenotype to that of the wild-type strain (Figure 1b). As the control, the empty vector pUC18mini-Tn7T-Gm was integrated into chromosomes of RK5050 and the tapV, pilA, and rsp0189 mutants, which did not affect formation of twitching zones in either of the strains (data not shown). All these results confirm that TapV, but not Rsp0189, is essential for functional T4P in R. solanacearum.

2.3 | TapV is important for T4p-dependent swimming motility and root adherence, but negatively affects biofilm information in R. solanacearum

Adherence to various surfaces, swimming motility, and biofilm formation are also typical T4P-dependent properties in many bacteria. To determine whether TapV is required for these T4P-dependent properties in R. solanacearum, the swimming motility assay was carried out on semisolid agar plates. Swimming halos produced by the tapV and pilA mutants were significantly smaller than those produced by the wild-type strain, while the rsp0189 mutant produced similar swimming halos to the wild-type strain (Figure 2a). The biofilm formation assay was carried out in polystyrene microtitre plates (96-well plates). The tapV mutant formed more biofilm than the wild-type strain, while the rps0189 mutant exhibited similar ability on biofilm formation to the wild-type strain (Figure 2b), which was different from the pilA mutant (a known T4P-deficient mutant), which formed less biofilm
than the wild-type strain (Liu et al., 2001; Kang et al., 2002). It should be clarified that both the wild-type strain and mutants did not form pellicles at the air–liquid interface even though they were cultured in polystyrene microtitre plates without shaking overnight (data not shown), confirming that these observed biofilms are due to surface adhesion.

To investigate the impact of TapV on adherence to host cells, roots of hydroponically cultured tomato plants were dipped into bacterial suspension and adherent cells were quantified by dilution plating. Although the tapV mutant exhibited similar ability to the wild-type strain to adhere to tomato roots at 16 hr post-inoculation (hpi), its ability to adhere to tomato roots was significantly impaired during 20–24 hpi, which is consistent with the pilA mutant (a known T4P-deficient mutant) exhibiting significantly impaired ability to adhere to tomato roots 16–24 hpi compared to the wild-type strain (Figure 2c). All of these results confirm that TapV, but not Rsp0189, is important for T4P-dependent swimming motility and root adherence, while TapV negatively affects biofilm information in R. solanacearum.

**FIGURE 1** Involvement of TapV on twitching motility of *Ralstonia solanacearum*. (a) Schematic and comparison of TapV (Rsc1986 in reference strain GMI1000, 962 amino acids, AA) and its homologue Rsp0189 (673 amino acids). TapV and Rsp0189 contain a peptidoglycan (PG)-binding domain (LysM, red) and a predicted tetratricopeptide repeat domain (TPR, grey). The figures between TapV and Rsp0189 refer to amino acid identities of two regions between TapV and Rsp0189.

(b) Twitching motility assay of strains. (a) wild-type refers to the wild-type strain (RK5050), (b) Δrsp0189 refers to RQ6069 (RK5050, Δrsp0189), (c) ΔtapV refers to RQ5703 (RK5050, ΔtapV), (d) ΔtapV + tapV refers to RQC0343 (RQ5703 with complementary tapV), (e) ΔpilA refers to RQ6055 (RK5050, ΔpilA), and (f) ΔpilA + pilA refers to RQC0347 (RQ6055 with complementary pilA). A bacterial suspension of 10 µl at an OD600 of 0.1 was dropped onto the surface of 1% broth agar on sterile slides and kept at 28 °C for 24 hr with coverslips. Twitching zones were observed with a light microscope (Olympus CX21) equipped with a 40× objective. Each assay was carried out with three biological replicates including four replications per trial. Typical twitching zones could be observed on all samples from the wild-type strain and complementary strains, but not on any sample from the tapV or pilA mutants. The empty vector pUC18mini-Tn7T-Gm was subjected to system control, which did not affect formation of twitching zones in either of the strains (data not shown), and a representative result is presented.
2.4 | Expression of the T3SS is dependent on TapV both in vitro and in planta, but is T4P independent

TapV was originally screened as one of the T3SS-regulating candidates by transposon mutagenesis, in which the expression profile of T3SS in \( R. \) solanacearum was monitored by \( \text{popA-lacZYA} \) fusion (Zhang et al., 2013). The \( \text{popA} \) gene is located upstream of the \( \text{hrp} \) regulon that belongs to T3Es and is directly controlled by HrpB. The \( \text{popA-lacZYA} \) fusion exhibits an identical expression profile to the \( \text{hrp} \) regulon under different conditions and this fusion does not affect the infection process of OE1-1 toward host plants (Zhang et al., 2013). The T3SS is not expressed in rich medium but is induced in \( \text{hrp} \)-inducing medium (nutrient-limited medium) (Cennac et al., 2004; Yoshimochi et al., 2009), and we assessed \( \text{popA} \) expression in \( \text{hrp} \)-inducing medium. Consistent
with those in transposon mutants, popA expression in the tapV mutant was significantly impaired in hrp-inducing medium (71 versus 316 Miller units of the wild-type reporter strain RK5050), and complementary tapV could completely restore impaired popA expression to that of RK5050 (Figure 3a). On the contrary, the pilA and rsp0189 mutants exhibited similar expression levels of popA as RK5050 (Figure 3a), indicating that TapV, but not PilA and Rsp0189, is required for T3SS expression in R. solanacearum. The requirement of TapV for T3SS expression is T4P independent.

Plant signals can greatly enhance T3SS expression to much higher levels compared with that in hrp-inducing medium (Valls et al., 2006; Yoshimochi et al., 2009). We further investigated whether TapV is required for T3SS expression in planta. Tobacco leaves were infiltrated with a bacterial suspension at an OD₆₀₀ of 0.1, and bacterial cells were recovered from infiltrated leaves at 6–24 hpi for the enzyme assay. Consistent with above result in hrp-inducing medium, popA expression in tobacco leaves was significantly impaired with tapV deletion during 6–24 hpi, but not in the pilA or rsp0189 mutants (Figure 3b), confirming that TapV is important for T3SS expression both in vitro and in planta, and the requirement of TapV for T3SS expression is T4P independent.

