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A putative LysR-type transcriptional regulator PrhO positively regulates the type III secretion system and contributes to the virulence of *Ralstonia solanacearum*

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SUMMARY

LysR-type transcriptional regulators (LTTRs) are ubiquitous and abundant amongst bacteria and control a variety of cellular processes. Here, we investigated the effect of Rsc1880 (a putative LTTR, hereafter designated as PrhO) on the pathogenicity of *Ralstonia solanacearum*. Deletion of *prhO* substantially reduced the expression of the type III secretion system (T3SS) both *in vitro* and *in planta*, and resulted in significantly impaired virulence in tomato and tobacco plants. Complementary *prhO* completely restored the reduced virulence and T3SS expression to that of the wild-type. Moreover, PrhO-dependent T3SS and virulence were conserved amongst *R. solanacearum* species. However, deletion of *prhO* did not alter biofilm formation, swimming mobility and *in planta* growth. The expression of some type III effectors was significantly reduced in *prhO* mutants, but the hypersensitive response was not affected in tobacco leaves. Consistent with the key regulatory role of HrpB on T3SS, PrhO positively regulated the T3SS through HrpB. Furthermore, PrhO regulated *hrpB* expression via two close paralogues, HrpG and PrhG, which are two-component response regulators and positively regulate *hrpB* expression in a parallel manner. However, deletion of *prhO* did not alter the expression of *phcA*, *prhJ* and *prhN*, which are also involved in *hrpB* regulation. In addition, PrhO was expressed in a cell density-dependent manner, but negatively repressed by itself. No regulation was observed for HrpB, PhcA and PrhN on *prhO* expression. Taken together, we genetically demonstrated that PrhO is a novel virulence regulator of *R. solanacearum*, which positively regulates T3SS expression through HrpG, PrhG and HrpB and contributes to virulence.

Keywords: hrp regulation, LTTRs, pathogenicity, *Ralstonia solanacearum*, type III secretion system.

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INTRODUCTION

The LysR-type transcriptional regulators (LTTRs), which contain an N-terminal helix-turn-helix (HTH) DNA-binding domain and a C-terminal co-inducer-binding domain, are one of the largest families of regulators (more than 4000 orthologues) in diverse bacteria, archaea and algae (Maddocks and Oyston, 2008; Reen *et al.*, 2013; Schell, 1993). Extensive research has generally characterized LTTRs as extremely abundant, similar sized (300–350 amino acids), positively or negatively auto-regulated, and global regulators of diverse cellular processes (Heroven *et al.*, 2004; Maddocks and Oyston, 2008). LTTRs activate or repress transcription by affecting the efficiency of transcription initiation, and hence control a great variety of cellular processes, including metabolism, the oxidative stress response, antibiotic resistance, quorum sensing, cell motility and virulence (Hernández-Lucas *et al.*, 2008; Maddocks and Oyston, 2008; Sperandio *et al.*, 2002). Most LTTRs regulate the transcription of closely linked or genes in operon, whereas some regulate multiple unlinked genes (Maddocks and Oyston, 2008; Schell, 1993). To date, numerous LTTRs have been identified to be important for the virulence of many pathogenic bacteria of humans, animals and plants (Habdas *et al.*, 2010; Huang *et al.*, 1998; Jin *et al.*, 2011; Rashid *et al.*, 2016). For instance, BexR, MexT and MvfR regulate genes for virulence and antibiotic resistance in *Pseudomonas aeruginosa* (Deziel *et al.*, 2005; Jin *et al.*, 2011; Keith *et al.*, 2009; Reen *et al.*, 2013). In *Escherichia coli*, O157, QseA and QseD integrate several signalling networks and contribute to virulence (Habdas *et al.*, 2010; Kendall *et al.*, 2010). In the phytopathogenic bacterium, *Ralstonia solanacearum*, PhcA, a quorum-sensing-dependent LTTR, has been well characterized as a global regulator that controls the expression of diverse virulence-related genes, including those involved in plant cell wall degradation, motility, synthesis of extracellular polysaccharide (EPS) and the type III secretion system (T3SS) (Bhatt and Denny, 2004; Brumbley *et al.*, 1993; Genin *et al.*, 2005; Huang *et al.*, 1995).

Ralstonia solanacearum, the causal agent of bacterial wilt of plants, is a Gram-negative, soil-borne, vascular bacterium which

causes severe losses in many economically important plants worldwide (Genin, 2010). As a vascular bacterium, it generally invades the xylem vessels through root wounds or natural openings (Vasse *et al.*, 1995). Once inside the xylem vessels, *R. solanacearum* proliferates extensively and produces a huge amount of EPS slime, which severely blocks sap flow in xylem vessels and causes rapid stunting and wilting of plants (Denny, 1995; Roberts *et al.*, 1988). In addition to EPS, the syringe-like T3SS is essential for the infection process of *R. solanacearum* in host plants (Boucher *et al.*, 1987; Galán and Wolf-Watz, 2006; Lindgren, 1997). Bacteria use it to inject virulence factors, called type III effectors (T3Es), into host cytosol to subvert host defence and cause diseases (Cunnac *et al.*, 2004; Fujiwara *et al.*, 2016; Popa *et al.*, 2016; Tasset *et al.*, 2010). *Ralstonia solanacearum* is highly heterogeneous, which might be responsible for its extremely broad range of host species (Genin and Denny, 2012). The T3SS in *R. solanacearum* is encoded by approximately 20 genes located in the hypersensitive response and pathogenicity (*hrp*) gene cluster (Arlat *et al.*, 1992), and is highly conserved amongst *R. solanacearum* species (Coll and Valls, 2013; Genin and Denny, 2012). Moreover, *R. solanacearum* harbours an unusually large repertoire of T3Es (average of more than 70 T3Es per strain and total of more than 110 T3Es amongst *R. solanacearum* species), which is extremely more abundant than those of any closely related phytopathogenic bacterium, i.e. 20–30 T3Es in *Pseudomonas syringae*, 10–15 T3Es in *Erwinia* spp. and approximately 40 T3Es in *Xanthomonas* spp. To date, about 30 T3Es have been functionally characterized in the interaction between *R. solanacearum* and host cells (Coll and Valls, 2013; Peeters *et al.*, 2013; Tasset *et al.*, 2010).

The T3SS and entire T3Es in *R. solanacearum* are directly controlled by the master regulator HrpB, an AraC family transcriptional regulator (Angot *et al.*, 2006; Mukaihara *et al.*, 2004, 2010). The expression of *hrpB*, T3SS and T3Es is repressed in nutrient-rich medium, but activated in nutrient-limited medium, which might mimic plant apoplastic fluids, and can be increased to much higher levels when in contact with host plants or when invading host plants (Arlat *et al.*, 1992; Clough *et al.*, 1997; Marenda *et al.*, 1998; Yoshimochi *et al.*, 2009b). Two close paralogues, HrpG and PrhG, which belong to the OmpR/PhoB family of two-component response regulators, positively regulate *hrpB* expression in a parallel manner (Plener *et al.*, 2010; Zhang *et al.*, 2013). Plant signals or certain mimic signals are presumed to be recognized by an outer membrane protein PrhA, and transferred to HrpG through the PrhA–PrhR/I–PrhJ–HrpG signalling cascade (Marenda *et al.*, 1998; Valls *et al.*, 2006). HrpG and PrhG respond to host signals by phosphorylation at certain residues and greatly enhance *hrpB* expression, but the regulation mechanism remains to be further elucidated (Yoshimochi *et al.*, 2009b). Moreover, a well-characterized global LTTR regulator, PhcA, negatively regulates *hrpB* expression, which is activated at high cell density and binds to the promoter of *prhI/R* genes to repress their expression; this, in turn, shuts down the expression of *prhJ*, *hrpG*, *hrpB* and T3SS (Genin *et al.*, 2005; Yoshimochi *et al.*, 2009a). Different from HrpG, PrhG is independent of the PrhA cascade, and is positively regulated by PhcA and PrhN (Zhang *et al.*, 2017, 2011).

In order to further elucidate the regulation of T3SS, we generated a *popA-lacZYA* fusion, which belongs to T3Es, to monitor the expression profiles of T3SS in *R. solanacearum*. The generated reporter strains exhibited identical virulence and T3SS expression to those of wild-type strains under different conditions (Yoshimochi *et al.*, 2009b; Zhang *et al.*, 2013). We screened 43 T3SS-related candidates in the OE1-1 strain with transposon mutagenesis (Zhang *et al.*, 2013). Amongst them is Rsc1880, a putative LTTR (hereafter designated as PrhO). The expression level of the T3SS was substantially reduced in *prhO* transposon mutants. In total, 63 transcriptional regulators are predicted to be LTTRs in the genome of the OE1-1 strain, but only PhcA has been functionally characterized to date. Here, we investigated the role of PrhO in the regulation of T3SS expression and virulence in *R. solanacearum*.

RESULTS

PrhO positively regulates the T3SS expression of *R. solanacearum* species both *in vitro* and *in planta*

Previously, we used a *popA-lacZYA* fusion in OE1-1 (RK5050) to monitor T3SS expression and screened several T3SS-related candidates (Zhang *et al.*, 2013). Here, we generated a *prhO* in-frame-deleted mutant RK5738 (RK5050, Δ*prhO*) to confirm its effect on T3SS expression. Consistent with observations in transposon mutants, *popA* expression was substantially reduced in RK5738 (97 versus 293 Miller units of RK5050) in *hrp*-inducing (sucrose) medium, and complementary *prhO* completely restored the reduced *popA* expression to that of RK5050 (Fig. 1a), confirming that PrhO positively regulates T3SS expression of OE1-1 in *hrp*-inducing medium.

