RpoN2- and FliA-regulated fliTX is indispensible for flagellar motility and virulence in Xanthomonas oryzae pv. oryzae

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Abstract

Background: Bacterial blight of rice caused by Xanthomonas oryzae pv. oryzae (Xoo) is one of the most important crop diseases in the world. More insights into the mechanistic regulation of bacterial pathogenesis will help us identify novel molecular targets for developing effective disease control strategies. A large flagellar gene cluster is regulated under a three-tiered hierarchy by σ^{54} factor RpoN2 and its activator FleQ, and σ^{28} factor FliA. A hypothetical protein gene fliTX is located upstream of rpoN2, however, how it is regulated and how it is related to bacterial behaviors remain to be elucidated.

Results: Sequence alignment analysis indicated that FliTX in Xoo is less well conserved compared with FliT proteins in Escherichia coli, Salmonella typhimurium, and Pseudomonas fluorescens. Co-transcription of fliTX with a cytosolic chaperone gene fliS and an atypical PilZ-domain gene flgZ in an operon was up-regulated by RpoN2/FleQ and FliA. Significantly shorter filament length and impaired swimming motility were observed in ΔfliTX compared with those in the wildtype strain. ΔfliTX also demonstrated reduced disease lesion length and in planta growth in rice, attenuated ability of induction of hypersensitive response (HR) in nonhost tobacco, and down-regulation of type III secretion system (T3SS)-related genes. In trans expression of fliTX gene in ΔfliTX restored these phenotypes to near wild-type levels.

Conclusions: This study demonstrates that RpoN2- and FliA-regulated fliTX is indispensible for flagellar motility and virulence and provides more insights into mechanistic regulation of T3SS expression in Xoo.

Keywords: Xanthomonas oryzae pv. oryzae, Flagellar motility, Pathogenicity, Induction of hypersensitive response, T3SS

Background

Bacterial leaf blight caused by Xanthomonas oryzae pv. oryzae (Xoo) is a major bacterial disease of rice in Asian countries, which can lead to 20%–50% yield loss in rice production [1]. Xoo has been used as a model pathogen to study the molecular mechanism of bacterial pathogenesis in monocotyledonous plants [2, 3]. Now, we have learned that Xoo produces multiple virulence factors, such as exopolysaccharide (EPS), extracellular enzymes, adhesins, and the type III secretion system (T3SS) and its effectors [1, 4, 5]. HrpG and HrpX are the two master regulators to control the expression of hrp genes and type III effector genes [6]. Moreover, some other regulators controlling the expression of these virulence factors have also been identified [6–8]. One of the important regulators is alternative sigma factor σ^{54} encoded by rpoN2 [9, 10]. Deletion of rpoN2 significantly reduces virulence and flagellar motility, yet how exactly RpoN2 regulates these virulence phenotypes in Xoo remains unknown [10].

The flagellum is the main motor organ in bacteria, which helps bacteria move toward favorable conditions and become infectious [11–13]. The flagellum consists of three parts, the basal body, the hook, and the filament. The regulatory network of flagellar gene transcription is quite complicated and fascinating. In Escherichia coli and Salmonella typhimurium, over 650 genes involved in flagellum assembly are organized into a hierarchy of three classes [14–16]. The FlhDC encoded by the class I gene flhDC is the master regulator and controls the transcription of class II genes [17]. The class II genes products include most of flagellum structural components and alternative sigma factor FliA.
(σ28). FliA regulates the transcription of class III genes, which encode the hook-associated proteins FlgK and FlgL, the anti-σ28 factor FlgM, the flagellar cap FliD, the flagellin FliC and other proteins involving in chemosensory signal transduction [18, 19]. The flagellar gene cluster of Pseudomonas aeruginosa has a four-tiered hierarchy of transcriptional regulation. Class I genes encode the σ54 factor RpoN and σ28-dependent transcriptional activator FleQ. Class II genes include the two-component system fleSR and the σ28 factor fliA. The transcription of fleSR and fliA are regulated by RpoN and FleQ. Class III genes are regulated by FleR and are necessary for completion of the basal-body hook structure. Class IV genes are transcribed by FliA and encode the flagellin and some chemotaxis proteins [20, 21].