2.5 | TapV greatly contributes to the pathogenicity of R. solanacearum in host plants

Both the T3SS and T4P play important roles in the pathogenicity of R. solanacearum, so we decided to evaluate whether TapV and Rsp0189 contribute to the pathogenicity of R. solanacearum toward host plants. Two host plants of tomato and tobacco plants were subjected to virulence assay with inoculation methods of soil-soaking, which mimics natural invasion through roots, and petiole inoculation, which enables direct invasion into xylem vessels. Note that tobacco plants were inoculated with leaf infiltration, which enables direct invasion into intercellular spaces of tobacco leaves. RK5050 (the wild-type strain) eventually killed all test plants, while the tapV mutant was significantly less virulent than RK5050, which killed only approximately 25% of test plants to 25 days post-inoculation (dpi) regardless of different host plants and inoculation methods. Complementary tapV completely restored its virulence to that of the wild-type strain in both tomato and tobacco plants (Figure 4a–d), confirming that TapV greatly contributes to the pathogenicity of R. solanacearum in host plants.

The virulence of the pilA and rsp0189 mutants was also assessed in tomato and tobacco plants. Only the pilA mutant exhibited similar pathogenicity to the tapV mutant, which was significantly less virulent than RK5050 in test plants regardless of different host plants and inoculation methods. This is consistent with previous reports on some known R. solanacearum T4P-deficient mutants (Liu et al., 2001; Kang et al., 2002), while the rsp0189 mutant exhibited similar pathogenicity to RK5050 (Figure 4a–d). It is worthwhile noting that the tapV and pilA mutants exhibited almost equivalently reduced virulence compared with RK5050 in tomato and tobacco plants regardless of inoculation method.

2.6 | TapV positively affects T3SS expression via the PhcA–TapV–PrhG–HrpB pathway

In R. solanacearum, the T3SS is directly controlled by a master regulator HrpB, and hrpB expression is positively regulated by two close
paralogs of HrpG and PrhG in a parallel way. Expression of hrpG is positively regulated by a signalling cascade of PrhA–PrhIR–PrhJ, while expression of prhG is independent of this cascade, which is positively regulated by PrhN and PhcA, a global regulator (Genin, 2010; Hikichi et al., 2017). We generated tapV mutants from reporter strains of RK5046 (hrpB-lacZYA), RK5120 (hrpG-lacZAY), RK5212 (prhG-lacZAY), RK5124 (prhJ-lacZAY), RK5134 (prhA-lacZAY), RK5619 (phcA-lacZAY), and RK5043 (phcA-lacZAY) to ascertain how TapV affects T3SS expression. Expression of hrpB and the T3SS was not activated in rich medium but was induced in hrp-inducing medium (Cunnac et al., 2004; Yoshimochi et al., 2009), and we assessed hrpB expression in hrp-inducing medium. Deletion of tapV substantially impaired hrpB expression in hrp-inducing medium (61 vs. 186 Miller units of RK5046 [hrpB-lacZYA]) (Figure 5a), confirming that the impact of TapV on T3SS is mediated through the master regulator HrpB (Figure 5a). Furthermore, deletion of tapV substantially decreased prhG expression in both hrp-inducing and rich media (150 vs. 2,907 Miller units of RK5212 [prhG-lacZAY] in hrp-inducing medium and 483 vs. 2,144 Miller units of RK5212 in rich medium), but there was no alteration on hrpG expression with tapV deletion in either medium (Figure 5a,b), confirming that the impact of TapV on hrpB expression is mediated through PrhG, but independent of the signalling cascade of PrhA–PrhIR–PrhJ–HrpG.

We recently demonstrated that PhcA and PrhN positively regulate prhG expression (Zhang et al., 2013, 2015), and we evaluated whether TapV affects their expression. Deletion of tapV did not affect expression of prhN and phcA in either medium (Figure 5a,b),
and we turned to evaluate whether PhcA and PrhN affect tapV expression. A tapV-lacZYA reporter fusion was generated and integrated into chromosome of OE1-1 (the wild-type strain) with the Tn7-based chromosomal integration system to generate the reporter strain RQC0341. Note that integration of tapV-lacZYA did not affect the basal growth rate of each mutant (data not shown). Deletion of phcA substantially impaired tapV expression in both rich and hrp-inducing media (713 vs. 2,524 Miller units of RQC0341 in rich medium and 633 vs. 1519 Miller units of RQC0341 in hrp-inducing medium) (Figure 5c), indicating that expression of tapV is positively regulated by PhcA. This is consistent with previous reports that the Phc confinement-sensing system

![Figure 5](image-url)

**FIGURE 5** Effect pathway of TapV on the type III secretion system (T3SS). (a) and (b) Effect of tapV deletion on expression of a subset of regulatory genes in hrp-inducing medium (a) and nutrient-rich medium (b). Expression of hrpB and the T3SS was not expressed in rich medium, but induced in hrp-inducing medium, and the hrpB expression was assessed in hrp-inducing medium (a). The ΔtapV refers to deletion of tapV from each reporter strain of RK5046 (hrpB-lacZYA), RK5120 (hrpG-lacZAY), RK5212 (prhG-lacZAY), RK5124 (prhJ-lacZAY), RK5134 (prhA-lacZAY), RK5619 (prhN-lacZAY), and RK5043 (phcA-lacZAY). (c) Expression of tapV-lacZYA with deletion of phcA or prhN. The ΔphcA and ΔprhN refer to deletion of phcA or prhN from the reporter strain of RQC0341 (OE1-1, tapV-lacZYA), respectively. Cells were grown in hrp-inducing medium or nutrient-rich medium to an OD600 of about 0.1 and subjected to the β-galactosidase assay (the in vitro enzyme assay). The mean values from all three biological replicates were averaged and are presented with SD (error bars). The statistical significance between parent strains (WT) and tapV mutants (ΔtapV) was assessed using a post hoc Dunnett test following analysis of variance. Significance level, **p < .01
positively controls pilA expression in *R. solanacearum* (Kang et al., 2002), whereas deletion of prhN did not affect tapV expression in either medium (Figure 5c), indicating that both TapV and PrhN positively regulate prhG expression in a parallel way. All of these results suggest that TapV positively affects T3SS expression via the PhcA–TapV–PrhG–HrpB pathway.