Ralstonia solanacearum species are highly heterogeneous, and strains usually show different properties in pathogenicity. For example, strain OE1-1 is virulent in both tomato and tobacco plants, whereas strain GMI1000 is virulent in tomato plants, but elicits a hypersensitive response (HR) in tobacco leaves. PrhOs are highly conserved in *R. solanacearum* species that exhibit more than 90% identity at the amino acid level. We therefore deleted *prhO* from GF001 (GMI1000, *popA-lacZYA*) to evaluate its role in GMI1000. Consistent with the observations in OE1-1, *popA* expression was substantially reduced in GF0094 (GF001, Δ*prhO*) (Fig. 1a). As expected, OE1-1 PrhO completely restored the reduced *popA* expression in GF0094 to that of GF001 (Fig. 1a), suggesting that the PrhO-dependent expression of T3SS is conserved amongst *R. solanacearum* species.

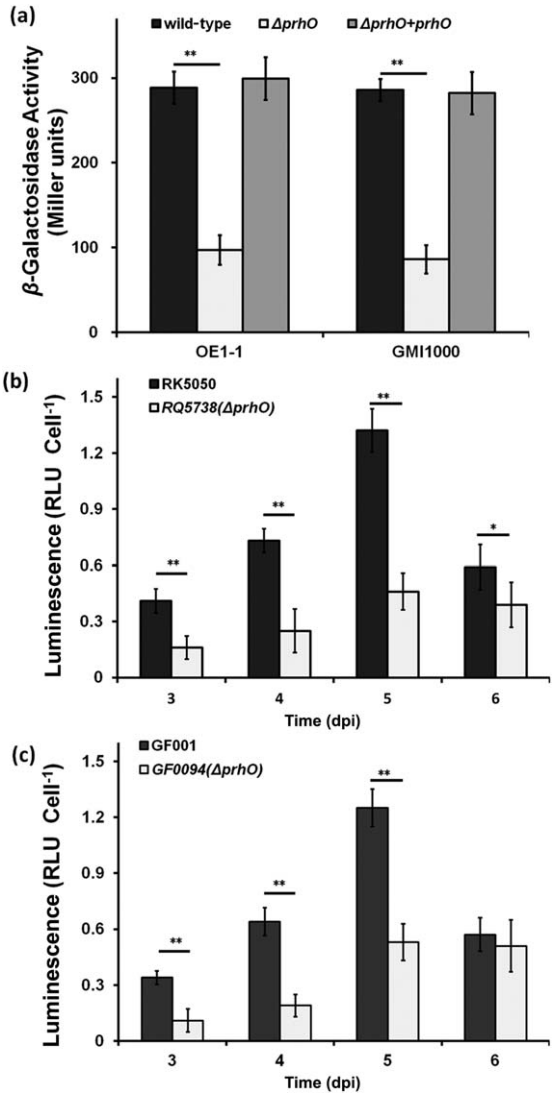

Fig. 1 Expression of *popA* in *Ralstonia solanacearum* strains. (a) Expression of *popA* in OE1-1 (left) and GMI1000 (right) derivatives grown in *hrp*-inducing medium. (b) Expression of *popA* in OE1-1 derivatives *in planta*. (c) Expression of *popA* in GMI1000 derivatives *in planta*. Black column, RK5050 (OE1-1, *popA-lacZYA*) or GF001 (GMI1000, *popA-lacZYA*); white column, *prhO* mutants RQ5738 (RK5050, Δ*prhO*) or GF0094 (GF001, Δ*prhO*); gray column, *prhO* complemented strains RQC0090 (RQ5738 + *prhO*) or RQC0095 (GF0094 + *prhO*). (a) Cells were grown in *hrp*-inducing medium to an optical density at 600 nm (OD_{600}) of approximately 0.1 and subjected to β-galactosidase assay. Enzymatic activities are presented in Miller units. (b, c) Tomato plants were inoculated with *R. solanacearum* strains using petiole inoculation and stem species were removed at 3–6 days post-inoculation (dpi) for enzyme assay *in planta* with a Galacto-Light Plus kit (Life, USA). Cell numbers were quantified by dilution plating and luminescence was evaluated using a GloMax20 luminometer (Promega, USA). Enzymatic activity was presented as luminescence normalized by the cell number. RLU, relative luminescence unit. The mean values of four independent experiments with four replications per trial were averaged and presented with standard deviation (SD) (error bars). Significance level: $*P < 0.05$; $**P < 0.01$ (*t*-test).

T3SS expression can be increased to a much higher level *in planta* than that in *hrp*-inducing medium. We therefore evaluated whether T3SS expression *in planta* was altered with *prhO* deletion. When directly inoculated into xylem by petiole inoculation, *R. solanacearum* causes wilting and death in tomato plants at 3 and 7 days post-inoculation (dpi), respectively. Hence, bacterial cells were collected daily from petiole-inoculated tomato stems at 3–6 dpi and subjected to enzyme assay. The two *prhO* mutants, including derivatives of OE1-1 (RK5738) (Fig. 1b) and GMI1000 (GF0094) (Fig. 1c), exhibited significantly reduced T3SS expression at 3–5 dpi ($P < 0.01$) compared with that of wild-type strains in tomato stems (Fig. 1b,c). At 6 dpi, T3SS expression in GF0094 was not changed ($P = 0.31$) (Fig. 1c), but slightly reduced in RK5738 ($P < 0.05$) (Fig. 1b). Our data indicate that the *in planta* expression of *R. solanacearum* T3SS is also positively regulated by PrhO.

PrhO positively regulates the expression of some T3Es in *R. solanacearum*

The *R. solanacearum* species complex harbours more than 110 T3Es, with some playing important roles during the infection process in host plants. PopA is a T3E, and we therefore evaluated whether the expression of T3Es was altered with *prhO* deletion. Total RNA was isolated from GF001 (GMI1000, *popA-lacZYA*) and GF0094 (GF001, Δ*prhO*), and mRNA levels of *ripAA*, *ripB*, *ripD*, *ripE*, *ripO*, *ripPI*, *ripR*, *ripTAL*, *ripW* and *ripX* (*popA*) were quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Consistent with results from the enzyme assay, the mRNA level of *ripX* was significantly reduced in GF0094 relative to that of the wild-type strain GF001 ($P < 0.01$) (Fig. 2). The mRNA levels of *ripAA*, *ripB*, *ripD*, *ripE*, *ripO*, *ripPI* and *ripW* were also significantly reduced ($P < 0.01$), and those of *ripR* and *ripTAL* were slightly reduced ($P < 0.05$), compared with that of the wild-type strain GF001 (Fig. 2), suggesting that the expression of some T3Es is also positively regulated by PrhO in *R. solanacearum*.

PrhO contributes to the virulence of *R. solanacearum* in host plants

T3SS and some T3Es play important roles in the pathogenicity of *R. solanacearum*, and we therefore evaluated the contribution of PrhO to pathogenicity. With soil-soaking inoculation, which mimics natural invasion through roots, wild-type strains caused wilting and death of most tomato plants within 10 dpi (Fig. 3a,b). In contrast, the two *prhO* mutants, including derivatives of OE1-1 (Fig. 3a) and GMI1000 (Fig. 3b), caused the eventual death of only about half of the tested tomato plants, and about half of the tomato plants remained healthy or only slightly wilted up to 15 dpi (Fig. 3a,b). With petiole inoculation, by which bacteria could invade xylem vessels directly, significantly reduced virulence was also observed in RQ5738 (Fig. 3c). When complemented with PrhO, the reduced virulence in *prhO* mutants was completely

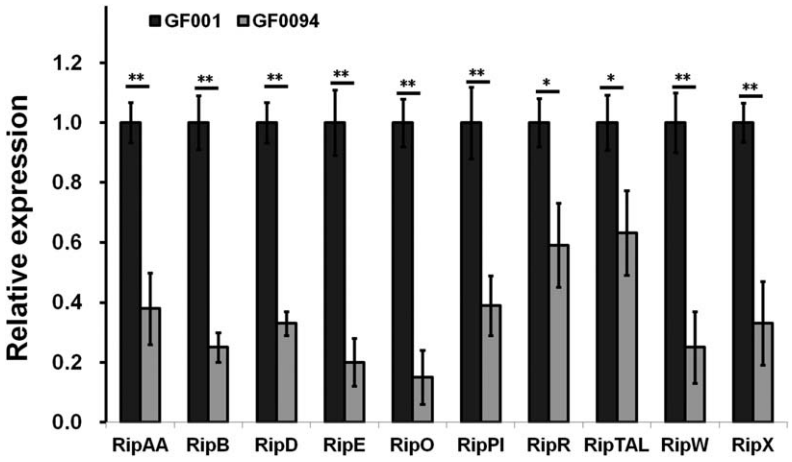

Fig. 2 Relative expression of selected type III effector (T3E) genes in *Ralstonia solanacearum*. Total RNA was isolated from GF001 and *prhO* mutant (GF0094), and mRNA levels of representative T3E genes in *hrp*-inducing medium were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). *serC* was selected as reference for normalization. Normalized values of GF0094 were divided by those of GF001 and relative values (relative expression) are presented. Mean values of at least three independent trials were averaged and are presented with standard deviation (SD) (error bars). Statistical significance was assessed between normalized values of GF001 and GF0094. Significance level: $*P < 0.05$; $**P < 0.01$ (*t*-test).

