The putative sensor histidine kinase PhcK is required for the full expression of phcA encoding the global transcriptional regulator to drive the quorum-sensing circuit of Ralstonia solanacearum strain OE1-1

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Abstract
A gram-negative plant-pathogenic bacterium Ralstonia solanacearum strain OE1-1 produces and extracellularly secretes methyl 3-hydroxymyristate (3-OH MAME), and senses the chemical as a quorum-sensing (QS) signal, activating QS. During QS a functional global transcriptional regulator PhcA, through the 3-OH MAME-dependent two-component system, induces the production of virulence factors including a major extracellular polysaccharide EPS I and ralfuranone. To elucidate the mechanisms of phcA regulation underlying the QS system, among Tn⁵-mutants from the strain OE1-1, we identified a mutant of RSc1351 gene (phcK), encoding a putative sensor histidine kinase, that exhibited significantly decreased QS-dependent cell aggregation. We generated a phcK-deletion mutant (ΔphcK) that produced significantly less EPS I and ralfuranone than the wild-type strain OE1-1. Quantitative reverse transcription PCR assays showed that the phcA expression level was significantly down-regulated in the ΔphcK mutant but not in other QS mutants. The transcriptome data generated with RNA sequencing technology revealed that the expression levels of 88.2% of the PhcA-positively regulated genes were down-regulated in the ΔphcK mutant whereas the expression levels of 85.9% of the PhcA-negatively regulated genes were up-regulated. Additionally, the native phcK-expressing complemented ΔphcK strain and the ΔphcK mutant transformed with phcA controlled by a constitutive promoter recovered their cell aggregation phenotypes. Considered together, the results of this study indicate that phcK is required for full phcA expression, thereby driving the QS circuit of R. solanacearum strain OE1-1. This is the first report of the phcA transcriptional regulation of R. solanacearum.

Keywords
PhcA, PhcK, quorum sensing, Ralstonia solanacearum
Bacterial cells produce and extracellularly secrete quorum-sensing (QS) signals, which are small diffusible molecules that enable cell-to-cell communication for the cooperative regulation of physiological processes (Ham, 2013). Bacteria monitor QS signals to track changes in their cell numbers and activate QS. Additionally, QS-activated bacterial cells synchronously control the expression of genes beneficial for vigorous replication and adaptations to environmental conditions (Rutherford and Bassler, 2012). Moreover, cell-to-cell signalling through QS signals helps regulate the virulence of pathogenic bacteria (Ham, 2013).

The gram-negative, soilborne bacterium *Ralstonia solanacearum* infects more than 250 plant species worldwide, and is responsible for a potentially devastating bacterial wilt disease on them (Mansfield et al., 2012). The bacteria first invade the intercellular spaces of plant roots (Vasse et al., 1995; Araud-Razou et al., 1998; Hikichi et al., 2017). After invading the intercellular spaces of plant roots, *R. solanacearum* cells attach to the surface of host cells (Mori et al., 2016), where they avoid plant innate immune responses (Nakano et al., 2013; Hikichi et al., 2017; Kiba et al., 2018) and grow vigorously to activate QS (i.e., *phc* QS; Hikichi et al., 2017). This leads to the formation of mushroom-shaped biofilms, which are essential for the virulence of *R. solanacearum* (Mori et al., 2016, 2018a). From the mushroom-shaped biofilms, the planktonic bacterial cells are released to invade xylem vessels (Mori et al., 2016; Hikichi et al., 2017), after which the bacteria systemically spread and multiply throughout the xylem (Vasse et al., 1995; Hikichi et al., 2017) to activate *phc* QS and induce wilting symptoms on infected tomato plants (Genin and Denny, 2012).

Some gram-negative bacteria regulate gene expression with the use of two-component systems that are composed of a sensor histidine kinase and a response regulator (Capra and Laub, 2012). Sensor histidine kinases are transmembrane proteins containing a histidine phosphotransfer domain and an ATP-binding domain. Response regulators are cytosolic proteins containing multidomains with a receiver domain and effector domains involved in DNA binding. The sensor histidine kinase detects a particular environmental signal to autophosphorylate a specific histidine residue, and then phosphorylates an aspartate residue on the receiver domain of the cognate response regulator. The phosphorylated response regulators stimulate or repress expression of target genes.

The strain AW1 of *R. solanacearum* uses methyl-3-hydroxy-malate (3-OH PAME) as the QS signal (Flavier et al., 1997). In contrast, *R. solanacearum* strains OE1-1 and GMI1000 produce methyl-3-hydroxy-O-myrurate (3-OH MAME) as the QS signal (Kai et al., 2015; Ujita et al., 2019). The QS signal, 3-OH MAME or 3-OH PAME, is synthesized by the methyltransferase PhcB and is sensed through sensor histidine kinase PhcS to activate the *phc* QS. The genome sequences confirm that the *phcBSRQ* operon consists of *phcB*, *phcS*, *phcR*, and *phcQ* in the genome of AW1 (Clough et al., 1997), similar to the strains OE1-1 (Kai et al., 2015) and GMI1000 (Salanoubat et al., 2002). Clough et al. (1997) suggests a model of the *phc* QS signalling pathway in which PhcS senses 3-OH PAME, phosphorylating itself. A threshold concentration of 3-OH PAME induces the ability of PhcS to phosphorylate the cognate response regulator PhcR. The phosphorylated PhcR releases PhcA, resulting in functional PhcA. The functional PhcA regulates the expression of *phc* QS-dependent genes (Genin and Denny, 2012). Although this model has been mostly accepted, it has not been validated experimentally and the mechanism by which PhcA is activated during the *phc* QS signalling pathway is still poorly understood. Furthermore, there is relatively little available information regarding the regulation of *phcA*, which is required for elucidating the regulatory mechanisms of *phc* QS.

