**Research Letter**

**prhKLM genes of Ralstonia solanacearum encode novel activators of hrp regulon and are required for pathogenesis in tomato**

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**Abstract**

The genes in the hrp regulon encode the proteins composing type III secretion system in *Ralstonia solanacearum*. The hrp regulon is positively controlled by HrpB, and hrpB expression is activated by both HrpG and PrhG. We have identified three genes, prhK, prhL, and prhM, which positively control the hrp regulon in strain OE1-1. These genes are likely to form an operon, and this operon is well conserved in the genera *Ralstonia* and *Burkholderia*. This indicates that the operon is not specific to the plant pathogens. Mutations in each of these three genes abolished hrpB and prhG expression. prhK, prhL, and prhM mutant strains lost pathogenicity toward tomato completely, and they were less virulent toward tobacco. PrhK and PrhL share sequence similarity with alolphanate hydrolase and PrhM with LamB. This suggests that the three gene products are not transcriptional regulators in the strict sense, but regulate hrp regulon indirectly. This novel class of virulence-related genes will mark the beginning of new findings regarding the overall infection mode of *R. solanacearum*.

**Introduction**

*Ralstonia solanacearum* (Yabuuchi *et al.*, 1995) is a Gram-negative, soil-borne vascular phytopathogen that causes wilt diseases in >200 plant species (Schell, 2000). hrp (hypersensitive response and pathogenicity) genes encode the component proteins of type III secretion system (T3SS) and are essential for the pathogenicity of *R. solanacearum* (Kanda *et al.*, 2003a). Bacteria use the T3SS to interact with host plants, and to inject virulence factors, the so-called type III effectors, into the host cytosol (Galan & Collmer, 1999).

The hrp genes are clustered together and form the hrp regulon (Van Gijssegem *et al.*, 1995). This regulon is repressed in nutrient-rich media (Arlat *et al.*, 1992). Nutrient-poor conditions, which may mimic conditions in the intracellular spaces of plants, induce a 20-fold increase in the expression of hrp regulon (Genin *et al.*, 1992). Plant signals stimulate the expression of operons belonging to the hrp regulon by another 10–20-fold relative to the expression in nutrient-poor conditions (Marenta *et al.*, 1998). The hrp regulon is positively regulated by the AraC-type transcriptional regulator HrpB (Genin *et al.*, 1992). Plant signals are perceived by the outer-membrane receptor PrhA, and are transduced to HrpG through PrhR/PrhI and PrhJ (Aldon *et al.*, 2000). HrpG then activates the expression of hrpB (Brito *et al.*, 1999). The HrpG homolog, PrhG, is also a two-component response regulator for the activation of hrpB (Pleren *et al.*, 2010). On the other hand, the hrp regulon is negatively regulated by a global virulence regulator, PhcA, in a quorum sensing-dependent manner (Genin *et al.*, 2005). PhcA binds to the promoter region of the prhIR operon, and represses the expression of prhIR. In turn, this shuts down the expression of all downstream genes (Yoshimochi *et al.*, 2009a).

In a previous study (Y. Zhang, unpublished data) using transposon mutagenesis, we isolated prh (positive regulation of hrp regulon) genes, which positively regulate the expression of hrp regulon, from the Japanese strain OE1-1. In the prhK, prhL, and prhM mutants, the expression of hrp regulon was completely abolished. prhK, prhL, and prhM do not belong to any of the known pathogenicity gene families in plant pathogenic bacteria. The aim of this study was to shed light on how the three genes regulate the hrp regulon. We also uncovered the involvement of the three genes in the pathogenicity of *R. solanacearum* in a couple of host plant species.
Materials and methods

Bacterial strains, culture media, and growth condition

The *R. solanacearum* strains, derivatives of the Japanese strains OE1-1 (phytype 1, race 1, biovar 3) (Kanda et al., 2003a) and RS1002 (phytype 1, race 1, biovar 4) (Mukaihara et al., 2004) used in this study, are listed in Table 1. *Escherichia coli* strains DH12S (Invitrogen) and S17-1 (Simon et al., 1983) were grown in Luria–Bertani (LB) medium at 37°C. *Ralstonia solanacearum* strains were grown at 28°C in rich B medium or hydroponic plant culture medium supplemented with 2% sucrose (sucrose medium) (Yoshimochi et al., 2009b). Antibiotics were added at the following concentrations: ampicillin (Ap, 100 μg mL⁻¹), gentamicin (Gm, 20 μg mL⁻¹), kanamycin (Km, 50 μg mL⁻¹), and polymyxin B (PB, 50 μg mL⁻¹).