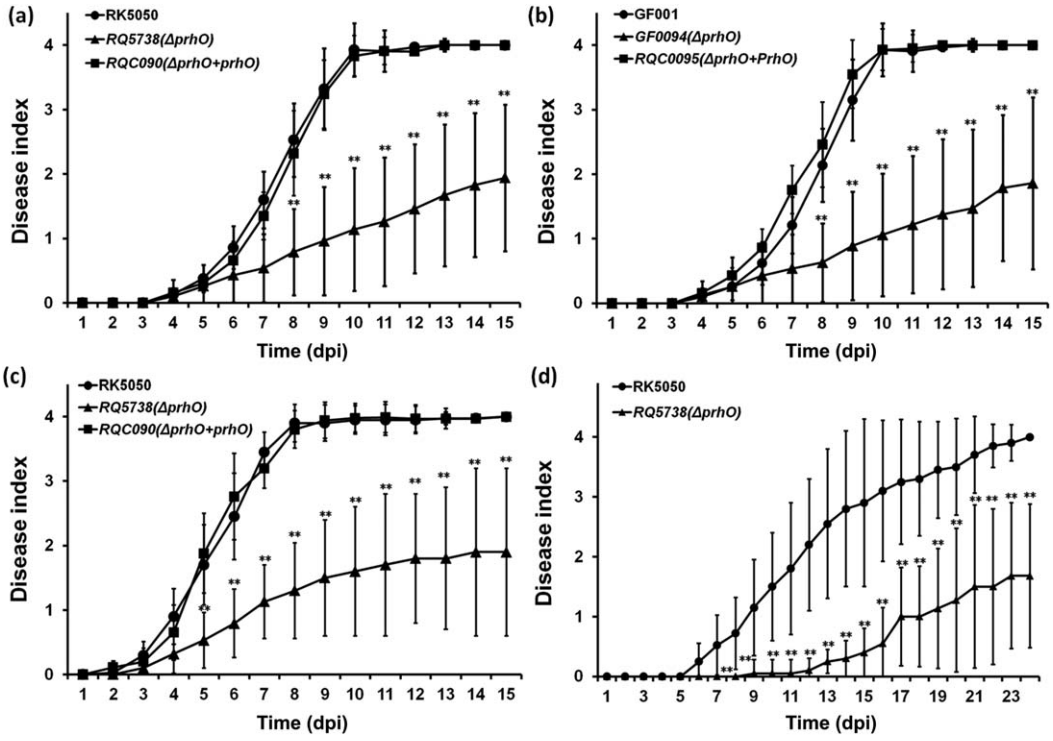

Fig. 3 Pathogenicity test. *Ralstonia solanacearum* strains used: (a, c, d) OE1-1 derivatives; (b) GMI1000 derivatives. Test plants: (a, b, c) tomato plants; (d) tobacco plants. Inoculation methods: (a, b) soil-soaking inoculation; a bacterial suspension was poured into the soil of plants at a final concentration of 10^7 colony-forming units (cfu)/g of soil; (c) petiole inoculation; about 3 μL of bacterial suspension at 10^8 cfu/mL was dropped onto the freshly cut petiole surface; (d) leaf infiltration in tobacco leaves; about 50 μL of bacterial suspension at 10^8 cfu/mL was infiltrated into tobacco leaves with a blunt-end syringe. Filled circles, wild-type strains; filled triangles, *prhO* mutants; filled squares, *prhO* mutants complemented with OE1-1 *prhO*. Plants were inspected daily for wilting symptoms and scored on a disease index scale of 0–4 (0, no wilting; 1, 1%–25% wilting; 2, 26%–50% wilting; 3, 51%–75% wilting; 4, 76%–100% wilting or death). dpi, days post-inoculation. Each assay was repeated in four independent trials and each trial contained at least 12 plants. Mean values of all results were averaged and are presented with standard deviation (SD) (error bars). Significance level: $*P < 0.05$; $**P < 0.01$ (*t*-test).

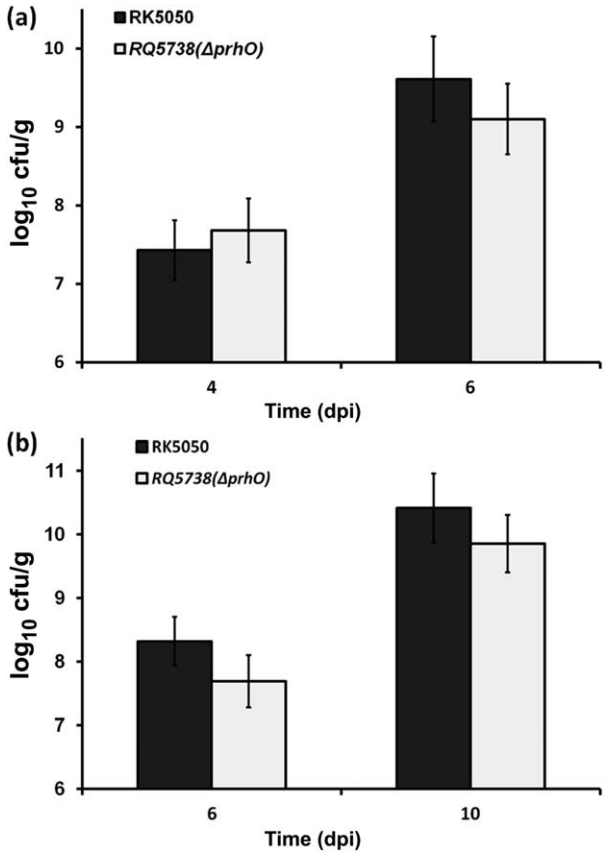

Fig. 4 Bacterial growth *in planta*. Tomato plants were inoculated with OE1-1 derivatives using petiole inoculation (a) and soil-soaking inoculation (b), and stem species were removed for quantification by dilution plating. Black column, RK5050; gray column, *prhO* mutant (RQ5738). cfu, colony-forming unit; dpi, days post-inoculation. Each assay was repeated in four independent trials and each trial contained at least 12 plants. Mean values of all results were averaged and are presented with standard deviation (SD) (error bars).

restored to that of the wild-type (Fig. 3a–c). We also evaluated the effect of PrhO on the virulence of OE1-1 in tobacco plants. With leaf infiltration, significantly reduced virulence was also observed in RQ5738, as about half of the RK5Q38-inoculated tobacco plants remained healthy or slightly wilted even up to 24 dpi (Fig. 3d). These results confirm that PrhO is important for the virulence of *R. solanacearum* species in different host plants.

PrhO is not required for bacterial growth *in planta* or HR elicitation in tobacco leaves

As an avascular pathogen, extensive proliferation in xylem vessels is one of the most important pathogenicity determinants in *R. solanacearum*. We therefore evaluated whether the impaired virulence of *prhO* mutants was a result of defective bacterial growth in host plants. With petiole inoculation of wild-type strains, tomato plants normally started to wilt at 3 dpi and died at 7 dpi; hence, we collected and quantified RK5738 cells from tomato stems at 4 and 6 dpi, respectively. RQ5738 exhibited similar bacterial growth to that of RK5050 in tomato stems (Fig. 4a). With soil-soaking inoculation, no difference was observed in bacterial growth between RQ5738 and RK5050 in tomato stems (Fig. 4b), suggesting that PrhO is not required for the bacterial growth of *R. solanacearum* in host plants.

GMI1000 elicits HR in tobacco leaves, and some T3Es are responsible for HR elicitation. We therefore evaluated whether *prho* deletion could alter the HR elicitation of GMI1000 in tobacco leaves. The generated *popA-lacZYA* strain (GF001) exhibited identical HR development to GMI1000 in tobacco leaves (Zhang *et al.*, 2017). Moreover, tobacco (*Nicotiana tabacum* BY) leaves were infiltrated with GF0094 and GF001, and the development of necrotic lesions was investigated periodically. It was intriguing that GF0094 exhibited almost identical development of necrotic lesions to that of GF001 in tobacco leaves (Fig. 5), indicating that PrhO is not involved in the HR elicitation of GMI1000 in tobacco leaves.

PrhO positively regulates T3SS expression through HrpG and PrhG to HrpB

The expression of T3SS and T3Es is directly controlled by HrpB. We thus deleted *prhO* from RK5046 (OE1-1, *hrpB-lacZYA*) to evaluate its impact on *hrpB* expression. Our results showed that *hrpB* expression was significantly reduced in RQ5940 (*hrpB-lacZYA*, Δ*prhO*) in *hrp*-inducing medium, and complementary PrhO completely restored *hrpB* expression to that of RK5046 (Fig. 6a), suggesting that the regulation of PrhO on T3SS is mediated through HrpB.

The expression of *hrpB* is positively regulated by both HrpG and PrhG in a parallel manner. Furthermore, we deleted *prhO* from RK5120 (OE1-1, *hrpG-lacZYA*) and RK5212 (OE1-1, *prhG-lacZYA*) to evaluate its impact on the expression of *hrpG* and *prhG*. *hrpG* expression was almost abolished in RQ5978 (RK5120, Δ*prhO*) in both rich medium (51 versus 1222 Miller units of RK5120) and *hrp*-inducing medium (32 versus 343 Miller units of RK5120) ($P < 0.01$) (Fig. 6b). Similarly, the expression of *prhG* was also significantly reduced in RQ5980 (RK5212, Δ*prhO*), but only in *hrp*-inducing medium (190 versus 3197 Miller units of RK5212) ($P < 0.01$) (Fig. 6c).