In this study, we screened Tn5-mutants and identified a mutant exhibiting significantly decreased *phc* QS-dependent cell aggregation activity. In this mutant, a transposon was inserted in the *RSc1351* gene (*phcK*) encoding a putative sensor histidine kinase. We then analysed the regulatory effects of *phcK* on *phc* QS.

## RESULTS

### 2.1 A transposon mutant exhibits significantly decreased cell aggregation activity

We first created more than 1,000 *R. solanacearum* strain OE1-1 transposon mutants with the EZ-Tn5 < KAN-2 > Tnp Transposome Kit, and visually assayed their extracellular polysaccharide (EPS) productivity on Hara-Ono medium (Hara and Ono, 1983). Among mutants that produced less EPS than the wild-type strain OE1-1, KUKW-51 (Table 1) exhibited significantly less *phc* QS-dependent cell aggregation activity compared with strain OE1-1 (*t* test, *p* < .05; Figure S1). A nucleotide sequence analysis in a Blastp search by NCBI (https://blast.ncbi.nlm.nih.gov/blast.cgi) revealed that the transposon was inserted into the *RSc1351* gene.

### 2.2 RSc1351 gene encodes a putative sensor histidine kinase, PhcK

An analysis of the deduced RSc1351 amino acid sequence with the BLAST algorithm confirmed the presence of a histidine kinase domain at amino acid positions 185–264 as well as a histidine kinase-like ATPase domain with 16 ATP-binding sites (amino acid positions 286, 290, 293, 320, 322, 324, 326, 338–341, 358, 360, 367, 368, and 370) and two G-X-G motifs (amino acid positions 324–326 and 338–340) between amino acids 279 and 376 (Figure S2a). On the basis of membrane protein secondary structures predicted with the SOSUI program (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html), RSc1351 was designated as a membrane protein with four transmembrane helices (amino acid positions 40–61 [primary type] and 63–85, 101–121, and 132–153 [secondary type]). Furthermore, the KinasePhos 2.0 program (http://kinasephos2.mbc.nctu.edu.tw/) predicted that the histidine residue at amino acid position 205 is phosphorylated. These findings suggested that the RSc1351 gene
encodes a putative sensor histidine kinase (hereafter designated as \( \text{phcK} \)).

### 2.3 \( \text{phc} \) QS-dependent phenotypes of the \( \text{phcK} \)-deleted mutant

We then generated the \( \text{phcK} \)-deleted mutant \( \Delta \text{phcK} \) (Table 1) from strain OE1-1, as well as the complemented \( \Delta \text{phcK} \) mutant strain \( \text{phcK-comp} \) (Table 1), and analysed their cell aggregation activity. The \( \Delta \text{phcK} \) mutant exhibited significantly fewer cell aggregates than the strain OE1-1 (\( t \) test, \( p < .05 \); Figure 1a), similar to \( \text{phcB} \)-deleted mutant (\( \Delta \text{phcB} \); Table 1; Kai et al., 2015) and the \( \text{phcA} \)-deleted mutant (\( \Delta \text{phcA} \); Table 1; Mori et al., 2016). There were no significant differences between the \( \text{phcK} \)-comp and the OE1-1 strains with regard to cell aggregation.

\( \text{R. solanacearum} \) strain OE1-1 produces EPS I in a \( \text{phc} \) QS-dependent manner (Mori et al., 2016). The \( \Delta \text{phcK} \) mutant produced significantly less EPS I than the OE1-1 strain (\( t \) test, \( p < .05 \); Figure 1b), similar to the \( \Delta \text{phcB} \) and \( \Delta \text{phcA} \) mutants. The \( \text{phcK-comp} \) strain produced EPS I quantity similar to the strain OE1-1. The \( \text{epsB} \) gene is included in the \( \text{eps} \) operon and is required for EPS I biosynthesis (Huang and Schell, 1995). We then analysed expression of \( \text{epsB} \) in \( \text{R. solanacearum} \) strains by quantitative reverse transcription PCR (RT-qPCR) assays.

### Table 1 Strains and plasmids used in this study

| Relevant characteristics | Source |
|--------------------------|--------|
| **Plasmids**             |        |
| pUC118                   | Takara Bio |
| pUC118KUKW-51            | This study |
| pMD20                    | Takara Bio |
| pMD20delta-phcK          | This study |
| pMD20phcK                | This study |
| pMD200480prophecA        | This study |
| pMD20delta-1352          | This study |
| pK18mobsacB              | Kvitko and Collmer (2011) |
| pdelta-phcK              | This study |
| pdelta-1352              | This study |
| pUC18-mini-Tn7T-Gm       | Choi et al. (2005) |
| pTNS2                    | Choi et al. (2005) |
| pUC18-mini-Tn7T-Gm-phcK  | This study |
| p0480prophecA            | This study |
| **Escherichia coli strain** |        |
| DH5α                     | Takara Bio |
| **Ralstonia solanacearum strains** |        |
| OE1-1                    | Kanda et al. (2003) |
| KUKKW-51                 | This study |
| \( \Delta \text{phcB} \)  | Kai et al. (2015) |
| \( \Delta \text{phcA} \)  | Mori et al. (2016) |
| \( \Delta \text{phcK} \)  | This study |
| \( \Delta \text{RSc1352} \) | This study |
| \( \text{phcK-comp} \)   | This study |
| \( \text{phcA-comp\DeltaphcK} \) | This study |