FliT is a key chaperone in the flagellar assembly and operation, which interacts with several flagellar proteins, including the filament-cap FliD, the export apparatus components FliI (ATPase), FliJ and FlhA, and the master regulator FlhDC [22–27]. FliT binds to the cognate substrates to not only prevent them from degradation and aggregation in the cytoplasm, but also efficiently transfer them to the export apparatus [28]. The structural analysis has showed that FliT adopts an auto-inhibited conformation, in which both the substrate- and FlhA-binding sites are occluded. Formation of FliT-substrate complex activates its binding to FlhA and thus targeting of the complex to the export gate [29]. In addition, FliT acts as a negative regulator of flagellar regulon and inhibits the binding of FlhDC to the promoter DNA [27, 30]. Interestingly, deletion of fliT does not affect the swimming ability in S. typhimurium, but significantly reduces motility properties in P. fluorescens [23, 31]. Moreover, disruption of fliT induces the expression of Salmonella pathogenicity island 1 (SPI1) genes, implying the potential role of FliT in bacterial virulence [32].

Our previous study has showed that over 60 contiguous flagellar genes forming a large gene cluster in Xoo PXO99A encode proteins with various functions, including structural components, protein export apparatus, regulatory factors, post-translational modification enzymes, and chemotaxis proteins [10]. These genes were tightly regulated under a three-tiered hierarchy by σ54 factor RpoN2, and transcriptional activator FleQ, and σ28 factor FliA. Interestingly, a hypothetical protein gene PXO_06168, named as fliTX, has been revealed upstream of rpoN2 and downstream of fliS, which is very similar location of the fliT genes in the genome of S. typhimurium and P. fluorescens. However, how fliTX is regulated and how it is related to bacterial behaviors, such as flagellar motility and virulence, remain to be elucidated.

In this study, we characterized the regulation and biological functions of fliTX. Promoter activities and quantitative real-time polymerase chain reaction (qRT-PCR) assays demonstrated that the transcription of fliTX was up-regulated by RpoN2, FleQ and FliA. In frame deletion of fliTX led to significant changes in flagellar motility, pathogenicity on rice, hypersensitivity on tobacco, and T3SS-related gene transcription, suggesting that FliTX plays key roles in controlling flagellar motility and virulence in Xoo.

**Results**

**Identification, deletion and complementation of fliTX**

Our previous study showed that there is a flagellar regulon containing over 60 contiguous genes in the genome of Xoo strain PXO99A, which are regulated by RpoN2 and FleQ [10]. Upstream of rpoN2, there were five genes encoding a filament cap protein FlID (PXO_06166), a cytolsic chaperone FlIS (PXO_06167), a hypothetical protein FliTX (PXO_06168), a non-canonical PilZ-domain protein FlgZ (PXO_06169), and a DNA-binding response regulator (PXO_06170) (Fig. 1a). The intergenic distances of neighboring genes are 150 bp, 9 bp, –1 bp, and 71 bp, respectively. Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed to determine whether these five genes form an operon. The fragments containing junctions of fliS-fliTX and fliTX-flgZ were obtained using the Xoo cDNA as the template (Fig. 1a), indicating that fliS, fliTX and flgZ are co-transcribed in an operon. Sequence alignment analysis indicated that FliTX was less well conserved compared with FliT proteins in Escherichia coli, Salmonella typhimurium, and Pseudomonas fluorescens (Fig. 1b). To further identify the role of FliTX in Xoo, an in-frame deletion mutant ΔfliTX and its complementary strain ΔfliTX were generated as described in the Materials and Methods. DNA sequencing analysis showed that the corresponding region of fliTX was completely deleted in ΔfliTX.

**fliTX is transcriptionally up-regulated by RpoN2, FleQ and FliA**

To identify whether and how fliTX is regulated in Xoo, the promoter activities of the fliS-fliTX-FlgZ operon were examined by measuring β-galactosidase activity of the fliSp-lacZ fusion in ΔrpoN2, ΔfleQ, ΔfliA, and the relevant complementary strains. β-galactosidase activity of fliSp-lacZ was significantly reduced in ΔrpoN2, ΔfleQ, and ΔfliA compared with that in the wild type, and restored in the relevant complementary strains (Fig. 2a). qRT-PCR analysis showed that transcripts of fliS, fliTX, and FlgZ were dramatically decreased in ΔrpoN2, ΔfleQ, and ΔfliA compared with that in wild type (Fig. 2b), indicating that the transcription of the fliS-fliTX-FlgZ operon was regulated by RpoN2, FleQ, and FliA. Consistent with our previous report [10], the transcription of fliA was also significantly decreased in ΔrpoN2 and ΔfleQ (Fig. 2b). These results strongly suggest that RpoN2/FleQ regulate the transcription of the fliS-fliTX-FlgZ operon via FliA in Xoo.
FliTX is required for flagellar motility and filament production