The expression of *hrpG* is positively regulated by the PrhA–PrhI/R–PrhJ signalling cascade, but negatively regulated by PhcA-mediated PrhI/R. However, the expression of *prhG* is positively regulated by PhcA and PrhN, but independent of the PrhA cascade. We thus deleted *prhO* from RK5043 (OE1-1, *phcA-lacZYA*), RK5124 (OE1-1, *prhJ-lacZYA*) and RK5619 (OE1-1, *prhN-lacZYA*) to evaluate its impact on the expression of *phcA*, *prhJ* and *PrhN*. However, deletion of *prhO* did not alter the expression levels of *prhJ, phcA* or *prhN* in either rich or *hrp*-inducing medium (Fig. 6d–f). Taken together, these data indicate that PrhO

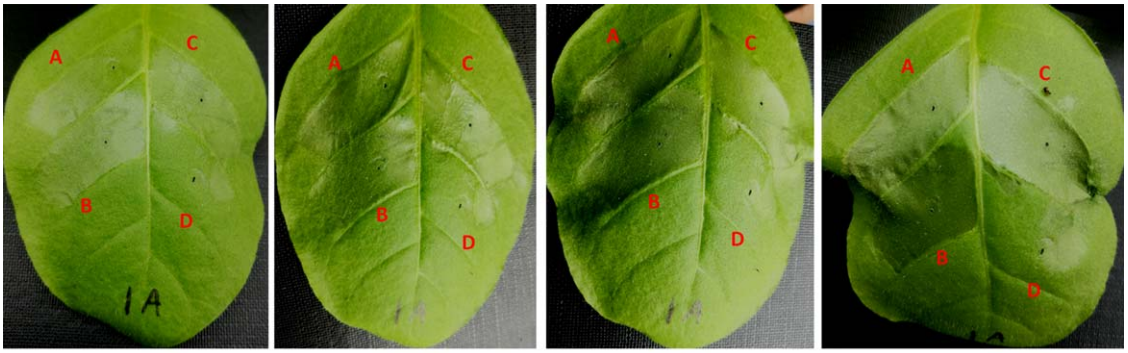

Fig. 5 Hypersensitive response (HR) test. Approximately 50 μL of bacterial suspension at 10^8 colony-forming units (cfu)/mL was infiltrated into tobacco leaves with a blunt-end syringe. (A) GF001 (GMI1000, *popA-lacZYA*); (B) GF0094 (GF001, Δ*prhO*); (C) RQC0095 (GF0094 complemented with *prhO*); (D) distilled water. The development of necrotic lesions was observed periodically and photographs were taken. Each experiment was repeated four times and each treatment contained four plants. The results presented are from a representative experiment, and similar results were obtained in all experiments.

positively regulates T3SS expression through HrpG and PrhG to HrpB, but not through the PrhA–PrhI/R–PrhJ signalling cascade, PhcA or PrhN.

PrhO is not required for biofilm formation and swimming motility in *R. solanacearum*

EPS production, biofilm formation and swimming motility are also important for the virulence of *R. solanacearum* in host plants and are globally controlled by PhcA (a representative LTTR in *R. solanacearum*). The *prhO* mutants remained mucoid on agar plates, indicating that EPS production was not abolished with *prhO* deletion. In polystyrene microtitre plates, RQ5738 exhibited biofilm formation similar to that of the wild-type in rich medium (Fig. 7a). On semi-solid motility agar plates, RQ5738 produced swimming halos similar to those of the wild-type strain. All of these results suggest that PrhO is not required for EPS production, biofilm formation or swimming motility in *R. solanacearum*.

PrhO is expressed in a cell density-dependent manner and negatively represses it expression, but there is no feedback regulation from PhcA, PrhN or HrpB

PhcA is a well-characterized LTTR in *R. solanacearum* which is quorum-sensing dependent. We constructed a *prhO-lacZYA* reporter strain (RQ5985) to evaluate its expression profile at different cell densities. The expression of *prhO* increased constantly with cell density in both rich medium (Fig. 8a) and *hrp*-inducing medium (data not shown), indicating that PrhO is expressed in a cell density-dependent manner. As PhcA globally regulates the expression of several virulence-related genes, we thus generated a *pchA* deletion mutant (RQ5987) from RQ5985 (OE1-1, *prhO-lacZYA*) to evaluate its impact on *prhO* expression. RQ5987 and RQ5985 exhibited similar expression levels of *prhO* in both rich and *hrp*-inducing medium (Fig. 8b). Moreover, *prhO* expression was not changed with *prhN* deletion in both rich and *hrp*-inducing medium (Fig. 8b), suggesting that *prhO* expression is independent of PhcA and PrhN.

Many LTTRs are auto-regulated, and we thus introduced the complementary *prhO* into RQ5985 (OE1-1, *prhO-lacZYA*) to evaluate its auto-regulation. Interestingly, the complementary *prhO* significantly decreased *prhO* expression, but only in *hrp*-inducing medium (Fig. 8c), suggesting that *prhO* expression is negatively regulated by itself under *hrp*-inducing conditions. Plener *et al.* (2010) reported that HrpB could regulate its expression through certain unknown mediators. We thus generated a *hrpB* deletion mutant (RQ5991) from RQ5985 (OE1-1, *prhO-lacZYA*) to evaluate whether PrhO is involved in the feedback regulation of HrpB. However, Q5991 and RQ5985 exhibited similar expression levels of *prhO* in both rich and *hrp*-inducing medium (Fig. 8c), suggesting that *prhO* expression is negatively regulated by itself, but there is no feedback regulation from PhcA, PrhN or HrpB.

DISCUSSION

LTTRs are abundant regulators in diverse bacteria, archaea and algae, which globally control a great variety of cellular processes, including pathogenicity (Maddocks and Oyston, 2008; Reen *et al.*, 2013; Schell, 1993). With genome searching, 63 transcriptional regulators were predicted to be LTTRs in OE1-1, but only PhcA has been functionally well characterized in *R. solanacearum* (Brumbley *et al.*, 1993; Genin *et al.*, 2005; Yoshimochi *et al.*, 2009a). In the present study, we provide genetic evidence to demonstrate that PrhO, a putative LTTR, is a novel virulence regulator, which positively regulates T3SS expression and contributes to the virulence of *R. solanacearum* in host plants.

T3SS is essential for pathogenicity in many pathogenic bacteria of humans, animals and plants (Galan and Wolf-Watz, 2006; Hueck, 1998). To date, numerous LTTRs have been identified to

