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Comparative Genomic Analysis Identifies FleQ and GcbB as Virulence-Associated Factors in *Pseudomonas syringae* pv. *tabaci* Strains

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ABSTRACT

Pseudomonas syringae pv. *tabaci* (Pta) is an important plant pathogen, which causes wildfire disease in *Nicotiana* species. However, the genetic basis underlying strain-level differences in virulence remains largely unresolved. To address this, we performed a comparative genomic analysis between a highly virulent strain Pta6605 and a less virulent strain Pta7375. Despite high overall genome similarity, we identified key single-nucleotide polymorphisms, including premature stop-codon mutations in seven open reading frames in Pta7375. Notably, point mutations in two regulatory genes, such as *fleQ*, which encodes a transcription factor essential for flagellar biogenesis and biofilm formation, and *gcbB*, which encodes a GGDEF domain-containing diguanylate cyclase responsible for cyclic dimeric guanosine monophosphate (c-di-GMP) synthesis, were implicated in virulence disparity. Functional analyses using deletion and locus replacement mutants in the Pta6605 background revealed that the disruption of *fleQ* markedly reduced motility, flagellin production, c-di-GMP accumulation, biofilm formation and virulence level mirroring the Pta7375 phenotype. The *gcbB* replacement mutant showed reduced disease symptom development, although c-di-GMP levels remained comparable to the Pta6605 wild type. Locus replacement between strains confirmed that a point mutation in *fleQ* was the primary driver of reduced motility and flagellin expression in Pta7375. These findings indicate that the reduced virulence of Pta7375 is associated with impaired regulation of flagella-related genes and disruption of the FleQ-mediated c-di-GMP signalling, underscoring the value of comparative genomics in disentangling the complex regulatory networks that govern virulence in plant pathogens.

1 | Introduction

Pseudomonas syringae, a globally significant plant pathogen, comprises over 50 pathovars with distinct host specificities and collectively infects nearly all economically important crops (Xin

et al. 2018). Phylogenetically classified into 13 phylogroups, this species exhibits remarkable genetic diversity, reflecting its adaptations to various hosts and environmental niches (Xin et al. 2018). Despite extensive studies on its molecular pathogenicity, the genetic basis of strain-level differences in virulence,

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particularly in host colonisation and disease progression, remains poorly understood (Helmann et al. 2019; Lindeberg et al. 2008). Identifying the specific genes driving these variations is challenging because of structural genomic plasticity among strains.

Pseudomonas syringae initiates infection by entering host tissues through stomata or wounds, relying on flagellar motility (O'Malley and Anderson 2021; Xin et al. 2018). Once established, it deploys virulence factors, such as type III effectors, for suppressing plant immunity, along with phytotoxins, exopolysaccharides and biofilm matrix for evading antimicrobial defence (Budil and Lišková 2024; Xin et al. 2018). The bacterial transition from a motile to a sessile lifestyle is tightly regulated by cyclic dimeric guanosine monophosphate (c-di-GMP), a bacterial second messenger that modulates biofilm formation, motility and virulence (Wang et al. 2023). FleQ, which has a Rec domain, an AAA+/ATPase σ 54 interaction domain, and a DNA-binding helix-turn-helix (HTH) motif, is a transcription factor that plays a central role in controlling bacterial lifestyle (Su et al. 2015). FleQ orchestrates the shift between motility and sessility by inversely regulating the flagellar (planktonic growth) and biofilm-associated (sessile growth) genes (Oladosu et al. 2024). In *Pseudomonas fluorescens* and *Pseudomonas putida*, FleQ governs adhesion proteins, such as LapA and MapA, balancing surface attachment and dispersal (Blanco-Romero et al. 2018; Pastora and O'Toole 2023). Critically, FleQ interacts with c-di-GMP, which fine-tunes its transcriptional activity; elevated c-di-GMP levels repress motility genes while activating biofilm formation (Xiao et al. 2021). However, the functional interplay between FleQ and c-di-GMP in plant pathogens remains underexplored, particularly given the redundancy of the GGDEF/EAL-domain proteins that regulate c-di-GMP levels (Nie et al. 2020).

Pseudomonas amygdali pv. *tabaci* (formerly *P. syringae* pv. *tabaci* [Pta]) is a gram-negative bacterium that causes wildfire disease in tobacco (Kashihara et al. 2022). The genetic basis underlying strain-level differences in virulence of Pta remains largely unresolved. The dynamic plasticity of plant-pathogenic bacterial genomes, coupled with extensive mutational variation, often makes the identification of causative genes by comparative genomics challenging. However, we believe that identifying genes that determine the differences in virulence is important for elucidating the control mechanisms of the virulence of plant-pathogenic bacteria.

In this study, we performed comparative genomics for identifying genes that play important roles in virulence by comparing the genomes of a highly virulent strain, *Pta6605*, and a less virulent strain, *Pta7375*. Given the overall similarity in their genome architectures, our investigation centered on analysing single-nucleotide polymorphisms that may underlie differences in virulence. The primary objective was to assess the roles of two candidate genes, *fleQ* (involved in c-di-GMP signalling) and *gcbB* (which encodes a protein with a GGDEF domain), in modulating virulence. To achieve this, we conducted functional analyses using deletion and locus replacement mutants to determine *fleQ* and *gcbB* contribute to virulence in *Pta6605*.

2 | Results

2.1 | Comparative Genomic Analysis Between *Pta6605* and *Pta7375*

To elucidate the causes of the differences in virulence between strains, we investigated the genetic difference in virulence between the highly virulent *Pta6605* strain and the less virulent *Pta7375* strain. The virulence factors of *Pta6605* have been studied, but no reports have been made on *Pta7375* due to its low virulence. *Pta6605* caused severe symptoms in the host *Nicotiana tabacum* 'Xanthi' as observed in the flood assay (Figure 1a). *Pta6605* demonstrated high motility in both the swimming and swarming assays (Figure 1b,c). In contrast, *Pta7375* exhibited reduced virulence in Xanthi in the flood assay (Figure 1a) and displayed significantly low motility (Figure 1b,c). Given the established direct relationship between flagellar function and virulence in *Pta6605* (Ichinose et al. 2003), we examined the accumulation of flagellar proteins by western blotting. The results revealed a significant reduction in flagellin levels in *Pta7375* compared to that in *Pta6605* (Figure 1d). Furthermore, biofilm production was markedly low in *Pta7375* (Figure 1e). Comparison of in vitro growth between *Pta6605* and *Pta7375* strains showed no difference in initial growth, but a clear difference in optical density (OD) was observed at 24 h. By 36 h, both strains reached similar OD values (Figure S1).

To elucidate the genetic determinants underlying the divergent phenotype of *Pta6605* and *Pta7375*, we performed a comparative genomic analysis. Their genomes exhibited 99% overall nucleotide identity and conserved syntenic organisation (Figure 1f). We identified three strain-specific large insertion events. In addition, we catalogued approximately 350 polymorphic sites within the shared genome (Table 1), comprising 43 nonsynonymous single-nucleotide variants (SNVs), 39 small insertions/deletions (<20 bp) and 15 premature stop codons predicted to truncate open reading frames (ORFs). Collectively, these structural and sequence-level variations were likely to underlie the observed differences in virulence between the two strains. Detailed analysis revealed there are frameshift mutations leading to premature stop codons in seven ORFs of *Pta7375* (Figure 1g). These ORFs encode an alpha/beta-hydroxylase (Pta6605_RS01805), an autotransporter (Pta6605_RS01830), two hypothetical proteins (Pta6605_RS01865 and Pta6605_RS12405), a dehydrogenase (Pta6605_RS07205), a sigma-54-dependent Fis family transcription factor (*fleQ*; Pta6605_RS09960) and a protein containing a GGDEF domain (*gcbB*, Pta6605_RS17490). Phylogenetic analysis revealed that the GGDEF domain-containing protein was a homologue of *Pseudomonas fluorescens* Pf0-1 GcbB (Newell et al. 2011) (Figure S2).

