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RpoN1 (sigma factor 54) contributes to the virulence of *Paracidovorax citrulli* by regulating the expression of type IV pili PilA

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Abstract

The σ^{54} factor (RpoN), a significant transcriptional regulatory factor, plays crucial roles in regulating virulence, motility, biofilm formation, and the utilization of carbon and nitrogen sources in pathogenic bacteria. However, the function of RpoN has not been identified in *Paracidovorax citrulli* (formerly *Acidovorax citrulli*). To investigate this, we constructed a *rpoN1* deletion mutant and a corresponding complement strain in the background of *P. citrulli* strain xjl12. The *P. citrulli rpoN1* deletion mutant displayed attenuated virulence in melon. RNA-Seq analysis revealed that *rpoN1* is involved in regulating the expression of certain pathogenicity-associated genes related to the secretion system, biofilm formation, and motility. Phenotypic analysis demonstrated that the *rpoN1* deletion mutant of *P. citrulli* significantly attenuated biofilm formation, twitch motility, swarming motility, cotyledon colonization, and seed colonization. However, swimming motility was significantly enhanced in the *rpoN1* mutant. As expected, qRT-PCR assays indicated that the type IV pili-related gene *Aave_4679* (*pilA*) was barely expressed in the *rpoN1* mutant, and western blot analysis revealed that RpoN1 positively regulated the expression of *pilA*. Additionally, bacterial one-hybrid assays and electrophoretic mobility shift assays indicated that RpoN1 directly binds to the promoter of *pilA*. Our investigation revealed that RpoN1 is essential for the virulence of *P. citrulli* and provides valuable insights into the physiology and pathogenic mechanisms of bacterial fruit blotch.

Keywords *Paracidovorax citrulli*, RpoN, Virulence, *pilA*, Motility

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Background

Bacterial fruit blotch (BFB) is a seed-borne disease caused by *Paracidovorax citrulli* (formerly *Acidovorax citrulli*) (Schaad et al. 1978) and poses a significant threat to cucurbit production, primarily affecting watermelons and melons, on a global scale (Willems et al. 1992). In recent years, research on the pathogenic molecular mechanisms of *P. citrulli* has primarily focused on the Type III secretion system (T3SS) (Jiwénez-Guerrero et al. 2020; Zhang et al. 2020; Ji et al. 2022), Type VI secretion system (T6SS) (Tian et al. 2015b; Fei et al. 2022), polar flagella (Bahar et al. 2011), and quorum sensing (Wang et al. 2016). Additionally, the Type IV pilus (T4P) is known to contribute to the pathogenicity of *P. citrulli* (Bahar et al. 2009; Yang et al. 2023). T4P is widely

present on the surface of bacteria and can be dynamically retracted. This dynamic activity is essential for various bacterial functions, including cell adhesion, biofilm formation, twitching motility, genetic material uptake, and virulence (Craig et al. 2004; Nudleman and Kaiser 2004).

RpoN, also known as σ^{54}, is a subunit of RNA polymerase that meticulously controls gene expression by recognizing specific promoter elements. RpoN plays a crucial role in regulating carbon and nitrogen metabolism and is involved in various bacterial functions, including flagellar synthesis, T4P, bacterial growth, motility, biofilm formation, T3SS, and virulence regulation (Hutcheson et al. 2001; Tian et al. 2015a; Li et al. 2020; Yu et al. 2020). However, the impact of RpoN on *P. citrulli* remains largely unknown.

To characterize the function of RpoN in *P. citrulli*, we identified *Aave_0419* (*rpoN1*) and *Aave_1899* (*rpoN2*) from the *P. citrulli* AAC00-1 genome (GenBank accession number NC_008752). We constructed deletion mutants of *rpoN1* and *rpoN2* and investigated the transcript levels of both using RNA-seq. Interestingly, we found that the deletion of *rpoN1* severely downregulated the expression of *pilA* (*Aave_4679*), which was not found in *ΔrpoN2* (Additional file 1). In this study, we focused on examining the regulatory mechanism of RpoN1 on the virulence of *P. citrulli*. Phenotypic experiments, RNA-seq, qRT-PCR, and western blot analyses revealed that *rpoN1* positively regulated the transcription of *pilA*. Additionally, bacterial one-hybrid and electrophoretic mobility shift assays (EMSA) demonstrated that RpoN1 directly regulates the expression of *pilA*, influencing biofilm formation, twitching motility, swarming motility, and virulence in *P. citrulli*.

Results

The presence of two σ^{54} factors in *Paracidovorax citrulli*

Two RpoN-encoding genes, *Aave_0419* (*rpoN1*) and *Aave_1899* (*rpoN2*), were identified in the genome of *P. citrulli* AAC00-1 through comparison of their amino acid sequences with those of other pathogenic bacteria. The full length of *rpoN1* is 1590 bp, encoding 529 amino acids, while the full length of *rpoN2* is 1407 bp, encoding 468 amino acids. A BLASTP sequence homology analysis was conducted using NCBI BLAST. Multiple sequence alignment revealed that the amino acid sequences of RpoN1 and RpoN2 were highly conserved in *Escherichia coli*, *Pseudomonas ogarae*, *Xanthomonas oryzae* pv. *oryzae*, and *Pseudomonas syringae*. Compared with the above bacteria, the amino acid sequence similarities of RpoN1 (RpoN2) were 41.54% (38.01%), 41.46% (36.99%), 36.86% (33.97%), and 40.21% (38.01%), respectively. (Additional file 2: Figure S1).

RNA-Seq analysis reveals RpoN1's role in regulating secretion systems, biofilm formation, and motility

In this study, we examined the transcriptional regulation of RpoN1 in *P. citrulli* using RNA-Seq. Compared to the wild-type strain, *ΔrpoN1* mutant exhibited 140 differentially expressed genes (DEGs), with 61 genes upregulated and 79 genes downregulated (Additional file 2: Figure S2). These DEGs are associated with motility, the secretion system, and biofilm formation in *P. citrulli* (Additional file 3: Table S1). The RNA-Seq results were validated through qRT-PCR using eight selected DEGs (Fig. 1). A comprehensive list of all DEGs is available in Additional file 1.

Among the DEGs listed in Additional file 3: Table S1, the expression of several genes associated with T6SS increased, including the gene *Aave_1465*, which encodes the type VI secretion system tube protein Hcp and negatively regulates biofilm formation in *P. citrulli* (Fei et al. 2022). Additionally, the expression of genes related to Type II secretion system (T2SS) and chemotaxis decreased in *ΔrpoN1*. Moreover, the expression levels of genes involved in the movement, such as *pilA*—a key gene for the formation of type IV pili (Yang et al. 2023)—and various genes related to flagellar assembly, also declined (Additional file 3: Table S1). Notably, among all downregulated genes, the expression of the type IV pili-related gene *pilA* was the most significantly reduced, with a log2-fold change value of −5.613 ($P < 0.05$) (Additional