β-Galactosidase assay

The β-galactosidase assay was performed as previously described (Yoshimochi et al., 2009b). Enzyme activities were measured at least in triplicate, and averages are presented with SEs.

| Table 1. *Ralstonia solanacearum* strains used in this study |
|-------------------------------------------------------------|
| Strain | Relative characteristics | Reference source |
| OE1-1 | Wild-type race 1 biovar 3 | Kanda et al. (2003a) |
| RK5043 | OE1-1 phcA-lacZYA | This work |
| RK5046 | OE1-1 hrpB-lacZYA | Yoshimochi et al. (2009b) |
| RK5050 | OE1-1 popA-lacZYA | Yoshimochi et al. (2009b) |
| RK5120 | OE1-1 hrpG-lacZYA | Yoshimochi et al. (2009b) |
| RK5204 | RK5046 ΔprhK | This work |
| RK5206 | RK5046 ΔprhK | This work |
| RK5208 | RK5050 ΔprhL | This work |
| RK5210 | RK5046 ΔprhL | This work |
| RK5212 | OE1-1 prhG-lacZYA | This work |
| RK5253 | RK5050 ΔprhM | This work |
| RK5255 | RK5046 ΔprhM | This work |
| RK5256 | RK5120 ΔprhM | This work |
| RK5258 | RK5212 ΔprhM | This work |
| RK5260 | RK5120 ΔprhL | This work |
| RK5262 | RK5212 ΔprhL | This work |
| RK5264 | RK5120 ΔprhK | This work |
| RK5268 | RK5043 ΔprhM | This work |
| RK5270 | RK5043 ΔprhL | This work |
| RK5281 | RK5212 ΔprhK | This work |
| RK5363 | RK5050 ΔRsc2168 | This work |
| RK5366 | RK5050 ΔRsc2167 | This work |
| RS1002 | Wild-type race 1 biovar 4 | Mukaihara et al. (2004) |
| RK10001 | RS1002 popA-lacZYA | This work |
| RK10009 | RK10001 ΔprhM | This work |
| RK10012 | RK10001 ΔprhL | This work |
| RK10015 | RK10001 ΔprhK | This work |

Construction of prhK, prhL, and prhM deletion mutants

Plasmids designed to create deletion mutants were based on pK18mobsacB (Schäfer et al., 1994). This resulted in plasmids pK18d2171, pK18d2170, and pK18d2169. The construction of the clones is described in detail in the Supporting Information, Appendix S1. pK18d2171, pK18d2170, and pK18d2169 were transferred from *E. coli* S17-1 into *R. solanacearum* RK5050 (popA-lacZYA), RK5046 (hrpB-lacZYA), RK5120 (hrpG-lacZYA), RK5212 (prhG-lacZYA), and RK5043 (phcA-lacZYA). Deletion strains were generated through consecutive homologous recombination events.

A popA-lacZYA reporter strain of RS1002, RK10001, was constructed using the pK18mobsacB-based plasmid ppop3 (Yoshimochi et al., 2009b). Deletion mutants of RK10001 were constructed using the same techniques as described for RK5050.

Complementation analyses

Genes were cloned into pUC18-mini-Tn7T-Gm (Choi et al., 2005). A detailed cloning procedure is described in Appendix S1. The plasmids, together with a transposase-containing helper plasmid pTNS2, were electroporated into the OE1-1 mutants. The genes on pUC18-mini-Tn7T-Gm were specifically inserted into a single attTn7 site downstream of the *glmS* gene (Yao & Allen, 2007). The transformant cells were selected on BG agar media supplemented with Gm and PB. Insertion into the attTn7 site was confirmed by colony PCR using primer pair glmS down and Tn7R or Tn7L and rsc0179 upper (Table S1).

Bacterial population in plants

A 2-μL aliquot of a bacterial cell suspension (10⁷ CFU mL⁻¹) was placed on the freshly cut surface of a tomato plant petiole (*Solanum lycopersicum* cv. Moneymaker). After incubation for up to 5 days, stem pieces (1 cm in length) were removed above the cut petiole, weighed, and crushed at 3000 r.p.m. for 60 s with a 5-mm-diameter zirconia bead using a Micro Smash MS-100 (TOMY SEIKO). Cell suspensions were diluted and spread on B agar supplemented with glucose and PB, and the number of colonies was counted after a 2-day incubation at 28°C.

