Received: 6 November 2019 | Revised: 22 January 2020 | Accepted: 24 January 2020

DOI: 10.1111/mpp.12920

ORIGINAL ARTICLE

Molecular Plant Pathology WILEY

The RpoN2-PilRX regulatory system governs type IV pilus gene transcription and is required for bacterial motility and virulence in *Xanthomonas oryzae* pv. *oryzae*

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Funding information
This work was supported by grants from the National Science Foundation of China (31600105) to C. Yu and the National Science Foundation of China (31870120 and 31972255) to C. He and H. Chen, respectively.

Abstract

The type IV pilus (T4P), a special class of bacterial surface filament, plays crucial roles in surface adhesion, motility, biofilm formation, and virulence in pathogenic bacteria. However, the regulatory mechanism of T4P and its relationship to bacterial virulence are still little understood in *Xanthomonas oryzae* pv. *oryzae* (Xoo), the causal pathogen of bacterial blight of rice. Our previous studies showed that the σ^{54} factor RpoN2 regulated bacterial virulence on rice in a flagellum-independent manner in Xoo. In this study, both yeast two-hybrid and pull-down assays revealed that RpoN2 directly and specifically interacted with PilRX, a homolog of the response regulator PilR of the two-component system PilS-PilR in the pilus gene cluster. Genomic sequence and reverse transcription PCR (RT-PCR) analysis showed 13 regulons containing 25 genes encoding T4P structural components and putative regulators. A consensus RpoN2-binding sequence $GGN_{10}GC$ was identified in the promoter sequences of most T4P gene transcriptional units. Electrophoretic mobility shift assays confirmed the direct binding of RpoN2 to the promoter of the major pilin gene *pilAX*, the inner membrane platform protein gene *pilCX*, and *pilRX*. Promoter activity and quantitative RT-PCR assays demonstrated direct and indirect transcriptional regulation by RpoN2 of the T4P genes. In addition, individual deletions of *pilAX*, *pilCX*, and *pilRX* resulted in significantly reduced twitching and swimming motility, biofilm formation, and virulence in rice. Taken together, the findings from the current study suggest that the RpoN2-PilRX regulatory system controls bacterial motility and virulence by regulating T4P gene transcription in Xoo.

KEYWORDS

σ^{54}, motility, PilRX, type IV pilus, virulence, *Xanthomonas oryzae* pv. *oryzae*

1 | INTRODUCTION

In bacteria, the σ^{54} factor is an important transcriptional regulator. It binds to core RNA polymerase (RNAP) and recognizes specific promoter regions, thereby regulating the transcription of a variety of functional genes. Further studies showed that σ^{54} factor binds to the highly conserved sequence $GGN_{10}GC$ at positions −24/−12 relative to the transcription start site of target genes (Barrios *et al.*, 1999; Yang *et al.*, 2015). Previous studies also showed that the σ^{54} factor is required for flagellum-dependent motility, nitrogen utilization, the type III secretion system, and virulence in plant-pathogenic bacteria (Hendrickson *et al.*, 2000a; 2000b; Alarcon-Chaidez *et al.*, 2003; Kazmierczak *et al.*, 2005; Tian *et al.*, 2015; Lardi *et al.*, 2015). Due to the complex regulatory network and diverse biological functions of σ^{54}, it is important to identify the downstream genes of the σ^{54} factor and to further clarify its regulatory pathways.

Unlike other σ factors, the regulatory function of σ^{54} is dependent on transcriptional activators (enhancer-binding proteins, EBPs). EBPs generally contain three domains: an N-terminal signal-sensing domain, whose main function is to perceive signals and regulate the activity of transcription activators; a central AAA^+ domain, whose main function is to interact with the σ^{54} factor and hydrolyse ATP to release energy; and a C-terminal DNA-binding domain, whose main function is to bind to gene promoters (Studholme and Dixon, 2003). The central AAA^+ domain is the most conserved domain and exists in all EBPs. The σ^{54} factor regulates gene transcription by interacting with EBPs. First, σ^{54} and RNAP bind to a target gene promoter to form a closed complex, and the EBP binds to the target gene upstream of the promoter sequence. Second, the EBP AAA^+ domain interacts with the σ^{54} factor via DNA looping. Finally, ATP hydrolysis by the AAA^+ domain results in opening of the σ^{54}-RNAP complex, and then target gene transcription is activated (Schumacher *et al.*, 2006). Therefore, it is necessary to identify EBPs for analysis of σ^{54} regulatory pathways.

Type IV pili (T4Ps), nanomachines with extracellular, transenvelope, and cytoplasmic components, are observed in a large variety of gram-negative bacteria and several gram-positive bacteria (Giltner *et al.*, 2012). The extracellular fibre is about 6–9 nm in diameter and many times longer than a cell. In most species, the fibre can be extended and retracted. It is comprised primarily of subunits known as pilins; while the N-terminal region of all pilins is highly conserved, the remainder of any given pilin can vary widely, and these variations are associated with differences in pilin function (Hospenthal *et al.*, 2017). T4Ps provide bacteria with a link to their external environments by enabling them to attach to host cells. Moreover, T4Ps directly bind to extracellular double-stranded DNA via their tip and mediate DNA internalization through retraction has also been reported (Ellison *et al.*, 2018). Therefore, T4Ps facilitate surface and host cell adhesion, colonization, biofilm formation, twitching, a form of surface-associated motility facilitated by cycles of extension and retraction, and adhesion for bacteria. Importantly, T4Ps are crucial virulence factors for many human pathogens. These functions have been studied mostly in animal-pathogenic bacteria. However, we know much less about their functions in plant-pathogenic bacteria.

In *Pseudomonas aeruginosa*, PilR-PilS, located in the T4P gene cluster, is a two-component system (TCS) and regulates transcription of the major pilin gene *pilA* (Hobbs *et al.*, 1993). In this TCS, PilR is a transcription activator that can activate the transcription of *pilA* by directly binding to its promoter; PilS is an atypical sensor histidine kinase with six transmembrane segments (Jin *et al.*, 1994). On detection of an activating signal, PilS undergoes autophosphorylation on a conserved His residue in the cytoplasmic kinase domain, and then the phosphate is transferred to PilR, resulting in the activation of downstream genes (Kilmury and Burrows, 2016). However, PilR, but not PilS, regulates the expression of *pilA* in *Xanthomonas axonopodis* pv. *citri* (Yang *et al.*, 2004), indicating that there are different working modes of PilR-PilS between *Pseudomonas* and *Xanthomonas*. The functions of the PilR-PilS and T4P genes are poorly understood in other *Xanthomonas* strains.

In a previous study, we found that RpoN2 and FleQ regulate the flagellar system in *Xanthomonas oryzae* pv. *oryzae* (Xoo) (Tian *et al.*, 2015). The gene *rpoN2* is located in the central region of the flagellar regulon and transcribed in an operon with *fleQ*. On the top class of the four-tiered transcriptional cascade, RpoN2/FleQ controls the expression of regulatory genes, structural genes, and the flagellin gene *fliC* in the Xoo flagellar gene cluster. Interestingly, deletion of *rpoN2* caused a significant decrease in the virulence of Xoo, while the absence of *fleQ* did not alter the virulence of the bacterium (Tian *et al.*, 2015). This result indicates that RpoN2 most probably regulates Xoo virulence independent of controlling flagellar gene expression by interacting with other EBPs. Therefore, in this study, we hoped to identify new EBPs related to virulence and reveal the regulatory mechanism of RpoN2 on virulence in Xoo.

2 | RESULTS

2.1 | RpoN2 directly and specifically interacts with PilRX

To study the regulatory mechanism of RpoN2 on virulence in Xoo, we searched for potential EBPs of RpoN2 in the Xoo genome (RefSeq: NC_010717.2; INSDC: CP000967.2) on National Center for Biotechnology Information (NCBI). As described in the Experimental Procedures section, BLAST and conserved domain analysis of candidate EBPs showed that there are five FleQ paralogs with an AAA^+ domain that can interact with RpoN2 in Xoo, namely PXO_02717, PXO_03020, PXO_03564, PXO_03965, and PXO_04881 (PilR-like protein in Xoo, PilRX) (Figure S1). In our previous study, the absence of FleQ did not affect Xoo virulence (Tian *et al.*, 2015). PXO_02717 is a propionate catabolism operon regulatory protein, and PXO_03020, PXO_03564, and PXO_03965 are NtrC family proteins. These proteins are involved in bacterial growth and nitrogen metabolism (Schumacher *et al.*, 2013; Brown *et al.*, 2014). In *P. aeruginosa*, PilR and PilS are a TCS

pair that regulate T4P synthesis, twitching motility, and virulence (Farinha *et al*., 1993; Kilmury and Burrows, 2018). Therefore, we hypothesized that PilRX is an EBP for RpoN2 and is involved in the RpoN2-dependent regulatory pathway on virulence in Xoo. To confirm this hypothesis, we first analysed the interaction between RpoN2 and PilRX by yeast two-hybrid (Y2H) and glutathione-S-transferase (GST) pull-down assays. For the Y2H assay, we constructed the plasmids pGADRpoN2, pGADPilRX, pGADPilSX, pGBKRpoN2, pGBKPilRX, and pGBKPilSX to express the fusion proteins AD-RpoN2, AD-PilRX, AD-PilSX, DBD-RpoN2, DBD-PilRX, and DBD-PilSX, respectively. These plasmids were transformed into *Saccharomyces cerevisiae* strains, and protein expression was validated by western blotting analysis. Interaction between RpoN2 and PilRX was screened for via growth on quadruple drop-out (QDO) medium lacking the amino acids Trp, Leu, His, and Ade. The results showed that the yeast diploids containing DBD-RpoN2/AD-PilRX and DBD-PilRX/AD-RpoN2 grew well on QDO medium and that the positive controls DBD-PilRX/AD-PilSX and DBD-PilSX/AD-PilRX also grew well, while the negative controls DBD/AD-RpoN2, DBD/AD-PilRX, and DBD/AD-PilSX did not grow (Figure 1a). For the GST pull-down assay, GST-PilRX, SUMO-His_6-RpoN2, SUMO-His_6-PilSX, GST tag only, and SUMO-His_6 tag only were expressed and purified (Figure S2). The GST pull-down assay was performed as described in the Experimental Procedures section. The results showed that SUMO-His_6-RpoN2 and SUMO-His_6-PilSX were detected when GST-PilRX was present in the mixture but were not detected when the GST tag only was present in the mixture, indicating that RpoN2 and PilSX were able to pull down PilRX (Figure 1b). These results demonstrate that RpoN2 directly interacts with PilRX in Xoo.

