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Initial evidence on auto-regulation of *prhK* operon and its potential working model for the regulation of *hrp* regulon in *Ralstonia solanacearum*

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Ralstonia solanacearum is a causative agent of bacterial wilt in many plant species and has been studied as a model plant vascular pathogen. *hrp* regulon and its encoding type III secretion system are responsible for the pathogenicity of *R. solanacearum* on host plants. Transcriptional regulator HrpB controls *hrp* regulon, and both HrpG and PrhG regulate *hrpB* expression. PrhA-PrhR/I-PrhJ-HrpG signal cascade activates *hrpB* expression. While PrhG is independent of this cascade, it is activated by *prhk*, *prhL* and *prhM*, which form one operon. Each of these three genes is essential for the pathogenicity of *R. solanacearum* on host plants, but their products are not transcriptional regulators. They are well conserved in Betaproteobacteria and control some other virulence related genes rather than *prhG* and *hrp* regulon. Here, after evaluating the regulation between these three genes and some known virulence genes, we provide some initial evidence on the auto-regulation of this operon and proposed the potential working model of *prhK* operon on *hrp* regulon in *R. solanacearum*.

Key words: Bacterial wilt, *hrp* regulon, *prhK* operon, *R. solanacearum*, pathogenicity.

INTRODUCTION

Type III secretion system (T3SS) is conserved in many plant and animal bacterial pathogens and this syringe-like protein-secretion system is essential for their virulence and host specificity. Bacteria use T3SS to inject bacterial proteins, so-called type III effectors into host cytoplasm to subvert host defense signaling and facilitate bacterial colonization in host (Hueck, 1998; Galán & Collmer, 1999; Galán & Wolf-Watz, 2006; Zhou & Chai, 2008; Lewis *et al*., 2009). In bacterial plant pathogens, the T3SS is encoded by a cluster of approximately 20 *hrp* (hypersensitive response and pathogenicity) genes that are organized into several operons (Arnold *et al*., 2003; Valls *et al*., 2006).

Ralstonia solanacearum is a causative agent of bacterial wilt disease in almost 200 crop and ornamental plant species; it is soil-borne, Gram-negative, and has been studied as a model plant vascular pathogen (Yabuuchi *et al*., 1995; Schell, 2000; Denny, 2006). The construction of T3SS in *R. solanacearum* is strongly influenced by the growth environment. Its expression is repressed in nutrient-rich condition, well induced in nutrient-poor condition and greatly enhanced when stimulated with plant signals (Arlat *et al*., 1992; Genin *et al*., 1992; Mukaihara *et al*., 2004; Yoshimochi *et al*., 2009b). T3SS is positively controlled by HrpB, which belongs to the AraC-family transcriptional regulator. HrpB might directly bind to *hrpII* box, a conserved DNA motif in the promoters of most of its target ge-

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nes (Genin *et al*., 1992; Mukaihara *et al*., 2004). The transcription of *hrpB* is activated by HrpG and PrhG, which are close paralogs and both belong to the OmpR/PhoB subfamily of two-component response regulators (Plener *et al*., 2010; Zhang *et al*., 2013). The plant signals are presumed to be perceived by the outer membrane receptor protein PrhA and transferred to HrpB through PrhA-PrhR/I-PrhJ-HrpG signal cascade (Marenda *et al*., 1998; Brito *et al*., 1999; Aldon *et al*., 2000), while PrhG seems to be independent of this cascade (Plener *et al*., 2010; Zhang *et al*., 2013). The quorum sensing-dependent transcriptional activator PhcA negatively regulates *hrpG* expression via *prhIR*, while it positively regulates *prhG* expression (Clough *et al*., 1997; Genin *et al*., 2005; Yoshimochi *et al*., 2009a; Zhang *et al*., 2013). PhcA activity starts to increase at a cell density of 10^7 CFU ml^{-1} and reaches its maximum at 5×10^8 CFU ml^{-1}. We speculated that *R. solanacearum* might switch from HrpG to PrhG for *hrpB* activation in a cell density-dependent manner (Zhang *et al*., 2013). The regulation of *hrpG* has been well characterized, while the regulation of *prhG* remains to be elucidated.

