Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2018) 19(3), 579–592 DOI: 10.1111/mpp.12543

A ten gene-containing genomic island determines flagellin glycosylation: implication for its regulatory role in motility and virulence of *Xanthomonas oryzae* pv. *oryzae*

CHAO YU[1], HUAMIN CHEN[1], FANG TIAN[1], FENGHUAN YANG[1], XIAOCHEN YUAN[2], CHING-HONG YANG[2] AND CHENYANG HE[1,*]

[1]*State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China*
[2]*Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, USA*

SUMMARY

Flagellin glycosylation plays a crucial role in flagellar assembly, motility and virulence in several pathogenic bacteria. However, little is known about the genetic determinants and biological functions of flagellin glycosylation in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal pathogen of bacterial blight of rice. Here, the structure, regulation and functions of a ten-gene cluster *gigX* (*g*lycosylation *i*sland *g*enes of *Xoo*), which was embedded in a flagellar regulon, were characterized. *gigX*1 to *gigX*10 encoded putative enzymes or proteins involved in glycan biosynthesis and transfer, including a nucleotide sugar transaminase, an acyl-carrier protein (ACP), a 3-oxoacyl-ACP synthase, a 3-oxoacyl-ACP reductase, a dehydrogenase, an acetyltransferase, a ring hydroxylating dioxygenase, a hypothetical protein, a methyltransferanse and a glycosyltransferase, respectively. The *gigX* genes were co-transcribed in an operon and up-regulated by the upstream σ^{54} factor RpoN2 and transcriptional activator FleQ. In-frame deletion of each *gigX* gene affected flagellin glycosylation modification, meaning that the unglycosylated flagellin of the mutants was smaller than the glycosylated flagellin of the wild-type. No significant changes in flagellar filament and motility were observed in the Δ*gigX* mutants, among which only Δ*gigX*6 displayed increased swimming ability. Importantly, all mutants, except Δ*gigX*9, showed significantly increased virulence and bacterial growth in the susceptible rice cultivar IR24, and Δ*gigX*1 and Δ*gigX*10 showed enhanced type III secretion system (T3SS)-related gene expression. Moreover, the glycosylated flagellin of the wild-type induced higher H_2O_2 levels in rice leaves than did the unglycosylated flagellins of Δ*gigX*1 or Δ*gigX*10. Taken together, this study reveals that the *gigX* cluster determines flagellin glycosylation, and implicates the regulatory role of post-translational modification with the glycosylation, acetylation and methylation of flagellin in the regulation of motility and virulence of *Xoo*.

* *Correspondence*: Email: hechenyang@caas.cn

Keywords: flagellar motility, flagellin glycosylation, *gigX* cluster, immune response, virulence, *Xanthomonas oryzae* pv. *oryzae*.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is one of the most important bacterial blight pathogens of rice, leading to 20%–50% yield losses, and has become an ideal model to study the molecular mechanisms of bacterial pathogenicity on monocot plants (Nino-Liu *et al.*, 2006). A range of virulence-related factors, including extracellular polysaccharide (EPS) synthesis, biofilm formation, motility, extracellular cellulase, xylanase and adhesions, are produced by *Xoo* to facilitate its pathogenesis (Das *et al.*, 2009; White and Yang, 2009). In addition to these virulence factors, like many other Gram-negative bacteria, *Xoo* also possesses a type III secretion system (T3SS) to inject and deliver effectors into plant cells, some of which are critical determinants of pathogenic consequences during *Xoo*–rice interactions (Tsuge *et al.*, 2006; White and Yang, 2009). In the *Xoo* T3SS, HrpG and HrpX are two major regulatory proteins that control the expression of structural genes, including *hrcC*, *hrcU* and *hrcT*, and the secretion of a number of effector proteins (Song and Yang, 2010; White and Yang, 2009). Therefore, it is of both scientific and economic significance to elucidate the regulatory mechanisms underlying how the virulence factors are expressed in *Xoo*.

A flagellar gene cluster containing over 60 contiguous genes in the genome of PXO99^A has been identified previously; the genes putatively encode proteins with various functions, including structural components, protein export apparatus, regulatory factors, chemotaxis, GGDEF/EAL domain proteins, which are involved in the cyclic-di-guanosine monophosphate (cyclic-di-GMP) signal transduction pathway, and proteins for post-translational modification (Tian *et al.*, 2015). Within this gene cluster, a genomic island (GI) containing 10 genes was annotated to be responsible for flagellin glycosylation (Salzberg *et al.*, 2008).