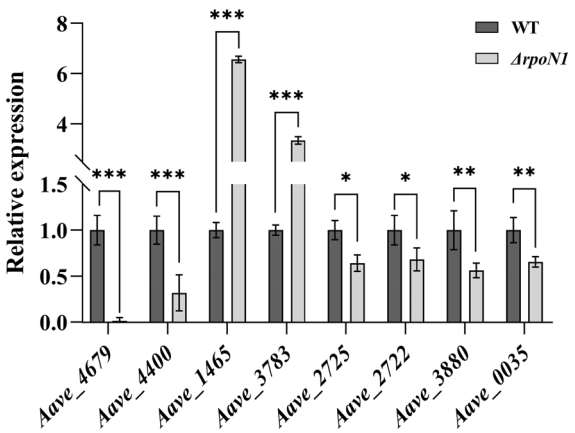

Fig. 1 Expression levels of selected genes between wild-type xjl12 and *ΔrpoN1*. To verify the accuracy of the RNA-Seq data, eight genes related to motility, T6SS, T2SS, and chemotaxis were selected from the list of differentially expressed genes (DEGs). These genes are identified as *Aave_4679*, *Aave_4400*, *Aave_1465*, *Aave_3783*, *Aave_2725*, *Aave_2722*, *Aave_3880*, and *Aave_0035*. The expression levels of these genes in the mutant strain *ΔrpoN1* and the wild-type strain xjl12 were monitored using qRT-PCR. The values represent the average of three independent experiments. Asterisks indicate significant differences between the samples (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$)

file 3: Table S1). These findings underscore the role of *rpoN1* in regulating the transcription of *pilA*.

Gene Ontology (GO) analysis indicated that the DEGs primarily engaged in biological functions such as bacterial pilus and flagellar assembly, endopeptidase activity, and tryptophan catabolism (Additional file 2: Figure S3). KEGG enrichment analysis identified pathways significantly enriched with DEGs, including flagellar assembly, biofilm formation, bacterial chemotaxis, and the bacterial secretion system (Additional file 2: Figure S4). Given these observations, RpoN1 appears to regulate various pathogenic factors in *P. citrulli*, particularly the regulation of T4P.

ΔrpoN1 and *ΔpilA* reduce biofilm formation in *Paracidovorax citrulli*

Previous studies have demonstrated that biofilms contribute to the virulence of *P. citrulli* and that *pilA* is essential for biofilm formation in this species (Yang et al. 2023). In this study, we assessed the effects of *rpoN1* and *pilA* on biofilm formation in LB medium. As illustrated in Fig. 2, compared to the wild-type strains, the biofilms of *ΔrpoN1* and *ΔpilA* completely disappeared but were restored in the complemented strains *ΔrpoN1*-C and *ΔpilA*-C. These findings indicate that *rpoN1* and *pilA* positively regulate the biofilm formation of *P. citrulli.*

rpoN1 and *pilA* regulate the motility of *Paracidovorax citrulli*

Previous studies have confirmed that motility is crucial to the virulence of *P. citrulli* and that *pilA* is essential for its twitching motility (Bahar et al. 2009, 2011; Yang et al. 2023). To investigate the role of RpoN1 in regulating twitching motility in *P. citrulli*, we measured the transparent halos surrounding the *ΔrpoN1* and *ΔpilA* colonies on NA plates. As shown in Fig. 3a, significantly reduced twitching motility was observed in the *ΔrpoN1* and *ΔpilA* strains compared with the wild-type and complemented strains *ΔrpoN1*-C and *ΔpilA*-C. Moreover, transmission electron microscopy (TEM) revealed that neither *ΔrpoN1* nor *ΔpilA* could produce pili (Fig. 3b). Furthermore, T4P can mediate the swarming motility of bacteria. In *Pseudomonas aeruginosa*, the *pilA*-deficient mutant strain exhibited enhanced swarming motility compared to the wild-type strain (Shrout et al. 2006). In contrast, the swarming motility of the *ΔrpoN1* and *ΔpilA* strains was dramatically reduced compared with that of the wild-type and complemented strains *ΔrpoN1*-C and *ΔpilA*-C. Notably, these motility phenotypes of *ΔrpoN1* and *ΔpilA* were highly similar (Fig. 3c; Additional file 2: Figure S5). These results indicate that *rpoN1* and *pilA* positively regulate both twitching and swarming motility in *P. citrulli*.

Interestingly, the swimming motility of the *ΔrpoN1* strain was greater than that of the wild-type strain (Fig. 3d; Additional file 2: Figure S6). These results suggest that *rpoN1* negatively regulates the swimming motility of *P. citrulli*. However, qRT–PCR results indicated that the expression levels of the flagella-related genes *flhA*, *flgM*, and *fliC* were significantly decreased in *ΔrpoN1*, whereas *fliE*, *fliJ*, and *fliK* were significantly increased (Fig. 3e). Therefore, the regulatory role of *rpoN1* on flagella warrants further investigation.

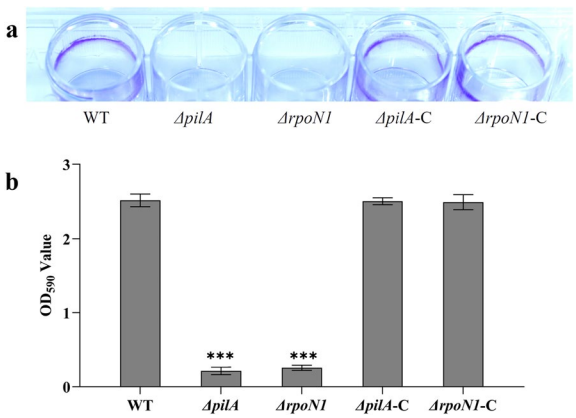

Fig. 2 *rpoN1* is essential for biofilm formation in *P. citrulli*. **a** Biofilm formation in wild-type (WT), *ΔrpoN1*, *ΔpilA*, and the complemented strains *ΔrpoN1*-C and *ΔpilA*-C. **b** Biofilm formation was visualized using crystal violet staining and quantified by measuring absorbance at 590 nm after ethanol suspension. The values represent the averages of three independent experiments, and *** indicates a highly significant difference between wild type and the tested samples ($P < 0.001$)

rpoN1 and *pilA* contribute to *Paracidovorax citrulli* virulence

To further investigate the regulatory mechanism of *rpoN1* on the virulence of *P. citrulli* in melon, we conducted cotyledon injection and spray inoculation trials using the wild-type strain, *ΔrpoN1*, *ΔpilA*, and their complemented strains *ΔrpoN1*-C and *ΔpilA*-C on melon seedlings. The results from the cotyledon injection indicated that by day 5, melon leaves inoculated with *ΔrpoN1* and *ΔpilA* exhibited only slight disease symptoms, which were significantly less severe compared to the wild-type strain (Fig. 4a). The disease indices (DIs) for the cotyledons of melon seedlings inoculated with wild-type xjl12, *ΔrpoN1*, *ΔpilA*, *ΔrpoN1*-C, and *ΔpilA*-C were 0.893, 0.301, 0.329, 0.876, and 0.872, respectively (Fig. 4b). Notably, the DIs of *ΔrpoN1* and *ΔpilA* were significantly lower than those of the wild-type strain, *ΔrpoN1*-C, and *ΔpilA*-C.