Previously, it was reported that three genes (namely *prhk*, *prhL* and *prhM*) are essential for the expression of *hrpB* and pathogenicity of *R. solanacearum* on host plants (Zhang *et al*., 2011). Only three genes were introduced back into mutants, and then the expression of *hrp* regulon and pathogenicity in each mutant were completely complemented; it is suggested that these three genes form one operon (*prhK* operon). *prhK* operon controls the expression of *hrp* regulon via PrhG, but it is independent of HrpG. Mutations in each of the three genes completely abolished the expression of *prhG*, *hrpB* and *hrp* regulons. Any of *prhk*, *prhL* and *prhM* deficient mutants completely lost pathogenicity on host plants, while *prhG* deficient mutant just shows weaker virulence than that in wild type (Plener *et al*., 2010; Zhang *et al*., 2013). This evidence supports the idea that PrhKLM controls some other virulence related genes rather than *prhG* gene and *hrp* regulon. These three genes are well conserved in Betaproteobacteria, and not specific to bacterial plant pathogens (Zhang *et al*., 2011).

From annotation data, PrhK and PrhL seem to be related to the urea amidolyase of *Saccharomyces cerevisiae* and PrhM is related to the utilization of lactam rings as a nitrogen source in fungi (Wang *et al*., 1997; Salanoubat *et al*., 2002). Their products do not seem to be transcriptional regulators, suggesting that they regulate the *hrp* regulon indirectly, and the regulation mechanism remains unrevealed. Then, we focused on the regulation between *prhK* operon and some known genes and proposed the potential working model of *prhK* operon on the regulation of *hrp* regulon expression.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth condition

Ralstonia solanacearum strains and *Escherichia coli* strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in Lysogeny broth (LB) medium at 37°C. *Ralstonia solanacearum* strains, derivatives of the Japanese strains OE1-1 (phylotype I, race 1, biovar 3) (Kanda *et al*., 2003) were grown at 28°C in rich B medium or hydroponic plant culture medium supplemented with 2% sucrose (*hrp*-inducing medium) (Yoshimochi *et al*., 2009b). Antibiotics were added in 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

β-galactosidase assay was performed as previously described (Yoshimochi *et al*., 2009b). β-galactosidase

TABLE 1. *Ralstonia solanacearum* stains used in this study

Strain	Relative characteristics	Source of reference
OE1-1	wild type, race 1 biovar 3	Kanda *et al.* (2003)
RK5283	OE1-1 *prhM-lacZYA*	Current work
RK5286	RK5283 *ΔprhG*	Current work
RK5288	RK5283 *ΔphcA*	Current work
RK5291	RK5283 *ΔprhK*	Current work
RK5294	RK5283 *ΔprhL*	Current work
RK5297	RK5283 *ΔphcA ΔprhK*	Current work
RK5299	RK5283 *ΔphcA ΔprhL*	Current work

activity was measured according to Miller (Miller, 1972) and values were measured at least in three independent experiments (average and standard error values are presented).

Construction of prhM-lacZYA reporter strain

1-kb *Eco*RI-*Hind*III fragment of pKS2169 (Zhang *et al*., 2011) was recloned into pK18mobsacB (Schäfer *et al*., 1994) to produce pK18-2169. The 6.4-kb *Sal*I-digested promoterless *lacZYA* fragment from pUC-lacZYA (Yoshimochi *et al*., 2009b) was inserted into *Sal*I site of pK18-2169 in the same direction as the *prhM* transcription, to construct pK18-2169-lacZYA (Km^r, $sucrose^s$). After confirming by sequencing with either primers M13-47 or LacAA2, pK18-2169-lacZYA was transferred from *E. coli* strain S17-1 (Simon, 1983) into *R. solanacearum* OE1-1 with consecutive homologous recombinations (Zhang *et al*., 2011). As a result, the reporter gene *lacZYA* was integrated into the chromosome of *R. solanacearum* OE1-1 at 610-bp (*Sal*I site) of *prhM* gene, which shows the same copy number and shares the same promoter as *prhK* operon, and RK5283 (*prhM-lacZYA*) was obtained.