Measurements of β-galactosidase activity in planta

β-Galactosidase activity in planta was determined using the Galacto-Light Plus kit (Applied Biosystems). The activity was measured using the GloMax 20/20 luminometer (Promega). Stem pieces inoculated with bacterial suspensions were crushed using the Micro Smash MS-100. The bacterial
suspensions were treated with 10 µL 0.1% sodium dodecyl sulfate (SDS) and 20 µL chloroform. A 70-µL aliquot of the reaction buffer [Galacto-Light Plus substrate with reaction buffer diluent (1:100)] was added to 20 µL of each SDS–chloroform-treated sample. After incubation at 25 °C for 30 min, 100 µL of accelerator II solution was added and chemiluminescence was measured. The luminescence was normalized to the cell number.

Virulence assays

Virulence assays were performed on wilt-susceptible tomato and tobacco plants (Nicotiana tabacum) using a soil-soak assay previously described by Yao & Allen (2007). Plants were incubated at 25 °C and examined daily. Each experiment included eight plants per treatment, and each assay was repeated four times.

Nucleotide sequence accession number

The nucleotide sequences presented in this study have been deposited in the DDBJ database under accession number AB558586.

Results and discussion

prhK, prhL, and prhM are essential for the expression of popA operon

In a previous study, we screened three genes, prhK, prhL, and prhM, for positive regulation of popA operon of R. solanacearum strain OE1-1 using transposon mutagenesis (Y. Zhang, unpublished data). prhK, prhL, and prhM are the orthologs of Rsc2171, Rsc2170, and Rsc2169, respectively, in R. solanacearum strain GM11000. According to MicrobeOnline Operon Predictions (http://www.microbesonline.org/operons/), prhK, prhL, and prhM form an operon along with Rsc2168 and Rsc2167 (Fig. 1). The nucleotide sequence of the 2.8-kb region revealed that prhK, prhL, and prhM encode proteins containing 215, 353, and 247 amino acids, respectively, and these proteins are 100% identical to Rsc2171, Rsc2170, and Rsc2169 from GM11000, respectively.

We constructed deletion mutants in RK5050 (popA-lacZYA), which resulted in RK5204 (∆prhK), RK5208 (∆prhL), and RK5253 (∆prhM). When these mutant cells were inoculated directly onto the cut petiole, the mutants colonized the stem less efficiently than did the wild type, with a difference of one to two orders of magnitude (Fig. S1). However, the growth pattern of deletion mutants was similar to that of the wild-type strain in B media or hrp-inducing sucrose media (data not shown). In sucrose medium, the expression level of popA operon was reduced to an almost basal level in all three mutants (Table 2). This demonstrates that prhK, prhL, and prhM are necessary for the expression of hrp regulon. Expression levels of popA-lacZYA in the Rsc2168 (RK5363) and Rsc2167 (RK5366) deletion mutants were 260 and 281 Miller units, respectively, which was not different from the levels in the wild type (RK5050, Table 2). These results indicate that these two genes do not function in the regulation of hrp regulon.

While the OE1-1 strain is pathogenic to tobacco, the Japanese isolate RS1002 (Mukaihara et al., 2004) is non-pathogenic to tobacco. Instead, it elicits a hypersensitive response (HR). We monitored the expression levels of popA operon in popA-lacZYA fusion strains of RS1002; RK10001 and the three deletion mutants of prhK, prhL, and prhM genes (Table 2). popA expression was reduced to an almost basal level in all three mutants, as was observed in the OE1-1 strain. This demonstrates that the functions of PrhK, PrhL, and PrhM are not strain-specific.