Since fliTX is located within the flagellar regulon, the function of FliTX in flagellar filament assembly and flagellum-dependent motility was investigated. The swimming ability of ΔfliTX was detected on the 0.25% agar semisolid plates. Compared with the wild type strain, ΔfliTX showed a much smaller swimming zone, and the defect was restored to near wild-type level in the complementary strain containing a plasmid to express the full length of fliTX in trans (Fig. 3a). To further determine whether deletion of fliTX affected the flagellar biogenesis in Xoo, the single-polar flagellum of various Xoo strains were observed by transmission electron microscope (TEM). The average length of the flagellum on ΔfliTX was significantly shorter than that on the wild type, and it was recovered to wild-type level in the complementary strain (Fig. 3b). These results indicate that FliTX is necessary for flagellar biogenesis and motility in Xoo.

ΔfliTX shows reduced pathogenicity and bacterial growth in rice

To demonstrate the function of fliTX in virulence, the pathogenicity of various Xoo strains on susceptible rice cultivar IR24 was tested by the leaf clipping method, and lesion lengths were measured 14 days post inoculation. Compared with the wild type, ΔfliTX caused much shorter disease lesion, which were restored in the complementary strain (Fig. 4a and b). Measuring bacterial population in the diseased leaves of rice showed that deletion of fliTX significantly led to reduced bacterial population in rice leaves, but complementation with fliTX in trans restored the bacterial growth in planta to near wild-type levels (Fig. 4c). These findings reveal that FliTX is required for virulence of Xoo in rice.

ΔfliTX is impaired in the ability to elicit hypersensitive response (HR) in tobacco

To unveil the role of FliTX in Xoo when interacting with nonhost plants, HR-inducing ability of Xoo strains on tobacco leaves was tested. Wild type strain induced the typical programmed cell death due to the hypersensitive responses (HR) in the non-host tobacco plants. In contrast, ΔfliTX completely lost such an ability to elicit HR, while the complementary strain caused a similar phenotype as the wild type strain (Fig. 5a). We then hypothesized that FliTX protein might be able to elicit HR. To test it, the recombinant protein FliTX-His6 was first expressed in E. coli strain BL21 and extracted from the
Fig. 2 Regulation of flITX transcription in Xanthomonas oryzae pv. oryzae. a β-galactosidase activity assay. Activities of the flIS promoter in Xoo strains were detected. The experiments were repeated three times, independently. b qRT-PCR analysis of genes in flIS operon and flIA in Xoo strains. The data represents the relative expression level of genes in PXO99A, ΔflIA, ΔflIEQ and ΔrpoN2. The error bar represents standard deviations from three biological repeats.

Fig. 3 Flagellar motility and filament production of Xanthomonas oryzae pv. oryzae strains. a Assay of swimming motility for PXO99A, ΔflITX and ΔflITX-C strains. The swimming zones are recorded after bacterial growth for 4 days on the semisolid plates at 28 °C. Error bars indicate standard deviation. Statistical significance is presented by asterisk (* P < 0.05, Student’s t-test). b Observation of filament for PXO99A, ΔflITX and ΔflITX-C strains using transmission electron microscopy.
Fig. 4 Virulence of Xanthomonas oryzae pv. oryzae strains in rice. a PXO99A, ∆fliTX and ∆fliTX-C strains were inoculated into 6-week-old rice leaves by using the leaf-clipping method. The disease symptoms were observed at 14 days post-inoculation. b The lesion lengths were recorded from 10 inoculated leaves for every strain. c Bacterial numbers in the top 20 cm of each lesion leaf were scored. Data represent the mean and standard deviations of three independent experiments, and the asterisk above the bars denote statistically significant differences (P < 0.05, Student’s t test).