### 2.7 TapV is important for dispersal in host plants, but not for in planta proliferation

Extensive proliferation in host plants is one of the most important pathogenicity determinants of *R. solanacearum*. We thus assessed whether TapV is required for in planta proliferation. Tobacco leaves were infiltrated with bacterial suspension at a concentration of $10^8$ cfu/ml and cell growth in tobacco leaves was assessed every other day until 6 dpi, when tobacco leaves became withered and dried. Both the wild-type strain and the tapV mutant exhibited similar proliferation in tobacco leaves from 2 to 6 dpi (Figure 6a), indicating that TapV is not required for intercellular proliferation of *R. solanacearum*.

Because the tapV mutant exhibited significantly less virulence than RK5050 in tobacco plants, we next assessed whether its impaired virulence was due to deficiency in migration from infiltrated leaves into petioles. Tobacco leaves were infiltrated with bacterial suspension at a concentration of $10^8$ cfu/ml and the cell number in petioles of the infiltrated leaves was measured every other day from 6 dpi, when RK5050 migrated into petioles, but not the tapV and pilA mutants, which could not be detected in petioles until 8 dpi. RK5050 could not be detected in petioles until 6 dpi with a density of approximately $2 \times 10^5$ cfu/g, and grew extensively to a maximum of approximately $4 \times 10^7$ cfu/g at 12 dpi, when petioles of infiltrated leaves became withered and dried (Figure 6b). The tapV and pilA mutants could not be detected in petioles of infiltrated leaves until 8 dpi with a densities of approximately $3 \times 10^5$ and $10^7$ cfu/g, respectively, which is about three orders of magnitude less than RK5050 (Figure 6b), indicating that the tapV and pilA mutants are strikingly deficient in migration from infiltrated leaves into petioles. Compared to RK5050, the tapV mutant grew much more slowly, reaching a density of approximately $10^7$ cfu/g at 14 dpi, when infiltrated tobacco leaves became withered, while the tobacco plants were still green (Figure 6b). Note that the RK5050-inoculated tobacco petioles became withered and fallen, and we did not assess bacterial growth in these petioles at 14 dpi (Figure 6b). The pilA mutant exhibited similar growth patterns to the tapV mutant in tobacco petioles of infiltrated leaves (Figure 6b). All of this confirmed that TapV is important for *R. solanacearum* to migrate from intercellular spaces into host petioles, which might be T4P dependent.

The tapV mutant exhibited significantly less virulence in petiole-inoculated tomato plants, by which *R. solanacearum* migrated into xylem vessels from tomato petioles. We further assessed the requirement of TapV for migration of *R. solanacearum* from inoculated tomato petioles into xylem vessels as shown in Figure 7. In brief, tomato plants were inoculated by dropping 2 µl of bacteria suspension onto the fresh-cut surface of petioles, and stems were periodically harvested for quantification of cell densities. The RK5050-inoculated petioles were harvested until 12 dpi, when tobacco petioles became withered and fallen, while petioles inoculated with tapV or pilA mutants were harvested until 14 dpi, when tobacco plants started slightly wilting, but remained green. Each assay was repeated with at least four biological replicates, including four replicates per trial. The mean values from all experiments were averaged and are presented with SD (error bars). The statistical significance between the wild-type strain (RK5050) and mutants (tapV mutant RQ5703 or pilA mutant RQ6055) was assessed using a post hoc Dunnett test following analysis of variance. Significance level, $**p < .01$.

![Figure 6](image-url)
site were set as position 0, then those 2 cm above and below position 0 were set as positions +2 and −2, respectively, then positions +4 and −4. Cell densities in stem sections were quantified at 3 dpi, when RK5050-inoculated tomato plants started wilting, and continued to be assessed on a daily basis for days 4, 5, and 6, when RK5050-inoculated tomato plants became wilted and died at 6 dpi (Figure 7b–d). RK5050 (the wild-type strain) grew well in tomato stems at 3 dpi, reaching a density of approximately $10^9$ cfu/g in stem sections at positions 0, −2, and −4 (below the inoculation site), and $10^7$–$10^8$ cfu/g in stem sections at positions +2 and +4 (above the inoculation site) (Figure 7a). At 3 dpi, the tapV mutant grew slowly to a density of approximately $10^4$ cfu/g in stem sections at positions 0 and −2 (below the inoculation site), while it was faintly detected in stem sections at positions +2, +4, and −4 with a density of less than $10^2$ cfu/g (Figure 7a). RK5050 grew quickly in stems and reached to a maximum of approximately $10^{10}$ cfu/g at 4–6 dpi, while the tapV mutant exhibited significantly impaired proliferation in different stem sections compared to RK5050 (Figure 7b–d). For instance, the tapV mutant grew to a density of approximately $5 \times 10^4$ cfu/g in stem sections at positions 0, −2, and +2 (around the region of 2 cm above and below the inoculated site) at 4 dpi, but approximately $10^2$ cfu/g in stem sections at positions +4 and −4 (Figure 7b). The tapV mutant grew to a density of approximately $10^6$ cfu/g in all stem sections at positions 0, −2, −4, +2, and +4 at 5 dpi (Figure 7c) and reached a maximum of approximately $10^9$ cfu/g in all stem sections at 6 dpi (Figure 7d), all of which were significantly less than those of RK5050 at the corresponding positions. Migration and proliferation of the pilA mutant in stem sections was also significantly impaired compared to that of the wild-type strain, but much higher than for the tapV mutant at 3–5 dpi (Figure 7b–d). These results confirm that TapV plays an important role in the migration of R. solanacearum from petioles into host xylem vessels.

2.8 TapV is important for expression of a subset of T3Es but not for elicitation of the hypersensitive response

Expression of abundant T3Es is directly controlled by HrpB, which is significantly impaired in the tapV mutant. We thus assessed whether
expression of T3Es was impaired with tapV deletion. In this study, a total of 11 T3Es, including RipAA, RipAR, RipB, RipD, RipE1, RipO, RipP1, RipR, RipTAL, RipW, and RipX (PopA as positive control), were selected for quantification of message RNA levels with quantitative reverse transcription PCR (RT-qPCR). Expression levels of these T3Es were significantly reduced in the tapV mutant (Figure S1), confirming that TapV is important for expression of a subset of T3Es in R. solanacearum.