After seven days of spray inoculation, the disease incidence of true leaves in plants inoculated with

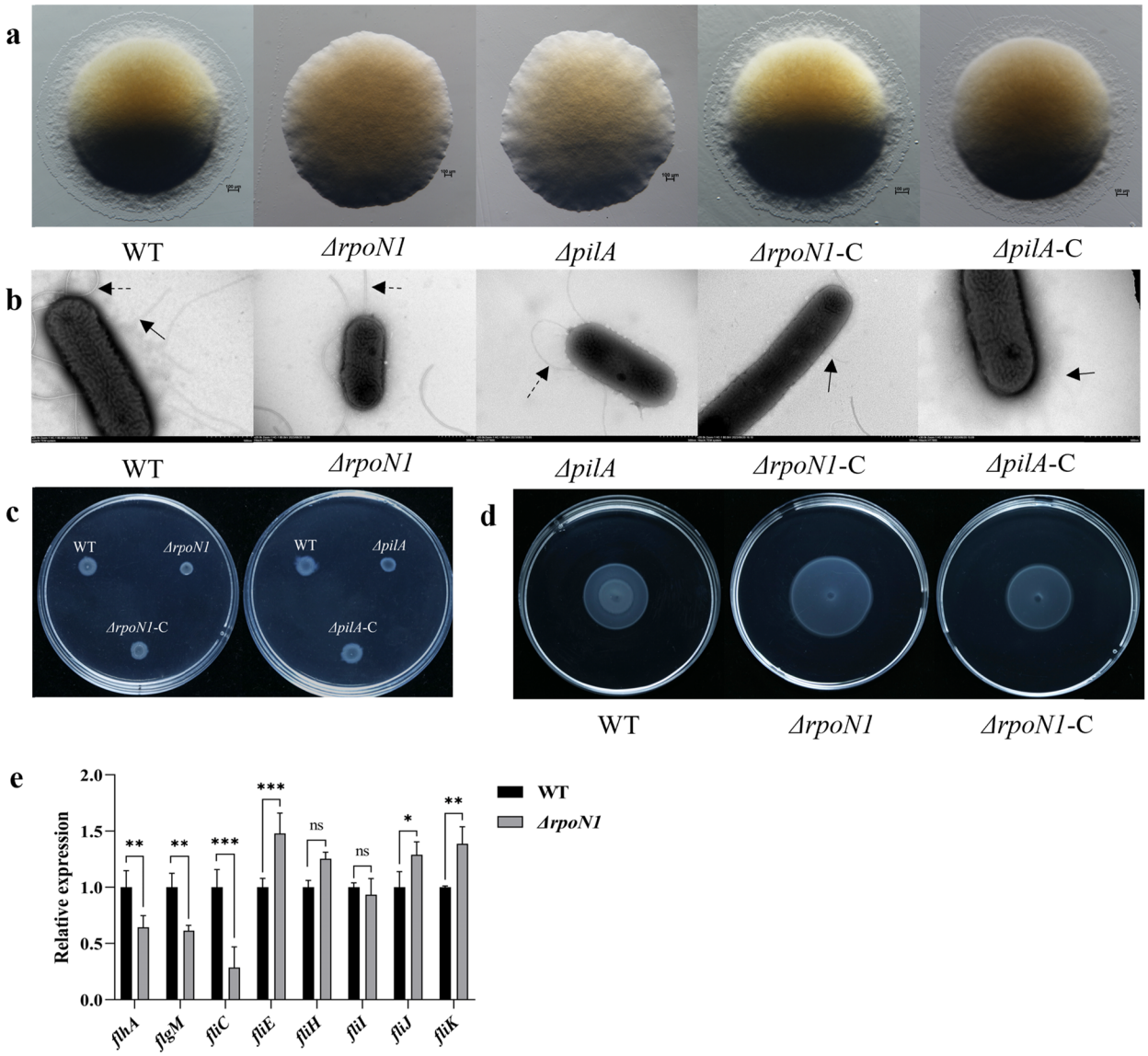

Fig. 3 The role of *rpoN1* in bacterial motility of *P. citrulli*. **a** Twitching motility of the wild type (WT), *ΔrpoN1*, *ΔpilA*, and complemented strains *ΔrpoN1*-C and *ΔpilA*-C of *P. citrulli*. The strains were spread on NA plates containing 1.0% agar. After 72 h, the colonies were photographed and observed using a stereoscope. **b** Transmission electron microscopy verified the presence of pili. Pili were observed in the wild type (WT) strain and the complemented strains *ΔrpoN1*-C and *ΔpilA*-C, while no pili were observed in the *ΔrpoN1* and *ΔpilA* strains. Solid arrows represent type IV pili, and dashed arrows represent the polar flagellum. Bars = 500 nm. **c** Swarming motility of the WT, *ΔrpoN1*, *ΔpilA*, and complemented strains *ΔrpoN1*-C and *ΔpilA*-C of *P. citrulli*. The strains were added dropwise to 0.6% (w/v) semisolid medium plates and incubated at 28°C for 48 h. **d** Swimming motility of the WT, *ΔrpoN1*, and complemented strains *ΔrpoN1*-C of *P. citrulli*. The strains were added dropwise to 0.3% (w/v) semisolid medium plates at 28°C for 48 h. **e** The expression levels of the flagella-related genes *flhA*, *flgM*, *fliC*, *fliE*, *fliH*, *fliI*, *fliJ*, and *fliK* in the wild-type and *ΔrpoN1* strains were measured by qRT-PCR. The values represent the average of three independent experiments. Asterisks indicate significant differences between the samples (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$)

ΔrpoN1 and *ΔpilA* was significantly lower compared to those inoculated with the wild-type xjl12 (Fig. 4c). The DIs for plants inoculated with the wild-type, *ΔrpoN1*, *ΔpilA*, *ΔrpoN1*-C, and *ΔpilA*-C strains were 0.421, 0.144, 0.121, 0.395, and 0.428, respectively (Fig. 4d).

In the seed-to-seedling transmission assays, seedlings from seeds inoculated with *ΔrpoN1* and *ΔpilA* exhibited weak symptoms and significantly lower mortality than those inoculated with the wild-type xjl12 and the complemented strains *ΔrpoN1*-C and *ΔpilA*-C (Fig. 4e). The

