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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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 σ54 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 GGN10GC 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
σ54, motility, PilRX, type IV pilus, virulence, Xanthomonas oryzae pv. oryzae
1 | INTRODUCTION

In bacteria, the σ54 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 σ54 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 σ54 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 the σ54 factor, it is important to identify the downstream genes of the σ54 factor and to further clarify its regulatory pathways.

Unlike other σ factors, the regulatory function of σ54 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 σ54 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 σ54 factor regulates gene transcription by interacting with EBPs. First, σ54 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 σ54 factor via DNA looping. Finally, ATP hydrolysis by the AAA+ domain results in opening of the σ54–RNAP complex, and then target gene transcription is activated (Schumacher et al., 2006). Therefore, it is necessary to identify EBPs for analysis of σ54 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 (Kimury 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 flfC 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, pGAD-PilSX, 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-His6-RpoN2, SUMO-His6-PilRX, GST tag only, and SUMO-His6 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-His6-RpoN2 and SUMO-His6-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).
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 σ54 factor, specifically binds to the highly conserved sequence GGN_{10}GC at positions −24/−12 relative to the transcription start sites of T4P genes. To investigate whether RpoN2 regulated the transcription of pilRX, pilAX, and pilCX directly, we constructed various mutants of RpoN2 and tested their promoter activity by reporter assays and reverse transcription PCR (RT-PCR). The results showed that RpoN2 specifically regulated the transcription of these genes in Xoo strains.
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 ΔrpoN2 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, ΔrpoN2, and its complemented strain. The results show that the expression levels of pilAX, pilCX, pilFX, pilGX, pilJX, pilMX, pilRX, and pilTX are remarkably reduced in ΔrpoN2 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 ΔpilRX. As we predicted, most of these genes were significantly down-regulated in ΔpilRX and restored in the complemented strain, except pilFX, which was down-regulated in ΔrpoN2 but not remarkably changed in ΔpilRX (Figure 4). In addition,
the expression of pilSX was not changed in ΔrpoN2 but was reduced in ΔpilRX (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 ΔrpoN2 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 ΔrpoN2, ΔpilRX, ΔpilAX, and ΔpilCX 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 ΔpilRX, ΔpilAX, and ΔpilCX 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 ΔpilRX, ΔpilAX, and ΔpilCX 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 ΔpilRX, ΔpilAX, and ΔpilCX 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, ΔpilRX, ΔpilAX, and ΔpilCX 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 ΔrpoN2, ΔpilRX, ΔpilAX, and ΔpilCX 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
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 $\sigma^{54}$ 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 $\sigma^{54}$ factors. Typically, EBPs have an N-terminal regulatory domain, a central A$\alpha^*$ domain that directly contacts $\sigma^{54}$, 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 A$\alpha^*$ 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 ($\sigma^{28}$ 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.
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 $\sigma^{54}$ 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 $\sigma^{54}$ factor and T4P genes between *Pseudomonas* and *Xanthomonas*. In addition, as shown in Figure 4, RpoN2-PilRX regulates the expression of 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 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 $\sigma^{54}$ 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 $\sigma^{54}$ factor and T4P genes between *Pseudomonas* and *Xanthomonas*. In addition, as shown in Figure 4, RpoN2-PilRX regulates the expression of 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
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 $\sigma^{24}$ 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 (*pilA*, *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 regulate Xoo virulence by controlling biofilm formation. However, deletion of pilRX (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 pil 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* pilus needs further study in the future.