Flagellin glycosylation is a complicated biochemical process of post-translational modification, which is generally encoded by a

number of clustered genes in several pathogenic bacteria (Chaban *et al.*, 2006; Taguchi *et al.*, 2006). For example, a GI containing 14 genes that determined flagellin glycosylation was first identified in *Pseudomonas aeruginosa* strain PAK, which encoded the enzymes and proteins involved in flagellin glycosylation, such as glycosyltransferase, acyl-carrier protein (ACP) and acetyltransferase (Arora *et al.*, 2001). The inactivation of genes in GI abolished flagellin glycosylation, indicating the involvement of GI in flagellin glycosylation (Arora *et al.*, 2001). In *Pseudomonas syringae* pv. *tabaci* 6605, a viosamine gene cluster contains six dTDP-mVio biosynthetic genes, including *vioA* for dTDP-viosamine aminotransferase, *vioM* for methyltransferase, *vioS* for 3-oxoacyl-ACP synthase, *vioB* for dTDP-viosamine acetyltransferase, *vioR* for 3-oxoacyl-ACP reductase and *acp* for ACP, and one mVio glycosyltransferase gene *vioT* (Nguyen *et al.*, 2009). dTDP-mVio is a substrate for VioT and is required for the incorporation of mVio into the flagellin glycan, demonstrating a viosamine metabolic pathway involved in flagellin glycosylation in *P. syringae* (Nguyen *et al.*, 2009; Yamamoto *et al.*, 2011). All of the genes displayed the corresponding homology in GI of *P. aeruginosa* strain PAK (Yamamoto *et al.*, 2011). In addition, three clustered genes, *fgt1*, *fgt2* and *orf3*, were found upstream of the flagellin gene *fliC* in *P. syringae* pv. *tabaci* 6605 (Taguchi *et al.*, 2006). Flagellins from the Δ*fgt1* and Δ*fgt2* mutants were not glycosylated and partially glycosylated, respectively, indicating that both genes encode glycosyltransferases, which are required for flagellin glycosylation (Taguchi *et al.*, 2006; Takeuchi, 2008). In addition to *P. syringae*, flagellin glycosylation has also been reported in the rice virulent strain K1 of *Acidovorax avenae*, in which four genes, *fgt*, *fmt*, *fcs* and *fst*, encoding putative glycosyltransferase, methyltransferase, carbamoylphosphate synthase and sugar transaminase, respectively, have been identified to regulate the glycosylation of flagellin (Hirai *et al.*, 2014). In *Xanthomonas campestris* pv. *campestris* (*Xcc*), there is a five gene-containing GI, including a glycosyltransferase gene *fgt*, a nucleotide sugar transaminase gene *vioA* and three hypothetical protein genes (Ichinose *et al.*, 2013). Moreover, flagellin glycosylation of *Burkholderia glumae* strain Pg-10, *Dickeya dadantii* strain 92-31, *Pantoea ananatis* strain AZ200124, *Pseudomonas cichorii* strain KN52, *Pectobacterium carotovorum* ssp. *carotovorum* strain EC1 and *Xcc* strain XcA has been demonstrated, suggesting that flagellin glycosylation is ubiquitous in a broad range of plant-pathogenic bacteria (Ichinose *et al.*, 2013).

Although flagellin glycosylation has been revealed in further pathogenic bacteria, in many cases, its biological function remains to be elucidated (Logan, 2006; Merino and Tomas, 2014). In general, flagellin glycosylation affects filament assembly, stability and flagellar motility (Asakura *et al.*, 2010; Guerry *et al.*, 2006; Taguchi *et al.*, 2006, 2008, 2010), virulence (Khodai-Kalaki *et al.*, 2015; Lithgow *et al.*, 2014), host specificity, recognition and innate immune evasion (Logan, 2006; Nothaft and Szymanski, 2010). Flagellin glycosylation has been implicated in the regulation of bacterial virulence and interactions with the host in several pathogenic bacteria (Arora *et al.*, 2005; Khodai-Kalaki *et al.*, 2015; Taguchi *et al.*, 2006). For instance, the *P. aeruginosa* mutant with unglycosylated flagellin was significantly attenuated in virulence to humans (Arora *et al.*, 2005). In *Burkholderia pseudomallei* and *Burkholderia thailandensis*, it has been suggested that flagellar glycosylation could be a mechanism used to modulate virulence, given the difference in glycan composition and virulence (Scott *et al.*, 2011). *Pseudomonas syringae* pv. *tabaci* flagellin glycosylation was also required for the full virulence of the pathogen by evasion of the host plant surveillance system (Taguchi *et al.*, 2006, 2008, 2009, 2010). *Burkholderia cenocepacia* flagellin glycosylation contributed to the evasion of the human innate immune response, but not to innate immune recognition in *Arabidopsis thaliana* (Hanuszkiewicz *et al.*, 2014; Khodai-Kalaki *et al.*, 2015). In most cases, flagellin glycosylation plays a positive role in bacterial virulence expression and helps the bacterial pathogen to evade host plant innate immunity.