2.2 | T4P genes in Xoo

T4Ps are common surface appendages and important factors involved in a wide variety of functions, including surface attachment, twitching motility, and biofilm formation, in bacteria. Over 40 genes controlling T4P biogenesis and function have been found in *P. aeruginosa*, including the TCS genes *pilR/pilS* and the major pilin gene *pilA* (Farinha *et al*., 1993; Jin *et al*., 1994). To study T4P biogenesis and function in Xoo, we searched pilus-related genes (from *pilA* to *pilZ*) on NCBI and found 25 candidate T4P genes distributed throughout the Xoo genome (Figure 2a). The proteins encoded by these genes can be divided into four groups: (a) structural components, pilus filament including a major pilin (PilAX) and minor pilins (PilEX, PilVX, PilWX, and PilXX), outer member subcomplex proteins (PilFX and PilQX), and inner membrane platform proteins (PilCX, PilMX, PilNX, PilOX, and PilPX); (b) regulatory factors (PilRX, PilSX, PilGX, and PilHX); (c) ATPases (PilBX, PilTX, and PilUX); and (d) other proteins, prepilin peptidase (PilDX) and other pilus assembly-related proteins (PilIX, PilJX, PilLX, PilYX, and PilZX) (Table S1). In addition, sequence alignment results showed that most of these proteins were highly similar (sequence identity > 90%) to the T4P proteins in *X. oryzae* pv. *oryzicola* BLS256 but had low sequence identity to those (sequence identity < 50%) in *Pseudomonas syringae* pv. *tomato* DC3000 (Table S1).

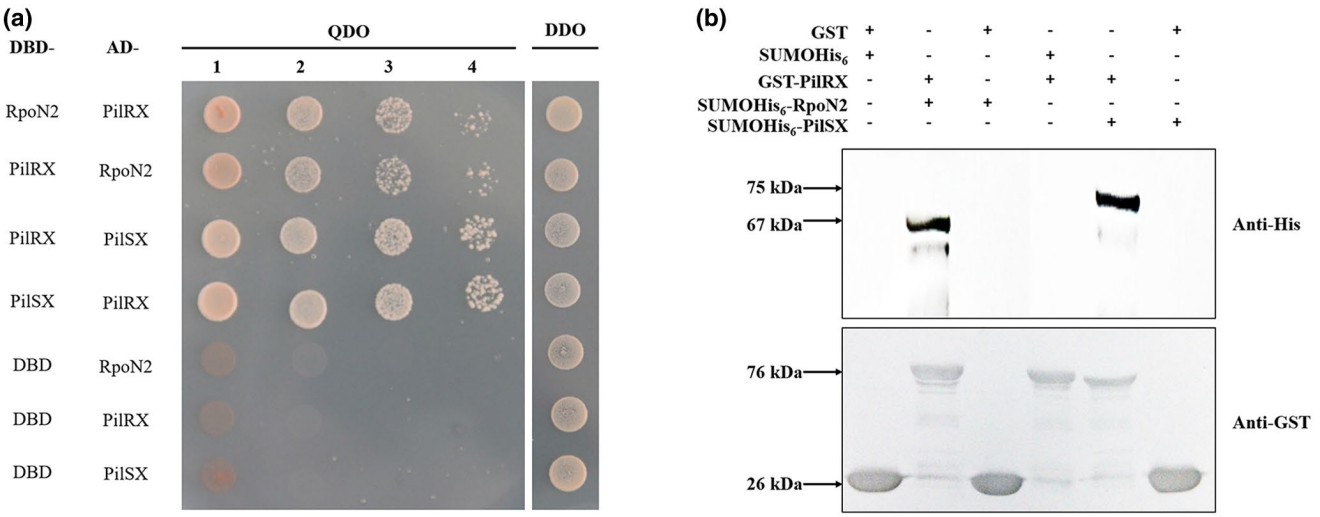

FIGURE 1 RpoN2 specifically interacts with PilRX in *Xanthomonas oryzae* pv. *oryzae*. (a) A yeast 2-hybrid assay showing the interactions between RpoN2 and PilRX. Y2HGold yeast strains carrying pGBKRpoN2 and pGBKPilRX were mated with Y187 yeast strains carrying pGADPilRX and pGADRpoN2, respectively. The mated strains were selected on double drop-out (DDO) plates. Diploids capable of growth on DDO plates were further examined on quadruple drop-out (QDO) plates for the interactions between test proteins. Serial dilutions (10-fold) were performed to evaluate the strength of the interactions observed. The interaction between PilRX and PilSX was performed as a positive control. Pictures were taken after the cells grew on DDO or QDO plates for 72 hr. (b) Glutathione-S-transferase (GST) pull-down analysis of the interaction between RpoN2 and PilRX. Purified GST-PilRX fusion protein was incubated with equal amounts of purified SUMO-His_6-RpoN2 or SUMO-His_6-PilSX and bound to glutathione-sepharose beads. Beads were then washed, and eluted proteins were analysed by western blotting using anti-His (upper) or anti-GST (lower) antibodies. The interactions of GST and SUMO-His_6-RpoN2, GST and SUMO-His_6-PilSX, and SUMO-His_6 and GST-PilRX were performed as negative controls. Protein size is labelled on the left of the membrane