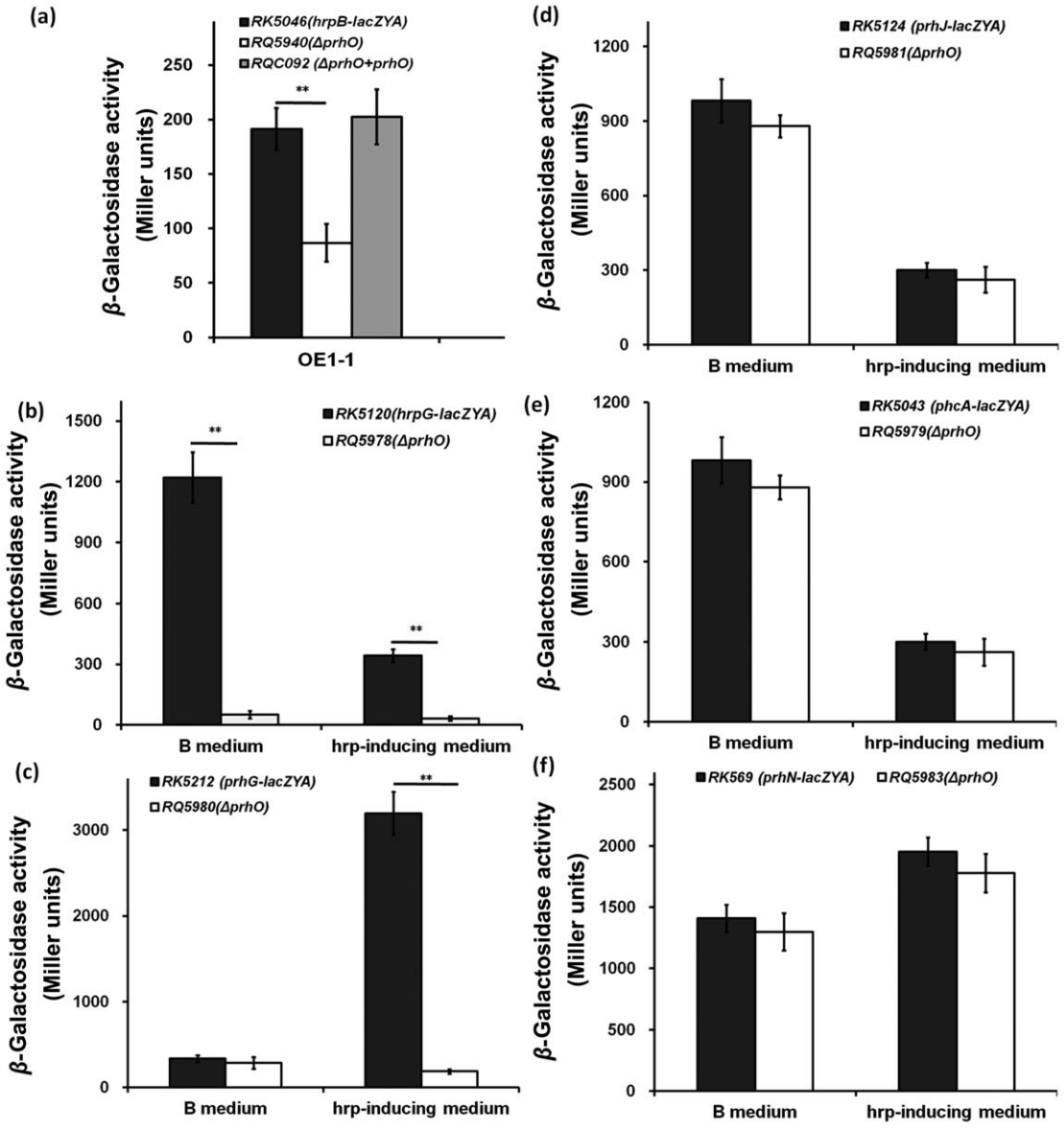

Fig. 6 Expression of certain known regulators. (a) *hrpB*; (b) *hrpG*; (c) *prhG*; (d) *prhJ*; (e) *phcA*; (f) *prhN*. Black column, reporter strains RK5046 (OE1-1, *hrpB-lacZYA*), RK5120 (OE1-1, *hrpG-lacZYA*), RK5212 (OE1-1, *prhG-lacZYA*), RK5124 (OE1-1, *prhJ-lacZYA*), RK5043 (OE1-1, *phcA-lacZYA*) and RK5619 (OE1-1, *prhN-lacZYA*); white column, *prhO* mutants RQ5940 (RK5046, Δ*prhO*), RQ5978 (RK5120, Δ*prhO*), RQ5980 (RK5212, Δ*prhO*), RQ5971 (RK5124, Δ*prhO*), RQ5979 (RK5043, Δ*prhO*) and RQ5983 (RK5619, Δ*prhO*); gray column, complemented strain RQC092 (RQ5940 + *prhO*). Cells were grown in rich medium or *hrp*-inducing medium to an optical density at 600 nm (OD_{600}) of approximately 0.1, and subjected to β-galactosidase assay. The mean values of all results were averaged and are presented in Miller units with standard deviation (SD) (error bars). Significance level: $^{**}P < 0.01$ (*t*-test).

control the T3SS in diverse pathogenic bacteria, such as BexR, MexT and MvfR in *Pseudomonas aeruginosa* (Deziel *et al.*, 2005; Jin *et al.*, 2011; Keith *et al.*, 2009; Reen *et al.*, 2013), QseA and QseD in enterohaemorrhagic *E. coli* K12 (Habdas *et al.*, 2010; Kendall *et al.*, 2010), GamR in *Xanthomonas oryzae* pv. *oryzae* (Rashid *et al.*, 2016) and PhcA in *R. solanacearum* (Huang *et al.*, 1998; Yoshimochi *et al.*, 2009a). The LTTR PrhO positively controls the T3SS in *R. solanacearum*, which is consistent with the impact of LTTRs on T3SS regulation. Deletion of *prhO* significantly reduced the T3SS expression of *R. solanacearum* both *in vitro* and *in planta*, but not the bacterial growth in host plants. However, the deletion of *prhO* did not alter EPS production, biofilm formation and swimming mobility, which are also important for the pathogenicity of *R. solanacearum*. Moreover, the extensive proliferation of *R. solanacearum* in the xylem vessels of host plants was not altered in *prhO* mutants, which is one of the most

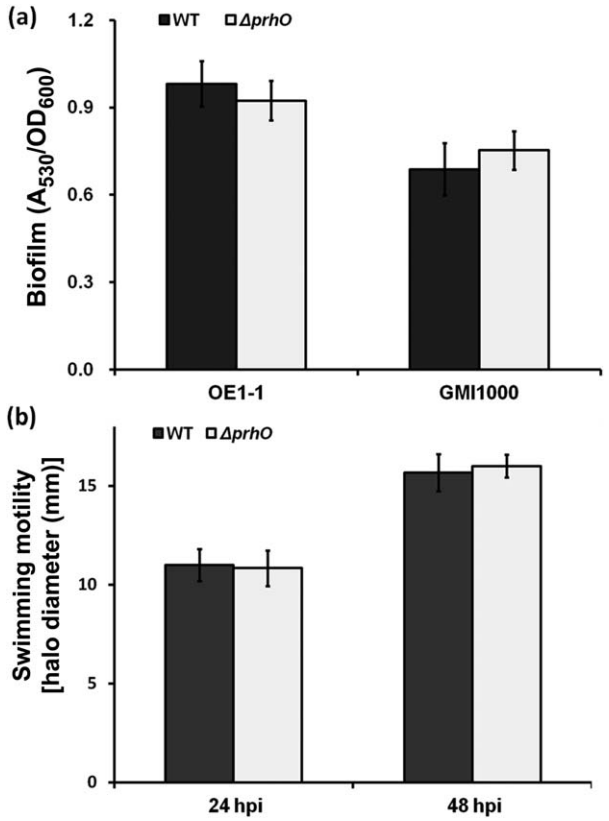

Fig. 7 Effect of PrhO on biofilm formation and swimming motility. (a) Biofilm formation. Left, OE1-1 derivatives; right, GMI1000 derivatives. Twenty microlitres of bacterial suspension at an optical density at 600 nm (OD_{600}) of 0.1 were inoculated into 180 μL of fresh rich medium and kept at 28 °C for 24 h without shaking. After staining with 0.1% crystal violet, biofilm formation was quantified with the absorbance at 530 nm (A_{530}) and normalized with OD_{600}. (b) Swimming motility. Three microlitres of bacterial suspension at $OD_{600} = 1.0$ were dropped onto 0.3% agar plates and kept at 28 °C for 48 h. Swimming motility was quantified as the halo diameter in millimetres. hpi, hours post-inoculation. Mean values of all results were averaged and are presented with standard deviation (SD) (error bars).

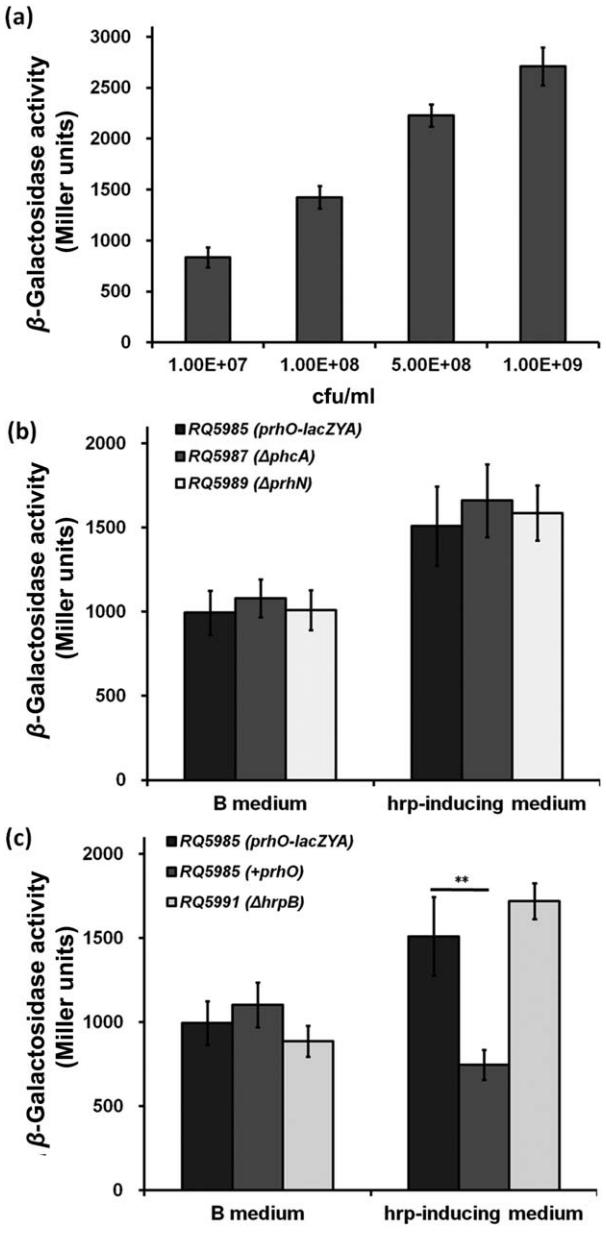

Fig. 8 *prhO* expression at different cell densities (a), in *phcA* and *prhN* mutants (b) and in the *prhO* complemented strain or *hrpB* mutant (c) in rich medium (left) and *hrp*-inducing medium (right). Mean values of all results were averaged and are presented with standard deviation (SD) (error bars). $^{**}P < 0.01$ (*t*-test).

important pathogenicity determinants in *R. solanacearum*. All of these data suggest that significantly reduced T3SS expression *in planta* is the probable cause of the impaired virulence of *prhO* mutants in host plants, not the defective growth in host plants. RNA sequencing (RNA-seq) is planned to further ascertain whether novel pathways are involved in the regulation of PrhO on the pathogenicity of *R. solanacearum* in host plants.

The expression levels of some T3Es were also significantly reduced in *prhO* mutants and some were responsible for the HR elicitation of *R. solanacearum* in resistant plants (Peeters *et al.*, 2013). It is intriguing that *prhO* mutants caused almost identical HR elicitation as GMI1000 in tobacco leaves. It should be noted that the expression of *popA* and some T3Es was significantly reduced, but not completely eliminated, in *prhO* mutants. This might be the result of a certain amount of residual *hrpB* expression in *prhO* mutants, and these T3Es could continue to elicit HR in resistant plants; this might be the reason why significantly impaired T3E expression did not delay HR elicitation of GMI100 in tobacco leaves.