Fig. 5 Hypersensitive cell death in tobacco induced by Xanthomonas oryzae pv. oryzae strains and FliTX protein. Cell suspensions of Xoo strains at OD600 of 0.1 (a) or recombinant FliTX protein (b) were infiltrated onto 6-week-old tobacco leaves. The ddH2O was used as control. The HR symptoms were detected and photographed at 24 h post-inoculation. At least four independent experiments were performed with similar results.
soluble fraction by using pre-equilibrated Ni2_resin. SDS-PAGE analysis demonstrated that FliTX-His6 was about 14 KDa in size (Additional file 1: Figure S1). Then, the purified protein was infiltrated into tobacco leaves at two different concentrations. HR in tobacco was strongly induced when FliTX-His6 was applied at the concentration of 4 μM, while no HR was observed when the concentration was reduced to 2 μM (Fig. 5b). The negative control of sterilized double-distilled water (ddH2O) did not cause HR either. These observations demonstrate that FliTX protein plays an important role in Xoo to elicit HR in nonhost tobacco.

**ΔfliTX was attenuated in T3SS-related gene expression**

The HR-inducing ability on nonhost and pathogenicity on host (Hrp) is closely related to T3SS in pathogenic bacteria [33, 34]. To understand the function of FliTX in T3SS in Xoo, transcripts of T3SS-related hrp genes were measured through qRT-PCR analysis. Compared with that in the wild type, transcription levels of hrpG, hrpX, hrpE and hpa1 were significantly decreased in ΔfliTX, and restored near to wild-type level in the complementary strain (Fig. 6a). Moreover, promoter activities of hrpG, hrpX and hpa1 revealed through flow cytometry analysis were dramatically reduced in ΔfliTX compared with that of the wild type. All promoter activities were restored to wild-type levels in the complementary strain (Fig. 6b). Since HrpG controls the transcription of other hrp genes via regulating hrpX expression in Xanthomonas [35], these results suggest that FliTX positively regulates the expression of T3SS in Xoo through the master regulator HrpG.

**Discussion**

In the current study, we identified a novel flagellar gene *fliTX*, determined its expression patterns, and assessed its functions in motility and virulence on rice through bioinformatics and genetic analysis. We demonstrated that the transcription of *fliTX* was positively regulated by RpoN2/FleQ and FliA. We also revealed that *fliTX* was indispensable for bacterial phenotypes, including flagellar motility, pathogenicity in rice, induction of HR in tobacco, and T3SS-related gene expression. Therefore, this study provides for the first time the mechanistic regulation of motility and virulence in Xoo.

An over 60 contiguous gene containing cluster has been shown to putatively encode flagellar proteins with various functions, including structural components, protein export apparatus, regulatory factors, post-translational modification enzymes/proteins, and chemotaxis proteins in Xoo [9, 10]. The flagellar assembly and operation are tightly regulated under a three-tiered hierarchy by RpoN2/FleQ and FliA [10]. Based on the gene location and transcription feature, we found that *fliTX* was transcribed in the *fliS-fliTX-flgZ* operon regulated by RpoN2/FleQ and FliA (Figs.1 and 2). This is quite different from the *fliD-flis-fliT* operon in other pathogenic bacteria including *E. coli* and *S. typhimurium* [36, 37]. The significantly reduced transcripts of *fliA* in ΔrpoN2 and ΔfleQ (Fig. 2b) suggest that regulation of transcription of the *fliS-fliTX-flgZ* operon by RpoN2/FleQ might be through FliA under a three-tiered hierarchy.

The varied functions of FliT in flagellar motility have been shown in several pathogenic bacteria. For example, FliT has been described as the filament-capping protein FliD substrate-specific chaperone in *S. typhimurium* [24]. Deletion of *fliD* inhibited the assembly of flagellin molecules onto the hooks, resulting in failure to filament biogenesis [37, 38]. However, no difference in swimming ability was observed between wildtype and the mutant [23]. In contrast, ΔfliT showed normal flagellar filaments but attenuated swimming motility in *P. fluorescens* F113 [31], suggesting that FliT might not act as a FliD chaperone. Our current observation that FliT was less conserved in the strains of *E. coli*, *S. typhimurium*, *P. fluorescens* and Xoo (Fig. 1) implicates that FliT may function differentially in flagellar motility in various bacteria. In this study, in frame deletion of *fliTX* led to significantly abnormal filaments and reduced swimming motility (Fig. 3), demonstrating that FliTX plays important roles in filament assembly and motility in Xoo. Further experiments are required to determine whether FliTX functions as a FliD chaperone to affect the flagellar motility in Xoo.