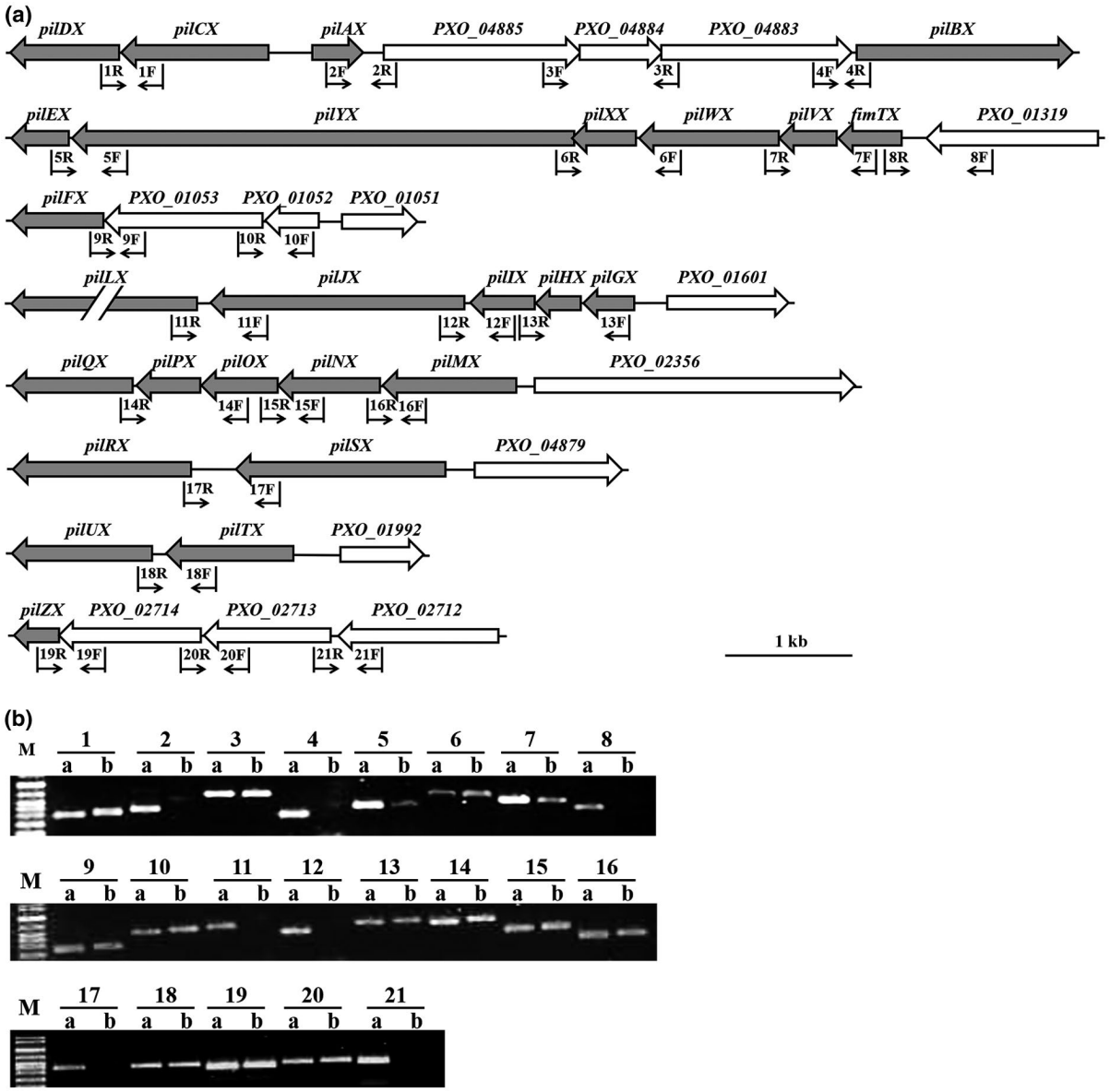

FIGURE 2 Thirteen transcriptional units are present in the *Xanthomonas oryzae* pv. *oryzae* pilus gene cluster. (a) Schematic diagram of the pilus gene cluster in the genome of the *X. oryzae* pv. *oryzae* (Xoo) strain PXO99^{A}. Open arrows indicate the length, location, and orientation of the open reading frames (ORFs). The number below the arrows indicates the primers (F, forward primer; R, reverse primer) used in reverse transcription (RT)-PCR. (b) RT-PCR assays. RT-dependent amplification of DNA fragments suggested that the pilus genes were transcribed in one operon. Genomic DNA (a) was used as a positive control and cDNA (b) was obtained as described in the Experimental Procedures section

The TCS proteins PilR and PilS have been reported to regulate the major pilin gene *pilA* in *P. aeruginosa* and in many other T4P-expressing bacteria (Hobbs *et al*., 1993; Wu and Kaiser, 1997; Kehl-Fie *et al*., 2009), but their roles in regulating T4P genes in Xoo are unknown. To assess the regulatory effects of RpoN2 and PilRX on T4P genes in Xoo, we first analysed the transcriptional units for these genes. As shown in Figure 2a, 21 pairs of primers spanning across neighbouring genes were designed to perform reverse transcription PCR (RT-PCR). Amplification of DNA fragments suggested that these genes were transcribed in one operon, and genomic DNA was used as a positive control. The results showed that these T4P genes were co-transcribed in 13 transcriptional units, and *pilAX*, *pilBX*, *pilCX*, *pilGX*, *pilJX*, *pilLX*, *pilMX*, *pilRX*, *pilSX*, *pilTX*, *fimTX*, *PXO_01052*, and *PXO_02713* were the first genes on those transcriptional units (Figure 2b). Next, we studied the regulatory effects of RpoN2 on T4P genes by testing the promoter activity and expression of these genes in Xoo strains.

2.3 | RpoN2 directly regulates the transcription of *pilRX*, *pilAX*, and *pilCX*

RpoN2, a σ^{54} factor, specifically binds to the highly conserved sequence $GGN_{10}GC$ at positions −24/−12 relative to the transcription

start site of target genes. To examine whether RpoN2 directly regulates T4P genes in Xoo, we first analysed the conserved RpoN2-binding sites in the promoter sequences of these transcriptional units. As the sequence logo showed, the conserved sequence $GGN_{10}GC$ was found in all promoters except that of *pilSX* (Figure 3a). Then, we performed an electrophoretic mobility shift assay (EMSA) to detect the binding between RpoN2 and these promoters. Interestingly, direct bindings were only confirmed between RpoN2 with *pilRX* promoter, *pilAX* promoter, and *pilCX* promoter (Figure 3b). With increasing of RpoN2 concentrations, the titre of bound RpoN2 increased (Figure 3b). No interaction was found between RpoN2 with promoters of other transcriptional units (Figure S3). These results indicate that RpoN2 specifically binds to the promoter regions of *pilRX*, *pilAX*, and *pilCX*. To confirm the regulation by RpoN2 of *pilRX*, *pilAX*, and *pilCX*, we examined the promoter activity of these genes by measuring the β-galactosidase activities of *pilRXp/pilAXp/pilCXp-lacZ* fusions in various strains. The results show that these β-galactosidase activities are significantly lower in Δ*rpoN2* than in the wild-type strain and are restored in the complemented strain (Figure 3c). These results suggest that RpoN2 directly regulates the expression of *pilRX*, *pilAX*, and *pilCX* in Xoo.

To further study the regulatory effects of RpoN2 on T4P genes in Xoo, we performed a quantitative RT-PCR (RT-qPCR) assay to examine the expression of the first genes in each transcriptional unit in the wild-type strain, Δ*rpoN2*, and its complemented strain. The results show that the expression levels of *pilAX*, *pilCX*, *pilFX*, *pilGX*, *pilJX*, *pilMX*, *pilRX*, and *filTX* are remarkably reduced in Δ*rpoN2* and restored in the complemented strain (Figure 4). In this study, PilRX was shown to be one of the EBPs for RpoN2. To confirm whether these genes are regulated by PilRX, we also determined the expression of these genes in Δ*pilRX*. As we predicted, most of these genes were significantly down-regulated in Δ*pilRX* and restored in the complemented strain, except *pilFX*, which was down-regulated in Δ*rpoN2* but not remarkably changed in Δ*pilRX* (Figure 4). In addition,

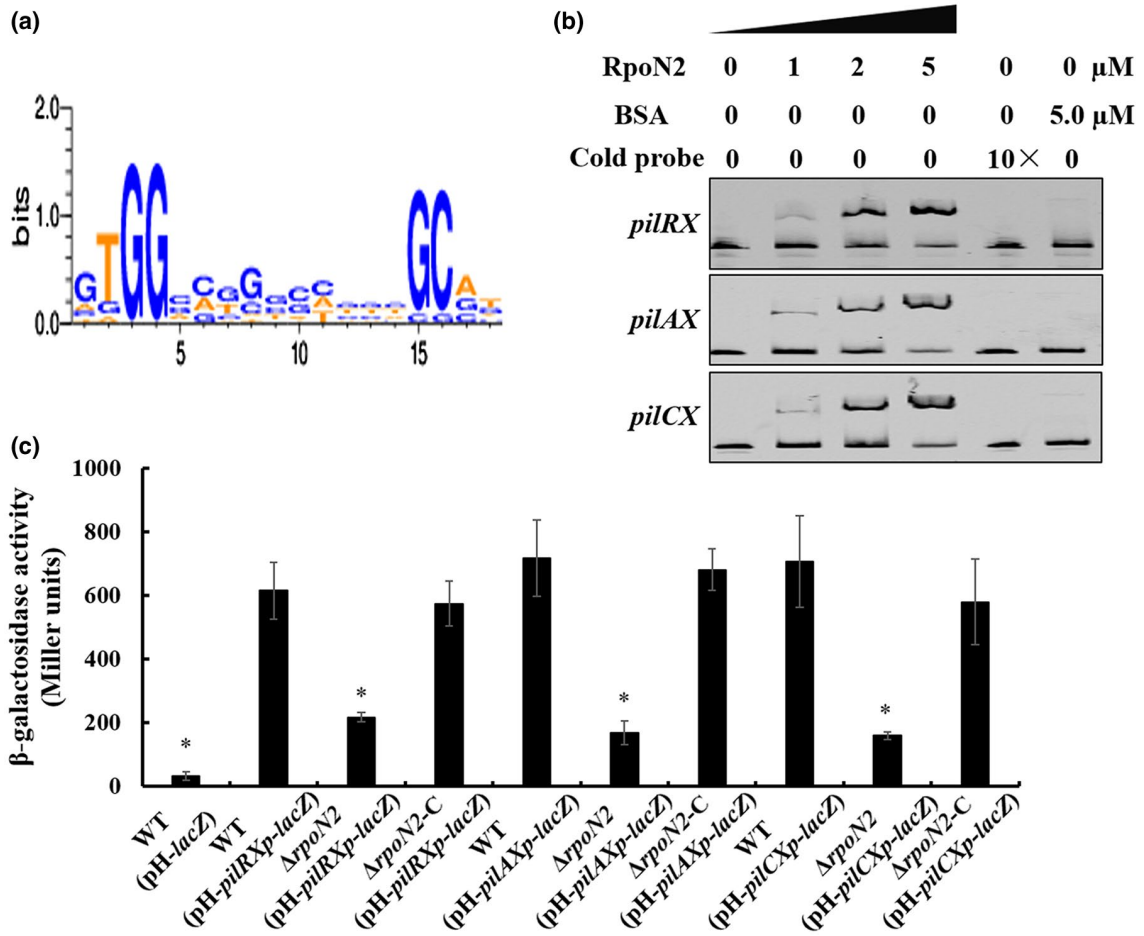

FIGURE 3 RpoN2 directly regulates the activities of the *pilRX*, *pilAX*, and *pilCX* promoters in *Xanthomonas oryzae* pv. *oryzae* (Xoo). (a) Conserved RpoN2-binding site analysis of pilus gene promoters. (b) The interactions between RpoN2 and pilus gene promoters were tested by electrophoretic mobility shift assay. Purified RpoN2 (0–5 μM) was incubated with 2 μM probe (FAM-labelled pilus gene promoter DNA regions) at 25 °C for 30 min, and the products were run on a native 4% (wt/vol) polyacrylamide gel in 0.5 × TBE buffer for approximately 1.5 hr at 100 V. A cold probe (unlabelled pilus gene promoter DNA regions) at 20 μM was used as a specific DNA competitor, and bovine serum albumen (BSA) at 5 μM was used as a nonspecific protein competitor. (c) β-Galactosidase activity assay of pilus gene promoters. The activities of the pilus gene promoters in Xoo strains were detected. The wild-type (WT) strain carrying the empty plasmid pH-*lacZ* was used as a negative control. The experiments were repeated independently three times. Error bars represent *SD* and asterisks indicate $p < .05$ (Student's *t* test)