Many genome-wide screens for pathogenesis-related genes in R. solanacearum have been performed, both experimentally and in silico. Examples of techniques used are transposon mutagenesis (Boucher et al., 1987; Lin et al., 2008), transposon-based screening of hrpB-dependent genes (Mukaihara et al., 2004), and in silico analysis of secreted proteins via the twin-arginine translocation system (Gonzalez et al., 2007). These analyses have identified T3SS-related hrp and effector genes, genes for type II secretion system (T2SS), flagellar and motility genes, pilus genes, and genes for biosynthesis of exopolysaccharide. Most of these genes are pathogen-specific. Although none of the screens reached

| Mutation | ∆prhK | ∆prhL | ∆prhM |
|----------|-------|-------|-------|
| OE1-1 popA-lacZYA | 307 (82) | 15 (3) | 13 (2) | 12 (2) |
| OE1-1 hrbB-lacZYA | 173 (30) | 21 (8) | 17 (1) | 16 (1) |
| OE1-1 hrgB-lacZYA | 190 (34) | 189 (27) | 201 (23) | 162 (8) |
| OE1-1 prhG-lacZYA | 3311 (48) | 286 (26) | 237 (24) | 225 (12) |
| OE1-1 phcA-lacZYA | 113 (8) | NT | 116 (5) | 108 (9) |
| RS1002 popA-sacZYA | 294 (41) | 10 (1) | 11 (1) | 10 (1) |

* Cells were grown to an OD600nm of about 0.1, which corresponds to 1.8 × 10^8 CFU mL^-1, in sucrose media, treated with SDS–chloroform and assayed. Mean values of at least six measurements are in Miller units with SEs in parentheses. NT, not tested.
saturation, some genes were identified as virulence determinants in multiple independent screenings. It is interesting that these three pathogenesis-related genes had not been identified, despite this long screening history.

The expression of hrpB is controlled by prhK, prhL, and prhM

Because HrpB controls the hrp regulon (Genin et al., 1992), we examined the influence of prhK, prhL, and prhM on the expression of hrpB. We constructed deletion mutants in RK5046 (hrpB-lacZYA), which resulted in RK5206 (ΔprhK), RK5210 (ΔprhL), and RK5255 (ΔprhM). In sucrose medium, the expression levels of hrpB were substantially reduced in the prhK, prhL, and prhM deletion mutants (Table 2). These data demonstrate that prhK, prhL, and prhM are necessary for the expression of hrpB.

The expression of prhG is controlled by prhK, prhL, and prhM

Expression of hrpB is activated by HrpG and PrhG (Brito et al., 1999; Plener et al., 2010). We examined the involvement of prhK, prhL, and prhM in the regulation of hrpB expression by hrpG and by prhG. We constructed deletion mutants of RK5120 (hrpG-lacZYA), which resulted in RK5264 (ΔprhK), RK5260 (ΔprhL), and RK5256 (ΔprhM), and of RK5212 (prhG-lacZYA), which resulted in RK5281 (ΔprhK), RK5262 (ΔprhL), and RK5258 (ΔprhM). Their expression levels were determined in sucrose medium. Whereas the expression levels of hrpG were similar in the wild type and the three deletion mutants, the expression level of prhG in each of the deletion mutants was reduced 10-fold compared with the level in the wild type (Table 2). The same results were obtained when the cells were incubated in nutrient-rich B media (data not shown). These results indicated clearly that the regulation of hrpB expression by prhK, prhL, and prhM is dependent on prhG but not on hrpG.

We have reported previously that the expression of prhG is positively regulated by PhcA (Y. Zhang, unpublished data). To examine the influence of prhL and prhM on the expression of phcA, we constructed deletion mutants of RK5043 (phcA-lacZYA), which resulted in RK5270 (ΔprhL) and RK5268 (ΔprhM). The expression levels of phcA were similar in the wild type and the prhL and prhM mutants (Table 2). This suggests that prhL and prhM are not involved in the regulation of phcA expression.

Complementation analyses

We used a Tn7-based broad-range bacterial cloning and expression system for complementation (Choi et al., 2005). When we tested this system for complementation in the hrpG mutant, HrpG function was completely recovered (data not shown). However, when prhK (in pUC2171), prhL (in pUC2170), and prhM (in pUC2169) were transposed into their corresponding mutants, the gene functions were not restored (Table 3), despite the fact that no polar effects were observed, and that the transgenes were under the control of their endogenous promoter. Even transforming RK5204 (ΔprhK) and RK5208 (ΔprhL) with two genes at once [prhK and prhL (in pUC2170)] did not complement these mutants (Table 3). Instead, all three genes, prhK, prhL, and prhM, were required at once to complement the three mutants (Table 3). We conclude that the coordinate expression of the three genes is likely to be necessary for the precise control of prhG expression. Based on the expression profile of prhK operon (Y. Zhang, unpublished data), PrhM may play a role in this coordination, although the exact function of PrhM remains to be elucidated.