**Notes:** This table lists the strains and plasmids used in this study. The relevant characteristics include the plasmid name, the source, and any additional details such as deletion or transposition events. The strains used include OE1-1, KUKKW-51, \( \Delta \text{phcB} \), \( \Delta \text{phcA} \), \( \Delta \text{phcK} \), \( \Delta \text{RSc1352} \), \( \text{phcK-comp} \), and \( \text{phcA-comp\DeltaphcK} \). The sources for these strains include Takara Bio and Kanda et al. (2003).
expression level in the ΔphcK mutant was significantly lower than that in OE1-1 (t test, p < .05; Figure 2), similar to the ΔphcB and ΔphcA mutants. There were no significant differences between the phcK-comp and the OE1-1 strains with regard to the epsB expression level.

The phc QS suppresses flagellar biogenesis, which results in inhibited *Ralstonia solanacearum* swimming motility (Tans-Kersten et al., 2001). The swimming motility of the ΔphcK mutant was significantly greater than that of the strain OE1-1 (t test, p < .05; Figure 1c), similar to the ΔphcB and ΔphcA mutants. There were no significant differences between the phcK-comp and the OE1-1 strains with regard to swimming motility. The RT-qPCR assays to assess the *fliC* expression level in *Ralstonia solanacearum* strains indicated *fliC* was more highly
expressed in the ΔphcK mutant than in the OE1-1 strain, similar to the ΔphcB and ΔphcA mutants (t test, \( p < .05 \); Figure 2). There were no significant differences between the phcK-comp and the OE1-1 strains with regard to the fliC expression level.

The production of ralfuranones in the OE1-1 strain is dependent on phc QS (Kai et al., 2014). The ΔphcK mutant produced significantly less ralfuranones than the OE1-1 and phcK-comp strains (Figure 3). Previous studies confirmed that PhcA during phc QS induces the expression of ralA, which encodes a ralfuranone synthase (Kai et al., 2014). We completed RT-qPCR assays to analyse the ralA expression level in R. solanacearum strains. The ralA expression level was significantly lower in the ΔphcK mutant than in OE1-1 (t test, \( p < .05 \); Figure 2), similar to the ΔphcB and ΔphcA mutants. There were no significant differences between the phcK-comp and the OE1-1 strains with regard to the ralA expression level.

The LecM production in a phc QS-dependent manner influences the stability of the extracellularly secreted 3-OH MAME content, thereby affecting phc QS (Hayashi et al., 2019a). We analysed the 3-OH MAME content of the R. solanacearum strains. The ΔphcK mutant produced significantly less 3-OH MAME than the OE1-1 strain (t test, \( p < .05 \); Figure 4a). The phcK-comp strain produced 3-OH MAME, similar to the strain OE1-1. We next completed RT-qPCR assays to analyse the lecM expression levels in R. solanacearum strains. The lecM expression level was significantly lower in the ΔphcK
mutant than in the OE1-1 (t test, p < .05; Figure 4b). There were no significant differences between the phcK-comp and the OE1-1 strains with regard to the lecM expression level.

2.4 | The phcK deletion led to a significantly reduced expression of phcA

The analysis of the phc QS-dependent phenotypes indicated that the phcK deletion led to a significantly reduced phc QS activity. We analysed the expression of phcB, phcA, and phcK in R. solanacearum strains grown in quarter-strength M63 medium (to OD$_{600}$ = 0.3) using RT-qPCR assays. The phcA expression level was significantly lower in the ΔphcK mutant than the OE1-1 strain (t test, p < .05; Figure 2), as well as the ΔphcB and phcK-comp strains. On the contrary, there were no significant differences between the OE1-1 and ΔphcB or ΔphcA strains with regard to the phcK expression levels. Additionally, there were no significant differences between the OE1-1 and ΔphcA, ΔphcK, or phcK-comp strains with regard to the phcB expression levels.

2.5 | phcK deletion leads to a change in phc QS-dependent gene regulation, similar to phcA deletion

The deletion of phcK led to changes in the expression of phc QS-dependent genes, such as epsB, ralA, and fliC, leading to altered phc QS-dependent phenotypes. In addition, the RT-qPCR assays indicated that the phcK deletion led to a significantly reduced expression of phcA. To analyse the effects of the phcK deletion on phc QS signalling, we performed an RNA-sequencing (RNA-Seq) transcriptome analysis of R. solanacearum OE1-1 and the ΔphcK mutant. We obtained 44.9, 46.0, and 44.2 as well as 47.0, 46.0, and 49.4 million 100-bp paired-end reads for the ΔphcK and OE1-1 strains, respectively, after which an iterative alignment mapped 37.1, 42.9, and 43.4 as well as 41.8, 43.8, and 48.3 million 100-bp paired-end reads, respectively, to the R. solanacearum strain GMI1000 reference genome (Salanoubat et al., 2002). The mapping of the OE1-1 RNA-Seq reads to the GMI1000 genome resulted in the identification of 4,189 protein-coding transcripts (Table S1).