Although flagellin glycosylation plays a crucial role in flagellar assembly, motility and virulence in several pathogenic bacteria, little is known about the genetic determinants and biological functions of flagellin glycosylation in *Xoo*. A ten-gene cluster named *gigX* (*g*lycosylation *i*sland *g*enes of *Xoo*), embedded in a flagellar regulon, was revealed in the *Xoo* genome (Salzberg *et al.*, 2008). Mutation of *PXO_00987*, encoding a putative acetyltransferase, affected flagellin glycosylation (Li *et al.*, 2015). In this study, the structure, regulation and functions of the *gigX* cluster were further characterized through genetic and biochemical analysis. The *gigX* genes were homologous with the GI genes found in *P. aeruginosa*, which encode enzymes and proteins involved in the glycosylation process. Co-transcription of *gigX* genes was upregulated by the transcriptional regulator FleQ and alternative sigma factor RpoN2. The deletion of each of these genes resulted in the complete abolishment of flagellin glycosylation, whereas flagellar assembly and motility were not affected in most mutants. Importantly, bacterial virulence on rice cultivar IR24 was significantly increased in most mutants with unglycosylated flagellin. This study provides further insights into the post-translational modification of flagellin, and implicates its role in the regulation of motility and virulence in plant-pathogenic bacteria.

RESULTS

gigX genes encode enzymes/proteins involved in the glycosylation process

In the genome of the wild-type strain PXO99^{A}, there are two identical copies of the flagellar gene cluster consisting of more than 60 genes encoding proteins with various functions, including structural components, protein export apparatus, regulatory