Several T3Es, such as RipAA and RipP1, have been experimentally validated to be responsible for hypersensitive response (HR) elicitation of R. solanacearum GMI1000 in tobacco leaves (Poueymiro et al., 2009; Peeters et al., 2013), so we assessed the requirement of TapV for HR elicitation of GMI1000 in tobacco leaves. Tobacco leaves were infiltrated with a cell suspension at OD600 of 0.1 and symptom development of necrotic lesions was investigated. The tapV (GF0067), pilA (GF0106), and rsp0189 mutants (GF0108) exhibited similar HR development to the parent strain GMI1000 in tobacco leaves (Figure S2), indicating that TapV, PilA, and Rsp0189 are not required for the HR elicitation of GMI1000 in tobacco leaves.

### 3 | DISCUSSION

In the present study, we provided multiple lines of evidence to demonstrate that TapV is a novel essential component for T4P functions in R. solanacearum. Although functions of T4P are diverse in different bacteria, T4P have been generally demonstrated to be important for twitching motility, biofilm formation, adhesion to host cells, DNA uptake, and bacteriophage infection (Burdman et al., 2011; Dunger et al., 2016). Twitching motility is one of the most representative T4P-dependent properties in many bacteria, and is confirmed to be abolished in T4P-deficient mutants (pilA mutants) in R. solanacearum (Liu et al., 2001; Kang et al., 2002; Wairuri et al., 2012). TapV contains the typical LysM domain, PTR domain, and transmembrane domain. Several proteins containing the LysM domain and PTR domain have been validated to be required for T4P assembly in different bacterial species that bind to peptidoglycan and associate strongly with components of T4P on the outer membrane (Bateman and Bycroft, 2000; Wehbi et al., 2011; Sieweringa et al., 2014). The tapV mutant exhibited the same phenotype as the pilA mutant on twitching motility, confirming that TapV is required for T4P assembly. TapV might bind to peptidoglycan and associate with some components of T4P on the outer membrane in R. solanacearum. Moreover, deletion of tapV significantly impaired the ability of R. solanacearum to adhere to host roots, migrate in host plants, and swim on the soft-agar surface, similarly to the phenotypes of the pilA mutant (T4P-deficient mutant). All this provides strong evidence to support the fact that TapV plays an essential role in functional T4P in R. solanacearum, while Rsp0189, the homolog of TapV, was not required for T4P even though they share 54% of identity at N-termini of about 300 amino acids containing the typical LysM domain (85% of identity at the typical LysM domain) and 82% of identity at the central region of 150 amino acids containing the TPR domain. The precise roles of TapV in T4P remain to be further elucidated in R. solanacearum, including how TapV interacts with T4P components to promote T4P assembly, and which residues or domains play important roles in the interaction between TapV and other T4P components.

Some T4P-dependent properties are also important requisites for the virulence of many pathogenic bacteria of animals and plants (Burdman et al., 2011; Dunger et al., 2016). It is not surprising that deletion of tapV significantly impaired the pathogenicity of R. solanacearum toward host tomato and tobacco plants. In R. solanacearum, several reports have revealed that T4P mutants exhibited pathogenicity-related phenotypes, including reduced autoaggregation, biofilm formation, and lack of attachment ability to tomato roots (Liu et al., 2001; Kang et al., 2002; Wairuri et al., 2012). Although the tapV mutant proliferated normally as the wild-type strain in the intercellular space of tobacco leaves where the bacterium was infiltrated, it exhibited a severely impaired ability to spread throughout host plants, including migration from the intercellular spaces of infiltrated leaves into petioles, and migration from petioles into xylem vessels of stems. Although the tapV mutant can eventually migrate into and extensively proliferate in xylem vessels of host plants, the growth rate of the tapV mutant in xylem vessels was significantly lower than that of the wild-type strain. Moreover, the proliferation of the tapV mutant in xylem vessels and tobacco petioles was less than that of the wild-type strain by more than one order of magnitude. We speculate that the impaired ability of the tapV mutant to spread and proliferate throughout xylem vessels is the main reason for its significantly impaired virulence. Given the fact that the pilA mutant, a known T4P-deficient mutant in R. solanacearum, exhibited similar phenotypes to the tapV mutant in planta proliferation and migration throughout xylem vessels, the impaired ability of the tapV mutant in proliferation and migration might be due to the T4P deficiency. This is consistent with the fact that Rsp0189 is independent of functional T4P and hence is not required for pathogenicity of R. solanacearum toward host plants.

Substantial evidence has demonstrated that the ability to adhere to the host cell surface is important for bacteria to initiate infection, and twitching motility is important for biofilm formation, both of which allow bacteria to efficiently colonize different niches (O’Toole et al., 2000; Chiang and Burrows, 2003; Kaiser, 2003; Klausen et al., 2003). The tapV and pilA mutants exhibited significantly impaired swimming ability, which is known to make a most important contribution to bacterial wilt virulence, especially in the early stages of host plant invasion and colonization (Tans-Kersten et al., 2004; Lowe-Power et al., 2018). It is consistent with our observation that the ability to adhere to tomato roots was significantly impaired in the tapV and pilA mutants. Given that the T4P makes its contribution to multiple stages of pathogenesis (Lowe-Power et al., 2018), and the tapV and pilA mutants exhibited significantly impaired twitching motility, one of the most representative T4P-dependent features, significantly impaired migration and proliferation of the tapV mutant in host plants might be due to deficient T4P in R. solanacearum. Intriguingly, the tapV mutant produced much more biofilm than the wild-type strain in polystyrene plates, which is different
to many T4P-deficient mutants (Burdman et al., 2011; Dunger et al., 2016). With observation by scanning electronic microscope, it was recently reported that R. solanacearum can produce mushroom-type biofilms on the surface of tomato cells after invasion into intercellular spaces (Mori et al., 2016, 2017). A similar observation also reported that twitching motility is required for biofilm mushroom-like caps in P. aeruginosa (Klausen et al., 2003). TapV might play different roles in biofilm formation under different conditions and sophisticated observation will be required to evaluate its impact on mushroom-type biofilm in host plants.