Construction of prhG, prhK, prhL or phcA deletion

Plasmids pK18d1023, pK18d2171, pK18d2170 and pK18dphcA were created from pK18mobsacB with corresponding gene deletion and used to construct deletion mutants (Zhang *et al*., 2011). They were transferred from *E. coli* S17-1 into *R. solanacearum* RK5283 (*prhM-lacZYA*) with consecutive homologous recombinations. After confirmation by sequencing, series of mutants were created with deletion: RK5286 (*ΔprhG*), RK5288 (*ΔphcA*), RK5291 (*ΔprhK*), RK5294 (*ΔprhL*), RK5297 (*ΔphcA*, *ΔprhK*) and RK5299 (*ΔphcA*, *ΔprhL*).

RESULTS

Expression of PrhK operon is independent on PrhG, but negatively regulated by PhcA

Since the 6.4-kb promoterless *lacZYA* is integrated into chromosome, *lacZYA* shows same copy number and shares the same promoter as *prhK* operon in *R. solanacearum*; then we can monitor the expression of the *prhK* operon using *prhM-lacZYA* reporter strain (RK5283) and evaluate its expression level in *prhG* deletion strain RK5286. The expression level of *prhM* was not different ($p > 0.05$) from that in wild type (Fig. 1) in both rich medium and *hrp*-inducing medium, indicating that no feedback regulation from PrhG on *prhK* operon.

At higher cell density the expression level of *prhM* decreased relatively to that at lower cell density (data not shown), and PhcA is activated in cell density dependent manner (Genin *et al*., 2005). Then, we evaluated the expression level of *prhM*, when *phcA* was deleted in RK5288; the expression level of *prhM* increased to about 3-fold but only in *hrp*-inducing medium (Fig. 1). These data indicate that PhcA negatively regulates the *prhK* operon but only in *hrp*-inducing medium.

Regulation between prhK, prhL and prhM gene

We examined the expression of *prhM* using deletion mutants derived from RK5283. *PrhK* deletion mutant (RK5291) expressed only one-third of the wild type of

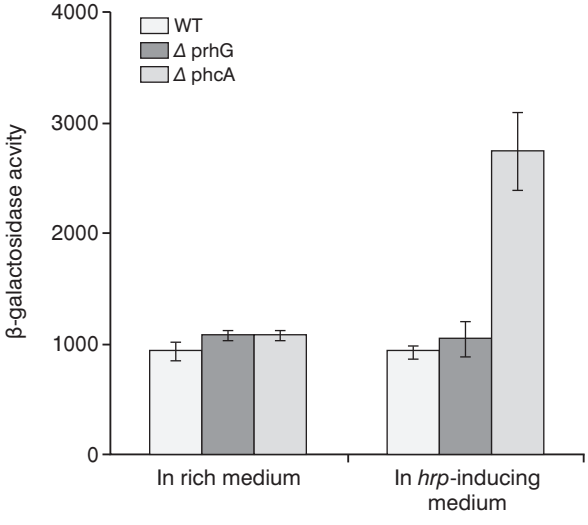

FIG. 1. Expression of *prhM* in *prhG* or *phcA* mutants. Bacterial cells of RK5283 (*prhM-lacZYA*), RK5286 (*prhM-lacZYA ΔprhG*), RK5288 (*prhM-lacZYA ΔphcA*), were incubated in rich medium and *hrp*-inducing medium to an OD600 of about 0.1. Cells were treated with SDS-chloroformed and β-galactosidase activity was measured in Miller units as described previously (Yoshimochi *et al*., 2009b). Values were averaged from at least six assays and standard error was calculated.