We focused on two candidate genes, *fleQ* and *gcbB*, that are potentially responsible for the reduced virulence of *Pta7375*. This is because both *fleQ* and *gcbB* are considered to contribute to c-di-GMP signalling, which functions as a second messenger. *fleQ* is involved in flagella formation and biofilm production in other *Pseudomonas* species (Oladosu et al. 2024; Su et al. 2015). In *Pta7375*, a single-base insertion mutation in *fleQ* resulted in a frameshift and a premature stop codon (Figure S3a), disrupting

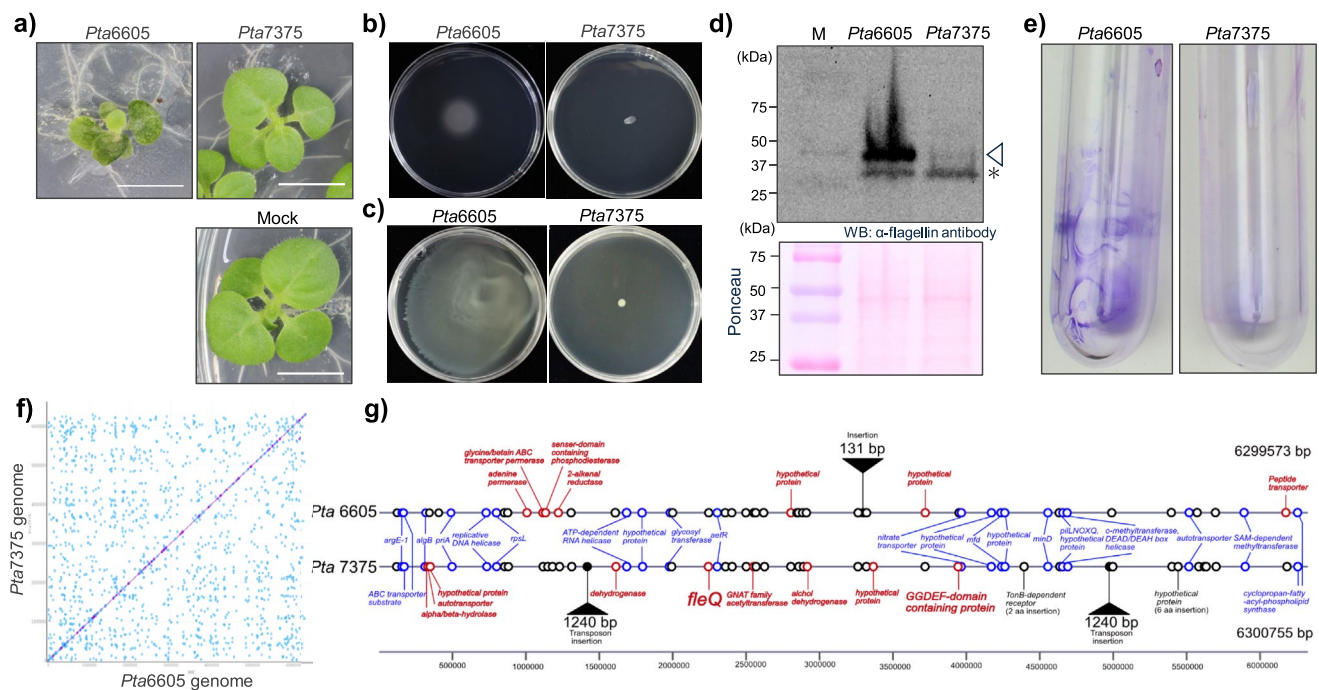


FIGURE 1 | Characterisation and comparative analysis of the genome sequences of *Pta6605* and *Pta7375*. (a) Disease symptoms of tobacco plants inoculated with *Pta6605* and *Pta7375* by using the flood method. The inoculum was adjusted to an OD₆₀₀ of 0.002 and contained 0.025% Silwet L-77. Photographs were captured at 4 days post-inoculation (dpi). Scale bar, 1 cm. (b) Swimming motility of *Pta6605* and *Pta7375* on MMMF agar for 24 h at 27°C. (c) Swarming motility of *Pta6605* and *Pta7375* on 0.3% SWM agar for 48 h at 23°C. (d) Analysis of flagellin levels in *Pta6605* and *Pta7375* by western blotting. Ponceau S staining indicates the loading control. White arrowhead indicates flagellin, and asterisk indicates nonspecific band. (e) Biofilm formation assay of *Pta6605* and *Pta7375*. Crystal violet staining of both strains in incubation tubes. (f) Comparison of the genome structures of *Pta6605* and *Pta7375* using Harr plot analysis. (g) Visualisation of the single-nucleotide polymorphisms (SNPs) and insertion regions between *Pta6605* and *Pta7375*. Red circles indicate stop codon insertions in open reading frames (ORFs); blue circles indicate amino acid substitutions in ORFs; and black circles indicate the positions of nucleotide insertion.

TABLE 1 | Single-nucleotide polymorphisms in *Pta7375* compared to *Pta6605*.

297 single-nucleotide substitutions	254 synonymous substitutions 43 nonsynonymous substitutions
54 single-nucleotide deletions	39 deletion sites, excluding stop-codon mutation 15 stop codon mutations

the essential DNA-binding HTH domain. Similarly, a single-base substitution in the *Pta6605*_RS17490 resulted in a stop codon and loss of the GGDEF domain, which is critical for c-di-GMP synthesis (Figure S3b). Based on these findings, we generated mutants targeting *fleQ* and *gcbB* to investigate whether these genes contribute to the virulence of *Pta6605* and to evaluate their roles in bacterial virulence.

2.2 | Phenotypic Characterisation of *fleQ* and *gcbB* Deletion Mutants in *Pta6605*

To determine whether *fleQ* and *gcbB* were responsible for the observed phenotype (motility, biofilm and virulence) in *Pta7375*, we generated Δ *fleQ*, Δ *gcbB* and Δ *fleQ* Δ *gcbB* double mutants in the *Pta6605* background. Construction of the

Δ *fleQ* mutant and its phenotypic characterisation, including *fleQ* and *gcbB* gene expression and flagellin protein accumulation, is shown in Figure S4. When *fleQ* and *gcbB* gene expression was examined in Δ *fleQ* and Δ *gcbB* mutants, it was revealed that the deletion of *fleQ* did not affect *gcbB* gene expression, and conversely, the deletion of *gcbB* did not affect *fleQ* gene expression, indicating that these genes do not regulate each other (Figure S4e,f). The *Pta6605* Δ *fleQ* and *Pta6605* Δ *fleQ* Δ *gcbB* mutants exhibited significantly reduced swimming and swarming motility, mirroring the phenotype of *Pta7375* wild-type (WT) (Figure 2a–c). In contrast, the *Pta6605* Δ *gcbB* mutant exhibited little to no effect on motility. Western blot analysis showed a clear flagellin band in the *Pta6605* WT but no detectable signal in the Δ *fliC* mutant (Figure 2d and Figure S4d). The Δ *fleQ* mutant similarly lacked any accumulation of Δ *fliC* protein (Figure 2d). In fact, the gene expression of *flagella-related* genes including *fliC* was decreased in the Δ *fleQ* mutant as compared with *Pta6605* WT (Figure S5c).

The *Pta6605* Δ *gcbB* mutant did not affect flagellar protein levels, and the *Pta6605* Δ *fleQ* Δ *gcbB* mutant showed a significant reduction, similar to that of *Pta7375* WT (Figure 2d). To verify the presence or absence of flagella, microscopic observations were conducted. Consistent with the western blot analysis results, no flagella were observed in Δ *fliC*, Δ *fleQ*, Δ *fleQ* Δ *gcbB* mutants and *Pta7375* WT, while flagella were confirmed in *Pta6605* WT and