It is interesting that TapV was found to positively affect T3SS expression, another essential pathogenicity determinant in R. solanacearum (Hikichi et al., 2007; Genin, 2010). It is intriguing that the pilA mutant exhibited similar expression levels of the T3SS both in vitro and in planta, indicating that the requirement of TapV for T3SS expression is T4P independent. TapV might play a novel role in affecting T3SS expression in plant pathogenic bacteria. It has been reported that expression of some T3SS-related genes was induced by bacterial contact to host cells mediated by T4P in P. aeruginosa (Yahr and Wolfgang, 2006). Expression of hrpB and T3SS is not activated until the bacterium comes into contact with host signals or some mimic signals (Marenda et al., 1998; Yoshimochi et al., 2009; Zhang et al., 2013). It was previously reported that a R. solanacearum GMI1000 hrpY mutant, deficient in contact with host cells with hrp pili, can adhere to suspension-cultured host cells and induce T3SS expression as the wild-type strain (Aldon et al., 2000; VanGijsegem et al., 2000). The T4P-dependent adhesion might enable R. solanacearum to make close contact with host cells, resulting in the induction of T3SS expression. HrpG and PrhG, two close paralogs of TCS response regulators, can respond to host signals and activate hrpB expression in a parallel way (Plener et al., 2010; Zhang et al., 2013). We previously reported that prhG expression was positively controlled by a global regulator PhcA, a central regulator in the global regulation network of pathogenicity that controls multiple genes directly or indirectly in R. solanacearum (Schneider et al., 2009; Genin and Denny, 2012; Zhang et al., 2013). Consistent with previous reports that pilA expression at the exponential phase of growth is positively controlled by PhcA in R. solanacearum (Kang et al., 2002), tapV expression was revealed to be positively regulated by PhcA, indicating that the impact of T4P on the T3SS is also mediated via PhcA. We further revealed that impact of TapV on the T3SS is mediated through the PhcA-TapV-PrhG-HrpB pathway, but independent of the PhrA-PrhIR-PrhJ-HrpG-HrpB signalling cascade, whereas no interaction was detected between expression of tapV and prhN, both of which might positively regulate prhG expression in a parallel way.

Expression levels of hrpB and a subset of T3Es were significantly impaired in tapV mutants, which is consistent with the fact that the master regulator HrpB directly controls entire T3SS and T3Es (Valls et al., 2006; Hikichi et al., 2007). The HrpB mutant is well known to be indisposed to proliferate in host plants and completely loses pathogenicity in host plants (Valls et al., 2006; Yoshimochi et al., 2009), while the tapV mutant exhibited equal proliferation to the wild-type strain in the intercellular spaces of tobacco leaves, indicating that weakly expressed hrpB might be enough to fulfill bacterial proliferation in host plants. This is consistent with our observation that HR elicitation of GMI1000 in tobacco leaves is not altered with tapV deletion. Note that expression of a subset of T3Es was substantially impaired in hrp-inducing medium, but not diminished, and expression of ripX (popA, one of the T3Es) in tobacco leaves remained at about a quarter to one third of expression levels of the wild-type strain. These weakly expressed T3Es might be enough to be recognized by tobacco plants and then trigger plant immunity. This is consistent with our previous reports that several T3Es substantially decreased mutants exhibiting identical HR elicitation as GMI1000 in tobacco leaves (Zhang et al., 2018).

In summary, our genetic results demonstrated TapV as a novel essential component for functional T4P in R. solanacearum that greatly contributes to the infection process in host plants. TapV plays important roles in the T4P-dependent properties of twitching motility, swimming motility, and host cell adhesion, and hence the tapV mutant is deficient in dispersal in host plants. Moreover, it was revealed that TapV positively affects the T3SS expression both in vitro and in planta through the PhcA-TapV-PrhG-HrpB pathway. This is the first report that T4P components affect T3SS expression, providing a novel insight into understanding the various biological functions of T4P and the complex regulatory pathway in T3SS in R. solanacearum.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains and growth conditions

Escherichia coli strains DH12S and S17-1 were grown at 37 °C in Luria Bertani medium for plasmid construction and conjugal transfer, respectively. R. solanacearum strains were grown at 28 °C in nutrient-rich medium (broth medium) or nutrient-limited medium (hrp-inducing medium, sucrose medium) (Yoshimochi et al., 2009). The R. solanacearum strains used in this study are listed in Table 1. They are derivatives of GMI1000 and OE1-1. OE1-1 is virulent in tomato and tobacco plants (Kanda et al., 2003), while GMI1000 is avirulent in tobacco plants and elicits a hypersensitive response in tobacco leaves (Poueymiro et al., 2009).

4.2 | Mutant generation with in-frame deletion of tapV, pilA, and rsp0189

Mutants with in-frame deletion of target genes were generated with pK18mobsacB-based homologous recombination as described previously (Zhang et al., 2015). In general, two DNA fragments flanking target genes were conjugated with joint PCR and cloned into pK18mobsacB to generate pK18dtapV, pK18dpilA, and pK18dosp0189, which were subjected to in-frame deletion of genes tapV, pilA, and rsp0189, respectively. After validating sequences, each plasmid was individually transferred into R. solanacearum by conjugation with S17-1, and desired the mutants (listed in Table 1) were generated and confirmed by colony PCR with respective primer pairs (listed in Table S1).
TABLE 1  Bacterial strains used in this study

| Strain     | Relative characteristics | References             |
|------------|--------------------------|------------------------|
| OE1-1      | Wild-type, race 1, biovar 3 | Kanda et al. (2003)    |
| RK5046     | OE1-1, hrpB-lacZYA        | Yoshimochi et al. (2009)|
| RK5050     | OE1-1, popA-lacZYA        | Yoshimochi et al. (2009)|
| RK5120     | OE1-1, hrpG-lacZYA        | Yoshimochi et al. (2009)|
| RK5212     | OE1-1, prhG-lacZYA        | Zhang et al. (2013)    |
| RQ5703     | RK5050, ΔtapV             | This study             |
| RQ6055     | RK5050, ΔpilA             | This study             |
| RQ6069     | RK5050, Δrsp0189          | This study             |
| RQC0343    | RQ5703, tapV complementation | This study             |
| RQC0347    | RQ6055, pilA complementation | This study             |
| RQC0347    | RQ5703, pilA complementation | This study             |
| RQC0348    | RQ5703, pilA complementation | This study             |
| RQC0349    | RQ5703, pilA complementation | This study             |
| GM1000     | Wild-type, race 1, biovar 4 | Salanoubat et al. (2002) |
| GF0067     | GM1000, ΔtapV             | This study             |
| GF0106     | GM1000, ΔpilA             | This study             |
| GF0108     | GM1000, Δrsp0189          | This study             |