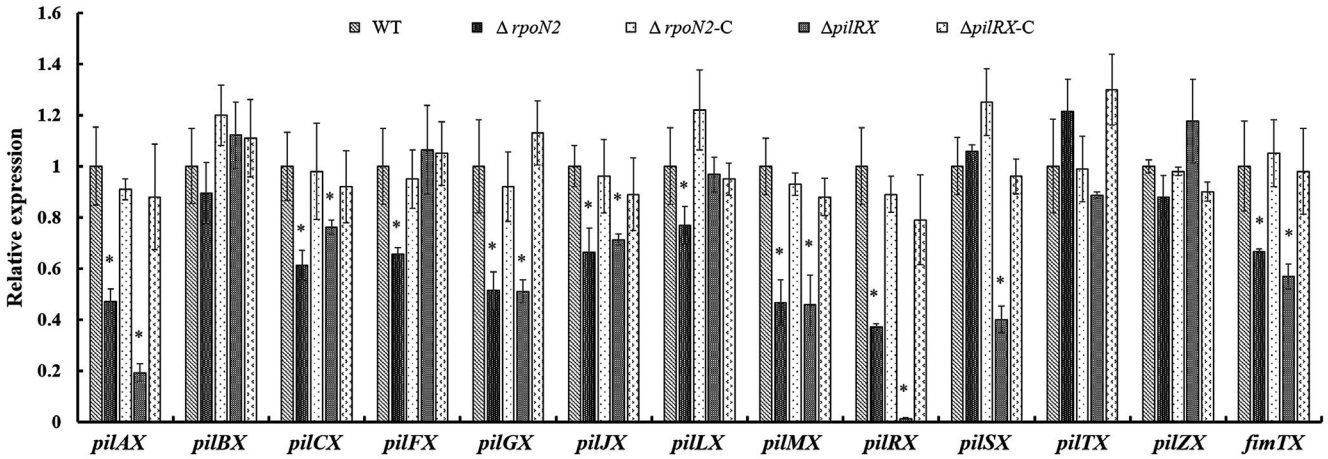

FIGURE 4 RpoN2 and PilRX regulate the expression of type IV pili (T4P) genes in *Xanthomonas oryzae* pv. *oryzae*. The first genes of 13 transcriptional units in the T4P gene cluster were chosen, and their expression in the wild-type strain, Δ*rpoN2*, Δ*rpoN2*-C, Δ*pilRX*, and Δ*pilRX*-C was tested by quantitative reverse transcription PCR. The experiments were repeated independently three times. Error bars represent *SD* and asterisks indicate $p < .05$ (Student's *t* test)

the expression of *pilSX* was not changed in Δ*rpoN2* but was reduced in Δ*pilRX* (Figure 4). These results suggest that RpoN2 and PilRX directly or indirectly regulate most of the T4P genes in Xoo.

2.4 | RpoN2 and T4P genes are required for twitching and swimming motility

It has been reported that the T4P is required for bacterial twitching motility, a form of surface-associated movement by extension and retraction of T4Ps (Mattick, 2002). To study the role of RpoN2 in regulating twitching motility in Xoo, we measured the zone of Δ*rpoN2* and some RpoN2-regulated T4P gene deletion mutants on peptone sucrose agar (PSA) plates. Compared with the wild-type strain, the twitching zones were significantly decreased in Δ*rpoN2*, Δ*pilRX*, Δ*pilAX*, and Δ*pilCX* and were restored in their complemented strains (Figure 5a,b). These results indicate that RpoN2 regulates twitching motility by controlling the expression of *pilRX*, *pilAX*, and *pilCX* in Xoo. In our previous study, RpoN2 was required for Xoo swimming motility, a form of flagellum-dependent motility, by interacting with FleQ (Tian *et al.*, 2015). To examine the relationship of T4P genes and swimming motility, we measured the swimming zones of these T4P gene deletion mutants on semisolid medium plates, as described in the Experimental Procedures section. Interestingly, compared with that in wild-type, the swimming zones in Δ*pilRX*, Δ*pilAX*, and Δ*pilCX* were remarkably reduced and restored in their complemented strains (Figure 5c,d). These results suggest that the T4P genes regulated by RpoN2 and PilRX are not only required for twitching motility but also for swimming motility in Xoo.

2.5 | RpoN2 and T4P genes are required for virulence and bacterial growth in rice

To further investigate the regulatory mechanism of RpoN2 on the virulence of Xoo in rice, the pathogenicity of Δ*pilRX*, Δ*pilAX*, and Δ*pilCX* and their complemented strains for the susceptible rice cultivar IR24 was tested by leaf-clipping inoculation. Disease symptoms were recorded by photography, and lesion lengths were measured at 14 days post-inoculation. As shown in Figure 6a,b, less severe bacterial blight symptoms and shorter lesions were observed with Δ*pilRX*, Δ*pilAX*, and Δ*pilCX* than with the wild-type strain, and these disease phenotypes were restored to near wild-type levels for the relevant complemented strains, indicating that these T4P genes are required for Xoo to cause disease on rice leaves. Similarly, compared with the wild-type strain, Δ*pilRX*, Δ*pilAX*, and Δ*pilCX* displayed a significant decrease in bacterial growth in rice leaf tissues (Figure 6c). Moreover, no remarkable differences in the bacterial growth rate in the wild-type and these T4P gene mutant strains were observed when grown in nutrient-rich M210 medium (Figure S4), suggesting that the reduced virulence of these T4P gene mutants was not caused by a change in growth in vitro. These results indicate that RpoN2 regulates the virulence of Xoo by controlling the expression of T4P genes.

2.6 | RpoN2 and T4P genes positively regulate biofilm formation

As *rpoN2* and T4P gene mutants displayed decreased motility and virulence, biofilm formation, the important motility- and virulence-related factor in Xoo, was tested in this study. As shown in Figure 7, compared with wild-type, biofilm formation was significantly decreased in Δ*rpoN2*, Δ*pilRX*, Δ*pilAX*, and Δ*pilCX* and restored in their complemented strains. This result indicates that RpoN2 and T4P positively regulate biofilm formation in Xoo.

3 | DISCUSSION

To elucidate the virulence regulatory mechanism of RpoN2 in Xoo, we identified the interaction of RpoN2 and PilRX, one of the TCS

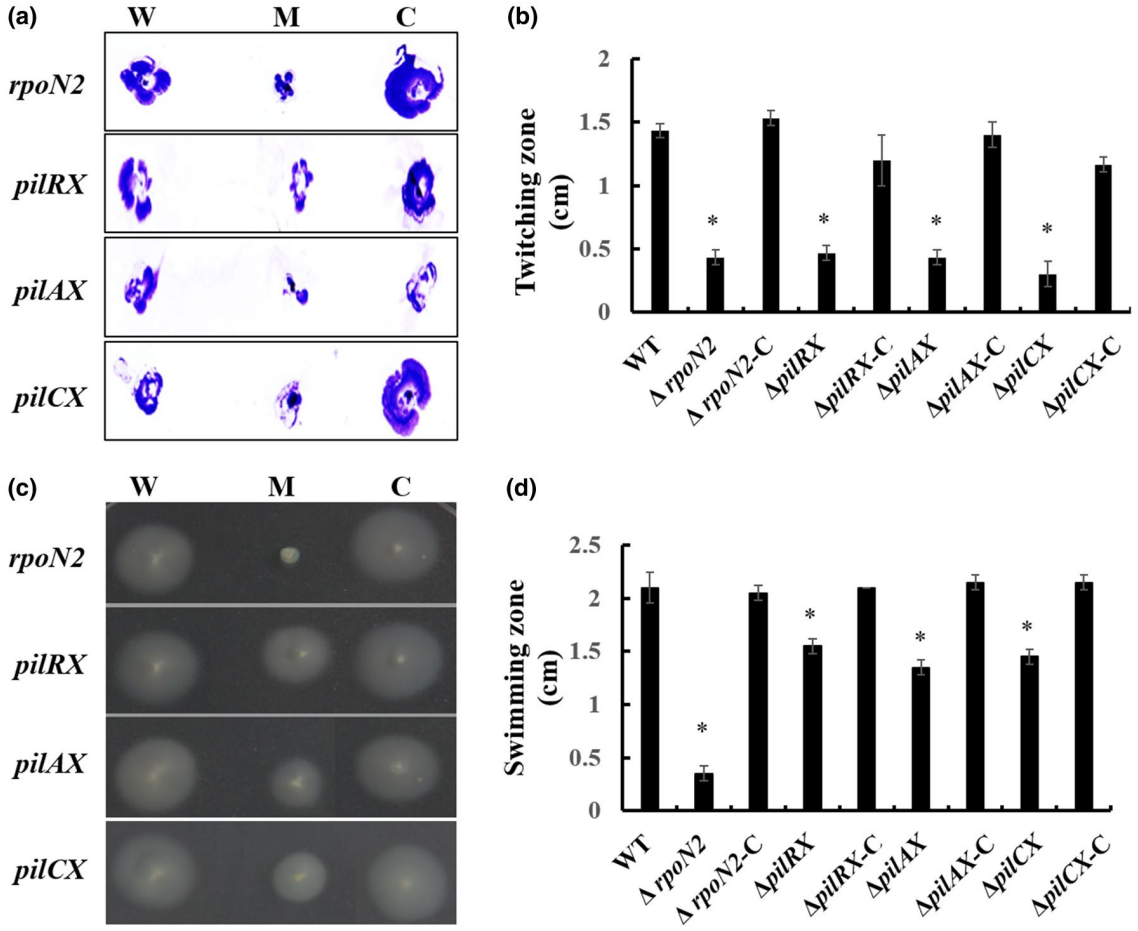

FIGURE 5 RpoN2, PilRX, PilAX, and PilCX are required for twitching and swimming motility in *Xanthomonas oryzae* pv. *oryzae*. (a) and (b) Twitching motility assay. The twitching zones were observed by crystal violet staining and recorded after bacterial growth for 4 days on plastic plates. (c) and (d) Swimming motility assay. The swimming zones were photographed and recorded after bacterial growth for 4 days on semisolid medium plates. W, wild-type; M, mutant; C, complemented strain. The values represent the average of three independent experiments. The error bars indicate *SD* and asterisks indicate $p < .05$ (Student's *t* test)