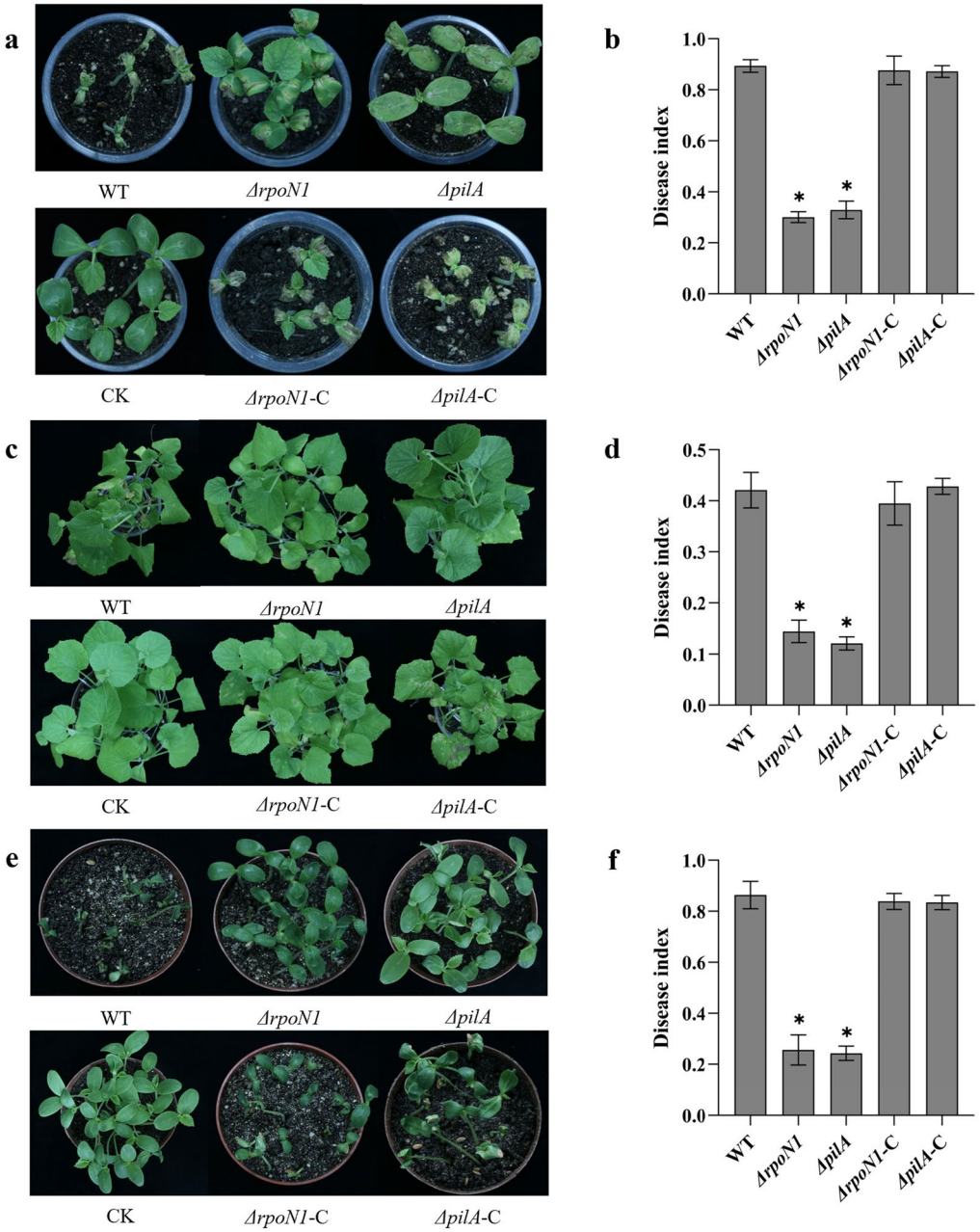

Fig. 4 The role of *rpoN1* in the virulence of *P. citrulli*. **a** Cotyledon injection inoculation assay. The cotyledons of melon seedlings were inoculated with a *P. citrulli* suspension (~ 1.0×10^3 CFU/mL). Double distilled water (ddH_2O) served as the control group (CK). Bacterial fruit blotch (BFB) symptoms of the seedlings were observed 5 days post-inoculation (dpi). **b** Disease index of cotyledon injection. **c** Spray inoculation assay. *P. citrulli* suspension (~1×10^8 CFU/mL) was sprayed onto the true leaves of melon plants, with ddH_2O as the CK. BFB symptoms were evaluated 7 days post-inoculation (dpi). **d** Disease index of plants subjected to true leaf spray inoculation. **e** Seed-to-seedling transmission assay. The effect of *rpoN1* on the seed-to-seedling transmission of *P. citrulli* in melons. Muskmelon seeds (n = 20) were soaked in a (~ 1.0×10^6 CFU/mL) bacterial suspension. BFB symptoms were observed 7 days after planting. **f** Seedling disease index of the seed-to-seedling transmission assay. The values in **b**, **d**, and **f** represent the average of three independent experiments. *** indicates a highly significant difference between wild type and the tested samples ($P < 0.001$)

DIs of seedlings inoculated with the wild-type, *ΔrpoN1*, *ΔpilA*, *ΔrpoN1*-C, and *ΔpilA*-C strains were 0.864, 0.256, 0.243, 0.838, and 0.831, respectively (Fig. 4f). These results indicate that *rpoN1* and *pilA* positively regulate the virulence of *P. citrulli*.

Deletion of *rpoN1* in *Paracidovorax citrulli* decreased bacterial growth in melon

In this study, the colonization ability of *P. citrulli* was evaluated through cotyledon and seed colonization. The colonization capacity of various strains was assessed by measuring the population of *P. citrulli* at 2, 24, 48, 72, and 96 h post-cotyledon inoculation. At 48 h post-inoculation, the populations of the *ΔrpoN1* and wild-type strains in melon cotyledons were 5.754×10^3 CFU/cm^2 and 2.75×10^4 CFU/cm^2, respectively. At 96 h post-inoculation, the population numbers of the *ΔrpoN1* and wild-type xjl12 strains were 5.047×10^6 CFU/cm^2 and 2.884×10^7 CFU/cm^2, respectively (Fig. 5a).

In assessing seed colonization ability, the population of *P. citrulli* in melon seeds was dynamically monitored over a period of 4 days. After 48 h of inoculation, the colony numbers of the *ΔrpoN1* and wild-type xjl12 strains were 4.753×10^4 CFU/g and 1.148×10^7 CFU/g, respectively. At 96 h post-inoculation, the colony numbers were 2.328×10^5 CFU/g and 1.936×10^8 CFU/g for the *ΔrpoN1* and wild-type xjl12 strains, respectively (Fig. 5b). These results indicate that the deletion of *rpoN1* significantly weakens the colonization ability of *P. citrulli* in melon seeds.

rpoN1 directly regulates the transcription of *pilA*

The results of RNA-seq and phenotypic assays established a foundation for the transcriptional regulatory effect of *rpoN1* on *pilA*. To further investigate the relationship between *rpoN1* and *pilA* in *P. citrulli*, we conducted qRT-PCR, western blot, bacterial one-hybrid, and electrophoretic mobility shift assays. The qRT-PCR results demonstrated that *pilA* expression in the *ΔrpoN1* strain was significantly down-regulated compared to the wild-type strain (Fig. 6a). Additionally, western blot analysis revealed that the deletion of *rpoN1* led to decreased PilA expression in *P. citrulli* (Fig. 6b). Furthermore, the bacterial one-hybrid assay confirmed the interaction between RpoN1 and the promoter of *pilA* (Wang et al. 2018). As shown in Fig. 6c, the cotransformed strains pBXcmT-*pilA* and pTRG-RpoN1 successfully grew on the screening media, whereas the negative control strains did not. Moreover, we validated the binding between RpoN1 and the *pilA* promoter through EMSA. As the concentration of RpoN1 increased, the amount of bound RpoN1 also increased (Fig. 6d). These findings suggest that RpoN1 interacts with the *pilA* promoter and directly regulates *pilA* transcription in *P. citrulli*.