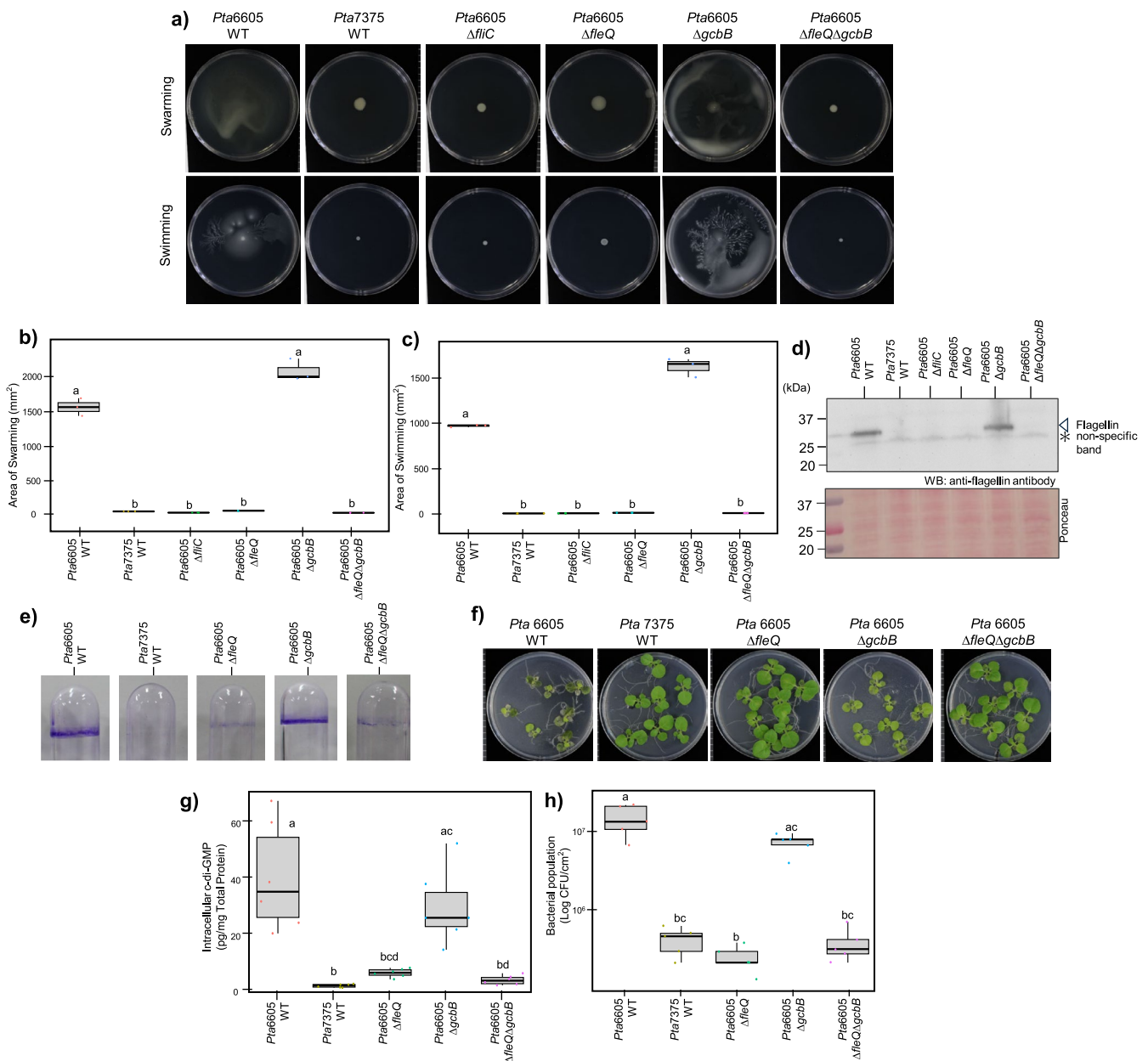


FIGURE 2 | $\Delta fleQ$, $\Delta gcbB$ and $\Delta fleQ\Delta gcbB$ mutant phenotypes compared to those of *Pta6605* wild type (WT). (a) Swarming motility (on SWM agar, 24 h, 27°C) and swimming motility (on MMMF agar 0.3%, 48 h, 23°C) of the deletion strains compared to those of *Pta6605* WT. (b) Area of swarming motility of the deletion strains compared to those of *Pta6605* WT. Motility area were quantified using Fiji (ImageJ) with scale calibration based on a ruler in each image. (c) Area of swimming motility of the deletion strains compared to those of *Pta6605* WT. Motility area were quantified using Fiji (ImageJ) with scale calibration based on a ruler in each image. (d) Detection of flagellin in the deletion mutants by WB. Ponceau S staining indicates the loading control. White arrowhead indicates flagellin, and asterisk indicates nonspecific band. (e) Biofilm formation assay on glass tube surface. (f) Disease symptoms on *Nicotiana tabacum* 'Xanthi' leaves inoculated with $\Delta fleQ$, $\Delta gcbB$, and $\Delta fleQ\Delta gcbB$ mutants (OD₆₀₀ = 0.002) by flood assay at 4 days post-inoculation (dpi). (g) Intracellular cyclic diguanylate (c-di-GMP) levels in $\Delta fleQ$, $\Delta gcbB$, and $\Delta fleQ\Delta gcbB$ mutants of *Pta6605* background. (h) Bacterial populations in Xanthi leaves at 3 dpi using the flood assay (optical density at 600 nm [OD₆₀₀] = 0.002). Data were analysed using the Kruskal–Wallis test followed by Dunn's post hoc test with Benjamini–Hochberg (BH) adjustment for multiple comparisons ($\alpha = 0.05$). Different letters above the bars/points indicate statistically significant differences between groups.

the *Pta6605* $\Delta gcbB$ mutant (Figure S6). Additionally, biofilm production was markedly decreased by the *Pta6605* $\Delta fleQ$ and $\Delta fleQ\Delta gcbB$ mutants (Figure S7). *Pta7375* WT had significantly lower c-di-GMP levels than that of *Pta6605* WT (Figure 2g). The *Pta6605* $\Delta fleQ$ mutant also exhibited lower c-di-GMP levels as compared with *Pta6605* WT. No significant reduction in

c-di-GMP accumulation was observed in the *Pta6605* $\Delta gcbB$ (Figure 2g). Although GcbB is presumed to function as a diguanylate cyclase (DGC), *gcbB* deletion did not affect c-di-GMP levels under this condition. Notably, the *Pta6605* $\Delta fleQ\Delta gcbB$ mutant displayed a significant decrease in c-di-GMP levels, resembling that of *Pta7375* WT.

We conducted an inoculation test on *N. tabacum* 'Xanthi', using a flood assay for assessing virulence (Figure 2f). Disease symptoms were observed in plants inoculated with *Pta6605* WT but not with the Δ *fleQ* mutant or *Pta7375* WT (Figure 2f). The Δ *gcbB* mutant exhibited a slight reduction in virulence compared to that of *Pta6605* WT (Figure 2f,h). When virulence was confirmed by performing an infiltration assay, severe disease symptoms were observed in *Pta6605* WT and the Δ *gcbB* mutant, but disease symptoms were suppressed in the Δ *fleQ* and the Δ *fleQ* Δ *gcbB* mutant (Figure S8). Both Δ *fleQ* and Δ *fleQ* Δ *gcbB* mutants showed a significant decrease in disease symptoms, similar to those observed in the *Pta7375* WT. These results indicate that *FleQ* is essential for the virulence of *Pta6605*, and that *GcbB* also contributes to virulence.

2.3 | Gene Swapping of *fleQ* Confirms Its Regulatory Role in Flagellin Production

To compare the effects of point mutations in *fleQ* and *gcbB* between *Pta7375* and *Pta6605*, we exchanged the *fleQ* and *gcbB* loci between these strains (Figure S9). To assess whether gene replacement affected expression levels, we examined *fleQ* and *gcbB* gene expression in the replacement strains (Figure S10). The expression of *fleQ* showed a tendency to decrease in *Pta7375* compared to *Pta6605*, and a similar trend was observed for *gcbB*; however, these differences were not statistically significant. These results confirmed that the gene replacement procedure itself did not cause dramatic changes in *fleQ* and *gcbB* gene expression levels. The effects of the replacement were investigated by evaluating the swimming ability of each replacement strain. As expected, *Pta6605 fleQ*⁷³⁷⁵ and *Pta6605 gcbB*⁷³⁷⁵*fleQ*⁷³⁷⁵ exhibited markedly reduced swimming motility compared to that of *Pta6605* WT (Figure 3a,b). *Pta6605 gcbB*⁷³⁷⁵ retained swimming motility similar to that of *Pta6605* WT. *Pta7375 fleQ*⁶⁶⁰⁵ and *Pta7375 gcbB*⁶⁶⁰⁵*fleQ*⁶⁶⁰⁵ recovered swimming motility comparable to that of *Pta6605* WT. *Pta7375 gcbB*⁶⁶⁰⁵ did not exhibit similar swimming motility to that of *Pta7375* WT (Figure 3a,b). We conducted western blot analysis to confirm whether the replacement mutants changed the production of flagellar proteins. *Pta6605 fleQ*⁷³⁷⁵ and *Pta6605 gcbB*⁷³⁷⁵*fleQ*⁷³⁷⁵ showed significantly reduced amounts of flagellin, whereas *Pta6605* WT and *Pta6605 gcbB*⁷³⁷⁵ produced high amounts of flagellin (Figure 3c). *Pta7375 fleQ*⁶⁶⁰⁵ and *Pta7375 gcbB*⁶⁶⁰⁵*fleQ*⁶⁶⁰⁵ recovered the high production of flagellin, whereas *Pta7375 gcbB*⁶⁶⁰⁵ still showed low levels of flagellin similar to that of *Pta7375* (Figure 3c). These results indicate that the *fleQ* mutation in *Pta7375* negatively affects motility and flagellar production. Additionally, we sought to identify other genes affected by deletion or single-nucleotide polymorphisms (SNPs) in *fleQ*⁷³⁷⁵. We scanned the *Pta6605* genome for the *FleQ*-binding motif (Baraquet and Harwood 2016) and found that at least two *flagella-related* genes, *flgA* and *fliL*, contain *FleQ*-binding motifs upstream near the start codon (Figure S5a,b). Reverse transcription-quantitative PCR (RT-qPCR) analysis revealed that the expression of *flgA* and *fliL* was decreased in both *Pta6605* Δ *fleQ* and *Pta6605 fleQ*⁷³⁷⁵ compared to *Pta6605* WT (Figure S5d,e). This result was further supported by the observation that *flgA* and *fliL* gene expression was recovered in *Pta7375 fleQ*⁶⁶⁰⁵ compared to *Pta7375* WT. These results indicate that even SNP in *fleQ*⁷³⁷⁵ can disrupt the transcription regulatory function of *FleQ*, leading to impaired activation of important *flagella-related* genes.