components in T4P gene clusters, characterized the regulation of T4P gene expression by RpoN2 and PilRX, and analysed the functions of RpoN2 and T4P in pilus- and flagellum-dependent motility, virulence, and biofilm formation in Xoo. The function and structure of T4P have been well studied in human-pathogenic bacteria (Mattick, 2002; Burrows, 2012), but they have been less studied in plant pathogens. To our immediate knowledge, this study is the first report to identify T4P genes that were directly regulated by RpoN2/PilRX and to investigate the role of T4P in motility, virulence, and biofilm formation in Xoo. In addition, these findings can help us to further understand the regulatory network of RpoN2 in Xoo.

It is well known that σ^{54} factors play important roles in bacteria that are dependent on EBPs. Therefore, identification of EBPs is the most efficient way to study the regulatory pathways of σ^{54} factors. Typically, EBPs have an N-terminal regulatory domain, a central AAA^+ domain that directly contacts σ^{54}, and a C-terminal DNA-binding domain (Schumacher *et al.*, 2006). In our work, six candidate EBPs (PXO_02717, PXO_03020, PXO_03564, PXO_03965, PilRX, and FleQ) containing the AAA^+ domain were identified. In Xoo, FleQ interacted with RpoN2 and controlled the flagellum-related phenotypes, except virulence to rice (Tian *et al.*, 2015; Yu *et al.*, 2018). The TCS PilR-PilS regulation of the major pilin gene *pilA* has been reported in many human-pathogenic bacteria, and the function of T4Ps in bacterial virulence has also been well described (Farinha *et al.*, 1993; Hobbs *et al.*, 1993; Strom and Lory, 1993). However, whether PilRX, the homolog protein of PilR in Xoo, has similar biological functions was unknown. In this study, the interaction of PilRX with RpoN2 was identified (Figure 1), and the roles of RpoN2-PilRX in regulating the expression of T4P genes (Figures 3 and 4) and controlling motility, virulence, and biofilm formation in Xoo were also demonstrated (Figures 5, 6 and 7). Therefore, we proposed a working model in which RpoN2 interacts with FleQ to regulate FliA (σ^{28} factor), thus controlling flagellum-dependent motility by regulating the expression of *fliC*, the flagellin gene, while it interacts with PilRX to regulate the expression of T4P genes, thus controlling pilus-dependent motility, virulence, and biofilm formation in Xoo (Figure 8). These results enriched the knowledge of the regulatory network of RpoN2 in bacteria. Whether the other four candidate EBPs in Xoo can interact with RpoN2 and are involved in RpoN2 regulatory pathways needs to be studied in the future.

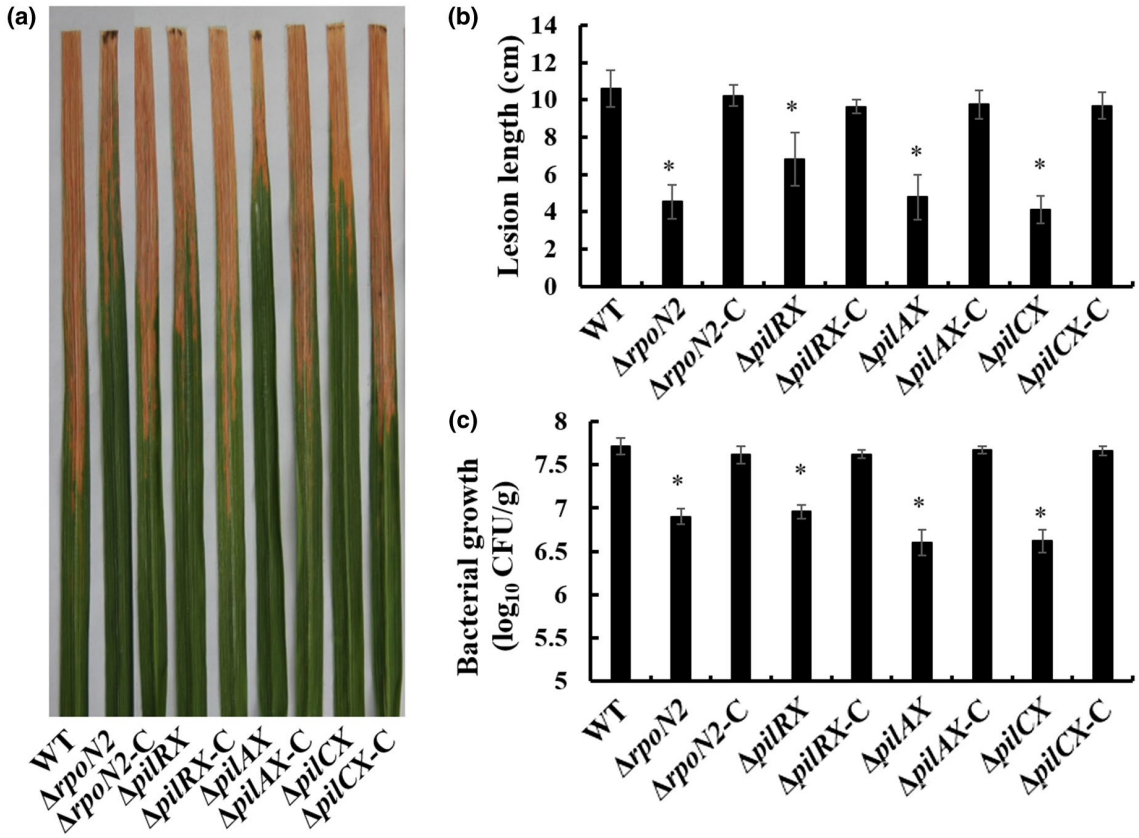

FIGURE 6 RpoN2, PilRX, PilAX, and PilCX positively regulate the virulence of *Xanthomonas oryzae* pv. *oryzae* (Xoo). (a) Disease symptoms were recorded by photography at 14 days after inoculation. Xoo strains were inoculated by leaf clipping after bacterial growth had reached an optical density at 600 nm (OD_{600}) of 0.8. (b) Disease lesion lengths of rice were measured at 14 days after inoculation. At least 10 leaves were assayed for each Xoo strain in each independent experiment. (c) The bacterial population was detected 14 days after inoculation. Three leaves were mixed as one sample for each strain. The experiments were repeated independently three times. Error bars represent *SD* and asterisks indicate $p < .05$ (Student's *t* test). WT, wild-type

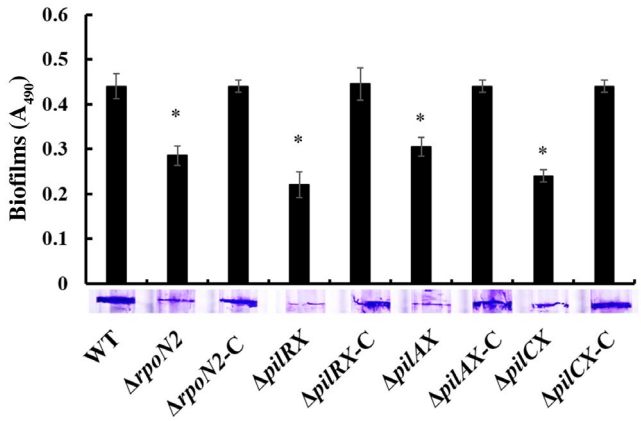

FIGURE 7 RpoN2, PilRX, PilAX, and PilCX are required for biofilm formation in *Xanthomonas oryzae* pv. *oryzae*. Biofilm formation was visualized by crystal violet staining and quantified by measuring the absorbance at 490 nm after suspension in ethanol. The values represent the average of three independent experiments. WT, wild-type strain. The error bars indicate the *SD* and asterisks indicate $p < .05$ (Student's *t* test)