Discussion

RpoN is a transcriptional regulator crucial for various biological functions in bacteria. In *Pseudomonas aeruginosa*, RpoN influences the expression of virulence factor, biofilm formation, and nitrogen source utilization (Hobbs et al. 1993; Cai et al. 2015). In *Vibrio cholerae*, RpoN controls the expression of genes related to motility and chemotaxis (Dong and Mekalanos 2012). However, its role in *Paracidovorax citrulli* (formerly *Acidovorax citrulli*) remains unclear. Our previous studies identified two genes encoding RpoN in *P. citrulli*, *Aave_0419* (*rpoN1*) and *Aave_1899* (*rpoN2*). In this study, we investigate the virulence regulatory mechanism of *rpoN1* in *P. citrulli*.

We analyzed the transcriptional level of *ΔrpoN1* using RNA-seq. The results revealed that *rpoN1* regulates the motility, the secretion system, and biofilm formation in *P. citrulli*. Notably, *pilA*, a major gene of T4Ps, was significantly down-regulated. Previous studies have shown that *pilA* is essential for biofilm formation and the twitching motility of *P. citrulli* (Bahar et al. 2009; Yang et al. 2023). In this study, *P. citrulli* was unable to form biofilms after the deletion of *rpoN1* or *pilA*, and its twitching motility was also weakened. Additionally, T4Ps are involved in mediating the swarming motility of pathogenic

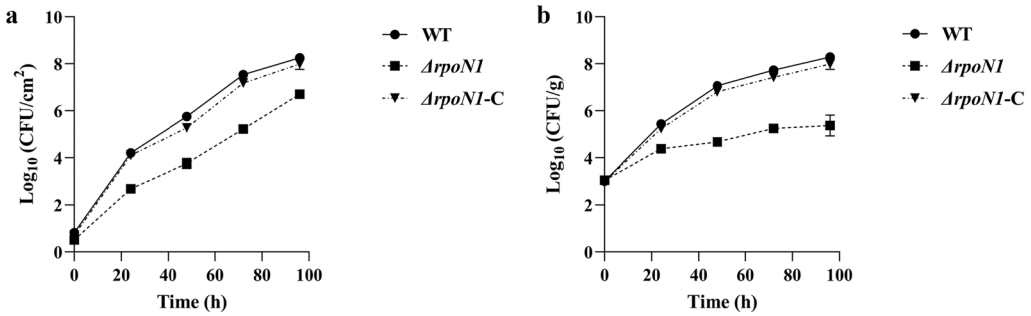

Fig. 5 Impact of *rpoN1* on the colonization ability of *P. citrulli* in melon. **a** Assessment of *P. citrulli* colonization on melon cotyledons. A bacterial suspension (~ 1×10^3 CFU/mL) for each strain was injected into melon seedling cotyledons. The bacterial population was quantified at 0, 24, 48, 72, and 96 h post-inoculation. **b** Assessment of *P. citrulli* colonization on melon seeds. A bacterial suspension (~ 1×10^3 CFU/mL) for each strain was injected into melon seeds. The bacterial population was quantified at 0, 24, 48, 72, and 96 h post-inoculation

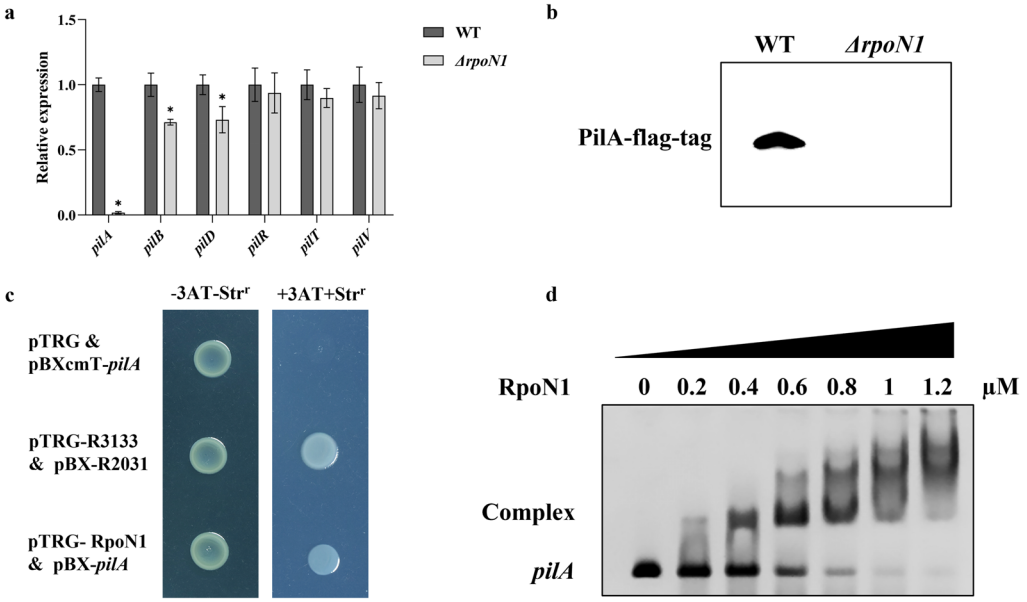

Fig. 6 RpoN1 directly regulates the expression of *pilA*. **a** The expression levels of pili-related genes *pilA*, *pilB*, *pilM*, *pilR*, *pilT*, and *pilV* in both the wild-type and *ΔrpoN1* strains were measured via qRT—PCR. The values represent the averages of three independent experiments. * denotes a significant difference between the two samples ($P < 0.05$). **b** The expression of PilA in the wild-type and *ΔrpoN1* strains was determined by western blotting. **c** The interaction between RpoN1 and the *pilA* promoter was assessed using a bacterial one-hybrid system. RpoN1 was cloned into vector pTRG, and the *pilA* promoter region as cloned into vector pBXcmT. Plasmids co-transformed into the bacterial strain are indicated on the left. **d** The interaction between RpoN1 and the *pilA* promoter was examined using an electrophoretic mobility shift assay. Purified RpoN1 (0 ~ 1.2 μM) was incubated with 200 ng of DNA (containing the *pilA* promoter region) at 25℃ for 30 min. The product was then resolved on a 5% (w/v) polyacrylamide gel in 0.5 × Tris–borate-EDTA (TBE) buffer at 90 V for approximately 2 h

bacteria. Mutant strains of *pilA* in *P. aeruginosa* exhibited enhanced swarming motility compared with wild type strains (Shrout et al. 2006). In *P. citrulli*, the swarming motility of *ΔrpoN1* and *ΔpilA* was significantly reduced compared with the wild-type strain. Furthermore, the pili of the *ΔrpoN1* and *ΔpilA* strains were absent, as observed by TEM. These findings suggest that *rpoN1* regulates biofilm formation and motility in *P. citrulli*, and is involved in regulating the expression of *pilA*.