The read counts obtained for each sample were expressed as the number of fragments per kilobase of open reading frame per million fragments mapped (FPKM) normalized prior to identifying differentially expressed genes. Genes were considered to be differentially expressed if they exhibited log$_2$(fold changes) of ≥2 or ≤−2. Relative to the corresponding expression levels in the OE1-1 strain, we detected 426 genes with significantly down-regulated expression (positively PhcK-regulated genes), and 171 genes with significantly up-regulated expression (negatively PhcK-regulated genes) in the ΔphcK mutant (Figure 5 and Table S1). Interestingly, phcA was included in the positively PhcK-regulated genes. On the other hand, the FPKM values of other phc QS-related genes, phcB and phcS, in the ΔphcK mutant did not significantly change compared to those in strain OE1-1.

We previously completed the transcriptome analysis of the ΔphcA mutant generated with RNA sequencing technology (Mori et al., 2018b). Among the positively PhcK-regulated genes, the expression levels of 330 genes were included in the 374 genes positively regulated by PhcA; EPS I production-related genes (i.e., eps operon, epsR, and xpsR), ralfuranone synthesis-related genes
(raiA and ralD), type VI secretion system-related genes, plant cell wall-degrading enzyme genes (cbhA, egl, and pme), and one gene encoding effector (ripG5) secreted via the type III secretion system (Figure 5a and Table S2). Additionally, the expression levels of 116 genes among the negatively PhcK-regulated genes were included in 135 genes negatively regulated by PhcA: flagellar motility-related genes (fliC), chemotaxis-related genes (che), some type III secretion-related genes (hrcC, hrcJ, hrpJ, and hrpK), some genes encoding effectors (ripA2, ripAB, ripAC, ripF1, and ripX) secreted via the type III secretion system, and chemotaxis-related genes (Figure 5b; Table S3).

The expression levels of PhcA-dependent regulated genes in the ΔphcK mutant were positively correlated with the expression levels in the ΔphcA mutant (Figure 5c).

2.6 | The RSc1352 gene-deleted mutant exhibits phc QS-dependent phenotypes similar to the wild-type strain OE1-1

The RSc1352 gene encodes a putative response regulator and is located immediately downstream from phcK (Salanoubat et al., 2002). The transcriptome analysis using RNA-Seq indicated that the phcK deletion led to a significantly decreased expression of the Rsc1352 gene (Table S1). We then generated an RSc1352 gene-deleted mutant (ΔRSc1352) from the wild-type strain OE1-1 and assessed its phc QS-dependent phenotypes. There were no significant differences between the ΔRSc1352 mutant and the OE1-1 strain with regard to the phc QS-dependent cell aggregation (Figure 1a), EPS I productivity (Figure 1b), and swimming motility (Figure 1c). Furthermore, the RT-qPCR assays also indicated that the expression level of the RSc1352 gene in the ΔphcK strain was significantly reduced compared to that in the wild-type strain OE1-1 (Figure S3). The complemented ΔphcK mutant strain phcK-comp exhibited a similar expression level of the RSc1352 gene to the strain OE1-1.

2.7 | Complementation of the ΔphcK mutant with phcA controlled by a constitutive promoter

The data for RNA-Seq transcriptome analysis and RT-qPCR assays indicated that PhcK was required for the full phcA expression, leading to a change in regulation of PhcA-dependent genes. The RNA-Seq data also indicated that expression levels of the Rsc0480 gene...
and phcA were similar in the OE1-1 strain, and that the RSc0480 gene was expressed independently of phc QS (Table S1). To analyse the recovered phc QS-dependent phenotypes of ΔphcK strain when transformed with phcA with a phc QS-independent and constitutive active promoter, we constructed the p0480prophec recombinant plasmid carrying the promoter of the RSc0480 gene and phcA based on pUC18-mini-Tn7-T-Gm (Choi et al., 2005). The recombinant plasmid was inserted into the ΔphcK mutant to create the transformant phcA-compΔphcK. Subsequent RT-qPCR assays revealed phcA was more highly expressed in the phcA-compΔphcK mutant than in the ΔphcK mutant (t test, p < .05; Figure 6a). Additionally, the phcA-compΔphcK mutant exhibited significantly enhanced cell aggregation activity (t test, p < .05; Figure 6b) and EPS I production (p < .05, Figure 6c), and inhibited swimming motility (t test, p < .05; Figure 6d) compared to the ΔphcK mutant. These results suggest that complemented phcA expression leads to recovery of the phc QS-dependent phenotypes of ΔphcK mutant.

2.8 | Exogenous 3-OH MAME application enhances the cell aggregation but not the phcA expression of strain OE1-1

An earlier investigation proved that treatment with 0.1 μM 3-OH MAME enhances the phc QS-dependent cell aggregation of the OE1-1 and ΔphcB strains (Kai et al., 2015). While exposure to 3-OH
MAME significantly enhanced the cell aggregation of the OE1-1 strain similar to the ΔphcB strain, cell aggregation of the ΔphcK mutant did not change (t test, p < .05; Figure 7a). Exogenous 3-OH MAME application did not affect the phcA expression level of the ΔphcK mutant as well as the OE1-1 and ΔphcB strains (Figure 7b).

2.9 | Decreased virulence of the ΔphcK mutant

We inoculated 5-week-old tomato plants with R. solanacearum strains by immersing the roots in bacterial suspension. The tomato plants inoculated with the OE1-1 strain exhibited wilt symptoms at 5 days after inoculation (dai) and died by 10 dai (Figure 8a). The ΔphcK mutant was not virulent on tomato plants, whereas the virulence of the phcK-comp mutant was similar to that of the OE1-1 strain.