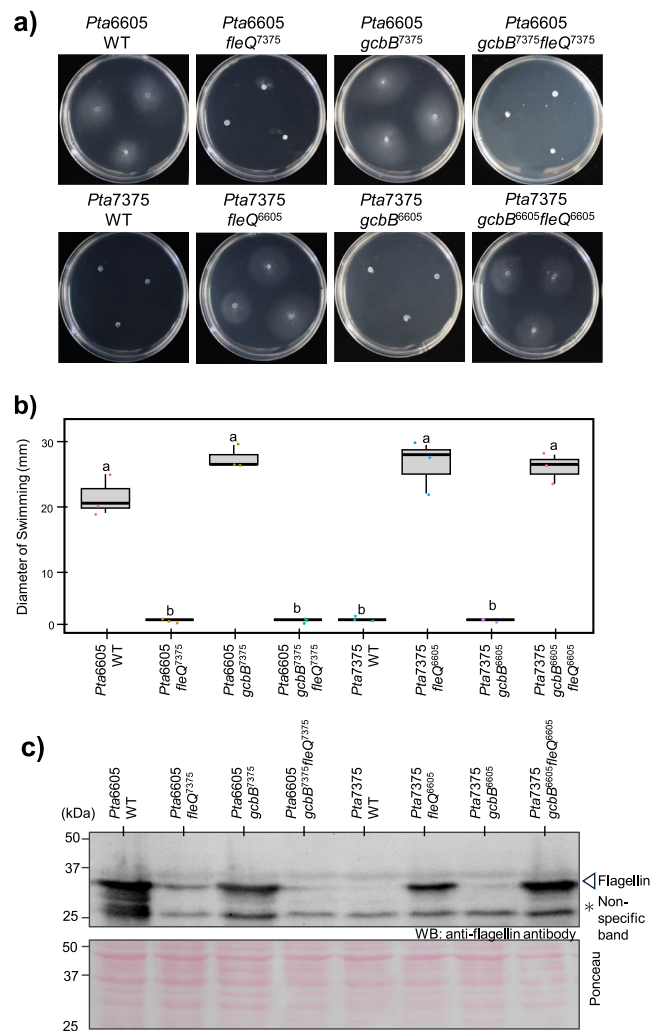


FIGURE 3 | Locus replacement of *fleQ* confirms its regulatory role in flagellin production. (a) Swimming motility of swapping mutants compared to those of *Pta6605* wild type (WT) and *Pta7375* WT. (b) Diameter of swimming motility of swapping mutants compared to those of *Pta6605* WT and *Pta7375* WT. Data were analysed using the Kruskal–Wallis test followed by Dunn's post hoc test with Benjamini–Hochberg (BH) adjustment for multiple comparisons ($\alpha = 0.05$). Different letters above the bars/points indicate statistically significant differences between groups. (c) Detection of flagellin in the swapping mutants by western blotting. Ponceau S staining indicates the loading control. White arrowhead indicates flagellin, and asterisk indicates nonspecific band.

2.4 | The Dual Mutation of *fleQ* and *gcbB* Reduces the Virulence of *Pta*

Because motility and flagellin production were consistent with our hypothesis for each replacement strain, we analysed biofilm formation, c-di-GMP levels, and virulence. In the biofilm assay, *Pta6605 fleQ*⁷³⁷⁵ and *Pta6605 gcbB*⁷³⁷⁵*fleQ*⁷³⁷⁵ replacement strains showed reduced biofilm formation compared to that of *Pta6605* WT; *Pta6605 gcbB*⁷³⁷⁵*fleQ*⁷³⁷⁵ showed the lowest extent of biofilm formation, similar to that of *Pta7375* WT (Figure S7). In contrast, *Pta7375 fleQ*⁶⁶⁰⁵ showed increased biofilm formation at 20 and 30 h compared to *Pta7375* WT. However, after 30 h incubation, *Pta7375 fleQ*⁶⁶⁰⁵ and *Pta7375*

*gcbB*⁶⁶⁰⁵*fleQ*⁶⁶⁰⁵ did not completely recover the biofilm accumulation to the level of *Pta7375*. These results suggest that mutations in *fleQ* play a crucial role in the reduced biofilm formation observed in *Pta7375*, while additional factors beyond *fleQ* and *gcbB* may also contribute to the complete biofilm formation.

*Pta6605 fleQ*⁷³⁷⁵ and *Pta6605 fleQ*⁷³⁷⁵*gcbB*⁷³⁷⁵ showed decreased amounts of c-di-GMP (Figure 4a), which was consistent with

biofilm formation. In contrast, *Pta7375 fleQ*⁶⁶⁰⁵ and *Pta7375 fleQ*⁶⁶⁰⁵*gcbB*⁶⁶⁰⁵ exhibited significantly increased c-di-GMP levels, but *Pta7375 gcbB*⁶⁶⁰⁵ did not (Figure 4a). These results suggest that FleQ functions as a major regulator of c-di-GMP levels in *Pta*.

Next, we tested the alterations, if any, in the virulence of the replacement strains using a flood assay. Development of disease symptoms was reduced in the *Pta6605 fleQ*⁷³⁷⁵,