The role of FliT in bacterial virulence has only been reported in *S. typhimurium*, in which the transcription of T3SS-containing SPI1 was induced upon disruption of *fliT*, and the repressive effect of *fliT* on SPI1 genes was completely abolished in ΔflhDC [32], indicating that FliT negatively regulates the virulence and related gene expression in the FlhDC-dependent manner in *Salmonella*. In this study, we demonstrated that in frame deletion of *fliTX* resulted in dramatically reduced lesion length and bacterial growth in rice (Fig. 4), and impaired HR-inducing ability in tobacco (Fig. 5a). It has been known that the T3SS plays critical roles in conferring pathogenicity on the host and triggering the HR on non-host plants by delivering effector proteins into plant cells [33, 34]. Meanwhile, in the current study, we showed that the expression of two T3SS regulator genes, *hrpG* and *hrpX*, were attenuated in the ΔfliTX mutant (Fig. 6). Thus, this study provides for the first time the experimental evidence that FliTX functions to promote the bacterial virulence via regulating T3SS gene expression in Xoo. Based on our previous demonstration that RpoN2 positively regulates the virulence on rice through an unknown manner [10], and the current observation that *fliTX* is up-regulated by RpoN2 and required for
the virulence in Xoo, it is reasonable to speculate that FliTX works in the RpoN2-dependent pathway to promote the bacterial pathogenesis in rice. More investigations are needed to confirm this hypothesis and further understand the regulatory mechanism of virulence by FliTX in Xoo.

For the assembly of bacterial flagellum for motility, the flagellar type III export apparatus utilizes both ATP and proton motive force to cross the cytoplasmic membrane and export flagellar proteins from the cytoplasm to the cell membrane [28]. FliT acts as the specific chaperone of the filament-capping protein FliD that is protected from degradation and aggregation in the cytoplasm and efficiently transferred to the distal end of the flagellar structure [26]. However, it remains mysterious whether and how the FliT protein is secreted. In addition, we showed that the recombinant FliTX protein induced HR in monhost tobacco leaves (Fig. 5b), implicating a potential role of FliTX in inducing plant defense responses. Therefore, it is required to further demonstrate whether and how FliTX is secreted into the plant cells during the induction of HR.

**Conclusions**

The fliTX gene is transcriptionally up-regulated by RpoN2/FleQ and FliA, and necessary for flagellar assembly and motility in Xoo. Deletion of fliTX led to significantly reduced virulence in rice, attenuated ability of induction of HR in tobacco and decreased hrp gene expression. RpoN2/FleQ- and FliA-regulated FliTX controls the bacterial pathogenesis via T3SS regulation with the unknown manner(s).
Methods

Bacterial strains and culture conditions

*Xanthomonas oryzae* pv. *oryzae* wildtype strain PXO99A and derived mutants were grown in peptone sucrose agar (PSA) medium [39] or M210 liquid medium [40] at 28 °C, *Escherichia coli* DH5α and BL21 strains were grown in Luria-Bertani (LB) medium at 37 °C. The antibiotics used were ampicillin (Ap), gentamicin (Gm), kanamycin (Km) and spectinomycin (Sp) at concentrations of 100, 50, 50, and 50 μg/mL, respectively. The bacterial strains and plasmids used in this study are listed in Table 1.

Bioinformatics analysis of *fliTX*

The domain organization of FliTX was analyzed using online software available at the SMART Website (http://smart.embl-heidelberg.de/). The amino acid sequences of FliTX were obtained from the National Center for Biotechnology Information (NCBI) website. BLASTP was using for searching the homology in *Xanthomonas* species. Relevant sequence alignment was performed using the DNAMAN software (Lynnon Biosoft, San Ramon, USA).

Generation of lacZ fusion and assay for β-galactosidase activity

The promoter region (−309 to −1) of fliS was amplified from PXO99A genomic DNA using specific primers fliSpF/R (Additional file 2: Table S1), and ligated into the HindIII and BamHI sites of the pHT304BZ vector containing a promoterless lacZ reporter gene [41]. Recombinant pHTpS was verified by DNA sequencing (Beijing Genomics Institute, Beijing) and treated with HindIII and KpnI, and the fragment containing fliS promoter region and the promoterless lacZ reporter gene was obtained and then cloned into plasmid pHM1 [42].