The effect of deletion of prhK, prhL, and prhM on disease development in tomato and tobacco

The pathogenicity of the mutants was tested by soil-soak inoculation. The popA mutant causes wilt in tomato plants (Kanda et al., 2003b). Tomato plants inoculated at the roots with RK5050 (popA-lacZYA) became wilted within 5 days.
postinoculation (dpi) and died by 12 dpi (Fig. 2a). None of the RK5050 prhK, prhL, or prhM mutants caused wilt in tomato plants (Fig. 2a). When the petiole inoculation method was used, the same phenotypes were observed (data not shown).

The other _R. solanacearum_ strain RK10001 caused the tomato plants to wilt even earlier than RK5050 (Fig. 2b). Unlike tomato plants inoculated with the OE1-1 mutants, tomato plants inoculated with the RS1002 prhK, prhL, or prhM mutants wilted eventually. However, all three mutants were less virulent than the wild type (Fig. 2b). RK10001 and the three mutants based on this strain elicited an HR with similar symptoms (data not shown). Although the prhKLM mutants drastically reduced the expression of _hrp_ regulon in both the OE1-1 and RS1002 mutants, the disease symptoms caused by pathogens with different genetic backgrounds showed large variation. This might explain why non-pathogen-specific genes such as these escaped the above-mentioned screens for pathogenesis-related genes.

Tobacco plants inoculated at their roots with RK5050 showed wilt symptoms sooner than the tomato plants (Fig. 2c). Although tobacco plants inoculated with RK5204 (ΔprhK) and RK5208 (ΔprhL) started to wilt at 4 dpi, they died later than the tobacco plants inoculated with RK5050, i.e. at 21 and 18 dpi, respectively. Tobacco plants inoculated with RK5253 (ΔprhM) showed wilt at 7 dpi, and died at 21 dpi (Fig. 2c). The three mutants displayed different levels of pathogenesis on the two host plants — tomato and tobacco. They were severely impaired in the colonization of tomato xylem vessels (Fig. S1), but proliferated in tobacco leaves only slightly slower compared with the wild type (data not shown). Different host plants displayed different symptoms, depending upon the infecting strain (Lin _et al._, 2008).

When a pUC7169 plasmid containing the three genes was transferred into each of the mutant strains, all three of the recombinant strains recovered pathogenicity to the wild-type level (Fig. 2d).

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**Fig. 2.** Pathogenicity tests. A bacterial suspension (10⁸ CFU mL⁻¹) was poured onto the soil of tomato plants (a, b) ( _Solanum lycopersicum_ cv. Moneymaker) or tobacco plants ( _Nicotiana tabacum_ ) (c, d) at a final concentration of 10⁷ CFU g⁻¹ of soil. Plants were inspected daily for wilt symptoms, and scored on a disease index scale from 0 to 4 (0, no wilting; 1, 1–25% wilting; 2, 26–50% wilting; 3, 51–75% wilting; and 4, 76–100% wilted or dead). (a, c) RK5050 (OE1-1 _popA-lacZYA_), closed circles; RK5204 (OE1-1 _popA-lacZYA ΔprhK_), squares; RK5208 (OE1-1 _popA-lacZYA ΔprhL_), triangles; RK5253 (OE1-1 _popA-lacZYA ΔprhM_), open circles. (b) RK10001 (RS1002 _popA-lacZYA_), closed circles; RK10015 (RS1002 _popA-lacZYA ΔprhK_), squares; RK10012 (RS1002 _popA-lacZYA ΔprhL_), triangles; RK10009 (RS1002 _popA-lacZYA ΔprhM_), open circles. (d) RK5050, closed circles; RK5204, complemented with pUC7169 ( _prhKLM_ ), squares; RK5208, complemented with pUC7169, triangles; RK5253, complemented with pUC7169, open circles. Each assay was repeated in four successive trials. Within each trial, we treated eight plants with each strain. The average and SE were calculated.
**In planta expression of popA operon**

Cell suspensions with high cell density of the popA-lacZYA reporter strain and the derived prhKLM mutants were infiltrated into tomato leaves, and the in planta popA expression was monitored up to 24 h postinoculation (hpi). Cell numbers did not change during this period, and gene expression was normalized to cell number. In the leaves, popA expression in the wild type increased until 18 hpi, and then fell slightly until 24 hpi (Fig. 3). Throughout the experiments, expression levels were substantially repressed in the prhK, prhL, and prhM mutants (Fig. 3).