The biogenesis and function of T4Ps have been well studied in *Pseudomonas*. PilA, the major pilus, is regulated by RpoN and PilR-PilS, and plays important roles in bacterial twitching motility and virulence to humans (Farinha *et al.*, 1993; Hobbs *et al.*, 1993), while the expression of *pilB*, *pilC*, and *pilD* does not depend on any of these transcriptional regulators (Koga *et al.*, 1993). Our previous study showed that there are two σ^{54} factors in Xoo, named RpoN1 and RpoN2, but whether they can regulate T4P genes was unknown (Tian *et al.*, 2015). In this study, we identified that RpoN2 regulates the expression of *pilAX* by interacting with PilRX (Figures 3 and 4). Interestingly, in contrast to the case in *Pseudomonas*, RpoN2-PilRX binds to the promoters of *pilCX* and *pilRX* and regulates their expression in Xoo (Figures 3 and 4). The low identity of T4P protein sequences (Table S1) also indicated the different regulatory relationships and biofunctions for σ^{54} factor and T4P genes between *Pseudomonas* and *Xanthomonas*. In addition, as shown in Figure 4, RpoN2-PilRX regulated the expression of the *pilGX/HX/IX*, *pilJX*, *pilMX/NX/OX/PX/QX*, and *fimTX-pilVX/WX/XX/YX/EX* operons in Xoo. A previous study showed that PilQX is required for Xoo twitching motility, biofilm formation, and virulence (Lim *et al.*, 2008; Dunger *et al.*, 2016). Homologous proteins that play roles in twitching motility and virulence in other bacteria have also been studied. For example, the PilG/H/L/J proteins are required for twitching signal transduction in *Acinetobacter baylyi* (Leong *et al.*, 2017), and PilG is involved in T4P-mediated transformation by interacting with PilQ and target DNAs in *Neisseria meningitidis* (Collins *et al.*, 2007; Frye *et al.*, 2015). PilM/N/O/P proteins form an inner membrane

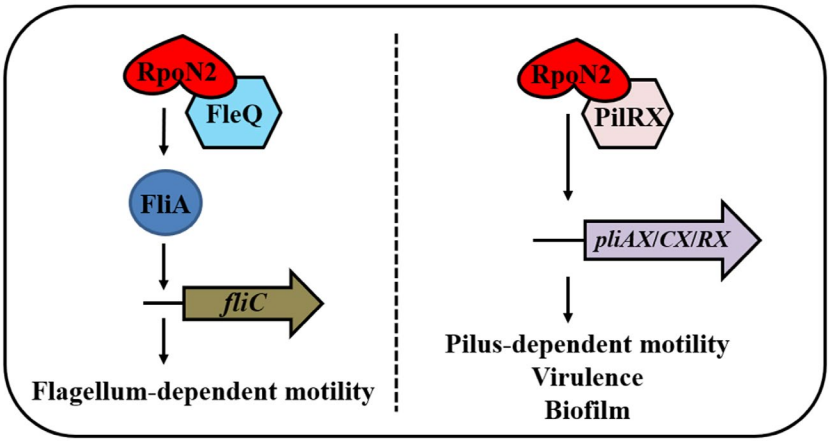

FIGURE 8 A simple model indicates the regulation of flagella and pili by RpoN2 in *Xanthomonas oryzae* pv. *oryzae*. Left, RpoN2 interacts with FleQ to regulate FliA (σ^{28} factor), thus controlling flagellum-dependent motility by regulating the expression of *fliC*, the flagellin gene. Right, RpoN2 interacts with PilRX to regulate the expression of T4P genes, thus controlling pilus-dependent motility, virulence and biofilm

complex that affects the stability of the *P. aeruginosa* T4P secretin (Ayers *et al*., 2009). Minor pilins encoded by *fimTX-pilVX/WX/XX/EX* and a nonpilin protein encoded by *pilYX* are required for T4P biogenesis and therefore T4P-mediated motility and virulence (Russell and Darzins, 1994; Alm *et al*., 1996; Alm and Mattick, 1996). Interestingly, an increasing number of studies have shown that these proteins have roles in virulence independent on functional pili (Bohn *et al*., 2009; Feinbaum *et al*., 2012; Marko *et al*., 2018). These results indicate that RpoN2-PilRX plays an important role in pilus-dependent or pilus-independent biological functions by directly or indirectly regulating T4P genes in Xoo.

Swimming and twitching are two forms of motility for bacteria: one is a flagellum-dependent movement in low-viscosity media, and the other is a pilus-dependent movement across solid and semisolid surfaces (Dasgupta *et al*., 2003; Burrows, 2012). In *P. aeruginosa*, swimming motility is controlled through a regulatory cascade that includes the σ^{54} factor RpoN, the transcriptional regulator FleQ, and the FleS-FleR TCS, while twitching motility is controlled by another TCS, PilR-PilS, that regulates the expression of major pilin gene *pilA* (Hobbs *et al*., 1993). Interestingly, a recent study showed that PilR-PilS controls the expression of some flagellum-related genes and affects swimming motility by positively regulating the transcription of *fleSR*, indicating the overlapping regulatory pathways between swimming and twitching motility in *P. aeruginosa* (Kilmury and Burrows, 2018). In our previous work, biosynthesis of the single polar flagellum was regulated by a hierarchical system that includes RpoN2 and FleQ (Tian *et al*., 2015). In this study, expression of T4P genes was regulated by RpoN2 (Figures 3 and 4), and swimming and twitching motilities were also reduced in the *rpoN2* and some T4P gene deletion mutants (Figure 5). Therefore, our results also support an overlapping regulatory function of RpoN2 for controlling the flagellum- and pilus-dependent motilities in Xoo.

Deletion mutant of flagellin (*fliC*) exhibited a significant defect in swimming motility and virulence in Xoo by dip inoculation, but showed similar virulence to wild-type by wound inoculation (Tian *et al*., 2015; Kumar Verma *et al*., 2018), indicating that orientational motility mediated by flagella contributes to Xoo entry inside rice leaves, but is not involved in the regulation of other virulence-associated functions. Interestingly, deletion of T4P genes (*pilAX*, *pilCX*, and *pilRX*) remarkably reduced Xoo twitching motility (Figure 5) and virulence by clipping inoculation (Figure 6), suggesting that T4Ps control not only pilus-dependent motility but also virulence-associated functions in Xoo. As an important virulence factor, biofilm has been studied in several *Xanthomonas* species. For example, deletion of *pilA* and *pilQ* significantly decreased biofilm formation and virulence of *Xanthomonas citri* subsp. *citri* and Xoo to rice, respectively (Lim *et al*., 2008; Dunger *et al*., 2014, 2016). In this study, we also demonstrated that PilAX, PilCX, and PilRX positively regulated biofilm formation and virulence in Xoo (Figures 6 and 7). These results suggest that T4Ps regulates Xoo virulence by controlling biofilm formation. However, deletion of *pilZX* (*PXO_02715*), which encodes a type IV fimbriae assembly protein, remarkably reduced virulence but did not affect biofilm formation (Yang *et al*., 2014), indicating some unknown virulence-associated factors regulated by T4P in Xoo.

Although the structure of pili has been studied in many phytopathogenic bacteria, there have been few reports of direct observation of bacterial pili, except in *Xylella fastidiosa*, in which an abundance of short pili and few long T4Ps were observed by transmission electron microscopy (TEM) (Meng *et al*., 2005; Cursino *et al*., 2011). Surprisingly, T4Ps were observed in *X. campestris* via immunoelectron microscopy (Ojanen-Reuhs *et al*., 1997), and extracellular pilin subunits on *X. citri* colonies were detected by inverted fluorescence microscopy (Dunger *et al*., 2014), but the direct observation of T4Ps in *Xanthomonas* by TEM has never been reported. In this study, we tried to observe the pili of the wild-type Xoo strain by TEM, but unfortunately we observed only the flagella on the surface of cells, and no pili were observed (Figure S5). Therefore, compared with those in *X. fastidiosa*, it is difficult to observe pili in *Xanthomonas* by TEM. How to effectively observe *Xanthomonas* pili needs further study in the future.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Xoo strains were grown in M210 liquid medium (0.8% casein