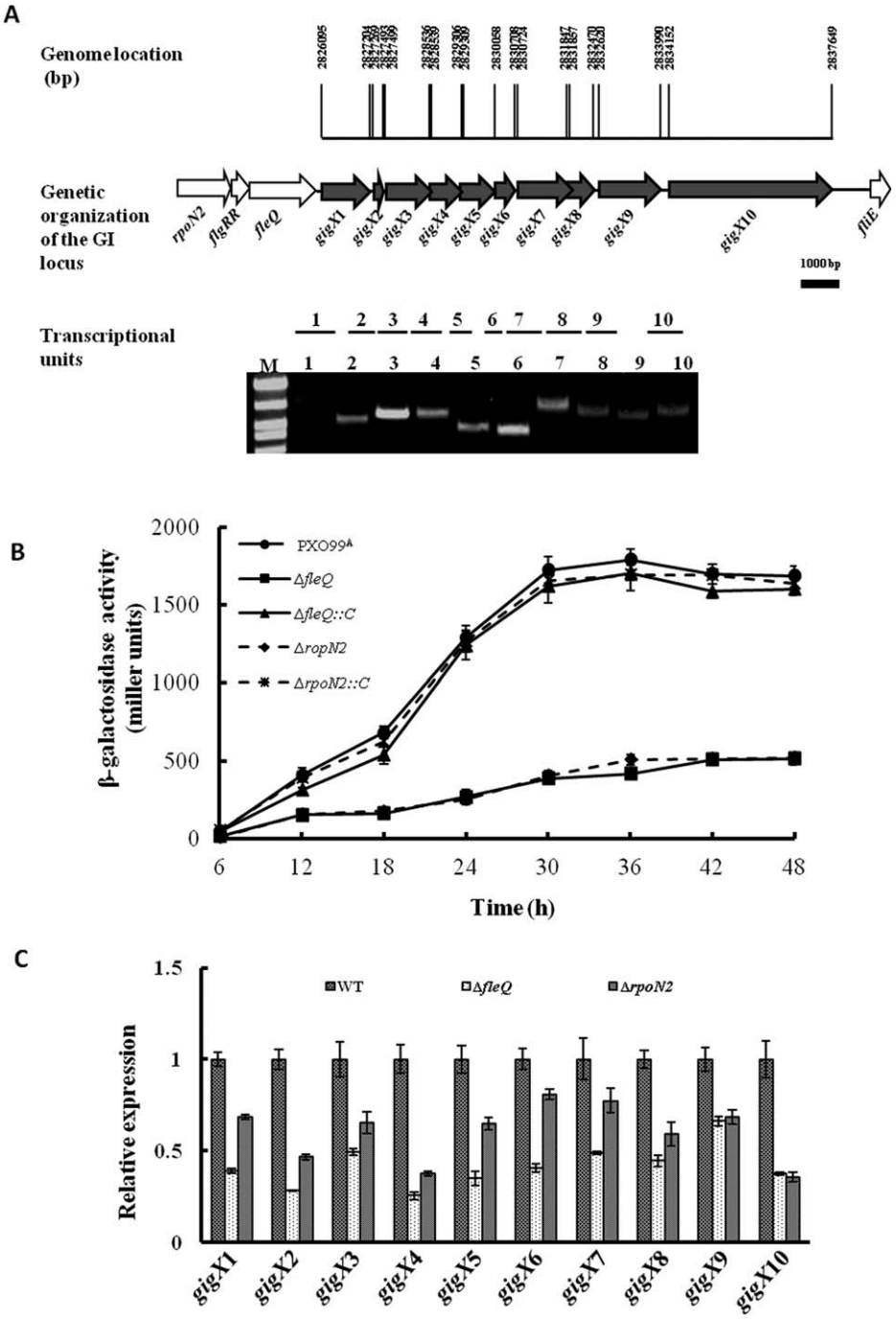

Fig. 1 Transcriptional regulation of *gigX* genes in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains. (A) Schematic diagram of the *gigX* cluster in the genome of *Xoo* strain PXO99^{A}. Open arrows indicate the length, location and orientation of the open reading frames (ORFs). The short lines below the arrows indicate the location and length of the reverse transcription polymerase chain reaction (RT-PCR) products. The bottom element shows the RT-PCR analysis of RNA isolated from PXO99^{A}. RT-dependent amplification of DNA fragments suggested that the genomic island (GI) genes were transcribed in one operon. (B) β-Galactosidase activity assay of the *gigX* promoter. The activity of the *gigX* promoter in PXO99^{A}, Δ*fleQ*, Δ*fleQ*-C, Δ*rpoN2* and Δ*rpoN2*-C was detected. The experiments were repeated three times, independently. (C) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *gigX* gene transcription in Δ*fleQ* and Δ*rpoN2*. The data represent the relative expression level of *gigX* in Δ*fleQ* and Δ*rpoN2*. The error bar represents standard deviations from three biological repeats. WT, wild-type.

factors and post-translational modification enzymes (Tian *et al.*, 2015). A putative glycosylation island that was embedded in the flagellar regulon consists of 10 genes, named *gigX*1–*gigX*10 (gene annotation #: PXO_00983–PXO_00992 and PXO_06174–PXO_06183). The genetic organization of this GI locus showed that the complete nucleotide sequence of the *gigX* genes was 11 555 bp in length and located between the upstream transcriptional regulator encoding gene *fleQ* and the downstream basebody encoding gene *fliE* (Fig. 1A). *gigX*1–*gigX*10 were annotated to encode a nucleotide sugar transaminase, ACP, 3-oxoacyl-ACP synthase, 3-oxoacyl-ACP reductase, acetyltransferase, hydroxylating dioxygenase, ring hydroxylating dioxygenase, hypothetical protein, methyltransferase and glycosyltransferase, respectively (Table 1). In addition, bioinformatics analysis showed that some of these genes were highly homologous to the GI genes in *P. aeruginosa* strain PAK and *P. syringae* pv. *tabaci* strain 6605,

Table 1 Similarities of glycosylation island genes in *Xanthomonas oryzae* pv. *oryzae* PXO99^A, *Pseudomonas aeruginosa* PAK and *Pseudomonas syringae* pv. *tabaci* 6605.