The bacterial population of the ΔphcK mutant at 3 dai in the tomato roots was significantly smaller than that of the OE1-1 strain (t test, p < .05; Figure 8b). There were no significant differences between the populations of OE1-1 and phcK-comp strains in the tomato roots.

In a plate-printing assay, we detected OE1-1 and phcK-comp in the inoculated roots and stems of tomato plants, whereas we did not observe ΔphcK beyond the inoculated roots (Figure 8c).

2.10 | Phylogenetic analysis of the deduced PhcK amino acid sequences among R. solanacearum strains

Regarding their phylogenetic relationships based on the PhcB and PhcS amino acid sequences, R. solanacearum strains have been divided into three groups according to their QS signal types (3-OH MAME type and 3-OH PAME type) and independently of their phylotypes, indicating that PhcB and PhcS have coevolved with the types of QS signals (Kai et al., 2015). To analyse the genetic variation of not only PhcK but also PhcA and PhcB among 37 R. solanacearum strains (phylotype I, 19 strains; phylotype IIA, 1 strain; phylotype IIB, 2 strains; phylotype III, 1 strain; and phylotype IV, including blood disease bacterial strain R229 and Ralstonia syzygii strain R24, 14 strains; Table S4), the deduced amino acid sequences of PhcK, PhcA, and PhcB were analysed with ClustalW and a phylogenetic tree was constructed with TreeView. The phylogenetic trees of PhcA indicated that 37 strains were divided into four clades, consistent with their phylotypes but not with their QS signal types, which were analysed according to the deduced PhcB amino acid sequences (Table S4 and Figure S2b). Based on the genome sequences in a Blastp search by NCBI and INRA (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi), we did not identify any phcK homologs based on the genome sequences of phylotype II strains than phylotype IIB strains shown in Table S4. Furthermore, we did not identify any phcK homologs from other phylotype II strains than phylotype IIB strains shown in Table S4. The phylogenetic tree of PhcK indicated that R. solanacearum strains were divided into three clades, consistent with their phylotypes (Table S4 and Figure S2b).

3 | DISCUSSION

The phc QS-deficient mutants exhibit significantly inhibited growth after invading the intercellular spaces of roots. Moreover, they are unable to invade xylem vessels and are no longer virulent (Hayashi et al., 2019a). Thus, the phc QS system affects R. solanacearum virulence (Genin and Denny, 2012; Hikichi et al., 2017). The results of the current study demonstrated that the deletion of phcK significantly

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**FIGURE 7** Influence of exogenously applied methyl 3-hydroxymyristate (3-OH MAME) on the cell aggregation (a) and phcA expression levels (b) of Ralstonia solanacearum strains OE1-1, phcK-deleted mutant (ΔphcK), and phcB-deleted mutant (ΔphcB). (a) Cells of R. solanacearum strains incubated in quarter-strength M63 medium in the wells of polyvinylchloride microtitre plates were stained with crystal violet. Cell aggregation was quantified based on absorbance at 550 nm (A550). The resulting value was normalized according to the number of cells (optical density at 600 nm, OD560). Bars indicate the standard errors. Asterisks indicate values that are significantly different from those of OE1-1 (t test, p < .05). (b) The R. solanacearum strains were grown in quarter-strength M63 medium until optical density at 600 nm (OD600) reached 0.3. Total RNA was then extracted from the bacterial cells. The rpoD gene was used as an internal control for quantitative reverse transcription PCR. Gene expression levels are presented relative to the rpoD expression level. The experiment was conducted at least twice using independently prepared samples and produced similar results. Data for a representative sample are provided. Bars indicate the standard errors.
down-regulated phcA, thereby down-regulating the expression levels of 88.2% of the PhcA-positively regulated genes and up-regulating the expression levels of 85.9% of the PhcA-negatively regulated genes. This led to a change of phcQS-dependent phenotypes of the ΔphcK mutant, similar to the ΔphcA mutant. Therefore, phcK is required for full phcA expression to help control the expression of phcQS-dependent genes, regulating the phcQS-dependent phenotypes. Furthermore, the ΔphcK mutant lost its systemic infectivity in tomato plants, resulting in a loss of virulence on tomato plants. These observations imply that PhcK is one of the phc regulatory elements, which are required for the systemic infectivity and the virulence of strain OE1-1.

The signal transduction systems with the two-component systems have been evolved to respond to environmental changes and are signal transduction regulatory circuits that comprise a membrane-bound sensor histidine kinase and a cytoplasmic response regulator (Gao and Stock, 2009). During QS, the activated response regulator usually functions as a transcription factor. In the R. solanacearum phc QS system, PhcB helps produce the QS signal, 3-OH MAME or 3-OH PAME (Kai et al., 2015; Ujita et al., 2019). Interestingly, phcK was required for the full phcA expression but not phcB expression. Extracellular 3-OH MAME content does not affect the expression of both phcK and phcA (Mori et al., 2018b; Hayashi et al., 2019a,2019b). Therefore, the PhcK-mediated transcriptional regulation of phcA may occur independently of the extracellular 3-OH MAME content. An analysis of the deduced amino acid sequences with a Blastp search by NCBI and the SOSUI program predicted that PhcK is a transmembrane sensor histidine kinase (Figure S2a). Clough et al. (1997) demonstrated on the phc QS signalling of strain AW1, which produces 3-OH PAME as the QS signal, that 3-OH PAME is not the only factor controlling these traits. It is thus thought that a putative sensor histidine kinase PhcK of strain OE1-1 may sense an unknown extracellular signal but
not 3-OH MAME, thereby contributing to full phcA expression. To our knowledge, this is the first report of the phcA transcriptional regulation of \textit{R. solanacearum}.