4.3 | Complementation analyses

Genetic complementation was performed with the pUC18-mini-Tn7T-Gm based site-specific chromosomal integration system (Tn7 insertion) as described previously (Choi et al., 2005; Zhang et al., 2011). In general, a DNA fragment, containing the target gene and an upstream region of about 600 bp, empirically harbouring a native promoter, was PCR amplified and cloned into pUC18-mini-Tn7T-Gm to generate pUCtapV and pUCpilA. After validating sequences, each complementary gene was integrated into the chromosome of corresponding mutants at a single attTn7 site (25 bp downstream of glmS) and the desired mutants were generated and confirmed by colony PCR with a primer pair of glmSdown and Tn7R (Zhang et al., 2011).

4.4 | Construction of reporter fusion of tapV-laZYA for promoter activity assay

Reporter strains with tapV-laZYA fusion were generated with the Tn7 insertion as described previously (Zhang et al., 2015). In general, promoterless lacZYA was fused to tapV at 54 bp after the start codon, in which 6 bp of nucleotide acids were replaced in KpnI by PCR for lacZYA insertion. The DNA fragment containing the promoter region (upstream region of about 600 bp) and the KpnI site was first cloned into pUC18-mini-Tn7T-Gm and then promoterless lacZYA was inserted to generate pUCtapV-lacZYA. After validating the sequence, the tapV-laZYA was integrated into chromosomes of different strains and the desired mutants were generated (Table 1).

4.5 | β-galactosidase assay

The β-galactosidase assay was performed to evaluate expression levels of lacZYA-fused genes both in vitro and in planta as described previously (Zhang et al., 2013). Enzyme activity in vitro was expressed in Miller units (Miller, 1992), and that in planta was normalized with luminescence divided by cell number (Zhang et al., 2013). Each assay was carried out with at least four biological replicates, and each trial included four replications. Mean values of all experiments were averaged with SD, and the statistical significance was assessed using a post hoc Dunnett test following analysis of variance (ANOVA).

4.6 | Virulence assay and HR test

Virulence assay was carried out on wilt-susceptible tomato plants (Solanum lycopersicum ‘Moneymaker’) and tobacco plants (Nicotiana tabacum ‘Bright Yellow’), which were grown at 25 °C for 2–3 or 3–4 weeks, respectively, and subjected for virulence assay (Yao and Allen, 2007; Zhang et al., 2013). Briefly, tomato plants were inoculated by the method of soil-soaking inoculation that mimics natural invasion through roots, and petiole inoculation that enables direct invasion into xylems vessels. Tobacco plants were inoculated by methods of soil-soaking and leaf-infiltration that enable direct invasion into intercellular spaces (Zhang et al., 2013). Each assay was carried out with at least four biological replicates and each trial included 12 plants. Wilt symptoms of plants were rated using a 1–4 disease index and the mean values of all experiments were averaged. The statistical significance was assessed using a post hoc Dunnett test following ANOVA.

The HR test was carried out in tobacco leaves of N. tabacum ‘Bright Yellow’ with leaf infiltration. In general, approximately 50 µl of bacterial suspension at a density of 10^8 cfu/ml was infiltrated into tobacco leaves with a blunt-end syringe and symptom development of necrotic lesions was recorded periodically (Zhang et al., 2015). Each test was carried out with four biological replicates including four leaves per trial and a representative result was presented.

4.7 | Observation of twitching motility

Twitching motility was observed as described previously (Turnbull and Whitchurch, 2014; Zhou et al., 2015). In brief, 10 µl of bacterial suspension at an OD_600 of 0.1 was dropped onto the surface of 1% broth agar on sterile slides, and kept at 28 °C for 24 hr with coverslips. The twitching zones were observed with a light microscope (Olympus CX21) equipped with a 40× objective. Each assay was carried out
with three biological replicates, including four replications per trial. Twitching zones were observed on all samples and a representative result was presented.

4.8 | Bacterial growth and dispersal in planta

Bacterial growth in planta was assessed as described previously (Zhang et al., 2013). In brief, bacterial cells were collected daily from tobacco leaves, tobacco petioles, and tomato stems for quantification with dilution plating. Cell densities in leaves and petioles (stems) were expressed in log_{10} cfu/cm² and log_{10} cfu/g, respectively.

The in planta dispersal was carried out in tobacco petioles and tomato stems, respectively. Briefly, approximately 50 µl of bacterial suspension at 10^8 cfu/ml was infiltrated into tobacco leaves and the tomato petioles of the infiltrated leaves were periodically harvested for quantification. For tomato petioles, 2 µl of bacterial suspension at a density of 10^6 cfu/ml was dropped onto the fresh-cut surface of tomato petioles. Tomato stems were harvested periodically and cut into sections of 2 cm in length. Stem sections (2 cm) around the inoculation site were set as position 0, and those 2 cm above and below position 0 were set as positions +2 and −2, respectively, and then positions +4 and −4. Cell densities in the stem sections were quantified with dilution plating and expressed in log_{10} cfu/g. Each assay was repeated for at least four biological replicates, including four replications per trial. Mean values of all experiments were averaged with SD, and the statistical significance was assessed using a post hoc Dunnett test following ANOVA.

4.9 | Bacterial attachment assays

Bacterial attachment was carried out on tomato roots as described previously (Liu et al., 2001). Briefly, the root systems of 3-week-old tomato plants with sterile culture were immersed in 10 ml of bacterial suspension at a density of 10^7 cfu/ml and kept at 25 °C without agitation for 36 hr. Lateral roots were then periodically harvested, gently dipped in water twice to remove unattached bacteria, and the cell number was quantified with dilution plating. Each assay was repeated for at least four biological replicates, including four replications per trial. Mean values of all experiments were averaged with SD, and the statistical significance was assessed using a post hoc Dunnett test following ANOVA.