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5α	*supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
BL21	For protein expression	Novagen
Saccharomyces cerevisiae		
Y2HGold	*MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, Δgal4, Δgal80, LYS2::GAL1$_{UAS}$-Gal1$_{TATA}$-His3, GAL2$_{UAS}$-Gal2$_{TATA}$-Ade2, URA3::MEL1$_{UAS}$-Mel1$_{TATA}$, AUR1-C MEL1*	Clontech
Y187	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, Δgal4, Δgal80, met–URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ, MEL1*	Clontech
Xanthomonas oryzae pv. *oryzae*		
PXO99^{A}	Wild-type strain, Philippine race 6	Laboratory collection
Δ*rpoN2*	*rpoN2* gene deletion mutant derived from PXO99^{A}	Our laboratory
Δ*pilRX*	*pilRX* gene deletion mutant derived from PXO99^{A}	This study
Δ*pilAX*	*pilAX* gene deletion mutant derived from PXO99^{A}	This study
Δ*pilCX*	*pilCX* gene deletion mutant derived from PXO99^{A}	This study
Δ*rpoN2*-C	Δ*rpoN2* containing plasmid pBBR-*rpoN2*, Ap^{r}	Our laboratory
Δ*pilRX*-C	Δ*pilRX* containing plasmid pBBR-*pilRX*, Ap^{r}	This study
Δ*pilAX*-C	Δ*pilAX* containing plasmid pBBR-*pilAX*, Ap^{r}	This study
Δ*pilCX*-C	Δ*pilCX* containing plasmid pBBR-*pilCX*, Ap^{r}	This study
Plasmid		
pGBKT7	Yeast two-hybrid vector to create fusion protein containing GAL4 DNA-binding domain, Km^{r}	Clontech
pGADT7	Yeast-two hybrid vector to create fusion protein containing the GAL4 activation domain, Ap^{r}	Clontech
pKMS1	Suicide vector carrying *sacB* gene for non-marker mutagenesis, Km^{r}	Li *et al.* (2011)
pKM-*pilRX*	pKMS1 derivative carrying a *pilRX* mutation, Km^{r}	This study
pKM-*pilAX*	pKMS1 derivative carrying a *pilAX* mutation, Km^{r}	This study
pKM-*pilCX*	pKMS1 derivative carrying a *pilCX* mutation, Km^{r}	This study
pBBR1MCS-4	Broad-host range expression vector, Ap^{r}	Kovach *et al.* (1995)
pBBR-*pilRX*	pBBR1MCS-4 carrying the full length of *pilRX*, Ap^{r}	This study
pBBR-*pilAX*	pBBR1MCS-4 carrying the full length of *pilAX*, Ap^{r}	This study
pBBR-*pilCX*	pBBR1MCS-4 carrying the full length of *pilCX*, Ap^{r}	This study
pHM1	Broad-host range expression vector, Sp^{r}	Hopkins *et al.* (1992)
pH-*lacZ*	pHM1 derivative carrying the promoterless *lacZ*, Sp^{r}	This study
pH-*pilRXp-lacZ*	pHM1 derivative carrying the promoter region of *pilRX* and promoterless *lacZ*, Sp^{r}	This study
pH-*pilAXp-lacZ*	pHM1 derivative carrying the promoter region of *pilAX* and promoterless *lacZ*, Sp^{r}	This study
pH-*pilCXp-lacZ*	pHM1 derivative carrying the promoter region of *pilCX* and promoterless *lacZ*, Sp^{r}	This study
pColdSUMO	Expression vector to generate an N-terminal SUMO-His$_{6}$ tag, Ap^{r}	Haigene
pCRpoN2	pColdSUMO carrying the coding sequence for RpoN2, Ap^{r}	This study
pCPilSX	pColdSUMO carrying the coding sequence for PilSX, Ap^{r}	This study
pGEX-6P-1	Expression vector to generate an N-terminal GST tag, Ap^{r}	GE Healthcare
pGPilRX	pGEX-6P-1 carrying the coding sequence for PilRX, Ap^{r}	This study
pGADRpoN2	pGADT7 carrying the full length of *rpoN2*, Km^{r}	This study
pGADPilRX	pGADT7 carrying the full length of *pilRX*, Km^{r}	This study
pGADPilSX	pGADT7 carrying the full length of *pilSX*, Km^{r}	This study
pGBKRpoN2	pGBKT7 carrying the full length of *rpoN2*, Km^{r}	This study
pGBKPilRX	pGBKT7 carrying the full length of *pilRX*, Km^{r}	This study
pGBKPilSX	pGBKT7 carrying the full length of *pilSX*, Km^{r}	This study

[a]Ap^{r}, Km^{r}, and Sp^{r} indicate resistant to ampicillin, kanamycin, and spectinomycin, respectively.

enzymatic hydrolysate, 0.5% sucrose, 0.4% yeast extract, 17.2 mM K_2HPO_4, 1.2 mM $MgSO_4.H_2O$, pH 6.5) or on PSA solid medium (1% peptone, 1% sucrose, 0.1% glutamate, pH 7.0) at 28 °C, and *Escherichia coli* strains were cultured in Luria Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.0) at 37 °C. The antibiotics ampicillin (Ap), spectinomycin (Sp), and kanamycin (Km) were used at 100, 100, and 50 μg/ml, respectively.

4.2 | Bioinformatic analysis

To identify new EBPs of RpoN2, the protein sequence of FleQ, a known EBP in our previous study, was downloaded from NCBI, then paralogs of FleQ were tested using BLAST in Xoo genome by NCBI BLAST, and candidate EBPs with total score more than 150 were chosen. The conserved domains of these proteins were analysed by the simple modular architecture research tool (SMART) (Letunic and Bork, 2018).

To study T4P genes in Xoo, 25 related genes (from *pilA* to *pilZ*) were searched on NCBI, and the sequences of these proteins were downloaded. Then the identity of these proteins in Xoo $PXO99^A$, *X. oryzae* pv. *oryzicola* BLS256, and *P. syringae* pv. *tomato* DC3000 were analysed by DNAMAN software (Lynnon Corp.).

To analysis the conserved binding sites of RpoN2, the promoter DNA regions of T4P transcriptional units (−300 to +50 upstream or downstream of the translation start [+1]) in Xoo genome were downloaded from NCBI, then the conserved sequence $GGN_{10}GC$ was searched on these promoter regions, and the sequence logo was made by WebLogo3 (http://weblogo.threeplusone.com/create.cgi) (Schneider and Stephens, 1990).

4.3 | Y2H assay

The Y2H assay was carried out using the Matchmaker Gold Y2H system (Clontech) as described, with minor modifications. The full-length sequence of *rpoN2*, *pilRX*, and *pilSX* was amplified using primer pairs Rpon2YF/RpoN2YR, PilRXYF/PilRXYR, and PilSXYF/PilSXYR, respectively. PCR products were cloned into pEASY (Transgen Biotech) for sequencing. The fragment was then digested and subcloned into the prey vector pGADT7 or the bait vector pGBKT7, resulting in pGADRpoN2, pGBKRpoN2, pGADPilRX, pGBKPilRX, pGADPilSX, and pGBKPilSX. The pGADT7-derived and pGBKT7-derived constructs were transformed into the *S. cerevisiae* Y187 and Y2HGold strains, respectively. Expression of fusion proteins in yeast strains was confirmed by western blotting using anti-cMyc primary antibodies for pGBKT7-derived constructs and anti-HA primary antibodies (Beijing Protein Institute) for pGADT7-derived constructs. Pairwise yeast mating was performed on yeast potato dextrose agar at 28 °C for 24 hr. The obtained diploids were selected on DDO plates (SD/−Leu/−Trp) and QDO plates (SD/−Leu/−Trp/−Ade/−His). Then, four 10-fold serial dilutions with sterile water were placed on QDO plates. The growth of these dilutions was checked after 3 days. A mating between the Y2HGold strain containing the empty pGBKT7 plasmid and Y187 containing pGADT7-derived constructs was performed as a negative control.

4.4 | Protein expression and purification

The DNA fragments of *rpoN2*, *pilRX*, and *pilSX* were amplified using specific primer pairs and these used primer pairs are listed in Table S2. PCR products were cloned into pEASY (Transgen Biotech) for sequencing. Fragments of *rpoN2*, *pilRX*, and *pilSX* were obtained by double enzyme digestion. Then, *rpoN2* and *pilSX* were subcloned into pColdSUMO, and *pilRX* was subcloned into pGEX-6P-1, resulting in pCRpoN2, pCPilSX, and pGPilRX, respectively. Finally, the plasmids were transformed into *E. coli* BL21 for protein expression. Protein purification was performed as described previously (Yang *et al.*, 2014). Briefly, the bacteria were cultured in LB liquid medium at 37 °C to an OD_{600} of 1.0, and isopropyl-thio-galactopyranoside was added at a final concentration of 0.3 mM. The bacterial cultures were then incubated at 16 °C for 12 hr and chilled to 4 °C. Then, the cells were collected by centrifugation at 12,000 × g for 10 min. The cell pellets were resuspended in phosphate-buffered saline (PBS), followed by sonication, and the soluble protein fractions were collected by centrifugation at 12,000 × g for 10 min and mixed with preequilibrated Ni2 + or GST resin (GE Healthcare) for 1 hr at 4 °C. The His-labelled proteins were extensively washed with buffer containing 20 mM Tris-HCl (pH 8.0), 350 mM NaCl, 0.5 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, and 30 mM imidazole, and subsequently eluted with buffer containing 300 mM imidazole. The GST-labelled protein was washed with PBS and subsequently eluted with washing buffer containing 10 mM glutathione and 50 mM Tris-HCl, pH 8.0. The fusion proteins were washed with PBS, collected, and then analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The SUMO-His6 tag expressed from the empty vector pColdSUMO and GST tag expressed from pGEX-6P-1 were purified using the same procedure as negative controls.

4.5 | GST pull-down assay

For the GST pull-down assay, purified RpoN2 was mixed with PilRX and then incubated in GST-binding columns for 4 hr at 4 °C in PBS. After washing with PBS four times, the proteins were eluted with washing buffer containing 10 mM glutathione and 50 mM Tris-HCl (pH 8.0), separated by SDS-PAGE, and then transferred to membranes for immunoblotting with either anti-His_6 or anti-GST primary antibodies (Huaxingbio). A goat antimouse antibody (Huaxingbio) conjugated with horseradish peroxidase was used as a secondary antibody and directly visualized by applying an Enhanced HRP-DAB Chromogenic Kit (Tiangen Biotech). The GST protein and SUMO-His_6 protein were used as negative controls, and PilSX was used as a positive control.

4.6 | RNA isolation and RT-PCR analysis

Bacterial strains were cultured in M210 medium at 28 °C to an OD_{600} of 1.0 and collected by centrifugation at 12,000 × g for 10 min. Then, the total RNA of these strains was isolated with an RNAprep pure Cell/Bacteria Kit (Tiangen Biotech) and treated with DNase (Tiangen Biotech). PCR was carried before reverse transcription action to make sure that genomic DNA had been removed completely. Then cDNA fragments were synthesized using a HiScript II RT SuperMix kit (Vazyme). The RT-PCR analysis was performed with primers designed using Primer Premier v. 5.0 software (PREMIER Biosoft) (Table S2). The genomic DNA of Xoo was used as a positive control.