PhcA is a global virulence regulator but does not have a response regulator receiver domain (Genin and Denny, 2012). In contrast, phcK seems to be in an operon with the RSc1352 gene encoding a putative cognate response regulator associated with PhcK. However, the RSc1352 gene deletion did not affect the phc QS-dependent phenotypes. The complimented expression level of the RSc1352 gene in the phcK-comp strain suggests that the significantly reduced expression level of the RSc1352 gene in the ΔphcK is not due to a polar effect of the phcK deletion, and that phcK is also required for the full expression of the RSc1352 gene. If PhcK functions as a sensor histidine kinase in the phc QS signalling pathway, an unknown cognate response regulator but not the RSc1352 protein associated with PhcK may be involved in the phcA transcriptional regulation.

During the phc QS, functional PhcA regulates the phc QS-dependent genes (Genin and Denny, 2012). The hydrolysis of 3-OH PAME by β-hydroxypalmitate methyl ester hydrolase suppresses the phc QS-dependent EPS production of \textit{R. solanacearum} strain AW1-3 (Shinohara et al., 2007). In addition, methyl ester hydrolases suppress the phc QS-dependent EPS production of \textit{R. solanacearum} GM10000 (Lee et al., 2017). These data suggest that the functional PhcA levels may depend on the production and sensing of QS signals (Genin and Denny, 2012). It is postulated that the sensor histidine kinase and response regulator hybrid transcription regulator protein PhcR cognate with PhcS is involved in the regulation of QS-dependent genes (Genin and Denny, 2012). However, the mechanism by which the PhcS-PhcR two-component system controls the activity of PhcA is not known. The PhcK-involved transcriptional regulation of phcA independent of the extracellular 3-OH MAME content suggests that the 3-OH MAME-dependent activated PhcS-PhcR two-component system may be involved in functional PhcA but not the phcA transcriptional regulation. Therefore, the regulation of phc QS-dependent genes through functional PhcA may be dependent on the extracellular 3-OH MAME content.

\textit{R. solanacearum} strains synthesize 3-OH MAME or 3-OH PAME as QS signals via the methyltransferase PhcB. These signals are sensed by the sensor histidine kinase PhcS (Kai et al., 2015; Ujita et al., 2019). Though the comparative genomic analysis reveals conservation of phcB, phcS, and phcA among \textit{R. solanacearum} strains (Ailloud et al., 2015; Bocsanczy et al., 2017), the phylogenetic trees based on the PhcB and PhcS amino acid sequences reveals that \textit{R. solanacearum} strains are divided into two groups according to their QS signal types (Kai et al., 2015; Hikichi et al., 2017). This suggests that the types of QS signals do not reflect the locations from which they were isolated and the host plants from which the strains were isolated. On the contrary, a phylogenetic analysis involving the deduced amino acid sequences of PhcK and PhcA indicated that \textit{R. solanacearum} strains can be divided according to their phylotypes (Table S4 and Figure S2b). We detected the phcK homologs of phylotype I and IV strains and only two phylotype IIIB strains, but not phylotype III strains. Although the phc QS system involving the production and sensing of 3-OH MAME or 3-OH PAME is conserved among \textit{R. solanacearum} strains, there may be diversity in the phcA transcriptional regulation among strains, especially the phylotype II and phylotype III strains. It is thus thought that the ancestors of \textit{R. solanacearum} might have first coevolved the QS signal synthase (PhcB) and its receptor (PhcS), and then evolved the additional levels of regulation acting on the downstream regulator PhcA on their phylotypes for the adaptation to new and different environments. This highlights the unique evolution of the QS signal-mediated signalling pathways of the phc QS system in \textit{R. solanacearum}.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, and growth conditions

\textit{R. solanacearum} strains (Table 1) were routinely grown in quarter-strength M63 medium (Cohen and Rickenberg, 1956) at 30°C. \textit{Escherichia coli} strains were grown in Luria-Bertani medium (Hanahan, 1983) at 37°C. Selective media contained kanamycin (50 µg/ml) and gentamycin (50 µg/ml).

4.2 | General DNA manipulations

We used standard techniques (Sambrook et al., 1989) as the DNA manipulations, and determined DNA sequences using the Automated DNA Sequencer Model 373 (Applied Biosystems), after which and DNA sequences were analysed with the DNASYS-Mac program (Hitachi Software Engineering).

4.3 | Phylogenetic analysis of Phc proteins

We aligned the deduced amino acid sequences of PhcB (464–468 amino acids), PhcA (347 amino acids), and PhcK (414 amino acids) of \textit{R. solanacearum} strains, after which the ClustalW program (DNA Data Bank of Japan; http://www.ddbj.nig.ac.jp/search/clus talw-j.html) was used to construct phylogenetic trees according to the neighbour-joining method (Saitou and Nei, 1987), with genetic distances computed with Kimura’s two-parameter model (Kimura, 1980). The phylogenetic trees were drawn with TreeView (https://treeview.co.uk/download-file/?v=%22).

4.4 | Transposon mutagenesis

The EZ-Tn5 < KAN-2 > Tnp Transposome Kit (Lucigen) was used to generate \textit{R. solanacearum} OE1-1 mutants with transposons.
inserted into their genomes according to the manufacturer's instructions. We selected the mutant clones in plates containing Hara–Ono medium (Hara and Ono, 1983) supplemented with kanamycin.