**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 ($\sigma^{28}$ 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.
| Strain or plasmid | Relevant characteristics | 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, Δgal4, Δgal80, LYS2::GAL1UAS-Gal1TATA-His3, GAL2UAS-Gal2TATA-Ade2, URA3::MEL1UAS-Mel1TATA, AUR1-C MEL1 | Clontech           |
| Y187             | MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, Δgal4, Δgal80, met-URA3::GAL1UAS-GAL1TATA-lacZ, MEL1 | Clontech           |
| **Xanthomonas oryzae pv. oryzae** |                    |                     |
| PXO99A           | Wild-type strain, Philippine race 6 | Laboratory collection |
| ΔploN2           |  ΔploN2 gene deletion mutant derived from PXO99A | Our laboratory       |
| ΔpilRX           | ΔpilRX gene deletion mutant derived from PXO99A | This study           |
| ΔpilAX           | ΔpilAX gene deletion mutant derived from PXO99A | This study           |
| ΔpilCX           | ΔpilCX gene deletion mutant derived from PXO99A | This study           |
| ΔploN2-C         | ΔploN2 containing plasmid pBBR-ΔploN2, Ap′ | Our laboratory       |
| ΔpilRX-C         | ΔpilRX containing plasmid pBBR-ΔpilRX, Ap′ | This study           |
| ΔpilAX-C         | ΔpilAX containing plasmid pBBR-ΔpilAX, Ap′ | This study           |
| ΔpilCX-C         | ΔpilCX containing plasmid pBBR-ΔpilCX, Ap′ | This study           |
| **Plasmid**      |                          |                     |
| pGBK77           | Yeast two-hybrid vector to create fusion protein containing GAL4 DNA-binding domain, Km′ | Clontech           |
| pGADT7           | Yeast two-hybrid vector to create fusion protein containing the GAL4 activation domain, Ap′ | Clontech           |
| pKMS1            | Suicide vector carrying sacB gene for non-marker mutagenesis, Km′ | Li et al. (2011)   |
| pKM-ΔpilRX       | pKMS1 derivative carrying a pilRX mutation, Km′ | This study           |
| pKM-ΔpilAX       | pKMS1 derivative carrying a pilAX mutation, Km′ | This study           |
| pKM-ΔpilCX       | pKMS1 derivative carrying a pilCX mutation, Km′ | This study           |
| pBRR1MCS-4       | Broad-host range expression vector, Ap′ | This study           |
| pBRR-ΔpilRX      | pBRR1MCS-4 carrying the full length of pilRX, Ap′ | This study           |
| pBRR-ΔpilAX      | pBRR1MCS-4 carrying the full length of pilAX, Ap′ | This study           |
| pBRR-ΔpilCX      | pBRR1MCS-4 carrying the full length of pilCX, Ap′ | This study           |
| pHM1             | Broad-host range expression vector, Sp′ | Hopkins et al. (1992) |
| pH-ΔlacZ         | pHM1 derivative carrying the promoterless lacZ, Sp′ | This study           |
| pH-ΔpilRx-ΔlacZ  | pHM1 derivative carrying the promoter region of pilRX and promoterless lacZ, Sp′ | This study           |
| pH-ΔpilAx-ΔlacZ  | pHM1 derivative carrying the promoter region of pilAX and promoterless lacZ, Sp′ | This study           |
| pH-ΔpilCp-ΔlacZ  | pHM1 derivative carrying the promoter region of pilCX and promoterless lacZ, Sp′ | This study           |
| pColdSUMO        | Expression vector to generate an N-terminal SUMO-His6 tag, Ap′ | Haigene             |
| pCRpoN2          | pColdSUMO carrying the coding sequence for RpoN2, Ap′ | This study           |
| pCPIpilSX        | pColdSUMO carrying the coding sequence for PilSX, Ap′ | This study           |
| pGEX-6P-1        | Expression vector to generate an N-terminal GST tag, Ap′ | GE Healthcare       |
| pGPIpilRX        | pGEX-6P-1 carrying the coding sequence for PilRX, Ap′ | This study           |
| pGADRpoN2        | pGADT7 carrying the full length of rpoN2, Km′ | This study           |
| pGADpilRX        | pGADT7 carrying the full length of pilRX, Km′ | This study           |
| pGADpilSX        | pGADT7 carrying the full length of pilSX, Km′ | This study           |
| pGBKpilN2        | pGBK77 carrying the full length of rpoN2, Km′ | This study           |
| pGBKpilRX        | pGBK77 carrying the full length of pilRX, Km′ | This study           |
| pGBKpilSX        | pGBK77 carrying the full length of pilSX, Km′ | This study           |

*Ap′, Km′, and Sp′ indicate resistant to ampicillin, kanamycin, and spectinomycin, respectively.*
enzymatic hydrolysate, 0.5% sucrose, 0.4% yeast extract, 17.2 mM K$_2$HPO$_4$, 1.2 mM MgSO$_4$, 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 PXO99A, 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$_{down}$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 pair Rpon2YF/RpoN2YR, PIIRXYF/PIIRXYR, and PIISXYF/PIISXYR, 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 pGBKTK7, resulting in pGADR-poN2, pGBKpilRX, pGBKpilRX, pGBPilSX, and pGBPilSX. The pGADT7-derived and pGBKTK7-derived constructs were transformed into the S. cerevisiae Y187 and Y2HGolGold strains, respectively. Expression of fusion proteins in yeast strains was confirmed by western blotting using anti-cMyc primary antibodies for pGBKTK7-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 pGBKTK7 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 pilRX were subcloned into pColdSUMO, and pilRX was subcloned into pGEX-6P-1, resulting in pCRpN2, pCPI5SX, and pGPiRX, 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 x 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 x g for 10 min and mixed with preequilibrated Ni$_2$ + 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-His$_6$ 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 PIIRX 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 PIISX 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 re-
duced 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 soft-
ware (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/Gel-
Shift 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 PXO99A 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 PXO99A ΔpoN2, and a complemented strain. The strains trans-
formed with pHM1-P were selected by resistance to spectinomy-
cin. 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−ΔΔCt method (Livak and Schmittgen, 2001), and three biological rep-
licates 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 PXO99A
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 PXO99A by elec-
troporation. The transformants were first selected on NAN (nutri-
tient 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 gen-
erate 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 recom-
binant 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 cul-
tured 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) crys-
tal 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 ex-
periments 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 grown 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.

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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, SUMOHis6-RpoN2. 3, GST-PilRX. 4, SUMOHis6-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 x 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 (OD₆₀₀) 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 ddH₂O. 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 PXO99A, X. oryzae pv. oryzicola BLS256 and Pseudomonas syringae pv. tomato DC3000

TABLE S2 Primers used in this study

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