The expression of the T3SS and entire T3Es in *R. solanacearum* is directly controlled by the key regulator HrpB which binds directly to the plant-inducible promoter (PIP) motif present in the

promoters of the target genes (Cunnac *et al.*, 2004; Mukaihara *et al.*, 2010). PrhO positively regulates T3SS expression via HrpB, which is consistent with the key regulatory role of HrpB on T3SS. Expression of *hrpB* is activated by two close paralogues, HrpG and PrhG, in a parallel manner. Our results showed that the expression of *hrpG* and *prhG* was almost eliminated with *prhO* deletion, suggesting that PrhO positively regulates the expression of both *hrpG* and *prhG*, and consequently regulates the expression of *hrpB*. However, it remains to be further ascertained whether PrhO regulates directly the expression of both *hrpG* and *prhG*. Further experiments are planned to identify the direct regulating targets of PrhO and to reveal its linear regulation on T3SS. Moreover, it is intriguing how PrhO simultaneously regulates the expression of *hrpG* and *prhG*, as they exhibit distinctly different expression properties. The most remarkable is that the expression level of *hrpG* is much higher in rich medium than in *hrp*-inducing medium or when co-cultivated with *Arabidopsis thaliana* seedlings (Yoshimochi *et al.*, 2009b), whereas the expression level of *prhG* is much lower in rich medium than in *hrp*-inducing medium (Zhang *et al.*, 2013). Moreover, the global regulator, PhcA, negatively regulates *hrpG* expression, but positively regulates *prhG* expression (Yoshimochi *et al.*, 2009b; Zhang *et al.*, 2013). *prhO* deletion did not alter the expression of *prhJ*, *phcA* and *prhN*, which are known to be important for the expression of *hrpG* and *prhG* (Hikichi *et al.*, 2007; Zhang *et al.*, 2015), indicating that the regulation of PrhO on the expression of *hrpG* and *prhG* is independent of these known regulating pathways. Recently, we have identified a plant-derived compound, umbelliferone, which impairs T3SS expression via HrpG and PrhG, but independent of these known regulators (Yang *et al.*, 2017), supporting our speculation that certain novel pathways should be integrated for the expression of *hrpG* and *prhG* in *R. solanacearum*. It should be noted that HrpB is not expressed until contact with *hrp*-inducing medium or with host plants, whereas HrpG is best expressed in rich medium. As members of the OmpR/PhoB family of two-component response regulators, HrpG and PrhG respond to host signals by phosphorylation at certain residues and sequentially activate *hrpB* expression (Yoshimochi *et al.*, 2009b). It remains to be further ascertained whether PrhO is involved in the host signal response with HrpG and PrhG.

The LTTR PhcA globally controls many virulence-related factors other than the T3SS, including EPS synthesis, biofilm formation and cell motility, which are important for the pathogenicity of *R. solanacearum* in host plants (Brumbley *et al.*, 1993; Genin *et al.*, 2005). However, *prhO* deletion did not alter these phenomena, except the T3SS, reflecting the complexity of LTTRs in diverse cellular processes. LTTR PhcA expression is quorum-sensing dependent, and its activity is triggered at high cell density by a unique autoinducer, 3-hydroxy palmitic acid methyl ester (3-OH PAME) or methyl 3-hydroxymyristate (3-OH MAME) (Flavier *et al.*, 1997; Mori *et al.*, 2017). *prhO* expression increases constantly with increasing cell density, suggesting that PrhO expression is also quorum-sensing dependent. It remains to be further elucidated how PrhO responds to cell density. Most LTTRs have been reported to be negatively auto-regulated and to repress their own expression, frequently with divergent promoters mediating their regulating targets (Maddocks and Oyston, 2008; Schell, 1993). *prhO* expression was significantly decreased by itself, but unchanged with the deletion of *hrpB* and *phcA*, indicating that PrhO was negatively regulated by itself, but the regulation pathway remains to be further elucidated.

The *R. solanacearum* species complex is extremely heterogeneous (Genin and Denny, 2012), but PrhO is highly conserved (more than 90% amino acid identity) in all *R. solanacearum* species. Indeed, PrhO of OE1-1 could functionally substitute for that of GMI1000 in T3SS expression and virulence in different host plants, indicating that the function of PrhO in T3SS expression and virulence is conserved amongst *R. solanacearum* species. With National Center for Biotechnology Information (NCBI) BLAST, PrhO orthologues were also found in certain bacteria, such as *Pandoraea* species (about 70% similarity), *Bordetella holmesii* (about 60%) and *Pseudomonas fuscovaginae* (about 50%), whereas no orthologues (more than 30% similarity) were found in *Pseudomonas syringae*, *Xanthomonas campestris* or *Xanthomonas oryzae* species, which are known to be much more closely related to phytopathogenic *R. solanacearum*.

In summary, our genetic results demonstrate that PrhO, a putative LTTR, is a novel virulence regulator of *R. solanacearum*. PrhO positively regulates the expression of T3SS through HrpG and PrhG to HrpB, and contributes to virulence. Future investigations will aim to ascertain the biochemical role of LTTR PrhO and its regulation mechanism on the expression of *hrpG* and *PrhG*.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

The *R. solanacearum* strains used in this study are listed in Table 1. It should be noted that strain OE1-1 (Kanda *et al.*, 2003) is virulent in both tomato and tobacco plants, whereas strain GMI1000 (Salanoubat *et al.*, 2002) is virulent in tomato plants, but causes HR in tobacco leaves. *Ralstonia solanacearum* strains were grown at 28 °C in nutrient-rich medium (B medium) or in *hrp*-inducing medium (sucrose medium) (Yoshimochi *et al.*, 2009b). *Escherichia coli* DH12S and S17-1 were grown in Luria–Bertani (LB) medium at 37 °C for plasmid construction and conjugational transfer, respectively.

Construction of *prhO* in-frame-deleted mutants

Mutants with in-frame deletion of genes were generated via pK18mobsacB-based homologous recombination, as described previously (Zhang *et al.*, 2015). For plasmid construction, two DNA fragments (about 600 bp) flanking the *prhO* gene were PCR amplified from OE1-1 genomic

Table 1 Strains used in this study.

Strain	Relative characteristics	Reference source
OE1-1	Wild-type, race 1, biovar 3	Kanda *et al.* (2003)
RK5050	OE1-1 *popA-lacZYA*	Yoshimochi *et al.* (2009b)
RQ5738	OE1-1 *popA-lacZYA*, Δ*prhO*	This study
RQ5739	OE1-1, Δ*prhO*	This study
RQC090	RQ5738, *prhO* complementation	This study
RK5046	OE1-1 *hrpB-lacZYA*	Yoshimochi *et al.* (2009b)
RQ5940	OE1-1 *hrpB-lacZYA*, Δ*prhO*	This study
RQC092	RQ5940, *prhO* complementation	This study
GMI1000	Wild-type, race 1, biovar 4	Salanoubat *et al.* (20022)
GF001	GMI1000, *popA-lacZYA*	Zhang *et al.* (2017)
GF0094	GMI1000, *popA-lacZYA*, Δ*prhO*	This study
RQC0095	GF0094, *prhO* complementation	This study
RK5120	OE1-1 *hrpG-lacZYA*	Yoshimochi *et al.* (2009b)
RQ5978	OE1-1 *hrpG-lacZYA*, Δ*prhO*	This study
RK5212	OE1-1 *prhG-lacZYA*	Zhang *et al.* (2013)
RQ5980	OE1-1 *prhG-lacZYA*, Δ*prhO*	This study
RK5043	OE1-1 *phcA-lacZYA*	Yoshimochi *et al.* (2009b)
RQ5979	OE1-1 *phcA-lacZYA*, Δ*prhO*	This study
RK5124	OE1-1 *prhJ-lacZYA*	Yoshimochi *et al.* (2009b)
RQ5981	OE1-1 *prhJ-lacZYA*, Δ*prhO*	This study
RK5619	OE1-1 *prhN-lacZYA*	Zhang *et al.* (2015)
RQ5983	OE1-1 *prhN-lacZYA*, Δ*prhO*	This study
RQ5985	OE1-1 *prhO-lacZYA*	This study
RQ5987	OE1-1 *prhO-lacZYA*, Δ*phcA*	This study
RQ5989	OE1-1 *prhO-lacZYA*, Δ*prhN*	This study
RQ5991	OE1-1 *prhO-lacZYA*, Δ*hrpB*	This study
RQC0096	RQ5983, *prhO* complementation	This study

DNA with the primer pairs 1880A1B-1880B1C and 1880A2C-1880B2H, respectively. It should be noted that 1880B1C and 1880A2C are fully complemented and these two DNA fragments were mixed for the second round of PCR to generate the DNA fragment (about 1.2 kb) with primers 1880A1B and 1880B2H, in which the open-reading frame of *prhO* was absent. The generated DNA fragment was first cloned into pBluescript KS (+) to obtain pKSd1880, and subcloned into pK18mobsacB to obtain pK18d1880. After validating the sequence, pK18d1880 was transferred from *E. coli* S17-1 into *R. solanacearum* strains, and *prhO* mutants (listed in Table 1) were generated and confirmed by colony PCR with the primer pairs 1880A1B and 1880B2H. The primers used in this study are listed in Table S1 (see Supporting Information).