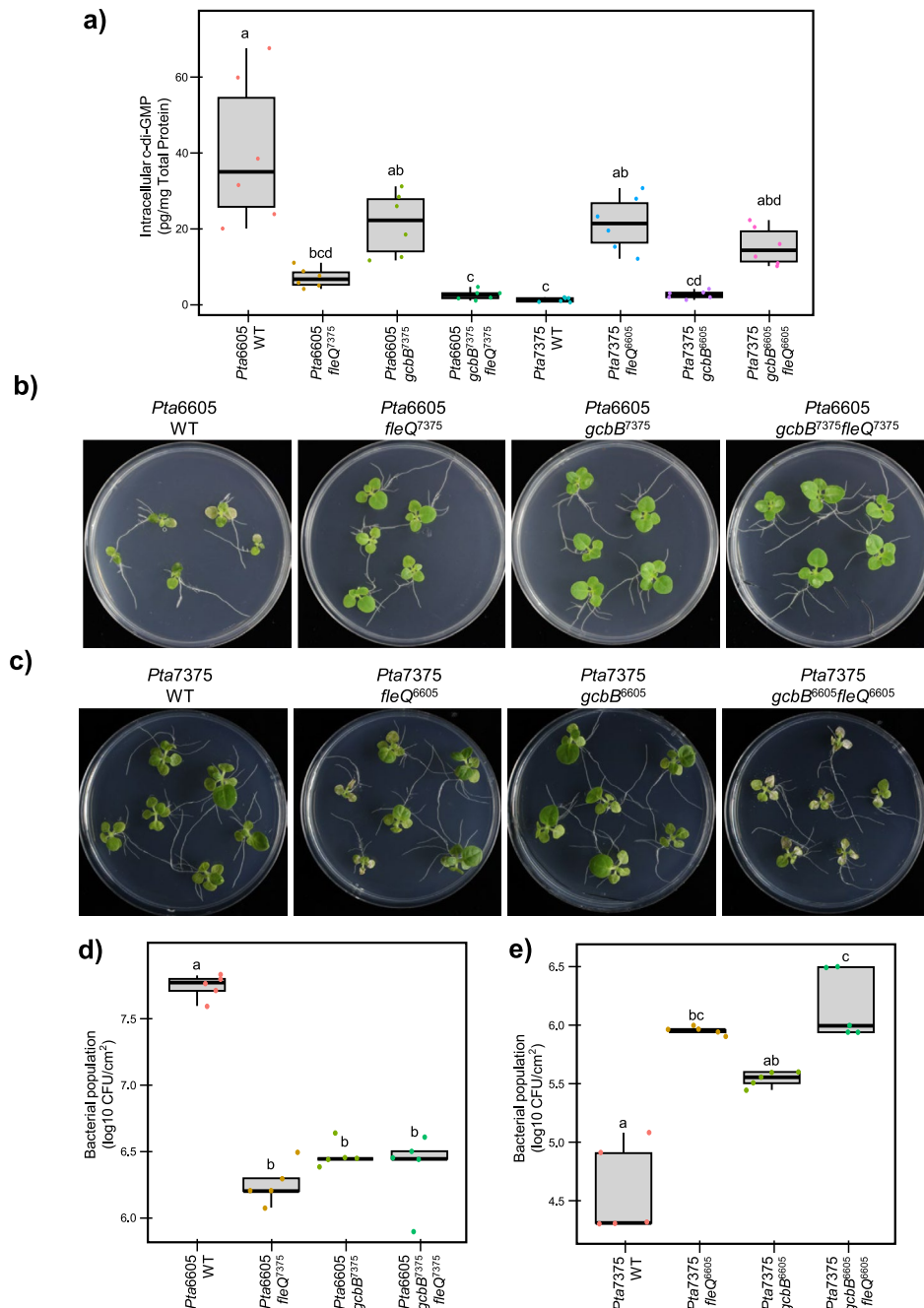


FIGURE 4 | Both FleQ and GcbB contribute to virulence in *Pseudomonas syringae* pv. *tabaci* strains. (a) Intracellular c-di-GMP levels in the swapping mutants. Different letters indicate significant differences. (b) Disease symptoms on *Nicotiana tabacum* 'Xanthi' leaves inoculated with the replacement mutants of *Pta6605* background (OD₆₀₀ = 0.002) at 4 days post-inoculation (dpi). (c) Bacterial populations in Xanthi leaves at 3 dpi using the flood assay (OD₆₀₀ = 0.002). (d) Bacterial populations in Xanthi leaves at 3 dpi using the flood assay (OD₆₀₀ = 0.08). Different letters indicate significant differences. (e) Disease symptoms on Xanthi leaves inoculated with the replacement mutants of *Pta7375* background (OD₆₀₀ = 0.08) at 4 dpi. Data were analysed using the Kruskal–Wallis test followed by Dunn's post hoc test with Benjamini–Hochberg (BH) adjustment for multiple comparisons ($\alpha = 0.05$). Different letters above the bars/points indicate statistically significant differences between groups.

the *Pta6605 gcbB*⁷³⁷⁵ and *Pta6605 gcbB*⁷³⁷⁵*fleQ*⁷³⁷⁵ strains (Figure 4b). Bacterial populations of *Pta6605 fleQ*⁷³⁷⁵, *Pta6605 gcbB*⁷³⁷⁵ and *Pta6605 gcbB*⁷³⁷⁵*fleQ*⁷³⁷⁵ were lower than that of *Pta6605* WT at 3 days post-inoculation (dpi) (Figure 4d). In contrast, none of the *Pta7375* replacement mutants showed recovery in disease symptom development at an initial concentration of 0.002 optical density value at 600 nm (OD₆₀₀) (data not shown). When the inoculum concentration was increased to OD₆₀₀ = 0.08, disease symptom development was observed for the replacement strains *Pta7375 fleQ*⁶⁶⁰⁵ and *Pta7375 gcbB*⁶⁶⁰⁵*fleQ*⁶⁶⁰⁵ compared to that for *Pta7375* WT (Figure 4c). In the *Pta7375* replacement mutants, the bacterial population at 3 dpi was higher than in *Pta7375* WT. Moreover, *Pta7375 gcbB*⁶⁶⁰⁵*fleQ*⁶⁶⁰⁵ exhibited a relatively high increase in the bacterial population (Figure 4e). Reintroduction of functional *fleQ* and *gcbB* into *Pta7375* restored motility. However, it only partially rescued virulence, suggesting the involvement of additional genes in virulence control.

3 | Discussion

In this study, we aimed to identify the determinants of differences in virulence among plant-pathogenic bacterial strains that would lead to a relatively deep understanding of the regulation of virulence in plant-pathogenic bacteria. Through comparative genomic analysis between the highly virulent strain *Pta6605* and the less virulent strain *Pta7375*, we attempted to identify the causative genes of the phenotypic differences between the *Pta6605* and *Pta7375* strains (virulence, motility, flagellin accumulation and biofilm formation). Comparative genomic analysis found point mutations in two crucial regulatory genes, *fleQ* and *gcbB*, in *Pta7375*. Deletion and replacement mutants revealed essential roles of these genes in regulating motility, biofilm formation and virulence. Our findings indicate that the genetic basis of attenuation of virulence in *Pta7375* is linked to the truncation of two pivotal genes, *fleQ* and *gcbB*.

FleQ is a well-studied transcriptional regulator, which controls the balance between motility and biofilm formation in various *Pseudomonas* species (Blanco-Romero et al. 2018; Nogales et al. 2015; Oladosu et al. 2024; Xiao et al. 2021). The *Pta6605 ΔfleQ* mutant led to a marked reduction in both swimming and swarming motility, effectively mimicking the phenotype of the less-virulent *Pta7375* strain. This observation is consistent with previous studies on *Pseudomonas aeruginosa* and *Pseudomonas putida*, where FleQ has been identified as a key regulator of flagellar biosynthesis and biofilm development (Blanco-Romero et al. 2018; Su et al. 2015). We found that deletion of *fleQ* in *Pta6605* and SNP in *fleQ* of *Pta7375* could disturb the production of flagellin protein FliC (Figures 2d, 3c, and Figure S4d). These results indicate that a SNP in the *fleQ* gene has an impact on the regulatory ability of FleQ. Thus, it is clear that FleQ serves as a regulator of flagellar motility in *Pta6605* and *Pta7375*. These findings are consistent with previous findings (Blanco-Romero et al. 2018; Matsuyama et al. 2016; Oladosu et al. 2024; Torres-Sánchez et al. 2023). To understand the molecular basis of FleQ dysfunction in *Pta7375*, we identified FleQ-binding motifs upstream of flagella-related genes (*flgA* and *fliL*) using FIMO (Find Individual Motif Occurrences) analysis.

Expression analysis confirmed that these genes, along with *fliC*, were downregulated in both *ΔfleQ* and *Pta6605 fleQ*⁷³⁷⁵ mutants (Figure S5), indicating that the SNP disrupts FleQ's transcriptional regulatory function.

Domain prediction analysis suggests that the SNP in *fleQ*⁷³⁷⁵ introduces a premature stop codon, resulting in loss of the helix-turn-helix DNA-binding domain (Figure S3a). This truncation likely prevents FleQ from activating flagella gene transcription. However, detailed functional characterisation of the truncated FleQ protein and its interaction with the c-di-GMP signalling pathway remain to be investigated.

We also identified GcbB as a factor contributing to virulence. GcbB, a homologue of *P. fluorescens* Pf0-1, functions as a diguanylate cyclase responsible for c-di-GMP synthesis, which may regulate the transition between motile and sessile bacterial lifestyles (Dahlstrom et al. 2018; Newell et al. 2011). GcbB contains CHASE, PAS and GGDEF domains, which are essential for its function in c-di-GMP synthesis (Newell et al. 2011; Valentini and Filloux 2016). In *Pta7375*, a frameshift mutation in *gcbB* resulted in the loss of the GGDEF domain, which is essential for c-di-GMP synthesis (Dahlstrom and O'Toole 2017; Jiménez-Fernández et al. 2016; Martínez-Rodríguez et al. 2023; Whiteley and Lee 2015; Xiao et al. 2016). On the other hand, analysis using the *ΔgcbB* mutant and replacement strains demonstrated that *gcbB* contributes to disease symptom development and bacterial growth during inoculation. However, under the conditions analysed in this study, no significant effects on motility, biofilm production or c-di-GMP levels were observed. Indeed, because multiple proteins containing GGDEF domains exist in the *Pta* genome, although the *gcbB* identified in this study plays some role in virulence, more detailed analysis is required to elucidate its specific function. This series of analyses suggests that the *fleQ* mutation substantially contributes to the reduced virulence of *Pta7375* by impairing motility, flagellin production, c-di-GMP accumulation and biofilm formation. In contrast, the *gcbB* mutation appears to cause only a slight reduction in virulence and does not markedly alter most of the phenotypes examined in this study.