4.10 | Biofilm formation and swimming motility assay

Biofilm formation was assessed in 96-well polystyrene microtitre plates as described previously (Mori et al., 2016; Zhang et al., 2018). Briefly, 20 µl of bacterial suspension at an OD₆₀₀ of 1.0 was inoculated into 180 µl of fresh broth medium and kept at 28 °C for 24 hr without shaking. After staining with crystal violet, biofilm formation was quantified by measuring absorbance at 530 nm (A₅₃₀) and normalized with the cell number (OD₆₀₀). The swimming motility assay was carried out on semisolid media (0.3% agar plates) as reported previously (Kelman and Hruschka, 1973). The diameters of the swimming halos on semisolid media (28 °C for 48 hr) were measured. Each assay was repeated independently for four biological replicates, including three replications per trial. The mean values of all experiments were averaged with SD, and the statistical significance was assessed using a post hoc Dunnett test following ANOVA.

4.11 | RT-qPCR analysis

Expression levels of genes without lacZYA fusion were quantified by RT-qPCR analysis as described previously (Zhang et al., 2018). Briefly, total RNA was isolated by the TRIZol reagent method (Life Technologies) and cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). The One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara) was used for RT-qPCRs with the Applied Biosystems 7500 Real-Time PCR System. The primers used in this study were selected as previously described. Among them the serC gene was selected as reference for normalization of gene expression, and ripX was selected as positive control (Monteiro et al., 2012; Zhang et al., 2018). Each assay was carried out from RNA isolation with three biological replicates and each trial included four replications. The mean values of all experiments were averaged with SD, and the statistical significance between the wild-type strain and mutants was assessed using a post hoc Dunnett test following ANOVA.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Aldon, D., Brito, B., Boucher, C. and Genin, S. (2000) A bacterial sensor of plant cell contact controls the transcriptional induction of Ralstonia solanacearum pathogenicity genes. EMBO Journal, 19, 2304–2314.
Angot, A., Peeters, N., Lechner, E., Vailleau, F., Baud, C., Gentzbittel, L. et al. (2006) Ralstonia solanacearum requires F-box-like domain-containing type III effectors to promote disease on several host plants. Proceedings of the National Academy of Sciences of the United States of America, 103, 14620–14625.
Ill secretion system genes throughout plant infection. Microbiology, 158, 2107–2116.

Mori, Y., Inoue, K., Ikeda, K., Nakayashiki, H., Higashimoto, C., Ohnishi, K. et al. (2016) The vascular plant pathogenic bacterium Ralstonia solanacearum produces biofilms required for its virulence on the surfaces of tomato cells adjacent to intercellular spaces. Molecular Plant Pathology, 17, 890–902.

Mori, Y., Ishikawa, S., Ohnishi, H., Shimatani, M., Morikawa, Y., Hayashi, K. et al. (2017) Involvement of rafuraranones in the quorum sensing signaling pathway and virulence of Ralstonia solanacearum strain OE1-1. Molecular Plant Pathology, 19, 454–463.

Mukaihara, T., Tamura, N. and Iwabuchi, M. (2010) Genome-wide identification of a large repertoire of Ralstonia solanacearum type III effector proteins by a new functional screen. Molecular Plant-Microbe Interactions, 23, 251–262.

O'Toole, G.A., Gibbs, K.A., Hager, P.W., Phibbs, P.V. and Kolter, R. (2000) The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by Pseudomonas aeruginosa. Journal of Bacteriology, 182, 425.

Peeters, N., Carrère, S., Anisimova, M., Plener, L., Cazalé, A.C. and Genin, S. (2013) Repertoire, unified nomenclature and evolution of the type III effector gene set in the Ralstonia solanacearum species complex. BMC Genomics, 14, 859.

Plener, L., Manfredi, P., Vallis, M. and Genin, S. (2010) PrhG, a transcriptional regulator responding to growth conditions, is involved in the control of the type III secretion system regulon in Ralstonia solanacearum. Journal of Bacteriology, 192, 1011–1019.

Poueymiro, M., Cunuc, S., Barberis, P., Deslandes, L., Peeters, N., Cazalé-Noel, A.C. et al. (2009) Two type III secretion system effectors from Ralstonia solanacearum GM1000 determine host-range specificity on tobacco. Molecular Plant-Microbe Interactions, 22, 538–550.

Roberts, D.P., Denny, T.P. and Schell, M.A. (1988) Cloning of the egl gene of Pseudomonas solanacearum and analysis of its role in phytopathogenicity. Journal of Bacteriology, 170, 1445–1451.

Robinson, D.G., Oliviésson, P. and Hinz, G. (2005) Protein sorting to the storage vacuoles of plants: a critical appraisal. Traffic, 6, 615–625.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arelt, M., et al. (2002) Genome sequence of the plant pathogen Ralstonia solanacearum. Nature, 415, 497–502.

Schneider, P., Jacobs, J.M., Neres, J., Aldrich, C.C., Allen, C., Nett, M. et al. (2009) The global virulence regulators VsrAD and PhcA control secondary metabolism in the plant pathogen Ralstonia solanacearum. ChemBioChem, 10, 2730–2732.

Semmler, A.B., Whitchurgh, C.B., Leech, A.J. and Mattick, J.S. (2000) Identification of a novel gene, finV, involved in twitching motility in Pseudomonas aeruginosa. Microbiology, 146, 1321–1332.

Slewering, K., Jain, S., Friedrich, C., Webber-Birungi, M.T., Semchonok, D.A., Binzen, I. et al. (2014) Peptidoglycan-binding protein TsaP functions in surface assembly of type IV pili. Proceedings of the National Academy of Sciences of the United States of America, 111, E953–E961.

Skerker, J.M. and Berg, H.C. (2001) Direct observation of extension and retraction of type IV pili. Proceedings of the National Academy of Sciences of the United States of America, 98, 6901–6904.