Complementation analyses

Complementation analyses were performed with the pUC18-mini-Tn7T-Gm-based site-specific chromosome integration system (Choi *et al.*, 2005) with some minor modifications (Zhang *et al.*, 2015). The *prhO* gene containing a 500-bp upstream region, empirically harbouring its native promoter, was PCR amplified from OE1-1 genomic DNA with the primer pairs 1880A1B and 1880B3H. The DNA fragment was first cloned into pBluescript KS (+) to obtain pKS1880C, and subcloned into pUC18-mini-Tn7T-Gm to obtain pUCprhO. After validating the sequence, complementary *prhO* was specifically integrated into the *R. solanacearum* chromosome at a single *att*Tn*7* site (25 bp downstream of the *glmS* gene) and confirmed by colony PCR with the primer pairs glmsdown and Tn7R (Zhang *et al.*, 2011).

β-Galactosidase assay

The expression levels of genes fused with promoterless *lacZYA* were evaluated by the β-galactosidase assay (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 *in planta* was normalized by the luminescence divided by the cell number. Each assay was independently repeated in at least four experiments, and each treatment included four replications. The mean values of all experiments were averaged with the standard deviation (SD) and the statistical significance was assessed using a *post-hoc* Dunnett test following analysis of variance (ANOVA).

qRT-PCR analysis

The expression level of genes without *lacZYA* fusion was quantified by qRT-PCR analysis, as described previously (Zhang *et al.*, 2017). Representative T3E genes, *ripAA* (*avrA*), *ripB*, *ripD*, *ripE*, *ripO*, *ripPI* (*popPI*), *RipR*, *ripTAL*, *ripW* and *popA* (*ripX*), were selected for qRT-PCR analysis in this study. It should be noted that RipAA and RipPI have been well characterized to be responsible for HR development of GMI1000 in tobacco plants (Lavie *et al.*, 2002; Poueymiro *et al.*, 2009). *serC* was selected as reference for normalization and *ripX* was selected as positive control (Monteiro *et al.*, 2012). Total RNA was isolated from GF001 and *prhO* mutant (GF0094) and subjected to qRT-PCR analysis. Each assay was repeated independently from RNA isolation at least three times, and each trial included four replications. The mean values of all experiments were averaged with SD and the statistical significance was assessed using a *post-hoc* Dunnett test following ANOVA.

Virulence assay and HR test

Virulence assay and HR test were performed as described previously (Yao and Allen, 2007). Wilt-susceptible tomato plants (*Solanum lycopersicum* cv. Moneymaker) and tobacco plants (*Nicotiana tabacum* cv. Bright Yellow) were subjected to virulence assay with soil soaking, which mimics the natural invasion through the roots, and petiole inoculation, by which bacteria can invade directly the xylem vessels. Each assay was repeated independently four times with 12 plants per trial. Wilt symptoms of plants were inspected as the disease index (scale of 1–4) and the mean values of all experiments were averaged with SD. The statistical significance was assessed using a *post-hoc* Dunnett test following ANOVA.

The HR test was carried out in leaves of *N. tabacum* with leaf infiltration, and the symptom development of HR was recorded periodically. Each test was repeated independently four times with four plants per treatment. A representative result was presented.

Bacterial growth *in planta*

Bacterial growth *in planta* was assessed as described previously (Zhang *et al.*, 2013). Bacterial cells were collected daily from tomato stems and quantified by dilution plating. Each assay was repeated independently four times with 12 plants per treatment. The mean values of all experiments were averaged with SD and the statistical significance was assessed using a *post-hoc* Dunnett test following ANOVA.

Biofilm and swimming motility assay

Biofilm formation was performed in 96-well polystyrene microtitre plates as reported by Yao and Allen (2007) with some minor modifications (Mori *et al.*, 2016). Briefly, 20 μL of bacterial suspension at an optical density at 600 nm (OD_{600}) of 1.0 was inoculated into 180 μL of fresh B medium and kept at 28 °C for 24 h without shaking. After staining with crystal violet, biofilm formation was quantified by measuring the absorbance at 530 nm (A_{530}), and normalized by the cell number (OD_{600}). The swimming motility assay was carried out on semi-solid medium (0.3% agar plates), as reported previously (Kelman and Hruschka, 1973). The diameters of the swimming halos on semi-solid medium (28 °C for 48 h) were measured. Each assay was repeated independently at least four times and each trial included three replications. The mean values of all experiments were averaged with SD and the statistical significance was assessed using a *post-hoc* Dunnett test following ANOVA.

Construction of *prhO-lacZYA* reporter strain and auto-regulation analyses

A promoterless *lacZYA* fragment was inserted 22 bp downstream of the start codon of *prhO* to construct the *prhO-lacZYA* fusion, in which six nucleotides (GCCTTT) were replaced by GGTACC (*Kpn*I) by PCR for *lacZYA* insertion. Two DNA fragments flanking the insertion site were amplified from OE1-1 genomic DNA with the primer pairs 1880A1B and 1880B1K, and 1880A2K and 1880B3H, respectively. It should be noted that the primers 1880b1K and 1880A2K were fully complemented, and these two DNA fragments were mixed for the second round of PCR with the primer pairs 1880A1B and 1880B3H to generate the DNA fragment which contained the *Kpn*I site for *lacZYA* insertion. The generated DNA fragment was finally subcloned into pk18mobSacB to obtain pK18prhO. After validating the sequence, the *Kpn*I-digested promoterless *lacZYA* from pUClacZYA was inserted into pK18prhO to obtain pK18prhO-lacZYA. It should be noted that *lacZYA* maintains the same transcription direction as *prhO* and that they share the same promoter. After validating the sequence, pK18prhO-lacZYA was transferred from *E. coli* S17-1 into *R. solanacearum*, and the reporter strain RQ5985 (OE1-1 *prhO-lacZYA*) was generated and confirmed by colony PCR with the primer pairs 1880A1B and lacZR1. Based on RQ5985, mutants with deletion of *phcA*, *prhN* or *hrpB* were generated to evaluate their impact on *prhO* expression. Complementary PrhO was introduced into RQ5985 for the auto-regulation analyses.

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REFERENCES

Angot, A., Peeters, N., Lechner, E., Vailleau, F., Baud, C., Gentzbittel, L., Sartorel, E., Genschik, P., Boucher, C. and Genin, S. (2006) *Ralstonia solanacearum* requires F-box-like domain-containing type III effectors to promote disease on several host plants. *Proc. Natl. Acad. Sci. USA*, **103**, 14 620–14 625.

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

Bhatt, G. and Denny, T.P. (2004) *Ralstonia solanacearum* iron scavenging by the siderophore staphyloferrin B is controlled by PhcA, the global virulence regulator. *J. Bacteriol.* **23**, 7896–7904.

Boucher, C.A., Van Gijsegem, F., Barberis, P.A., Arlat, M. and Zischek, C. (1987) *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. *J Bacteriol.* **169**, 5626–5632.

Brumbley, S.M., Carney, B.F. and Denny, T.P. (1993) Phenotype conversion in *Pseudomonas solanacearum* due to spontaneous inactivation of PhcA, a putative LysR transcriptional activator. *J. Bacteriol.* **175**, 5477–5487.

Choi, K.H., Gaynor, J.B., White, K.G., Lopez, C., Bosio, C.M., Karkhoff-Chweizer, R.R. and Schweizer, H.P. (2005) A Tn7-based broad range bacterial cloning and expression system. *Nat. Methods*, **2**, 443–448.

Clough, S.J., Flavier, A.B., Schell, M.A. and Denny, T.P. (1997) Differential expression of virulence genes and motility in *Ralstonia (Pseudomonas) solanacearum* during exponential growth. *Appl. Environ. Microbiol.* **63**, 844–850.

Coll, N.S. and Valls, M. (2013) Current knowledge on the *Ralstonia solanacearum* type III secretion system. *Microb. Biotechnol.* **6**, 614–620.

Cunnac, S., Occhialini, A., Barberis, P., Boucher, C. and Genin, S. (2004) Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*: identification of novel effector proteins translocated to plant host cells through the type III secretion system. *Mol. Microbiol.* **53**, 115–128.

Denny, T.P. (1995) Involvement of bacterial polysaccharides in plant pathogenesis. *Annu. Rev. Phytopathol.* **33**, 173–197.

Deziel, E., Gopalan, S., Tampakaki, A.P., Lepine, F., Padfield, K.E., Saucier, M., Xiao, G. and Rahme, L.G. (2005) The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of N-acyl-L-homoserine lactones. *Mol. Microbiol.* **55**, 998–1014.

Flavier, A.B., Clough, S.J., Schell, M.A. and Denny, T.P. (1997) Identification of 3-hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*. *Mol. Microbiol.* **26**, 251–259.

Fujiwara, S., Kawazoe, T., Ohnishi, K., Kitagawa, T., Popa, C., Valls, M., Genin, S., Nakamura, K., Kuramitsu, Y., Tanaka, N. and Tabuchi, M. (2016) RipAY, a plant pathogen effector protein, exhibits robust γ-glutamyl cyclotransferase activity when stimulated by eukaryotic thioredoxins. *J. Biol. Chem.* **291**, 6813–6830.

Galán, J.E. and Wolf-Watz, H. (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature*, **444**, 567–573.

Genin, S. (2010) Molecular traits controlling host range and adaptation to plants in *Ralstonia solanacearum*. *New. Phytol.* **187**, 920–928.

Genin, S. and Denny, T.P. (2012) Pathogenomics of the *Ralstonia solanacearum* species complex. *Annu. Rev. Phytopathol.* **50**, 67–89.