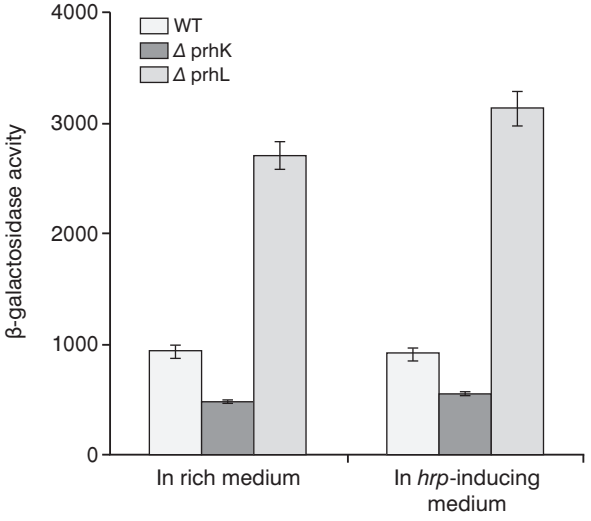

FIG. 2. Regulation between *prhK*, *prhL* and *prhM* gene. Bacterial cells of RK5283 (*prhM-lacZYA*), RK5291 (*prhM-lacZYA ΔprhK*), and RK5294 (*prhM-lacZYA ΔprhL*) were incubated in rich medium and *hrp*-inducing medium to an OD600 of about 0.1. Cells were treated with SDS-chloroformed and β-galactosidase activity was measured in Miller units. Values were averaged from at least six assays and standard error was calculated.

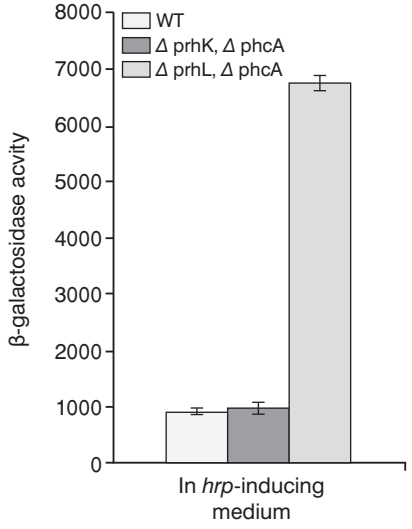

FIG. 3. Additive regulation of PhcA and PrhK, PrhL on *prhK* operon. Bacterial cells of RK5283 (*prhM-lacZYA*), RK5297 (*prhM-lacZYA ΔphcA ΔprhK*) and RK5299 (*prhM-lacZYA ΔphcA ΔprhL*) were incubated in *hrp*-inducing medium and β-galactosidase activity was measured in Miller units. Values were averaged from at least six assays and standard error was calculated.

prhM in both rich medium and *hrp*-inducing medium (Fig. 2), while *prhL* deletion mutant (RK5294) expressed *prhM* three times higher than that in wild type in both rich medium and *hrp*-inducing medium (Fig. 2).

Additive regulation of PhcA and PrhK, PrhL on prhK operon

When the *phcA* deletion was combined with the *prhK* or *prhL* deletion, the influence on the expression of *prhM* appeared to be additive. The expressions of *prhM* were sent back to the wild-type level in RK5297 (*ΔprhK*, *ΔphcA*) and completely derepressed in RK5299 (*ΔprhL*, *ΔphcA*) (Fig. 3).

DISCUSSION

From complementation analyses, coordinate expression of the three genes seems to be essential for the control of *prhG* expression, and PrhM may play a role in this coordination (Zhang *et al*., 2011). Although the exact function of *prhM* remains unknown, orthologs of the three genes have been detected in a wide range of bacteria. PrhK and PrhL are similar to KipI and KipA in *Bacillus subtilis*, respectively (Wang *et al*., 1997). PrhK is 55% similar to KipI C-terminal domain, which binds to KinA histidine kinase. KipA is an antagonist of KipI; it modulates the phosphorylation level of a response regulator Spo0F. KipI inhibits the autophosphorylation reaction of a specific histi-

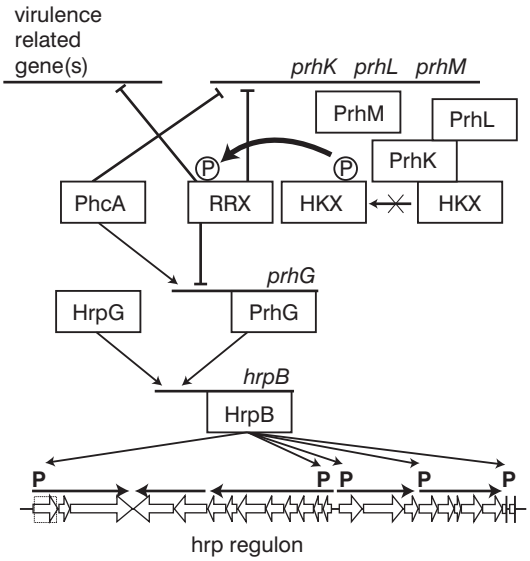

FIG. 4. Working model of regulation of *prhG* expression with PrhK, PrhL, and PrhM. Gene names are indicated on the thin lines. Boxed letters represent proteins. HKX and RRX are the predicted histidine kinase and response regulators, which are controlled by PrhK. Circled P indicates a phosphate group. P stands for a plant inducible promoter recognized by HrpB. Positive and negative regulations of transcription are indicated by (↓) and (⊥), respectively. Inhibition is shown by (×).