	PXO99^A				PAK		6605	
Gene	Cluster A	Cluster B	Size of product (amino acid)	Annotation	Cluster	% identity (amino acid)	Cluster	% identity (amino acid)
*gigX*1	PXO_06174	PXO_00992	369	Nucleotide sugar transaminase	*orfA*	55	*vioA*	64
*gigX*2	PXO_06175	PXO_00991	74	Acyl-carrier protein (ACP)	*orfB*	72	*acp*	20
*gigX*3	PXO_06176	PXO_00990	346	3-Oxoacyl-ACP synthase	*orfC*	81	*vioS*	31
*gigX*4	PXO_06177	PXO_00989	257	3-Oxoacyl-ACP reductase	*orfD*	38	*vioR*	38
*gigX*5	PXO_06178	PXO_00988	250	Dehydrogenase	–	–	–	–
*gigX*6	PXO_06179	PXO_00987	216	Acetyltransferase	*orfE*	44	*vioB*	32
gigX7	PXO_06180	PXO_00986	381	Ring hydroxylating dioxygenase α-subunit	*orfF*	35	–	–
gigX8	PXO_06181	PXO_00985	207	Hypothetical protein	*orfG*	25	–	–
*gigX*9	PXO_06182	PXO_00984	507	Methyltransferase	*orfL*	11	*vioM*	6
*gigX*10	PXO_06183	PXO_00983	1183	Glycosyltransferase	*orfN*	45	*vioT*	39

which were required for the protein glycosylation process (Table 1). Thus, these findings indicate that the *gigX* genes might be involved in the protein glycosylation process, such as glycan biosynthesis and transfer.

Co-transcribed *gigX* genes are regulated by σ^{54} factor RpoN2 and transcriptional activator FleQ

Alternative sigma factor σ^{54} RpoN and transcriptional activator FleQ have been shown to regulate flagellar gene expression in several bacteria (Prouty *et al.*, 2001). Our earlier work has shown that the expression of flagellar regulatory and structural genes is significantly down-regulated in the *rpoN2* and *fleQ* deletion mutants (Tian *et al.*, 2015). To assess the regulation by RpoN2 and FleQ of *gigX* genes, we first demonstrated that all *gigX* genes were co-transcribed in one operon belonging to one transcriptional unit using reverse transcription polymerase chain reaction (RT-PCR) analysis, and this transcriptional unit did not include *fleQ* (Fig. 1A). Next, bioinformatics analysis of the first *gigX* gene (*gigX*1) promoter sequence identified the NNGGN$_{10}$GCNN consensus sequence upstream of the transcription initiation site of *gigX*1, which is recognized by RpoN2-containing RNA polymerase, with the putative binding site centred at −12 and −24 nucleotides with a 10-nucleotide spacing between these consensus sequences. Thus, the *gigX*1 promoter was fused with the *lacZ* gene and the resulting *gigX*1-p::*lacZ* was analysed for activity by evaluation of the β-galactosidase assay in Δ*rpoN2*, Δ*fleQ* and relevant complementation strains. The β-galactosidase assay showed that the transcriptional activity of the *gigX*1 promoter decreased significantly in both Δ*rpoN2* and Δ*fleQ*, which could be restored to near wild-type level in the relevant complementation strains (Fig. 1B). Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) assay demonstrated that all *gigX* gene transcripts were remarkably reduced in Δ*rpoN2* and Δ*fleQ*, and restored in part in the relevant complementation strains (Fig. 1C). These results suggest that the regulation of co-transcribed *gigX* genes is positively controlled by RpoN2 and FleQ in *Xoo*.