| Table 1 Bacterial strains and plasmids used in this study |
|----------------------------------------------------------|
| **Strain or plasmid** | **Relevant characteristics** | **Source or Reference** |
|-----------------------|-----------------------------|------------------------|
| *Escherichia coli*     |                             |                        |
| DH5α                  | supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | [49]                   |
| BL21                  | For protein expression      | Novagen                |
| *Xanthomonas oryzae* pv. *oryzae* |                   |                        |
| PXO99A                | Wildtype strain, Philippine race 6 | Lab collection         |
| ΔfliTX                | fliTX gene deletion mutant derived from PXO99A | This study             |
| ΔfliTX-C              | Complementary strain of ΔfliTX, Ap′ | This study             |
| ΔpopN2                | popN2 gene deletion mutant derived from PXO99A, Gm′ | Our lab                |
| ΔpopN2-C              | Complementary strain of ΔpopN2, Ap′ | Our lab                |
| ΔfieO-Q               | fieO gene deletion mutant derived from PXO99A, Gm′ | Our lab                |
| ΔfieO-C               | Complementary strain of ΔfieO, Ap′ | Our lab                |
| ΔfiaA                 | fiaA gene deletion mutant derived from PXO99A, Gm′ | Our lab                |
| ΔfiaA-C               | Complementary strain of ΔfiaA, Ap′ | Our lab                |
| **Plasmid**           |                             |                        |
| pMD18-T               | Cloning vector, Ap′         | TaKaRa, Tokyo          |
| pKMS1                 | Suicidal vector carrying socB gene for non-marker mutagenesis, Km′ | [45]                   |
| pBHR1MCS-4            | Broad-host range expression vector, Ap′ | [50]                   |
| pHT304BZ              | Promoterless lacZ vector, Ap′ | [41]                   |
| pHfTPS                | pHT304BZ derivative carrying the promoter region of fliS, Ap′ | This study             |
| pHM1                  | Broad-host range expression vector, Sp′ | [42]                   |
| pH-fliS-sp-lacZ       | pHM1 derivative carrying the promoter region of fliS and promoterless lacZ, Sp′ | This study             |
| pET-28a               | Expression vector to generate a N-terminal His6 tag, Km′ | Haigene                |
| pET-fliTX             | pET-28a derivative carrying fliTX, Km′ | This study             |
| pPROBE-AT             | broad-host-range vector carrying a promoter-less gfp gene, Ap′ | [47]                   |
| pPhrpG                | pPROBE-AT derivative carrying the promoter region of hrpG and promoterless gfp, Ap′ | This study             |
| pPhrpX                | pPROBE-AT derivative carrying the promoter region of hrpX and promoterless gfp, Ap′ | This study             |
| pPhpa1                | pPROBE-AT derivative carrying the promoter region of hpa1 and promoterless gfp, Ap′ | Our lab                |

*Ap′, Km′, Sp′, and Gm′ indicate resistant to ampicillin, kanamycin, spectinomycin and gentamicin, respectively.*
the recombinant plasmid pH-\textit{fliSp-lacZ} was generated and introduced into PXO99\textsuperscript{A} and derived mutants. The resultant strains contained pH-\textit{fliSp-lacZ} were selected by resistance to spectinomycin and verified by polymerase chain reaction (PCR). For \(\beta\)-galactosidase assay, these Xoo strains were cultured in M210 liquid medium at 28 \(^\circ\)C and 200 rpm, and till an optical density (OD\textsubscript{600}) of 1.0, cells were collected by centrifugation at 12,000 g. The \(\beta\)-galactosidase activity was determined using the \(\beta\)-Galactosidase Enzyme Assay System (Promega, Wisconsin, USA). The experiments were repeated three times, independently.

\textbf{RNA isolation and qRT-PCR analysis}

RNA isolation and qRT-PCR analysis were performed as described previously [8]. Briefly, Xoo strains were grown in M210 liquid medium at 28 \(^\circ\)C till OD\textsubscript{600} of 0.8, and harvested by centrifugation at 12,000 g for analysis of gene expression. For T3SS-related gene assays, the harvested bacterial cells were sub-cultured in XOM2 medium [43] overnight at 28 \(^\circ\)C and collected again. Total RNA was extracted with RNAPrep pure Cell/Bacteria Kit (Tiangen, Beijing, China) and treated with DNase and cDNA was synthesized from total RNA using the FastQuant RT Super Mix (Tiangen, Beijing, China). RT-qPCR was performed using Quant qRT-PCR kit (Tiangen, Beijing, China) in Applied Biosystem’s 7500 (Applied Biosystems, Foster City, CA, USA) with gene specific primers, and \textit{gyrB} was used as a reference gene (Additional file 2: Table S1). The relative expression ratio was calculated using \(2^{-\Delta\Delta Ct}\) method [44]. These experiments were performed in three biological replicates and triplicate PCR.