To elucidate the roles of *rpoN1* and *pilA* in the pathogenicity of *P. citrulli* in melon, we assessed the virulence of *ΔrpoN1* and *ΔpilA* mutants through cotyledon injection, spray inoculation, and seed-to-seedling transmission assays. Our findings revealed that *rpoN1* and *pilA* positively regulate both the virulence and seed-to-seedling transmission capabilities of *P. citrulli*. Furthermore, we observed a reduction in the colonization ability of the *ΔrpoN1* mutant in melon. These results indicate that both *rpoN1* and *pilA* contribute significantly to the virulence of *P. citrulli.*

To further study the relationship between RpoN1 and *pilA* in *P. citrulli*, we conducted western blot analysis. The results indicated that the expression of PilA was significantly decreased in the *ΔrpoN1* mutant. These findings demonstrate that *rpoN1* regulates *pilA* transcription in *P. citrulli*. To determine whether RpoN1 directly influences motility and biofilm formation, thereby affecting the virulence of *P. citrulli*, we examined the interaction between RpoN1 and *pilA* using a bacterial one-hybrid system and EMSA. Our analysis revealed that RpoN1 can directly bind to the promoter of *pilA*. These results suggest that RpoN1 directly regulates the transcription of *pilA*, which impacts biofilm formation, twitching motility, swarming motility, and the virulence of *P. citrulli* (Fig. 7).

Flagella, a crucial pathogenic factor, influence the swimming motility and virulence of *P. citrulli* (Bahar et al. 2011). In this study, the swimming motility of *P.*

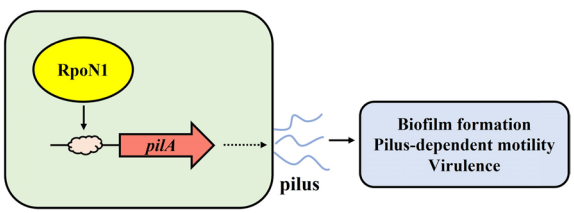

Fig. 7 A simple model shows that RpoN1 regulates *pilA* in *Paracidovorax citrulli*. RpoN1 regulates the expression of *pilA*, influencing biofilm formation, twitch motility, swarm motility, and the virulence of *Paracidovorax citrulli*

citrulli was enhanced in *ΔrpoN1* mutants. These results suggested that *rpoN1* negatively regulates the swimming motility of *P. citrulli*. However, qRT-PCR results showed that not all flagella-related genes were up-regulated. On the contrary, the expression levels of key flagella-related genes such as *flhA*, *flgM*, and *fliC* were significantly down-regulated. Additionally, in *Helicobacter pylori*, flagella can hijack pili proteins to control motility, and the deletion of *pilO* and *pilN* enhances migration ability in semisolid media (Liu et al. 2024). Based on these findings, the regulatory effect of *rpoN1* on the flagella of *P. citrulli* requires further investigation. Moreover, the σ^{54} factor relies on enhancer-binding proteins (EBPs) to regulate the transcription of various genes in bacteria (Gao et al. 2020). Therefore, exploring the regulatory network of the σ^{54} factor necessitates understanding EBPs. This study confirmed that RpoN1 directly regulates the expression of *pilA* in *P. citrulli*; however, identifying which EBP mediates RpoN1's regulation of *pilA* transcription warrants further study.

Conclusions

In this study, we identified two σ^{54} factors, RpoN1 and RpoN2, in *P. citrulli*. Our findings reveal that *rpoN1* regulates biofilm formation, bacterial motility, virulence, and colonization ability. Furthermore, we confirmed that RpoN1 directly influences biofilm formation, twitching motility, and swarming motility in *P. citrulli* by interacting with the promoter of *pilA*. Therefore, we demonstrated that RpoN1 affects virulence in *P. citrulli* by regulating the expression of the type IV pili-related gene *pilA*. In future studies, we will further investigate the regulatory network of RpoN1 in *P. citrulli*.

Materials and methods

Bacterial strains, growth conditions, and plant material

Bacterial strains and plasmids used in this study are listed in Table 1. *P. citrulli* was cultured in Luria–Bertani (LB) medium at 28°C (Chong 2001). All *Escherichia coli* strains were grown in LB medium at 37°C. The turbidity of the cell suspensions was quantified using optical density measurements at a wavelength of 600 nm, as determined by a spectrophotometer. The concentrations of the antibiotics used were 100 μg/mL rifampicin (Rif), 50 μg/mL kanamycin sulfate (Km), 50 μg/mL gentamycin sulfate (Gm), 100 μg/mL ampicillin (Amp), and 8 μg/

Table 1 Bacteria and plasmids used in this study

Strains and plasmids	Related properties	Sources
P. citrulli		
xjl12	Wild-type, Rif^R	This Laboratory
ΔrpoN1	*rpoN1* in-frame deletion mutant, Rif^R, Km^R	This study
ΔrpoN1-C	*rpoN1* complementation strain, containing pBBR-RpoN1, Rif^R, Km^R, Gm^R	This study
ΔpilA	*pilA* in-frame deletion mutant, Rif^R, Km^R	This study
ΔpilA-C	*pilA* complementation strain, containing pBBR-PilA, Rif^R, Km^R, Gm^R	This study
WT (pBBR- *pilA*-Flag)	Wild-type xjl12 containing pBBR- *pilA*-Flag, Rif^R, Gm^R	This study
ΔrpoN1 (pBBR- *pilA*-Flag)	*ΔrpoN1* containing pBBR- *pilA*-Flag, Rif^R, Km^R, Gm^R	This study
Escherichia coli		
DH5α	*Φ80 lacZDM15, D (lacZYA-argF) U169. recA1, endA1.thi-1*	TaKaRa, Dalian, China
BW20676	*Δpir pro hsdR, recA*	This Laboratory
XL1-Blue MRF´ Kan	D(mcrA)183, D(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1 gyrA96, relA1, lac, [F´proAB lacIqZDM15 Tn5 (KmR)]	Wang et al. (2018)
Plasmids		
pET30a	Multiple restriction sites, Km^R	This Laboratory
pEX18GM	Suicide vector with a *sacB* gene, Gm^R	Hoang et al. (1998)
pBBR1-MCS-5	Broad-host-range cosmid vector, Gm^R	Kovach et al. (1995)
pTRG	Plasmid used for protein expression in bacterial one-hybrid, Tet^R	Wang et al. (2018)
pBXcmT	Plasmid used for DNA cloning in bacterial one-hybrid assay, Chl^R	Wang et al. (2018)
pET30a- RpoN1	pET30a with the coding region of RpoN1, Km^R	This study
pTRG-RpoN1	pTRG with the coding region of RpoN1, Tet^R	This study
pBXcmT-*pilA*	pBXcmT with the *pilA* promoter region, Chl^R	This study

Rif^R, Gm^R, Km^R, Tet^R, and Chl^R indicate resistance to Rifamycin, Gentamicin, Kanamycin, Tetracycline, and Chloromycetin, respectively

mL streptomycin (Sm). Melon (cv. Huanghou) seeds were cultured in an artificial climate incubator at 25°C with 70% relative humidity (RH). The inoculated seedlings were transferred to a greenhouse set at 28°C with 80% RH.

Construction of deletion mutants and complemented strains of *Paracidovorax citrulli*

Deletion mutations of *rpoN1* and *pilA* were produced in *P. citrulli* through homologous recombination as previously described (Liu et al. 2019). Briefly, the upstream and downstream fragments of the target gene were amplified by PCR from *P. citrulli* AAC00-1 genomic DNA using specific primer pairs. The Km fragment was cloned from pET30a using Km primer pairs. A recombinant vector was constructed by ligating the three fragments into the suicide vector pEX18GM, which was then transferred into *E. coli* BW20676 for biparental mating with wild-type xjl12. Gene deletion mutants were obtained and validated by PCR using target gene-specific primers. The promoter positions of *rpoN1* and *pilA* were predicted using the Gene Promoter Prediction website (http://www.softberry.com/). The specific primer pair comp-F/R was employed to amplify the sequences containing the target gene and promoter. The fragment was ligated to pBBR1MCS-5 to construct a recombinant vector, which was then transferred into *E. coli* BW20676 for biparental mating with the *P. citrulli* mutants *ΔrpoN1* and *ΔpilA*. These complemented strains were further confirmed via PCR analysis with the relevant F/R primers. All sequences of primers used in this study are listed in Additional file 3: Table S2.