Deletion in each *gigX* gene affects flagellin glycosylation

To demonstrate the functions of the *gigX* cluster in flagellin glycosylation, in-frame deletion was performed for each of the *gigX* genes, and the flagellin proteins from the wild-type and mutant strains were extracted, purified and assayed for their glycosylation, as described in Experimental Procedures. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining analysis showed that the sizes of the flagellins in the mutants of each *gigX*, except *gigX*9 encoding a methyltransferase, were smaller than those of the wild-type PXO99^A, which was further confirmed by Western blot analysis (Fig. 2A). To determine that such a change in flagellin size was a result of protein glycosylation, a glycoprotein staining experiment was performed using a Pierce glycoprotein staining kit. As predicted, the flagellins from the wild-type and Δ*gigX*9 were glycosylated, whereas those from the other mutants were unglycosylated (Fig. 2A), indicating that deletion in the *gigX* genes abolished flagellin glycosylation. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) analysis showed that the unglycosylated flagellin of Δ*gigX*10 had a molecular mass about 3 kDa less than the glycosylated flagellin of the wild-type (Fig. 2B). In addition, although the wild-type and Δ*gigX9* flagellins showed the heterogeneous glycoform, the value of each peak tended to be small in Δ*gigX9* (Fig. 2B), indicating that GigX9 might function as a methyltransferase potentially involved in the methylation of flagellin and/or glycan. These findings reveal that all *gigX* genes determine the post-translational modification of flagellin in *Xoo*.

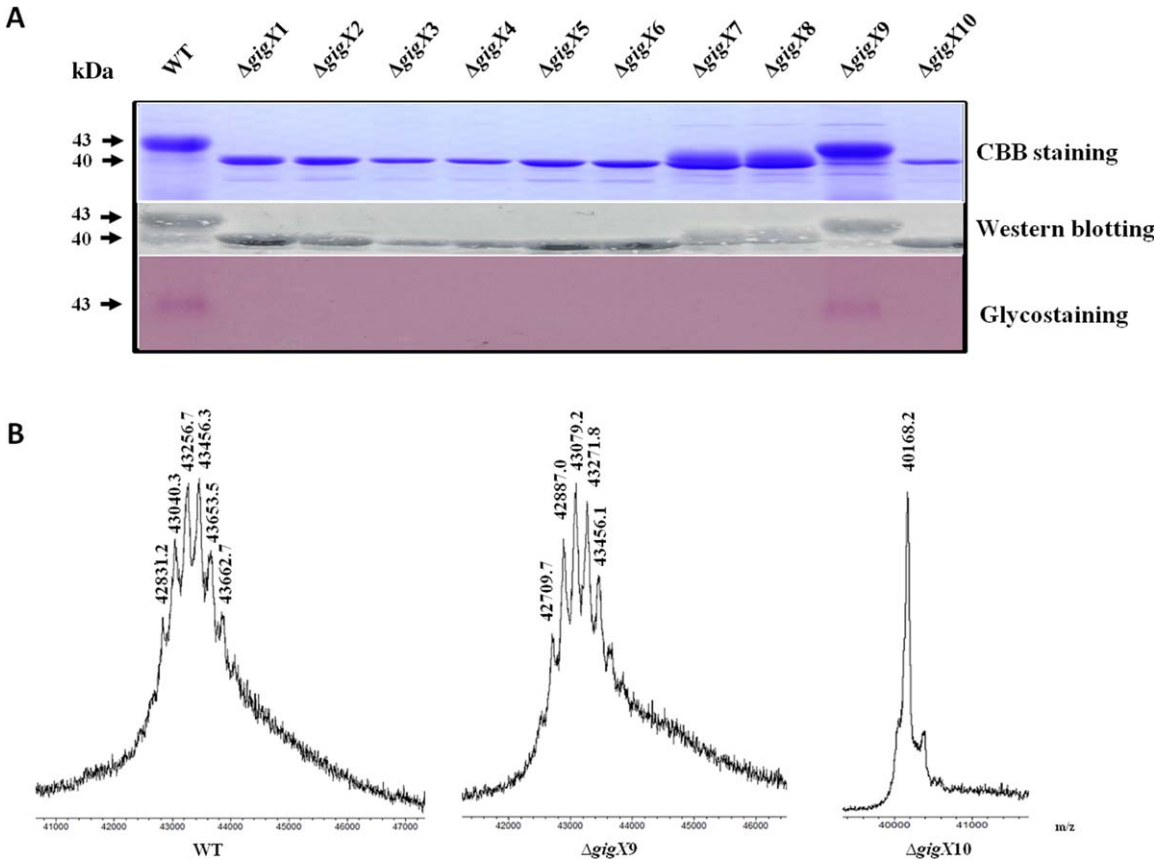

Fig. 2 Flagellin glycosylation in *Xanthomonas oryzae* pv. *oryzae* strains. (A) Detection of the purified flagellin proteins using Coomassie brilliant blue (CBB) staining, Western blotting and glycosyl moiety staining. (B) Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) analysis of flagellin proteins. The flagellins from the wild-type and Δ*gigX*9 displayed similar MS peaks with extensive heterogeneity in glycoform distribution. The molecular mass of flagellin from other *gigX* gene mutants was about 3 kDa less than that of the wild-type. WT, wild-type.