Strom, M.S., Nunn, D.N. and Lory, S. (1993) A single bifunctional enzyme, PiID, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proceedings of the National Academy of Sciences of the United States of America, 90, 2404–2408.

Strong, E.R. and Schimenti, J.C. (2010) Evidence implicating CCNB1IP1, a RING domain-containing protein required for meiotic crossing over in mice, as an E3 SUMO ligase. Genes, 1, 440–451.

Tans-Kersten, J.K., Brown, D. and Allen, C. (2004) Swimming motility, a virulence factor of Ralstonia solanacearum, is regulated by FlhDC and by the plant host environment. Molecular Plant-Microbe Interactions, 17, 686–695.

Turkull, L. and Whitchurgh, C.B. (2014) Motility assay: twitching motility. Methods in Molecular Biology, 11, 73.

Valls, M., Genin, S. and Boucher, C. (2006) Integrated regulation of the type III secretion system and other virulence determinants in Ralstonia solanacearum. PLoS Pathogens, 2, e82.

VanGijsegem, F., Vasse, J., Camus, J.-C., Marenda, M. and Boucher, C. (2000) Ralstonia solanacearum produces Hrp dependent pili that are required for PopA secretion but not for attachment of bacteria to plant cells. Molecular Microbiology, 36, 249–265.

Vasse, J., Frey, P. and Trigalet, A. (1995) Microscopic studies of intercellular infection and protoxyl invasion of tomato roots by Pseudomonas solanacearum. Molecular Plant-Microbe Interactions, 8, 241–251.

Wairuri, C.K., Van der Waals, J.E., Van Schalkwyk, A. and Theron, J. (2012) Ralstonia solanacearum needs Flp pilus for virulence on potato. Molecular Plant-Microbe Interactions, 25, 546–556.

Wall, D., Kolenbrander, P.E. and Kaiser, D. (1999) The Myxococcus xanthus pilQ (sglA) gene encodes a secretin homolog required for type IV pilus biogenesis, social motility, and development. Journal of Bacteriology, 181, 24–33.

Wall, D. and Kaiser, D. (2010) Type IV pili and cell motility. Molecular Microbiology, 32, 1–10.

Webhi, H., Portillo, E., Harvey, H., Shimkoff, A.E., Scheuwater, E.M., Howell, P.L. et al. (2011) The peptidoglycan-binding protein FinV promotes assembly of the Pseudomonas aeruginosa type IV pilus secretin. Journal of Bacteriology, 193, 540–550.

Yahr, T.L. and Wolfgang, M.C. (2006) Transcriptional regulation of the Pseudomonas aeruginosa type III secretion system. Molecular Microbiology, 62, 631–640.

Yang, Y.C., Chou, C.P., Kuo, T.T., Lin, S.H. and Yang, M.K. (2004) PiIR enhances the sensitivity of Xanthomonas axonopodis pv. citri to the infection of filamentous bacteriophage Cp. Current Microbiology, 48, 251–261.

Yao, J. and Allen, C. (2007) The plant pathogen Ralstonia solanacearum needs aerotaxis for normal biofilm formation and interactions with its tomato host. Journal of Bacteriology, 189, 6415–6424.

Yoshimochi, T., Zhang, Y., Kiba, A., Hikichi, Y. and Ohnishi, K. (2009) Expression of hrpG and activation of response regulator HrpG are controlled by distinct signal cascades in Ralstonia solanacearum. Journal of General Plant Pathology, 75, 196–204.

Zhang, Y., Chen, L., Takehi, Y., Kiba, A., Hikichi, Y. and Ohnishi, K. (2013) Functional analysis of Ralstonia solanacearum PrhG regulating the hrp regulon in host plants. Microbiology, 159, 1695–1704.

Zhang, Y., Kiba, A., Hikichi, Y. and Ohnishi, K. (2011) prhKLM genes of Ralstonia solanacearum encode novel activators of hrp regulon and are required for pathogenesis in tomato. FEMS Microbiology Letters, 317, 75–82.

Zhang, Y., Li, J., Zhang, W., Shi, H., Luo, F., Hikichi, Y. et al. (2018) A putative LysR-type transcriptional regulator PrhO positively regulates the type III secretion system and contributes to the virulence of Ralstonia solanacearum. Molecular Plant Pathology, 19, 1808–1819.

Zhang, Y., Luo, F., Wu, D., Hikichi, Y., Kiba, A. and Igarashi, Y. et al. (2015) PrhN, a putative MarR family transcriptional regulator, is involved in positive regulation of type III secretion system and full virulence of Ralstonia solanacearum. Frontiers in Microbiology, 6, 357.

Zhou, X., Qian, G., Chen, Y., Du, L., Liu, F. and Yuen, G.Y. (2015) PiIG is involved in the regulation of twitching motility and antifungal antibiotic biosynthesis in the biological control agent Lysobacter enzymogenes. Phytopathology, 105, 1318–1324.
**SUPPORTING INFORMATION**
Additional Supporting Information may be found online in the Supporting Information section.

**FIGURE S1** Relative expression of T3Es genes in the tapV mutant. Strains were grown in hrp-inducing medium to an OD$_{600}$ of about 0.1 and total RNA was isolated. The cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser and mRNA levels of representative T3Es genes were determined by RT-qPCR with reference gene as serC for normalization. Normalized values of tapV mutant were divided by those of wild-type strain (WT) and relative values (relative expression) were presented. Mean values of at least three biological replicates were averaged and presented with SD (error bars). Statistical significance between the wild-type strain and prhP mutants was assessed using a post hoc Dunnett test following ANOVA. Significance level, **$p < .01$**

**FIGURE S2** HR test. Approximate 50 µl of bacterial suspension at $10^8$ cfu/ml was infiltrated into tobacco leaves with a blunt-end syringe. (a) GMI1000, the wild-type strain, (b) GF0067 (GMI1000, ΔtapV), (c) GF0106 (GMI1000, ΔpilA), (d) GF0108 (GMI1000, Δrsp0189), and (e) distilled water. Development of necrotic lesions was observed periodically and pictures were taken. Each experiment was repeated at least for four times and each treatment contained four plants. The results presented are from a representative experiment, and similar results were obtained in all experiments.

**TABLE S1** Primers used in this study

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