Transcriptome sequencing and data analysis

To elucidate the regulatory mechanism of *rpoN1* in *P. citrulli*, RNA-Seq analysis was conducted by Shanghai Personalbio Technology Co., Ltd. (Shanghai, China). In summary, total RNA was extracted using Trizol reagent (Invitrogen Life Technologies). The RNA quality and integrity were assessed using a nanodroplet spectrophotometer (Thermo Scientific) and a Bioanalyzer 2100 system (Agilent). rRNA was removed from the total RNA with the Zymo-Seq Ribo Free Total RNA Library Kit. The AMPure XP system (Beckman Coulter, Beverly, CA, USA) was employed to purify the library fragments, selecting DNA fragments between 400 and 500 bp. Illumina PCR primer cocktails were utilized to selectively enrich DNA fragments bearing linker molecules at both ends during 15 cycles of PCR. Following purification, the product was quantified on a Bioanalyzer 2100 system (Agilent) using Agilent's high-sensitivity DNA assay. Subsequently, the sequencing library was sequenced on the Nova Seq 6000 platform. Differentially expressed mRNAs were analyzed using DESeq (v1.38.3). Transcripts with $|\log_2\text{FoldChange}| > 1$ and $P\text{-value} < 0.05$ were considered differentially expressed mRNAs. GO enrichment analysis of differentially expressed genes was executed using topGO, with the *P* value calculated using the hypergeometric distribution method (significance threshold: $P\text{-value} < 0.05$), to identify GO terms with significant enrichment and determine the primary biological functions associated with these genes. KEGG pathway enrichment analysis of differentially expressed genes was performed with Cluster Profiler (v4.6.0), focusing on significantly enriched pathways with $P\text{-values} < 0.05$. Each strain was analyzed in three biological repetitions.

Quantitative real-time PCR analysis

In this study, total RNA from *P. citrulli* was quickly extracted using Total RNA Extractor (Trizol) reagent (Shengon Biotech, Shanghai, China). The quantitative real-time (qRT)-PCR system was set up with a ChamQ Universal SYBR kit (Vazyme, Nanjing, China). Reverse transcription was performed using the Hiscript III RT SuperMix for qPCR (Vazyme, Nanjing, China). The qRT–PCR was carried out on an ABI PRISM 7500 real-time PCR instrument (Applied Biosystems). The reaction program was as follows: 95°C for 30 s (1 cycle), 95°C for 10 s, and 60°C for 30 s (40 cycles). In this study, the 16S ribosomal RNA gene was employed as the internal reference gene, and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The experiments were performed three times with three biological replicates per gene. The primers utilized for the selected genes in this assay are detailed in Additional file 3: Table S2.

Biofilm formation assay

The biofilm formation assay was conducted following a previously described method (Wang et al. 2022). All strains were cultured overnight in LB broth at 28°C and adjusted to an OD_{600} of 1.0 after two washes with sterilized water. Then, 40 µL of the bacterial suspension was added to 4 mL of LB broth in a polystyrene 12-well plate, which was maintained at 28°C. After 48 h, the bacterial suspensions were gently aspirated, washed three times with sterile water, and incubated at 80°C for 20 min. The biofilms were stained with a 1% crystal violet solution for 50 min and subsequently dissolved in anhydrous ethanol. The OD_{590} values were measured using an enzyme marker. This experiment was repeated three times.

Bacterial motility assay

Twitching motility was measured for each *P. citrulli* strain using a previously described method (Wang et al. 2022). Briefly, the samples were washed twice with sterile water, adjusted to an OD_{600} of 1.0, diluted to 1.0×10^5 CFU/mL,

and then spread evenly on 1.0% (w/v) NA solid medium plates. After incubation at 28°C for 72 h, the morphological characteristics of the colonies were observed using a stereo fluorescence microscope (Nikon). These experiments were repeated three times.

The swarming motility assay was performed as described previously (Liu et al. 2016). Briefly, for the swarming assay, all strains were cultured overnight in LB broth at 28°C and adjusted to an OD_{600} of 0.3. Then, 3 μL of the bacterial suspension was added dropwise to 0.6% (w/v) semisolid medium plates. The diameter of the swarming halos was measured on agar plates after 48 h of incubation at 28°C. For the swimming motility assay, the medium was changed to 0.3% (w/v) semisolid medium. These experiments were conducted three times.

Transmission electron microscopy

Transmission electron microscopy (TEM) was utilized to observe the pili of cultured bacteria. The TEM specimens were prepared as previously described, with slight modifications. The strains for observation were incubated on 1.0% (w/v) NA plates at 28°C for 12 h. Appropriate amounts of sterile water were added to the plates and gently shaken to suspend the colonies. The copper mesh was immersed in the bacterial suspension, stained with 2% phosphotungstic acid for 5 min, and then baked for 30 min. The bacterial pili were observed using a Hitachi-7650 transmission electron microscope at 80 kilovolts (kV).

Virulence assays

Three previous approaches have been employed to explore the effects of *rpoN1* and *rpoN2* on the virulence of *P. citrulli* (Liu et al. 2019).

Cotyledon inoculation assay: A gradient dilution of the cultured strains was prepared to achieve a concentration of approximately 1.0×10^3 CFU/mL. The strains were then injected into the cotyledons of well-developed melons over a 7-day growth period. The inoculated melon seedlings were incubated at 28°C with 80% RH. Symptoms of BFB were monitored at 24-h intervals post-inoculation.

True leaf spray inoculation: Melon seedlings bearing their third true leaf were chosen for spray inoculation in this study. Each cultured strain was washed twice with sterile water, and the OD_{600} was adjusted to 0.3. At least 50 mL of the bacterial suspension was transferred to sterile spray bottles and sprayed uniformly on both the front and back surfaces of the melon leaves. The inoculated seedlings were incubated at 28°C with 100% RH for 48 h, followed by incubation at 80% RH. BFB symptoms were observed seven days post-inoculation.

Seed-to-seedling transmission assay: Twenty dewy melon seeds were placed in 5 mL sterile centrifuge tubes, and 2 mL of bacterial suspensions with a concentration of approximately 1.0×10^6 CFU/mL were added. The mixture was then gently shaken for 4 h. After air-drying the bacterial liquids, the seeds were planted in a greenhouse maintained at 28°C with 80% RH. Symptoms of BFB were observed seven days post-planting. These experiments were repeated three times.