FleQ regulates c-di-GMP signalling by controlling the expression of diguanylate cyclase (DGC) genes that modulate c-di-GMP levels. For example, in *P. putida* KT2440, *fleQ* deletion significantly reduced transcription of the highly conserved *gcbA* gene across *Pseudomonas* species (Xiao et al. 2016). In vitro experiments showed that FleQ binds to the *gcbA* promoter region, and this binding is inhibited by increased c-di-GMP (Xiao et al. 2016). Therefore, we investigated whether FleQ regulates *gcbB* gene expression in *Pta6605*. However, no change in *gcbB* gene expression was observed in the *ΔfleQ* mutant (Figure S4f), suggesting that FleQ does not directly regulate *gcbB* transcription in *Pta*. Nevertheless, c-di-GMP levels were reduced in the *ΔfleQ* mutant (Figure 2g), suggesting that FleQ may regulate other DGC genes in *Pta6605*, thereby influencing overall c-di-GMP levels.

Beyond the molecular role of FleQ, it is critical to consider why less virulent alleles (*Pta7375*) would be maintained in field populations. One important driver is human-mediated selection through roguing practices: by removing plants

showing conspicuous disease symptoms (infected by highly virulent strains), growers inadvertently impose negative selection on genotypes that cause prominent lesions. Over time, strains with reduced lesion formation gain a relative fitness advantage because they evade removal (Murray-Watson and Cunniffe 2023). Although roguing has been studied extensively in the context of viral diseases (e.g., tomato yellow leaf curl virus), the same principle applies to bacterial pathogens: plants with large bacterial lesions are more likely to be pulled, reducing the effective inoculum of aggressive strains and favouring milder or silent infections that escape detection (Murray-Watson and Cunniffe 2023).

In addition, ecological theory predicts that lower virulence can be advantageous when transmission depends on subtle, chronic colonisation rather than rapid, lesion-driven dispersal. For example, work in other bacterial systems (*Staphylococcus aureus* in *Caenorhabditis elegans*) shows that in-host competition and host defence pressures drive the evolution of reduced virulence over successive passages (Ford et al. 2016). In the case of plant and *P. syringae* interactions, downregulation of flagellar genes (controlled by *FleQ* and further modulated by *AlgU*) has been shown to reduce plant immune activation (via FLS2 recognition of flagellin), thereby conferring a fitness advantage inside the host while simultaneously reducing conspicuous symptom development (Bao et al. 2020). Consequently, strains like *Pta7375* that bear *fleQ* alleles leading to attenuated motility may be selected both by host defences and by agricultural removal practices.

In summary, this study aimed to identify factors responsible for the virulence differences between two strains and identified two genes, *fleQ* and *gcbB*, through comparative genomic analysis. This represents a case where the identification of causal genes was facilitated by favourable conditions, including similar genomic structures and a low number of SNPs between the strains. Further analysis using these causal genes and mutants is expected to lead to the elucidation of gene networks involved in virulence regulation.

4 | Experimental Procedures

4.1 | Bacterial Strains and Growth Conditions

All bacterial strains used in this study are listed in Table 2. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics, as required. *Pta6605* and *Pta7375* (Taguchi et al. 2001) were grown at 27°C in King's B (KB) medium supplemented with necessary antibiotics (Table 2). For phenotypic analyses, liquid LB medium supplemented with 10 mM MgCl₂ was used to support optimal growth conditions.

4.2 | Plant Material and Growth Conditions

Nicotiana tabacum 'Xanthi' seeds were sown in a 1:1 mixture of Sakata Super Mix (Sakata Seed Corporation) and vermiculite (Asahi Kogyo Corporation). Plants were maintained under controlled conditions at 28°C with a photoperiod of 16 h of light

(90 μmol·m⁻²·s⁻¹) and 8 h of dark to ensure uniform growth and development for subsequent inoculation assays.

4.3 | Comparative Genomic Analysis

The complete genome sequences of *Pta6605* and *Pta7375* were retrieved from the National Center for Biotechnology Information and *Pseudomonas* genome databases (Accession Number AP024464) (Matsui et al. 2021). *Pta7375* was originally isolated from tobacco leaves in Nagasaki Prefecture, Japan (Taguchi et al. 2001). Complete genome sequencing of *Pta7375* was performed using a PacBio sequencer, as previously described (Matsui et al. 2021). The *Pta7375* genome was 6,201,939 bp in length, had an average GC content of 58.0%, and consisted of one circular chromosome (6,201,939 bp). Both genomes were automatically annotated using the DFAST pipeline (Tanizawa et al. 2018). For *Pta7375*, 5643 protein coding sequences, 66 tRNAs and 16 rRNA (5S, 16S and 23S rRNA) operons were predicted.

Comparative genomic analysis was conducted using Mauve v.2.4.0 with default settings for identifying genomic rearrangements, SNPs and insertion/deletion regions (Darling et al. 2004). Visualisation and confirmation of SNPs and insertion sites were performed using SnapGene (GSL Biotech), facilitating the identification of mutations potentially responsible for phenotypic differences between the strains. We deposited the complete genome sequence in GenBank (*Pta7375* accession number AP044048).

4.4 | Generation of Mutants

To generate both deletion and replacement alleles of *fleQ* and *gcbB*, approximately 1.5 kb of genomic sequence immediately upstream and downstream of each target locus was amplified from *Pta6605* or *Pta7375* by PCR. For deletion constructs, these flanking fragments were first cloned into pHSG396 (TaKaRa) via HindIII and EcoRI sites using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs), after which the *fleQ* open reading frame was excised by inverse PCR to produce the Δ*fleQ* fragment. This fragment was then subcloned into the suicide vector pK18mobsacBN (Kashihara et al. 2022; Schäfer et al. 1994). In parallel, flanking regions for *gcbB* were assembled directly into NotI-digested pK18mobsacBN by In-Fusion Assembly (TaKaRa) to create the Δ*gcbB* deletion vector. Replacement constructs were built in two steps: (1) full-length *fleQ* locus (5549 bp, including flanks) or *gcbB* (5400 bp, including flanks) loci were amplified and cloned into pHSG396 via HindIII/EcoRI or NotI sites as appropriate, and (2) the resulting inserts were transferred into pK18mobsacBN to yield pK18mobsacBN-*fleQ*⁶⁶⁰⁵, pK18mobsacBN-*fleQ*⁷³⁷⁵, pK18mobsacBN-*gcbB*⁶⁶⁰⁵ and pK18mobsacBN-*gcbB*⁷³⁷⁵. All constructs were verified by Sanger sequencing before use.

Deletion and replacement alleles were introduced into recipient strains by conjugation with *E. coli* S17-1 carrying the appropriate pK18mobsacBN derivative. After overnight mating on King's B agar, transconjugants were selected on KB plates supplemented with 50 μg/mL nalidixic acid and 50 μg/mL

TABLE 2 | Bacterial strains and plasmids.