### 4.5 | Analysis of the transposon-insertion site in the genomic DNA of mutants

To examine the transposon-insertion site of the mutant *R. solanacearum* strain KUKW-51, the PstI-digested genomic DNA fragments of the mutant were ligated into the pUC118 vector (Takara Bio). The resulting recombinant plasmids were inserted into *E. coli* DH5α cells (Takara Bio). The pUC118KUKW-51 recombinant plasmid (Table 1) in a kanamycin-resistant transformant was isolated and the insert was sequenced.

### 4.6 | Creation of a phcK-deleted mutant and a complementation construct

A 606-bp DNA fragment (delta-phcK-1) was amplified by PCR with the genomic DNA of strain OE1-1 as the template and primers delta-phcK-1-FW (5′-AAAAATGCGCTGCCGAGG-3′) and delta-phcK-1-RV (5′-AGCGATGGGCATCAGCGGCCTGCCTGATTG-3′). A 526-bp DNA fragment (delta-phcK-2) was amplified by PCR with the genomic DNA of strain OE1-1 as the template and primers delta-phcK-2-FW (5′-AAAAATGCCGTGCCGCCGAGG-3′) and delta-phcK-2-RV (5′-CCCCACCAGCGCCTTGATG-3′). Using the delta-phcK-1 and delta-phcK-2 sequences as templates, a 1,113-bp DNA fragment was amplified by PCR with primers delta-phcK-1-FW and delta-phcK-2-RV, and then cloned into the pMD20 vector (Takara Bio) to generate the pMD20delta-phcK recombinant plasmid (Table 1) in a kanamycin-resistant transformant. This plasmid was electroporated into OE1-1 competent cells, after which a kanamycin-sensitive and sucrose-resistant recombinant, ΔRSc1352, was selected.

### 4.7 | Creation of an RSc1352 gene-deleted mutant

A 741-bp DNA fragment (delta-1352-1) was amplified by PCR with the genomic DNA of strain OE1-1 as the template and primers delta-1352-1-FW (5′-AGCGCGAAGAGGCCGAGAAG-3′) and delta-1352-1-RV (5′-GGGGACGCTACATCGGTCAGGTTCCGGCGG-3′). A 907-bp DNA fragment (delta-1352-2) was amplified by PCR with the genomic DNA of strain OE1-1 as the template and primers delta-1352-2-FW (5′-AGCGCGAAGAGGCCGAGAAG-3′) and delta-1352-2-RV (5′-GGGGACGCTACATCGGTCAGGTTCCGGCGG-3′). Using the delta-1352-1 and delta-1352-2 sequences as templates, a 1,628-bp DNA fragment was amplified by PCR with primers delta-1352-1-FW and delta-1352-2-RV, and then cloned into the pMD20 vector to generate the pMD20delta-1352 recombinant plasmid (Table 1). This plasmid was electroporated into OE1-1 competent cells, after which a kanamycin-sensitive and sucrose-resistant recombinant, ΔRSc1352, was selected.

### 4.8 | Transformation of the ΔphcK mutant with *phcA* controlled by a constitutive promoter

To construct the p0480prophcA recombinant plasmid (Table 1) comprising prophcA under the control of the RSc0480 promoter, a 509-bp DNA fragment (ProRSc0480phcA-1) was amplified by PCR with the genomic DNA of strain OE1-1 as the template and primers ProRSc0480phcA-1-FW (5′-TACGCCCCAAATGCTCTCTCTCGG-3′) and ProRSc0480phcA-1-RV (5′-GACGTTGACCATTGGACATGGCTGTCTTCCTG-3′). Additionally, a 1,054-bp DNA fragment (ProRSc0480phcA-2) was amplified by PCR with the genomic DNA of strain OE1-1 as the template and primers ProRSc0480phcA-1FW2 and ProRSc0480phcA-2-RV (5′-GCCATGTCCAATGGTCAACGTCGATACCAAGCTG-3′). Using ProRSc0480phcA-1FW2 and ProRSc0480phcA-2-RV as templates, we amplified a 509-bp DNA fragment by PCR with primers ProRSc0480phcA-1-FW2 and ProRSc0480phcA-2-RV. The amplicon was then cloned into the pMD20 vector to generate the pdelta-1352 recombinant plasmid (Table 1). This plasmid was electroporated into OE1-1 competent cells, after which a kanamycin-sensitive and sucrose-resistant recombinant, ΔRSc1352, was selected.

### 4.9 | Transcriptome analysis based on RNA-Seq

Total RNA was extracted from *R. solanacearum* strains grown in quarter-strength M63 medium (until OD₆₀₀ = 0.3) with the High Pure RNA Isolation Kit (Roche Diagnostics), after which the ribosomal RNA was eliminated with the Ribo-Zero rRNA Removal Kit.
An oriented paired-end RNA-Seq (2 × 100 bp) analysis was completed with an Illumina HiSeq 2000 system by Hokkaido System Science (Sapporo, Japan). The selected inserts were 100 bp and the libraries underwent paired-end sequencing. Reads were trimmed with Cutadapt v. 1.1 (http://code.google.com/p/cutadapt/) and Trimmomatic v. 0.32 (http://www.usadelab.org/cms/?page=trimmomatic) prior to mapping with the TopHat v. 2.0.10 program (http://tophat.cbcb.umd.edu/). The read counts for each sample are presented as the FPKM, which was calculated with Cufflinks v. 2.2.1 (http://cole-trapnell-lab.github.io/cufflinks/). We conducted three biologically independent experiments for each strain.