\textbf{Protein expression and purification}

The FliTX expression and purification were performed as described previously [8]. Briefly, the coding region for \textit{fliTX} was amplified by PCR with primers TXF/R (Additional file 2: Table S1) and ligated to the middle vector pMD18-T for verification by DNA sequencing. Then the \textit{fliTX} fragment was digested from verified pMD18-T (Takara, Dalian, China) and confirmed by PCR analysis, resulting in the \textit{fliTX} gene were amplified by PCR from Xoo genomic DNA using primers \textit{fliTXLF/R} and \textit{fliTXRF/R}, respectively. The PCR products were first cloned into the middle vector pMD18-T (Takara, Dalian, China) and verified by sequencing. Then the upstream and downstream fragments of \textit{fliTX} were digested with corresponding restriction enzymes from the middle vectors and ligated into pKMS1. The final vector pKMS1 containing upstream and downstream fragments of \textit{fliTX} was introduced into Xoo by electroporation. The transformants were first selected on NAM medium (1% tryptone, 0.1% yeast extract, 0.3% peptone and 1.5% agar) with Km, and after continuous transfer cultured in NBN medium (1% tryptone, 0.1% yeast extract and 0.3% peptone) at least five times. Finally, the \(\Delta fliTX\) mutant was selected on NAS medium (1% tryptone, 0.1% yeast extract, 0.3% peptone, 10% sucrose and 1.5% agar) and further confirmed by PCR analysis. For complementation strain construction, the full length of \textit{fliTX} was amplified by PCR with primers \textit{fliTXF/R} and inserted into vector pMD18-T. After verifying by sequencing, \textit{fliTX} was digested from pMD18-T and ligated into pBBR1MCS-4. The final vector pBBR1MCS-4 containing \textit{fliTX} was electroporated into \(\Delta fliTX\) and confirmed by PCR analysis, resulting in the \(\Delta fliTX\) complementary strain (\(\Delta fliTX\)-C). The primers are listed in Additional file 2: Table S1.

\textbf{Motility assay and electron microscopy visualization of filament}

For motility assay, bacterial strains were cultured in M210 liquid medium till reached OD\textsubscript{600} of 1.0 and harvested by centrifugation at 12,000 g for 5 min. Cells were re-suspended in equal volume of ddH\textsubscript{2}O. Two microliters of bacterial suspension were spotted onto semisolid plates (0.03% peptone, 0.03% yeast extract and 0.25% agar) and incubated at 28 \(^\circ\)C. The diameters of the swimming zone were recorded after 4 days. The experiments were repeated three times with five replicates for each time. The TEM assay was performed as described previously [46]. Briefly, bacterial strains were grown on PSA plates at 28 \(^\circ\)C for 48 h, and cells were collected

(GE Healthcare, Piscataway, NJ, USA) for 1 h at 4 \(^\circ\)C. Finally, the FliTX protein combined to Ni was eluted with elution buffer (20 mM Tris-HCl, 350 mM NaCl, 0.5 mM EDTA, 10% glycerol, 5 mM MgCl\textsubscript{2} and 100 mM imidazole, pH 8.0) and dialyzed with 0.1× PBS. The purified FliTX was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and adjusted to 10 \(\mu\)M with 0.1 × PBS for the next experiments.