No changes in flagellar filament and motility occur in Δ*gigX*, except Δ*gigX*6

To investigate the roles of flagellin glycosylation in the flagellar motility of *Xoo*, the flagellar filament was visualized using transmission electron microscopy, and swimming and swarming zones were measured after bacterial growth at 28 °C for 2 and 4 days on 0.25% and 0.5% agar-containing plates, respectively. The flagellum of the Δ*gigX* mutants was found to be similar to that of the wild-type (Fig. 3A), suggesting that the deletion of *gigX* genes had no effect on flagellar assembly. Moreover, there was no significant difference in the average diameters of the swimming zone between the wild-type and mutants, except for Δ*gigX*6, which produced a larger swimming zone than the wild-type (Fig. 3B), indicating the inhibitory role of GigX6 in swimming motility. Furthermore, all Δ*gigX* mutants showed similar diameters of the swarming zone to the wild-type (Fig. 3C). Overall, these observations demonstrate that GigX might not be required for filament production and flagellar motility, with the exception of GigX6, which suppresses swimming motility in *Xoo*.

Deletion in each *gigX*, except *gigX*9, enhances virulence and bacterial growth in rice

To further determine the contribution of *gigX* to the virulence of *Xoo* in rice, the pathogenicity of Δ*gigX*1–Δ*gigX*10 and their complementation strains on susceptible rice cultivar IR24 was tested by leaf-clipping inoculation. The disease symptoms were recorded by photography and the lesion lengths were measured at 14 days post-inoculation. Compared with the wild-type PXO99^{A}, all Δ*gigX* mutants, except Δ*gigX*9, showed significantly enhanced ability to cause disease on rice leaves, including more severe bacterial blight symptoms (Fig. 4A) and longer lesions (Fig. 4B). The expression of each *gigX in trans* in the relevant mutants restored the disease phenotypes to near wild-type levels. Similarly, all Δ*gigX* mutants, except Δ*gigX*9, displayed a significant increase in bacterial growth in rice leaf tissues in comparison with the wild-type (Fig. 4C). Δ*gigX*9 showed the same virulent phenotype and *in planta* growth in rice as the wild-type. Moreover, assays for the bacterial growth rate of the wild-type and mutants demonstrated no significant differences when grown in nutrient-rich medium M210 (Fig. S1, see

Supporting Information), excluding the possibility that the enhanced virulence of the mutants was caused by a change in growth *in vitro*. Accordingly, these data indicate that the *gigX* genes, except *gigX*9, are involved in the virulence and *in planta* growth of *Xoo*.

Δ*gigX*1 and Δ*gigX*10 show enhanced T3SS-related gene expression

T3SS contributes to virulence through the injection of effector proteins into the cytosol of host cells in pathogenic bacteria (Jones and Dangl, 2006). Based on the observation of changes in virulence of the Δ*gigX* mutants, it was speculated that the functions of their T3SS might be affected. To test this hypothesis, the transcripts of two T3SS regulatory genes, *hrpG* and *hrpX*, one effector harpin gene, *hpa1*, and three structural genes, *hrcC*, *hrcU* and *hrcT*, were compared among the wild-type strain, Δ*gigX*1 and Δ*gigX*10 mutants, and relevant complementation strains. The expression of the T3SS-related genes was induced in XOM2, a nutrient-scarce minimal medium which has been considered to mimic growth conditions *in planta* (Furutani *et al.*, 2003), followed by the extraction of total RNA from the wild-type and mutants. The transcript levels of each gene in the mutants relative to that in the wild-type, which was designated as unity, were calculated. No significant change in the mRNA level of *hrpG* was found in either mutant; however, the transcripts of *hrpX*, *hrp1*, *hrcC*, *hrcU* and *hrcT* were significantly increased in Δ*gigX*1 and Δ*gigX*10 relative to the wild-type (Fig. 5), suggesting that either *gigX*1 or *gigX*10 has a negative effect on *hrp* gene expression. Complementation of Δ*gigX*1 and Δ*gigX*10 by *in trans* expression of *gigX*1 and *gigX*10, respectively, restored *hrp* gene expression to near wild-type levels (Fig. 5). These observations demonstrate that GigX1 and GigX10 negatively regulate T3SS expression at the transcriptional level.