4.10 | RT-qPCR

A RT-qPCR assay was completed with gene-specific primers (Table 2), the SYBR GreenER qPCR Reagent System (Invitrogen), and the 7300 Real-Time PCR System (Applied Biosystems), as previously described (Hayashi et al., 2019a). We normalized all values against the rpoD expression level as an internal standard for each cDNA sample (Narusaka et al., 2011; Mori et al., 2016). We did not observe any significant difference in the expression levels of rpoD among R. solanacearum strains. This experiment was conducted at least twice with independently prepared samples with eight technical replicates in each experiment and produced similar results. Data for a representative sample are provided.

4.11 | Ralfuranone productivity

R. solanacearum strains were grown for 4 days in 100 ml MGRL medium (Fujiwara et al., 1992) containing 3% sucrose and their ralfuranone productivity was analysed as previously described (Kai et al., 2014). We repeated the experiment three times with independently prepared samples.

4.12 | Extracellular 3-OH MAME content of R. solanacearum strains

R. solanacearum strains were grown in B medium (Clough et al., 1994) at 30°C for 4–6 hr, after which the extracellular 3-OH MAME content was analysed as previously described (Kai et al., 2015). We repeated the experiment three times with independently prepared samples.

4.13 | Cell aggregation

The aggregation of R. solanacearum cells grown in quarter-strength M63 medium without shaking was examined in vitro as previously described (Mori et al., 2016). Cell aggregation was quantified based on the absorbance at 550 nm (A550). The resulting value was normalized according to the number of cells (optical density at 600 nm, OD600). The experiment was repeated three times, each with seven technical replicates.

### TABLE 2 Primers used in the quantitative reverse transcription-PCR assays

| Gene   | Primer          | Nucleotide sequence (5′–3′)                                      |
|--------|-----------------|-----------------------------------------------------------------|
| rpoD   | rpoD-FW         | ATCGTCGAGCGCAACATCCC                                            |
|        | rpoD-RV         | AGATGGGAGTCTGCAGTCTCGTG                                          |
| epsB   | epsB-FW         | ATGGTCGAGCTGATGGATA                                              |
|        | epsB-RV2        | TGGACGTCTGATCGTCTC                                               |
| fliC   | fliC-FW2        | CAAACGCAAGTATCCGAGAAGCAGAC                                       |
|        | fliC-RV2        | ATGGAAAGTCTCGAGAAGCAGAC                                          |
| ralA   | ralA-FW         | GCCTGGAGTACAGGTCTGATGATA                                         |
|        | ralA-RV         | CGTCAGTACGAAACAGCG                                               |
| lecM   | fml-FW2         | GTATTACGGCTTCCCGCACAANC                                          |
|        | fml-RV2         | ATGGCAGTCGTGATCTGTC                                              |
| phcB   | phcB-FW3-514    | TACAAGATCAAGCAGCAGCTACCTCAAGCTG                                  |
|        | phcB-RV3-1011   | GTCTGTACAGCAGCTACCTCAAGCTG                                       |
| phcA   | phcA-FW5        | AGTCAGTCGTGATCTGTC                                               |
|        | phcA-RV5        | AGATCCTCTCAGCGAGTCTGTC                                           |
| phcK   | phcK-FW         | TGCATGTGGCTGTCTGATC                                              |
|        | phcK-RV         | CAGTGGACCGAATGCGCTGTC                                            |
| RSc1352 gene    | 1352-FW        | TGTTCTGAGCCGCCTACAG                                             |
|        | 1352-RV         | CACCTTCCAGAAGTGTGTC                                             |
4.14 | EPS I production

The EPS I production by *R. solanacearum* cells grown on quarter-strength M63 medium solidified with 1.5% agar was quantitatively analysed in an enzyme-linked immunosorbent assay (Agdia Inc.) as previously described (Mori et al., 2016). The EPS I productivity was quantified based on the absorbance at 650 nm (A650). This experiment was repeated three times, each with five technical replicates.

4.15 | Swimming motility

The swimming area diameters for the *R. solanacearum* strains incubated on quarter-strength M63 medium solidified with 0.25% agar were measured after incubation for 48 hr as previously described (Mori et al., 2018b). The experiment was repeated three times, each with five technical replicates.

4.16 | Virulence assay

Eight-week-old tomato plants (*Solanum lycopersicum* ‘Ohgata-Fukuju’) were inoculated with *R. solanacearum* strains (10^6 cfu/ml) according to a root-dipping inoculation procedure as previously described (Hayashi et al., 2019a). For each bacterial strain, 12 plants were treated in each trial and each assay was repeated in five successive trials. Plants were monitored daily for wilting symptoms, which were rated based on the following disease index scale: 0, no wilting; 1, 1%–25% wilting; 2, 26%–50% wilting; 3, 51%–75% wilting; 4, 76%–99%; and 5, dead.

We assessed the populations of *R. solanacearum* strains in inoculated tomato roots according to the observed growth on Hara–Ono medium as described by Hayashi et al. (2019a). Twelve plants were treated in each trial and each assay was repeated in five successive trials.

The behaviour of *R. solanacearum* strains in tomato plants inoculated with the root-dipping method was assessed as described by Hayashi et al. (2019a). A sample from each cut site (Figure 8b) was pressed onto Hara–Ono medium. Twelve plants were analysed in each trial and each assay was repeated in five successive trials.

4.17 | Statistical analysis

The means of all assays were analysed for significant differences between *R. solanacearum* strains with Student’s *t* test in Microsoft Excel.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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