4.7 | Electrophoretic mobility shift assay

The promoter DNA regions of pilus genes (−300 to +50 upstream or downstream of the translation start [+1]) were amplified by PCR using 5′ end FAM-labelled primers (Table S2). DNA binding was performed in a 10 μl reaction volume containing EMSA/GelShift Binding Buffer (Beyotime), 2 μM labelled DNA fragment, and 5 μM His_6-RpoN2 protein. The unlabelled gene promoter DNA region (20 μM) as a specific DNA competitor and bovine serum albumin (BSA, 5 μM) as a nonspecific protein competitor were used. After incubation at 25 °C for 30 min, the products were loaded onto a native 4% (wt/vol) polyacrylamide gel and electrophoresed in 0.5 × Tris-borate-EDTA (TBE) buffer for approximately 1.5 hr at 100 V. The fluorescence of samples was detected by a Typhoon FLA-5100 (Fuji film) at 488 nm.

4.8 | Generation of lacZ fusions and assay for β-galactosidase activity

DNA fragments (350 bp) of the *pilAX*, *pilCX*, and *pilRX* promoters were amplified from $PXO99^A$ genomic DNA using specific primers (Table S2) and ligated into a modified pHM1 vector containing a promoterless *lacZ* gene. The recombinant pHM1-P was introduced into $PXO99^A$, Δ*rpoN2*, and a complemented strain. The strains transformed with pHM1-P were selected by resistance to spectinomycin. These strains were grown as described above to measure the β-galactosidase activity in cellular extracts using a β-Galactosidase Enzyme Assay System (Promega). Assays were performed with three biological replicates.

4.9 | RT-qPCR assays

Bacterial strains were collected after growing on PSA at 28 °C for 24 hr. Total RNA isolation, DNase treatment, and cDNA synthesis were performed as described above. The gene-specific primers were designed using Primer Premier v. 5.0 software (PREMIER Biosoft) (Table S2), and the *gyrB* gene was used as a reference gene. RT-qPCR was performed using AceQ qPCR SYBR Green Master Mix (Vazyme) in a 7500 Sequence Detection System (Applied Biosystems). Following the manufacturer's instructions, cycling conditions were 95 °C, 5 min, and 40 cycles of 95 °C, 10 s 60 °C and 30 s. Then the raw data were downloaded and relative expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and three biological replicates and triplicate PCRs were tested for every sample.

4.10 | Construction of gene deletion mutants and complemented strains

The gene deletion mutants of T4P genes derived from $PXO99^A$ were generated by homologous recombination using the suicide vector pKMS1, as reported previously (Yu *et al*., 2018). In brief, the left and right arms of *pilAX*, *pilCX*, and *pilRX* were amplified by PCR from Xoo genomic DNA with the relevant F/R primers (Table S2). These fragments were ligated into suicide vector pKMS1, resulting in plasmids pKM-*pilAX*, pKM-*pilCX*, and pKM-*pilRX*. Then, pKM-*pilAX*, pKM-*pilCX*, and pKM-*pilRX* were introduced into $PXO99^A$ by electroporation. The transformants were first selected on NAN (nutrient agar without sucrose) medium (1% tryptone, 0.1% yeast extract, 0.3% peptone, and 1.5% agar) containing, followed by continuous transfer culture in NBN (nutrient broth without sucrose) medium three times. The candidates were screened on NAS (nutrient agar with 10% sucrose). The gene deletion mutant that could grow on NAS, but was sensitive to Km, was validated by PCR analysis. To generate the complemented strains, the coding region and promoter of *pilAX*, *pilCX*, and *pilRX* were amplified with the relevant F/R primers (Table S2) and inserted into vector pBBR1MCS-4, and the recombinant plasmids were electroporated into the relevant mutants for complementation analysis. Finally, these complemented strains were further confirmed by PCR analysis.

4.11 | Motility assays

A twitching motility assay was performed as described previously, with some modifications (Dunger *et al*., 2014). Briefly, Xoo strains were cultured on PSA at 28 °C for 24 hr, and then the freshly grown bacteria were collected with a sterile toothpick and stabbed through a new PSA plate to the plastic surface. After growth at 28 °C for 4 days, the agar was removed, and the Petri plate was stained with 0.1% (wt/vol) crystal violet (CV) at room temperature for 15 min. Then, the unbound dye was removed by rinsing three times with distilled water. Finally, the zone of twitching motility was photographed and recorded. The experiments were repeated independently three times.

For the swimming motility assay, the Xoo strains were grown in M210 medium at 28 °C to an OD_{600} of 0.8. The cells were harvested by centrifugation at 12,000 × g for 10 min and resuspended in an equal volume of distilled water, and then 2 μl of the bacterial suspension was inoculated onto semisolid medium plates (0.03% peptone, 0.03%

yeast extract, and 0.25% agar) and incubated at 28 °C for 4 days. The diameters of the bacterial swimming zones were photographed and measured. The experiments were repeated independently three times.

4.12 | Pathogenicity assay

The Xoo strains were grown in M210 medium at 28 °C to OD_{600} of 1.0, and the cells were harvested by centrifugation at 7,000 × g for 10 min and resuspended in equal volume of distilled water. The virulence of these strains was detected on susceptible rice (*Oryza sativa* subsp. *indica* "IR24") by leaf clipping, and the lesion lengths of 10 leaves were measured at 14 days after inoculation for every strain. For detection of bacterial population, three inoculated leaves were ground in distilled water with a mortar and pestle, and the mixture was diluted and spread onto PSA plates. The colonies of bacteria were counted after incubation at 28 °C for 72 hr. The experiments were repeated three times, independently.

4.13 | In vitro growth rate measurement

Xoo strains were growth in M210 medium overnight at 28 °C and transferred into 100 ml of fresh M210 medium. The concentrations of bacteria were adjusted to OD_{600} = 0.05. Then these strains were cultured at 28 °C for 200 rpm, and the growth curves were obtained by testing OD_{600} every 6 hr. The experiments were repeated three times, independently.

4.14 | Biofilm formation assay

The biofilm formation assay was performed as described previously (Yu *et al*., 2018). Briefly, bacterial strains were grown in M210 medium at 28 °C to OD_{600} of 0.5, and then 5 ml of bacteria was transferred to tubes and standing inoculated at 28 °C for 72 hr. The tubes were stained with 15 min at room temperature by adding 0.1% crystal violet and photographed after rinsing gently for three times with distilled water. Finally, the biofilm was dissolved with ethanol and the absorbance readings at 490 nm recorded. The experiments were repeated three times, independently.

4.15 | Statistical analysis

Motility zones, disease lesion length, bacterial population measurement, and relative gene expression were presented as means ± *SD*. Student's *t* test was performed with statistical significance set at the 0.05 confidence level.

ACKNOWLEDGMENTS

We thank our colleague Dr Chenyang He (Institute of Plant Protection, Chinese Academy of Agricultural Sciences) for his suggestions and revision of the manuscript. This work was supported by grants from the National Science Foundation of China (31600105) to C. Yu and the National Science Foundation of China (31870120 and 31972255) to C. He and H. Chen, respectively.

CONFLICT OF INTEREST

All authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

FIGURE S1 Conserved domain analysis of candidate EBPs in Xoo. The sequence of these candidate EBPs were download from NCBI website (https://www.ncbi.nlm.nih.gov/), and conserved domain was analysed by SMART (http://smart.embl.de/smart/set_mode.cgi?NORMAL=1)

FIGURE S2 Purified proteins of RpoN2, PilRX, PilSX and GST were detected by SDS-PAGE. 1, Marker. 2, SUMOHis$_6$-RpoN2. 3, GST-PilRX. 4, SUMOHis$_6$-PilSX. 5, GST tag. Protein size were labelled on the left of photo

FIGURE S3 Interactions between RpoN2 and pilus gene promoters were tested by EMSA. Purified RpoN2 at 5 μM was incubated with 2 μM probe (FAM-labelled pilus gene promoters DNA regions) at 25 °C for 30 min, and the products were run a native 4% (wt/vol) polyacrylamide gel in 0.5 × TBE buffer for about 1.5 hr at 100 V

FIGURE S4 Bacterial growth in vitro of Xoo strains. Bacterial growth in M210 medium was determined by measuring the optical density at 600 nm (OD_{600}) at 6-hr intervals. The experiments were repeated three times, independently. Error bars represent standard deviations

FIGURE S5 T4Ps were observed by transmission electron microscopy. The wild-type strain of Xoo (A and B) was grown on PSA medium at 28 °C for 48 hr, and suspended in ddH_2O. Then, the suspension was deposited onto grids coated with Formvar (Standard Technology), stained with 2% uranyl acetate for 30 s and dried for 10 min at room temperature. The pili were observed using a transmission electron microscope (H-7500, Hitachi)

TABLE S1 Similarities of T4P genes in *Xanthomonas oryzae* pv. *oryzae* PXO99^A, *X. oryzae* pv. *oryzicola* BLS256 and *Pseudomonas syringae* pv. *tomato* DC3000

TABLE S2 Primers used in this study

How to cite this article: Yu C, Nguyen D-P, Ren Z, et al. The RpoN2-PilRX regulatory system governs type IV pilus gene transcription and is required for bacterial motility and virulence in *Xanthomonas oryzae* pv. *oryzae*.
Molecular Plant Pathology. 2020;21:652–666.
https://doi.org/10.1111/mpp.12920