Bacterial colonization assay of melon cotyledons and seed

The seedling colonization of the wild-type strain of *P. citrulli* and its derived mutant strains was determined by infiltrating melon cotyledons and seeds. Bacterial cells at a concentration of 1.0×10^3 CFU/mL were injected into the cotyledons of melon plants (cv. Huanghou) using a sterile syringe. The inoculated melon seedlings were incubated in a growth chamber at 100% RH at 28°C for 0, 24, 48, 72, and 96 h. The inoculated melon cotyledons were then extracted into sterile centrifuge tubes with forceps, ground with 100 μL of sterile water, diluted in a gradient, and then evenly spread on LA plates containing the corresponding antibiotics. Colonies were counted after 24 to 96 h of incubation at 28°C. Seed colonization assays were conducted based on previous reports (Tian et al. 2015b). Melon seeds were sterilized using 70% ethanol, and 5 μL of the bacterial suspension at approximately 1×10^3 CFU/mL was injected into the seed openings. The seeds were subsequently placed in petri dishes lined with moistened sterile filter paper and incubated at 28°C. After 2, 24, 48, 72, and 96 h of incubation, the seeds were removed, placed in a sterile centrifuge tube, and 1 mL of sterile water was added. The mixture was vortexed and shaken for 10 min, then uniformly spread on LA plates containing the corresponding antibiotics after gradient dilution. Colonies were counted after incubation at 28°C for 24 to 96 h. These experiments were repeated three times.

Protein expression and purification

The DNA fragment of *rpoN1* was amplified by PCR using specific primers listed in Additional file 3: Table S2 and inserted into the pET30a plasmid to construct the recombinant vector pET30a-RpoN1-His. This vector was subsequently introduced into *E. coli* BL21 for protein expression. For protein purification, the bacteria were cultured in LB medium at 37°C and 220 rpm until the OD_{600} reached approximately 0.4. Isopropyl β-D-thiogalactoside was then added to a final concentration of 0.4 mM to induce expression for 12 h at 16°C and 220 rpm. The bacterial cells were harvested by centrifugation at $6000\times g$ for 10 min at 4°C and resuspended in 10 mL of 20 mM Tris–HCl (pH 7.4). The cells were lysed

using TieChui *E. coli* Lysis Buffer (ACE Biotechnology, Changzhou, China), and the lysates were incubated with pre-equilibrated Ni^{2+} at 4°C for 1 h. Proteins containing His tags were extensively washed with a buffer containing 20 mM Tris–HCl (pH 7.4) and 50 mM imidazole, and subsequently eluted with buffers containing 100 mM, 200 mM, and 300 mM imidazole, respectively. The purified protein was mixed with glycerol to a final concentration of 20% and stored at −80°C.

Western blot analysis

To clarify the expression of PilA in the absence of *rpoN1*, the plasmid pBBR-MCS5 carrying a Flag tag was ligated to the *pilA* fragment, which includes its native promoter, to construct recombinant vectors. These vectors were subsequently transfected into *P. citrulli* wild-type and *rpoN1* mutants. The overnight culture was adjusted to an OD_{600} of 1.0, and the cell precipitates were collected at 4°C and 6000×*g* for endocrine protein detection. The cell sediments were resuspended in 900 µL of 20 mM Tris–HCl buffer solution, and the cells were lysed with 100 µL of TieChui *E. coli* Lysis Buffer (ACE Biotechnology, Changzhou, China) for 5 min at 4°C. Protein supernatants were separated from the precipitates at 4°C and 8000×*g*, then heated at 100°C for 10 min to denature the proteins. Next, the protein supernatants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Red Bank, NJ, United States) using a semidry blot machine (Bio-RAD, CA, United States). After blocking with 5% milk containing 0.05% Tween in Tris buffer solution (TBST, pH 7.5) for 1 h at room temperature, the membrane was incubated with a monoclonal antibody specific for Flag tags (1:5000; Abmart, Shanghai, China) for 1 h. Detection was carried out using an HRP-conjugated anti-rabbit secondary antibody (No. M21002, Abmart, Shanghai, China). Immunoblots were developed with a HyGlo HRP ECL Detection Kit (MDBio Inc., Qingdao, China) and visualized using an automatic multifunction image analysis system Tanon-6,600 (Tanon, Shanghai, China). These experiments were conducted three times.

Bacterial one-hybrid assay

A bacterial one-hybrid reporter system comprising two plasmids, pTRG and pBXcmT, and the *E. coli* -Blue MRF' kan strain was employed to detect protein interactions between the transcriptional regulator RpoN1 and the promoter of *pilA* (Wang et al. 2018). Briefly, the fragment containing the *pilA* promoter was cloned into pBXcmT to construct the recombinant vector pBXcmT-*pilA*. Similarly, the fragment encoding RpoN1 was cloned into pTRG to construct the recombinant vector pTRG-RpoN1. Both recombinant vectors were then cotransfected into the *E. coli* XL1-Blue MRF' Kan strain. If direct physical binding occurs between RpoN1 and the *pilA* promoter, the transformed *E. coli* strains containing both pBXcmT-*pilA* and pTRG- RpoN1 will grow well on a selective medium. This medium is a minimal medium containing 5 mm 3-amino-1,2,4-triazole, streptomycin at 8 µg/mL, tetracycline at 12.5 µg/mL, chloramphenicol at 34 µg/mL, and Km at 30 µg/mL (Wang et al. 2018). Furthermore, cotransformants containing pBX-R2031 and pTRG-R3133 served as positive controls (Xu et al. 2016), while cotransformants containing empty pTRG and pBXcmT-*pilA* were used as negative controls. All cotransformants were spotted onto the selective medium and grown at 28°C for 3–4 days, and then photographed. These experiments were conducted three times.

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay was conducted to determine whether RpoN1 binds to the promoter of *pilA*. The DNA fragment of the *pilA* promoter was amplified by PCR using specific primers listed in Additional file 3: Table S2. DNA binding was carried out in a 20 µL reaction system containing 4 µL of EMSA Tris binding buffer, 1 µM His_6-RpoN1, and 200 ng of the DNA fragment. The binding reaction was performed at 25°C for 30 min. Subsequently, the samples were loaded onto a native 5% (w/v) polyacrylamide gel and electrophoresed in 0.5×Tris–borate-EDTA buffer at 90 V for approximately 2 h. The gel was then removed and soaked in developing solution for 5 min and visualized using a UV imager. These experiments were conducted three times.

Statistical analysis

All analyses were performed using SPSS 22.0 (SPSS Inc.). Analysis of variance (ANOVA) was used to determine the differences in biofilm assay, motility assay, disease index, and gene expression between treatments.

Abbreviations

BFB	Bacterial fruit blotch
DEGs	Differentially expressed genes
DIs	Disease indices
EBPs	Enhancer-binding proteins
EMSA	Electrophoretic mobility shift assay
PCR	Polymerase chain reaction
qRT-PCR	Quantitative Real-time PCR
RH	Relative humidity
RNA-Seq	RNA sequencing
TEM	Transmission electron microscopy
T2SS	Type II secretion system
T3SS	Type III secretion system
T4P	Type IV pilus
T6SS	Type VI secretion system

Supplementary Information

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Additional file 1.

Additional file 2.

Additional file 3.

Additional file 4.

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Author contributions
YT and BH designed the research; YZ, YT, and WJ prepared the materials; MS and LZ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available in the NCBI repository, https://www.ncbi.nlm.nih.gov/nuccore/NC_008752.1.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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