Genin, S., Brito, B., Denny, T.P. and Boucher, C. (2005) Control of the *Ralstonia solanacearum* type III secretion system (Hrp) genes by the global virulence regulator PhcA. *FEBS Lett.* **579**, 2077–2081.

Habdas, B.J., Smart, J., Kaper, J.B. and Sperandio, V. (2010) The LysR-type transcriptional regulator QseD alters type three secretion in enterohemorrhagic *Escherichia coli* and motility in K-12 *Escherichia coli*. *J. Bacteriol.* **192**, 3699–3712.

Hernández-Lucas, I., Gallego-Hernandez, A.L., Encarnacion, S., Fernandez-Mora, M., Martinez-Batallar, A.G., Salgado, H., Oropeza, R. and Calva, E. (2008) The LysR-type transcriptional regulator LeuO controls expression of several genes in *Salmonella enterica* serovar *typhi*. *J. Bacteriol.* **190**, 1658–1670.

Heroven, A.K., Nagel, G., Tran, H.J., Parr, S. and Dersch, P. (2004) RovA is autoregulated and antagonizes H-NS-mediated silencing of invasion and *rovA* expression in *Yersinia pseudotuberculosis*. *Mol. Microbiol.* **53**, 871–888.

Hikichi, Y., Yoshimochi, T., Tsujimoto, S., Shinohara, R., Nakaho, K., Kanda, A., Kiba, A. and Ohnishi, K. (2007) Global regulation of pathogenicity mechanism of *Ralstonia solanacearum*. *Plant Biotechnol.* **24**, 149–154.

Huang, J., Carney, B.F., Denny, T.P., Weissinger, A.K. and Schell, M.A. (1995) A complex network regulates expression of *eps* and other virulence genes of *Pseudomonas solanacearum*. *J. Bacteriol.* **177**, 1259–1267.

Huang, J., Yindeeyoungyeon, W., Garg, R.P., Denny, T.P. and Schell, M.A. (1998) Joint transcriptional control of *xpsR*, the unusual signal integrator of the *Ralstonia solanacearum* virulence gene regulatory network, by a response regulator and a LysR-type transcriptional activator. *J. Bacteriol.* **180**, 2736–2743.

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

Jin, Y., Yang, H., Qiao, M. and Jin, S. (2011) MexT regulates the type III secretion system through MexS and PtrC in *Pseudomonas aeruginosa*. *J. Bacteriol.* **193**, 399–410.

Kanda, A., Ohnishi, S., Tomiyama, H., Hasegawa, H., Yasukohchi, M., Kiba, A., Ohnishi, K., Okuno, T. and Hikichi, Y. (2003) Type III secretion machinery-deficient mutants of *Ralstonia solanacearum* lose their ability to colonize resulting in loss of pathogenicity. *J. Gen. Plant Pathol.* **69**, 250–257.

Keith, H.T., Vallet-Gely, I. and Simon, L.D. (2009) Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator. *PLoS Genet.* **5**, e1000779.

Kelman, A. and Hruschka, J. (1973) The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum*. *Microbiology*, **76**, 177–188. doi: 10.1099/00221287-76-1-177.

Kendall, M.M., Rasko, D.A. and Sperandio, V. (2010) The LysR-type regulator QseA regulates both characterized and putative virulence genes in enterohemorrhagic *Escherichia coli* O157:H7. *Mol. Microbiol.* **76**, 1306–1321.

Lavie, M., Shillington, E., Eguiluz, C., Grimsley, N. and Boucher, C. (2002) PopP1, a new member of the YopJ/AvrRxv family of type III effector proteins, acts as a host-specificity factor and modulates aggressiveness of *Ralstonia solanacearum*. *Mol. Plant–Microbe Interact.* **15**, 1058–1068.

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

Maddocks, S.E. and Oyston, P.C. (2008) Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology*, **154**, 3609–3623.

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

Miller, J.H. (1992) The lac system. In: *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria* (Miller, J.H., ed), pp. 43–80. Plainview, NY: Cold Spring Harbor Laboratory Press.

Monteiro, F., Genin, S., Van Dijk, I. and Valls, M. (2012) A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection. *Microbiology*, **158**, 2107–2116.

Mori, Y., Inoue, K., Ikeda, K., Nakayashiki, H., Higashimoto, C., Ohnishi, K., Kiba, A. and Hikichi, Y. (2016) The vascular plant-pathogenic bacterium *Ralstonia solanacearum* produces biofilms required for its virulence on the surfaces of tomato cells adjacent to intercellular spaces. *Mol. Plant Pathol.* **17**, 890–902. doi: 10.1111/mpp.12335.

Mori, Y., Ishikawa, S., Ohnishi, H., Shimatani, M., Morikawa, Y., Hayashi, K., Ohnishi, K., Kiba, A., Kai, K. and Hikichi, Y. (2017) Involvement of ralfuranones in the quorum sensing signalling pathway and virulence of *Ralstonia solanacearum* strain OE1-1. *Mol. Plant Pathol.* **19**, 454–463. doi: 10.1111/mpp.12537.

Mukaihara, T., Tamura, N., Murata, Y. and Iwabuchi, M. (2004) Genetic screening of Hrp type III-related pathogenicity genes controlled by the HrpB transcriptional activator in *Ralstonia solanacearum*. *Mol. Microbiol.* **54**, 863–875.

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. *Mol. Plant–Microbe Interact.* **23**, 251–262.

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., Valls, 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*. *J. Bacteriol.* **192**, 1011–1019.

Popa, C., Li, L., Gil, S., Tatjer, L., Hashii, K., Tabuchi, M., Coll, N.S., Ariño, J. and Valls, M. (2016) The effector AWR5 from the plant pathogen *Ralstonia solanacearum* is an inhibitor of the TOR signalling pathway. *Sci. Rep.* **6**, 27 058.

Poueymiro, M., Cunnac, S., Barberis, P., Deslandes, L., Peeters, N., Cazale-Noel, A.-C., Boucher, C. and Genin, S. (2009) Two type III secretion system effectors from *Ralstonia solanacearum* GMI1000 determine host-range specificity on tobacco. *Mol. Plant–Microbe Interact.* **14**, 538–550. doi: 10.1094/MPMI-22-5-0538.

Rashid, M.M., Ikawa, Y. and Tsuge, S. (2016) GamR, the LysR-type galactose metabolism regulator, regulates *hrp* gene expression via transcriptional activation of two key *hrp* regulators, HrpG and HrpX, in *Xanthomonas oryzae* pv. *oryzae*. *Appl. Environ. Microbiol.* **82**, 3947–3958.

Reen, F.J., Barret, M., Fargier, E., O'Muinneacháin, M. and O'Gara, F. (2013) Molecular evolution of LysR-type transcriptional regulation in *Pseudomonas aeruginosa*. *Mol. Phylogenet. Evol.* **66**, 1041–1049.

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. *J. Bacteriol.* **170**, 1445–1451.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J.C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thébault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J. and Boucher, C.A. (2002) Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature*, **415**, 497–502.

Schell, M.A. (1993) Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.* **47**, 597–626.

Sperandio, V., Li, C.C. and Kaper, J.B. (2002) Quorum-sensing *Escherichia coli* regulator A: a regulator of the LysR family involved in the regulation of the locus of enterocyte effacement pathogenicity island in enterohemorrhagic *E. coli*. *Infect. Immun.* **70**, 3085–3093.

Tasset, C., Bernoux, M., Jauneau, A., Pouzet, C., Brière, C., Kieffer-Jacquinod, S., Rivas, S., Marco, Y., Deslandes, L. and Dangl, J.L. (2010) Autoacetylation of the *Ralstonia solanacearum* effector PopP2 targets a lysine residue essential for RRS1-R-mediated immunity in *Arabidopsis*. *PLoS Pathog.* **6**, e1001202.

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

Vasse, J., Frey, P. and Trigalet, A. (1995) Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. *Mol. Plant–Microbe Interact.* **8**, 241–251.

Yang, L., Li, S., Qin, X., Jiang, G., Chen, J., Li, B., Yao, X., Liang, P., Zhang, Y. and Ding, W. (2017) Exposure to umbelliferone reduces *Ralstonia solanacearum* biofilm formation, transcription of type III secretion system regulators and effectors and virulence on tobacco. *Front. Microbiol.* **8**, 1234.

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

Yoshimochi, T., Hikichi, Y., Kiba, A. and Ohnishi, K. (2009a) The global virulence regulator PhcA negatively controls the *Ralstonia solanacearum hrp* regulatory cascade by repressing expression of the PrhIR signaling proteins. *J. Bacteriol.* **191**, 3424–3428.

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

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 Microbiol. Lett.* **317**, 75–82.

Zhang, Y., Chen, L., Yoshimochi, T., 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., Luo, F., Wu, D., Hikichi, Y., Kiba, A., Igarashi, Y., Ding, W. and Ohnishi, K. (2015) PrhN, a putative marR family transcriptional regulator, is involved in positive regulation of type III secretion system and full virulence of *Ralstonia solanacearum*. *Front. Microbiol.* **6**, 357. doi: 10.3389/fmicb.2015.00357.

Zhang, Y., Li, J., Zhang, W., Wang, R., Qiu, Q., Luo, F., Hikichi, Y., Ohnishi, K. and Ding, W. (2017) Ferulic acid, but not all hydroxycinnamic acids, is a novel T3SS inducer of *Ralstonia solanacearum* and promotes its infection process in host plants under hydroponic condition. *Front. Plant Sci.* **8**, 1595.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Primers used in this study.