\textbf{Gene deletion and complementation}

An in-frame gene deletion mutant \(\Delta fliTX\) derived from PXO99\textsuperscript{A} was constructed through homologous recombination by using the suicide vector pKMS1 [45]. About 900 bp upstream and 800 bp downstream fragments of \textit{fliTX} gene were amplified by PCR from Xoo genomic DNA using primers \textit{fliTXLF/R} and \textit{fliTXRF/R}, respectively. The PCR products were first cloned into the middle vector pMD18-T (Takara, Dalian, China) and verified by sequencing. Then the upstream and downstream fragments of \textit{fliTX} were digested with corresponding restriction enzymes from the middle vectors and ligated into pKMS1. The final vector pKMS1 containing upstream and downstream fragments of \textit{fliTX} was introduced into Xoo by electroporation. The transformants were first selected on NAM medium (1% tryptone, 0.1% yeast extract, 0.3% peptone and 1.5% agar) with Km, and after continuous transfer cultured in NBN medium (1% tryptone, 0.1% yeast extract and 0.3% peptone) at least five times. Finally, the \(\Delta fliTX\) mutant was selected on NAS medium (1% tryptone, 0.1% yeast extract, 0.3% peptone, 10% sucrose and 1.5% agar) and further confirmed by PCR analysis. For complementation strain construction, the full length of \textit{fliTX} was amplified by PCR with primers \textit{fliTXF/R} and inserted into vector pMD18-T. After verifying by sequencing, \textit{fliTX} was digested from pMD18-T and ligated into pBBR1MCS-4. The final vector pBBR1MCS-4 containing \textit{fliTX} was electroporated into \(\Delta fliTX\) and confirmed by PCR analysis, resulting in the \(\Delta fliTX\) complementary strain (\(\Delta fliTX\)-C). The primers are listed in Additional file 2: Table S1.)
and re-suspended with ddH2O, then one drop of suspension was deposited onto grids coated with Formvar (Standard Technology, Ormond Beach, FL, USA). The grids with bacteria were stained with 2% uranyl acetate for 30 s, and air drying for 10 min. The bacterial flagella were observed by TEM using Hitachi H-7500 electron microscope.

Pathogenicity test
As described above, bacterial strains were cultured in M210 liquid medium at 28 °C and 200 rpm till reached OD600 of 1.0, and collected by centrifugation at 12,000 g for 5 min, and re-suspended with equal volume of ddH2O. For the disease lesion length assay, bacterial cells were inoculated into leaves of 8-week-old rice (Oryza sativa ssp. indica) cultivar IR24 using the leaf-clipping method [8], and the lesion length was measured at 14 days post-inoculation. For the bacterial population assay, the top 2 cm of inoculated rice leaves were collected and weighted, then ground into 1 mL of ddH2O. The ground mixtures were optional diluted and spread onto the PSA plates. The bacterial colonies were counted after cultured in incubator with 28 °C for 72 h. At least 10 leaves were inoculated for each strain, and the experiments were repeated three times, independently.

Assay for induction of HR in tobacco
Xoo strains were grown in M210 liquid medium at 28 °C to OD600 of 1.0, and collected by centrifugation at 7000 g for 10 min. The cells were re-suspended with ddH2O, and adjusted to OD600 of 0.1. Then these bacterial cells or purified FliTX protein were inoculated into leaves of 6-week-old tobacco (Nicotiana benthamiana) using a needleless syringe. The HR symptoms were detected and photographed at 24 h post-inoculation. The experiments were repeated three times, independently.

Flow cytometry detection
The plasmid pHpa1 containing the hpa1 promoter region and a promoterless gfp gene was constructed in our previous studies [40]. Here two near 200-bp fragments containing the promoter region of hrpG or hrpX were PCR amplified using the primers hrpGpF/R or hrpXpF/R (Additional file 2: Table S1), and ligated to pPROBE-AT, a broad-host-range vector carrying a promoter-less AT, a broad-host-range vector carrying a promoter-less marker; 1: FliTX in the soluble fraction; 2: purified FliTX; 3: FliTX in the insoluble fraction. (TIFF 424 kb)

Additional file 1: Figure S1. Coomassie blue staining of the FliTX protein expressed and extracted from E. coli strain BL21. M: Molecular marker; 1: FliTX in the soluble fraction; 2: purified FliTX; 3: FliTX in the insoluble fraction. (DOCX 17 kb)

Additional file 2: Table S1. The primers used in this study. (DOO 17 kb)

Abbreviations
Ap: Ampicillin; ddH2O: Sterilized double-distilled water; EPS: Exopolysaccharide; Gm: Gentamicin; HR: Hypersensitive response; Km: Kanamycin; LB: Luria-Bertani; NCBI: National Center for Biotechnology Information; OD600: Optical density; PCR: Polymerase chain reaction; PSA: Peptone sucrose agar; qRT-PCR: Quantitative real-time polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; SDS: Standard deviations; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sp: Spectinomycin; STF: Salmonella pathogenicity island 1; T3SS: Type III secretion system; TEM: Transmission electron microscope; Xoo: Xanthomonas oryzae pv. oryzae

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its Additional files 1 and 2.

Authors’ contributions
CY and CYH designed the experiments; CY performed the experiments; CY, FT and CYH wrote the manuscript; All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

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

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