Bacterial strains/plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
DH5α	F ⁻ λ-ø80dLacZ ΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(r _K ⁻ m _K ⁺) supE44 thi-1 gyrA relA1	TaKaRa
S17-1	thi pro hsdR hsdR hsdM ⁺ recA(chr::RP4-2-Tc::Mu-Km::Tn7)	Schäfer et al. (1994)
<i>Pseudomonas syringae</i> pv. <i>tabaci</i> (Pta)		
Isolate 6605 (Pta6605)	Wild type isolated from tobacco, Nal ^r	Shimizu et al. (2003)
Isolate 7375 (Pta7375)	Wild type isolated from tobacco, Nal ^r	Taguchi et al. (2001)
Pta6605 Δ <i>fleQ</i>	Isolate 6605, Nal ^r , <i>fleQ</i> gene deletion	This study
Pta6605 Δ <i>gcbB</i>	Isolate 6605, Nal ^r , <i>gcbB</i> gene deletion	This study
Pta6605 Δ <i>fleQ</i> Δ <i>gcbB</i>	Isolate 6605, Nal ^r , <i>fleQ</i> and <i>gcbB</i> gene deletion	This study
Pta6605 <i>gcbB</i> ⁷³⁷⁵	Isolate 6605, Nal ^r , <i>gcbB</i> was swapped by <i>gcbB</i> locus of Pta7375	This study
Pta6605 <i>fleQ</i> ⁷³⁷⁵	Isolate 6605, Nal ^r , <i>fleQ</i> was swapped by <i>fleQ</i> locus of Pta7375	This study
Pta6605 <i>fleQ</i> ⁷³⁷⁵ <i>fleQ</i> ⁷³⁷⁵	Isolate 6605, Nal ^r , <i>gcbB</i> and <i>fleQ</i> were swapped by <i>gcbB</i> and <i>fleQ</i> locus of Pta7375	This study
Pta7375 <i>gcbB</i> ⁶⁶⁰⁵	Isolate 7375, Nal ^r , <i>gcbB</i> was swapped by <i>gcbB</i> locus of Pta 6605	This study
Pta7375 <i>fleQ</i> ⁶⁶⁰⁵	Isolate 7375, Nal ^r , <i>fleQ</i> was swapped by <i>fleQ</i> locus of Pta6605	This study
Pta7375 <i>fleQ</i> ⁶⁶⁰⁵ <i>gcbB</i> ⁶⁶⁰⁵	Isolate 7375, Nal ^r , <i>gcbB</i> and <i>fleQ</i> were swapped by <i>gcbB</i> and <i>fleQ</i> locus of Pta6605	This study
Plasmid		
pHSG396	A pUC type of cloning vector, Cm ^r	TaKaRa
pK18mobsacB	Small mobilizable vector, sucrose sensitive (<i>sacB</i>); Km ^r	Schäfer et al. (1994)
pK18mobsacBN	<i>NotI</i> site inserted between <i>PstI</i> and <i>HindIII</i> site of MCS in pK18mobsacB; Km ^r	Kashihara et al. (2022)
pK18mobsacBN-Δ <i>fleQ</i>	pK18mobsacBN construct for deletion of <i>fleQ</i>	This study
pK18mobsacBN-Δ <i>gcbB</i>	pK18mobsacBN construct for deletion of <i>gcbB</i>	This study
pK18mobsacBN- <i>fleQ</i> ⁶⁶⁰⁵	pK18mobsacBN construct for swapping of <i>fleQ</i> ⁶⁶⁰⁵	This study
pK18mobsacBN- <i>fleQ</i> ⁷³⁷⁵	pK18mobsacBN construct for swapping of <i>fleQ</i> ⁷³⁷⁵	This study
pK18mobsacBN- <i>gcbB</i> ⁶⁶⁰⁵	pK18mobsacBN construct for swapping of <i>gcbB</i> ⁶⁶⁰⁵	This study
pK18mobsacBN- <i>gcbB</i> ⁷³⁷⁵	pK18mobsacBN construct for swapping of <i>gcbB</i> ⁷³⁷⁵	This study

Abbreviations: Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Nal^r, nalidixic acid resistant.

kanamycin. To resolve single-crossover integrants and cure the vector backbone, colonies were counterselected on KB agar containing 50 μg/mL nalidixic acid and 10% (wt/vol) sucrose. Deletion mutants (Δ*fleQ*, Δ*gcbB* and the Δ*fleQ*Δ*gcbB* double mutant) were confirmed by colony PCR, while replacement strains were validated by restriction fragment length polymorphism (RFLP) analysis—using *Psp1406I* for *fleQ* swaps and *SfiI* for *gcbB* swaps (Figure S9). Double deletions or double replacements were generated by repeating the conjugation and selection procedure in a strain already carrying the first allele. A complete list of all strains, plasmids, primers and

mutants generated in this study is provided in Table 2 and Table S1.

4.5 | Phenotypic Assays

4.5.1 | Motility Assays

Swimming motility was assessed using MMMF medium (50 mM potassium phosphate [pH 5.7], 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂·6H₂O, 1.7 mM NaCl, 10 mM fructose and 10 mM

mannitol) solidified with 0.3% agar. Overnight cultures of Pta strains were centrifuged at 700g for 10 min, washed twice with 10mM MgSO₄ and adjusted to an OD₆₀₀ of 1.0. Aliquots (3μL) were spotted onto the centre of the agar plates and allowed to airdry. Plates were incubated at 27°C and photographed after 24 and 48 h to evaluate the swimming distance.

Swarming motility was measured using SWM medium [0.5% proteose peptone (Gibco Thermo Fisher Scientific), 0.3% yeast extract (BD Biosciences) and 0.45% agar (BD Biosciences)]. Preparation and incubation conditions of bacteria were identical to those used in the swimming motility assay. Swarming zones were documented after incubation at 27°C for 24 and 48 h. The area of swimming or swarming motility was quantified using Fiji (ImageJ, <https://fiji.sc/>). First, the image scale was calibrated using a ruler included in the motility assay image. The motility zone was then manually outlined using the Freehand Selection Tool to accurately trace irregularly shaped areas. The selected area was measured using the Analyse > Measure function to obtain the total area in mm².

4.5.2 | Biofilm Formation Assays

Biofilm formation was visualised using crystal violet staining (Shao et al. 2019). Overnight cultures of Pta strains were centrifuged at 700 g for 10 min, washed, and adjusted to an OD₆₀₀ of 0.1 in fresh KB medium containing 50 μg/mL Nal. Each bacterial strain was cultured in test tubes at 23°C without shaking. After incubation, unattached cells were removed by washing with sterile water. Biofilms were stained with 0.1% crystal violet for 30 min, thoroughly washed with sterile water, and airdried. Quantitative analysis of biofilm accumulation is described in the Appendix S1.

4.5.3 | Measurement of Intracellular c-di-GMP Levels

Intracellular c-di-GMP levels were measured using a c-di-GMP ELISA Kit (Cayman Chemical), as previously described (Liu et al. 2022). Pta strains were harvested after 24 h of growth by centrifugation at 13,000g for 2 min at 4°C, and washed twice with ice-cold phosphate-buffered saline (PBS). Cells were lysed using a B-PER Bacterial Protein Extraction Reagent (Thermo Fisher Scientific), incubated at room temperature for 10 min, and centrifuged at 13,000g for 5 min. Total protein concentration was determined using a Quick Start Bradford assay (Bio-Rad Laboratories). Intracellular c-di-GMP concentrations were normalised to the total protein content and expressed as picomoles per milligram of protein.

4.5.4 | Flagellin Detection by Western Blotting

Overnight cultures of Pta strains were adjusted to an OD₆₀₀ of 0.6 with PBS-T (PBS with 0.05% Tween 20) (TaKaRa). Aliquots (1 mL) were centrifuged at 800 g for 10 min, and cell pellets were resuspended in 60μL PBS-T. The samples were mixed with 4×sodium dodecyl sulphate (SDS) loading buffer and heated at 70°C for 20 min to denature proteins. Proteins were separated by electrophoresis on 15% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Membranes

were blocked and probed with rabbit anti-flagellin primary antibody (Shimizu et al. 2003) diluted 1:1000 in Can Get Signal Immunoreaction Solution 1 (Toyobo). Goat antirabbit IgG (H+L)-alkaline phosphatase conjugated (Bio-Rad Laboratories), diluted 1:10,000 in Can Get Signal Immunoreaction Solution 2, was used as the secondary antibody. Immunoreactive protein bands were detected using CDP-Star detection reagents (Cytiva) and signals were visualised using a ChemiDoc Touch imaging system (Bio-Rad Laboratories).

4.6 | Plant Inoculation Assays

4.6.1 | Flood Assay

The flood assay technique was adapted from the previous method (Ishiga et al. 2017). Overnight cultures of Pta strains were centrifuged at 700 g for 10 min and adjusted to an OD₆₀₀ of 0.002 or 0.08, with sterile distilled water containing 0.025% Silwet L-77 to facilitate bacterial adhesion. Tobacco plants grown on Murashige and Skoog medium were submerged in 30 mL of the inoculum to ensure complete leaf coverage. Excess inoculum was removed, and plants were airdried for 15 min. Plates were sealed with surgical tape and incubated at 22°C. Disease symptoms were observed and photographed at 4 dpi.