dine kinase KinA in a two-component signal transduction system (Jacques *et al*., 2008). Among the five aromatic residues that define the hydrophobic groove of KipI C-domain interacting with KinA, four residues are conserved in PrhK. Similarity of PrhL to KipA is 36%. PrhK and PrhL could be the histidine kinase inhibitor and the anti-inhibitor.

PrhG is one of the response regulators in *R. solanacearum* (Plener *et al*., 2010; Zhang *et al*., 2013) although no cognate histidine kinase is identified. We have demonstrated that the predicted phosphorylation site of PrhG is important for its activity (unpublished data), indicating that PrhG is likely to be phosphorylated. The two-component system including PrhG is the candidate for the target of the histidine kinase inhibitor PrhK. However, PrhK is likely to control not only the *hrp* regulon but also some other virulence-related genes. Taken all together, it is hypothesized that the two-component system (including PrhG) is not the target of PrhK inhibition. More than 40 histidine kinases and more than 50 response regulators are assigned in the *R. solanacearum* GMI1000 genome (Salanoubat *et al*., 2002). The functions of most kinases and response regulators are not yet characterized. PrhK could inhibit the phosphorylation of one of these uncharacterized histidine kinases and modulate the activity of the cognate response regulator. The histidine kinase controlled by PrhK using comprehensive deletion of histidine kinase genes is now sought.

Supposing that PrhK is a kinase inhibitor, the phosphorylation level of the sensor kinase could increase in the *prhK* mutant and the response regulator (RR), referred to as RRX could be more phosphorylated than that in wild type. *prhG* expression was repressed in the *prhK* mutant (Zhang *et al*., 2011), suggesting that RRX acts as a repressor of *prhG* expression. Although most of the response regulators activate the gene expression, some response regulators repress the gene expression. A response regulator PhoP activates the phosphate regulon and represses the nitrogen metabolism genes in *Streptomyces coelicolor* (Rodríguez-García *et al*., 2009). We have demonstrated that the expression level of *prhG* was decreased to almost zero in the *phcA* mutant (Zhang *et al*., 2013). *PrhG* expression is regulated positively by PhcA and negatively by RRX. Both regulators might competitively bind to the promoter region of *prhG*. A potential working model for the *prhG* regulation is proposed (Fig. 4).

The expression of the *prhM-lacZYA* reporter gene was repressed in the *prhK* mutant (Fig. 2), suggesting that RRX negatively regulates *prhK* operon. PhcA also negatively regulated *prhK* operon (Fig. 1). Since the repression of *prhK* by RRX and PhcA appeared to be additive, RRX and PhcA, if directly, independently bind to the promoter region. Supposing that PrhL is the antagonist of PrhK, *prhL* mutation releases the free PrhK much more than the wild type. The amounts of the phosphorylated active RRX are decreased. This hypothesis is in good agreement with the result that *prhL* mutant increased the expression of the *prhM-lacZYA* reporter gene (Fig. 2). On the other hand, the expression of *prhG* was not increased in *prhL* mutant (Zhang *et al*., 2011). It could be explained that the coordinate expression of three genes, *prhK*, *prhL*, and *prhM*, is likely to be necessary for the precise control of *prhG* expression, since only three genes were com-

plemented at the same time, the expression of *hrp* regulon and virulence on host plants were restored (Zhang *et al*., 2011).

In conclusion, the potential auto-regulation of *prhK* operon is proposed along with the working model of this operon on the regulation of *hrp* regulon and pathogenicity in *R. solanacearum* is proposed. However, further experimentation is necessary to reveal their functions and regulation mechanism(s).

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