**Putative functions of PrhK, PrhL, and PrhM**

All three genes (prhK, prhL, and prhM) of the prhK operon are well conserved among Betaproteobacteria. It is likely that in the genus Ralstonia, the operon contains three genes plus an additional two genes (RSc2168 and RSc2169) (Fig. 4). Except for Burkholderia glumae, the other three bacteria shown in Fig. 4 are not plant pathogens. This indicates that these three genes are quite common and are not specific to bacterial plant pathogens. Moreover, orthologs of these three genes have been detected in a wide range of bacteria, including E. coli.

RSc2171 and RSc2170, which are annotated as allophane hydrolase subunit 1 and 2, respectively (Salanoubat et al., 2002), are related to the urea amidolyase of Saccharomyces cerevisiae (Wang et al., 1997). In addition, Kipl and KipA in Bacillus subtilis, which modulate the phosphorylation level of the two-component response regulator Spo0F, are homologs of RSc2171 and RSc2170, respectively (Wang et al., 1997). PrhK is 55% similar to the KipI C-terminal domain, which binds to the KinA histidine kinase (Jacques et al., 2008). RSc2169 is annotated as a LamB/YcsF family protein. In fungi, LamB seems to be required for the utilization of lactam rings as a nitrogen source (Wang et al., 1997). From these annotation data, it does not seem likely that the gene products of prhK, prhL, and prhM are transcriptional regulators. Instead, regulation of hpr regulon by prhK, prhL, and prhM appears to be indirect. We think it is important to understand how PrhK, PrhL, and PrhM regulate hrb expression and will give this research priority in the future.

The expression level of prhG in the prhK, prhL, and prhM mutants was limited to approximately one-tenth of that in the wild type.

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**Fig. 3.** popA expression in the infiltrated leaves. RK5050 (wild type, black bars), RK5204 (ΔprhK, shaded bars), RK5208 (ΔprhL, gray bars), and RK5253 (ΔprhM, white bars) were infiltrated into tomato leaves at a cell concentration of 10⁹ CFU mL⁻¹. Leaf disks were taken from the infiltrated leaves every 6 hpi, and β-galactosidase activity was measured using Galacto-Light Plus containing chemiluminescent substrates. Each assay was repeated twice, and two plants received identical treatments within each trial. The average and SE were calculated.

**Fig. 4.** Sequence comparison of the prhK operons in the Betaproteobacteria. Similarities in deduced amino acid sequences between the Ralstonia solanacearum OE1-1 prhK operon and the corresponding operon in the indicated strain are expressed as percentages. The amino acid sequences were deduced from the nucleotide sequences of R. solanacearum OE1-1 (AB558586), Ralstonia pickettii 12D (CP001644), Ralstonia eutropha H16 (AM260479), Ralstonia eutropha J2315 (AM747720), and Burkholderia glumae BGR1 (CP001503).
the wild type (Table 2). These mutants lost pathogenicity toward tomato (Fig. 2a), just like the hrpG mutant. On the other hand, the prhG mutant itself is slightly less virulent than the wild type (Plener et al., 2010). While HrpG controls the expression of a number of virulence determinants and genes involved in adaptation to life in the host plant, PrhG controls very few specific targets other than the hrp regulon through hrpB activation (Valls et al., 2006; Plener et al., 2010). Therefore, we speculate that PrhKLM controls not only the prhG gene and the hrp regulon, but also other pathogenesis-related genes. Judging from the colony morphology and microscopic observation, exopolysaccharide production and motility in the prhKLM mutants were normal (data not shown). Genes for T2SS and genes encoding several extracellular plant cell wall-degrading enzymes, such as polygalacturonases, β-1,4-endoglucanase, and pectin methylesterase, are major virulence determinants (Mole et al., 2007). The aim is to monitor the expression levels of these genes in prhKLM mutants in the future to further investigate PrhKLM-controlled genes.

In conclusion, we have isolated a novel class of pathogenesis-related genes. These genes are among non-pathogenic bacteria from the genera *Ralstonia* and *Burkholderia*. The regulation mechanism of hrp regulon by these genes is still speculative. In the future, we plan to further elucidate the functions of PrhK, PrhL, and PrhM.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Cell growth in the stem.

**Table S1.** Primers used in this study.

**Appendix S1.** Materials and methods.

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