4.6.2 | Measurement of In Planta Bacterial Population

At 3 dpi, leaves from each treatment group were harvested and surface-sterilised by immersion in 70% ethanol. Using a sterile 4-mm-diameter cork borer, two circular discs were excised from each leaf and transferred into a sterile tube. The discs were thoroughly homogenised using a sterile pestle. Sterile water (1 mL) was added to each tube, and the homogenate was vortexed to ensure a uniform suspension. Serial 10-fold dilutions (10⁻¹–10⁻⁵) were prepared, and 5 μL of each dilution was spotted onto KB agar plates supplemented with 50 μg/mL Nal. After a 2-day incubation period at 27°C, colonies were counted, and the bacterial population was determined based on the dilution factor.

4.7 | Statistical Analysis

The data of motility, biofilm formation, c-di-GMP levels and plant inoculation assays data were analysed using the Kruskal–Wallis test followed by Dunn's post hoc test with Benjamini–Hochberg (BH) adjustment for multiple comparisons (α=0.05). All analyses were performed using R software (v. 4.4.1; <https://www.r-project.org/>) and RStudio (v. 2024.04.02+764; <https://posit.co/download/rstudio-desktop/>).

Author Contributions

M.T.H.: investigation, Writing – review and editing, Writing – original draft, Data curation, Validation, Visualization, Formal analysis. K.Y.: investigation. T.N.: investigation. S.A.: investigation, Data curation, Validation. S.M.: investigation, Validation, Data curation. K.S.: funding

acquisition, Software. N.S.: investigation, Supervision. M.Y.: supervision, Methodology, Investigation. Y.N.: supervision, Methodology. K.T.: supervision, Methodology. Y.L.: supervision, Resources, Funding acquisition, Writing – review and editing, Investigation, Methodology. H.M.: supervision, Resources, Writing – review and editing, Writing – original draft, Funding acquisition, Formal analysis, Project administration, Visualization, Validation, Methodology, Conceptualization, Investigation, Data curation, Software.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data have been deposited with links to BioProject accession number PRJDB1119 and PRJDB18907, and biosample accession number SAMD00892025 in the DDBJ BioProject database.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** Materials and Methods. **Figure S1:** In vitro growth assay of *Pta6605* and *Pta7375*. Each strain was grown in KB liquid medium containing nalidixic acid at 27°C with shaking. Initial concentration was adjusted to $OD_{595} = 0.1$. $N = 10$. **Figure S2:** Phylogenetic tree of GcbB proteins from various *Pseudomonas* strains based on their amino acid sequences. Amino-acid sequences of GcbB orthologs were retrieved from the Pseudomonas Ortholog Database (<https://pseudoluge.pseudomonas.com>). The phylogenetic tree was constructed using the neighbour-joining method. The *Pta* GcbB sequence is highlighted in blue, and the *P. fluorescens* Pf0-1 GcbB sequence in yellow. Numbers at the branch nodes represent bootstrap values. Text in parentheses indicates the locus tag. **Figure S3:** Mutations in *Pta7375* compared to *Pta6605*. (a) Mutation in *fleQ*. Unlike *Pta6605*, *Pta7375* has a deletion mutation of T. This mutation leads to a frameshift, generating a premature stop codon. This mutation results in the synthesis of truncated FleQ in *Pta7375*, which, based on domain prediction (<https://smart.embl.de/>), loses the helix-turn-helix (HTH) domain, an important DNA-binding domain in FleQ. (b) Mutation in *gcbB*. *Pta7375* has a substitution mutation of C to A, which leads to a premature stop codon. This mutation results in the synthesis of truncated GcbB in *Pta7375*, which, based on domain prediction, loses the GGDEF domain, an important domain for diguanylate cyclase activity required for synthesising cyclic diguanylate (c-di-GMP). **Figure S4:** Construction of the *Pta6605* Δ *fleQ* mutant and characterisation of flagellin protein accumulation. (a) Schematic representation of the generation of the *Pta6605* Δ *fleQ* mutant. The *fleQ* ORF was removed from the *Pta6605* genome. (b) Screening of *Pta6605* Δ *fleQ* mutant by colony PCR. Primer set HMP2365 and HMP2422 amplified a 1830 bp product from *Pta6605* WT and a 360 bp product from *Pta6605* Δ *fleQ* mutant. (c) RT-qPCR analysis of *fleQ* gene expression in *Pta6605* WT and *Pta6605* Δ *fleQ* mutants. (d) Detection of flagellin in the *Pta6605* Δ *fleQ* mutants by Western Blot. Ponceau staining indicates the loading control. Arrowhead indicates flagellin, and asterisk indicates non-specific band. (e) RT-qPCR analysis of *fleQ* gene expression in Δ *fleQ* and Δ *gcbB* mutants. (f) RT-qPCR analysis of *gcbB* gene expression in Δ *fleQ* and Δ *gcbB* mutants. **Figure S5:** Flagella related gene expression of *Pta* strains. (a) Sequence logo of FleQ binding site motif in *Pta*. (b) Representative flagella-related proteins and FleQ-binding motifs in their promoter sequences. Yellow highlights indicate FleQ-binding motifs, and blue highlights indicate translation start sites. (c)–(e) RT-qPCR analysis results of the *flagella-related* genes in replacement mutants are shown. Error bars indicate standard deviation (SD). **Figure S6:** Flagellar visualisation of *Pta* strains. Bacterial cultures grown on swimming medium for 72 h were stained for flagella using the Ryu protocol. Bacterial cells were imaged using a 100 $\times$ oil-immersion objective (Evident UPLFLN100XO2PH) on an Olympus IX70 light microscope. White arrowhead indicates flagella. Scale bar = 5 μ m. **Figure S7:** Biofilm accumulation at each time point among *Pta* mutants and replacement strains. Quantitative analysis of biofilm formation was performed at three time points (8, 20 and 30 h). (a) Deletion mutants, (b) *Pta6605* replacement mutants and (c) *Pta7375* replacement mutants. Bacterial cultures were grown in King's B (KB) medium supplemented with nalidixic acid at 27°C without agitation. Biofilm formation was assessed using 0.1% crystal violet staining. The bound crystal violet was subsequently dissolved in 30% (v/v) acetic acid, and the biofilm biomass was quantified by measuring the optical density at 595 nm (OD_{595}). Values shown in the graphs represent the mean OD_{595} of biological replicates for each strain at each time point. **Figure S8:** Disease symptom development in *Pta* Δ *gcbB*, Δ *fleQ* and Δ *fleQ* Δ *gcbB* mutants by infiltration assay. Each bacterial strain was adjusted to $OD_{600} = 0.002$ and inoculated by using needleless syringe, and disease symptom development was observed. Photographs show disease symptom development at 5 dpi. The experiment was performed three times with three independent plants inoculated for each strain. **Figure S9:** RFLP confirmation of replacement mutants of *fleQ* and *gcbB* (a) Schematic of the *fleQ* RFLP. The *fleQ* ORFs from *Pta6605* and *Pta7375* were amplified by PCR (primers HMP2446/HMP2447), generating a 999 bp fragment. *Psp1406I* cleaves the *Pta6605*-derived fragment into 607 bp and 392 bp bands. The *Psp1406I* recognition site is shown. (b) Electrophoresis of *fleQ* PCR products before (U) and after (D) *Psp1406I* digestion. 'U' denotes undigested PCR product; 'D,'

*Psp*1406I-digested. Lane numbers correspond to individual strains. (c) Schematic of the *gcbB* RFLP. The *gcbB* ORFs from *Pta*6605 and *Pta*7375 were amplified by PCR (primers HMP2530/HMP2531), generating a 715bp fragment. *SfcI* cleaves the *Pta*7375 derived fragment into 401bp and 314bp bands. The *SfcI* recognition site is shown. (d) Electrophoresis of *gcbB* PCR products. 'Undigested' indicated not digested PCR fragment as a control. Each lane shows *SfcI* digested PCR products. The *Pta*7375 *gcbB* serves as a positive control. Filled circles indicate the expected RFLP pattern. **Figure S10:** mpp70168-sup-0011-FigureS10.pdf. *fleQ* and *gcbB* gene expression of replacement mutants (a) Relative Expression of *fleQ* and (b) *gcbB* genes in replacement mutants was confirmed by RT-qPCR. *Pta*6605 Δ *fleQ* Δ *gcbB* mutants were used as negative controls for RT-PCR. *pcaG* gene was used as reference gene to normalise the relative gene expression. Statistical analysis was using the Kruskal–Wallis test followed by Dunn's post hoc test with Benjamini-Hochberg (BH) adjustment for multiple comparisons ($\alpha=0.05$). Different letters above the bars indicate statistically significant differences between groups. **Table S1:** Primer used in this study.