No changes in EPS production and biofilm formation are found in Δ*gigX*, except Δ*gigX*6

As the Δ*gigX* mutants displayed increased virulence in rice, two important virulence-related factors of *Xoo*, EPS production and biofilm formation, were investigated in this study. No significant differences in EPS secretion and biofilm formation were found between the wild-type and Δ*gigX* mutants, except Δ*gigX*6, which displayed significantly less EPS production and biofilm formation (Figs S2 and S3, see Supporting Information). These results suggest that GigX6, but not other GigXs, regulate EPS secretion and biofilm formation in *Xoo*.

Higher H_2O_2 level is induced by glycosylated flagellin in rice leaves

To assess the role of flagellin glycosylation in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in rice, the production of H_2O_2, one of the reactive oxygen species (ROS), in glycosylated or unglycosylated flagellin-treated rice leaves was measured via luminal chemiluminescence assay, with flg22, one of the typical PAMPs, used as a positive control. The wild-type glycosylated flagellin induced higher H_2O_2 levels in rice leaves than the Δ*gigX*1 or Δ*gigX*10 unglycosylated flagellins and flg22 (Fig. 6). These results suggest that *Xoo* glycosylated flagellin might elicit a stronger PTI in rice.

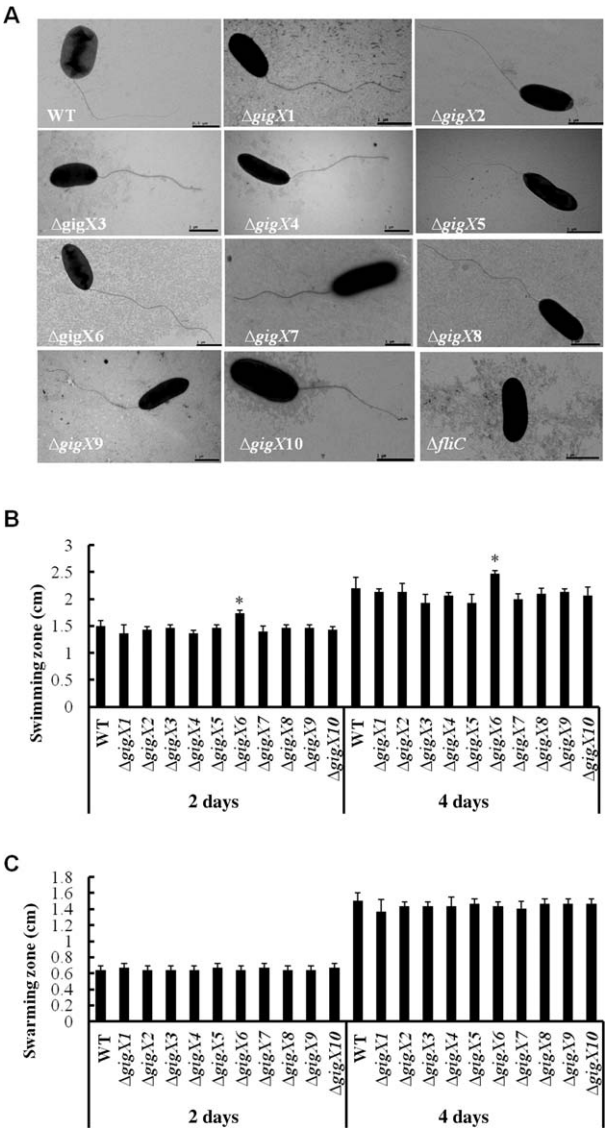

Fig. 3 Flagellar filament, swimming and swarming motility of *Xanthomonas oryzae* pv. *oryzae* strains. (A) Visualization of flagellar filament using scanning electron microscopy. (B) Measurement of swimming zones after bacterial growth for 2 and 4 days on semisolid plates (0.03% peptone, 0.03% yeast extract and 0.25% agar) at 28 °C. (C) Measurement of swarming zones after bacterial growth for 2 and 4 days on SWM plates (0.5% peptone, 0.3% yeast extract and 0.5% agar) at 28 °C. Error bars indicate standard deviations. The experiments were repeated three times, independently. WT, wild-type.

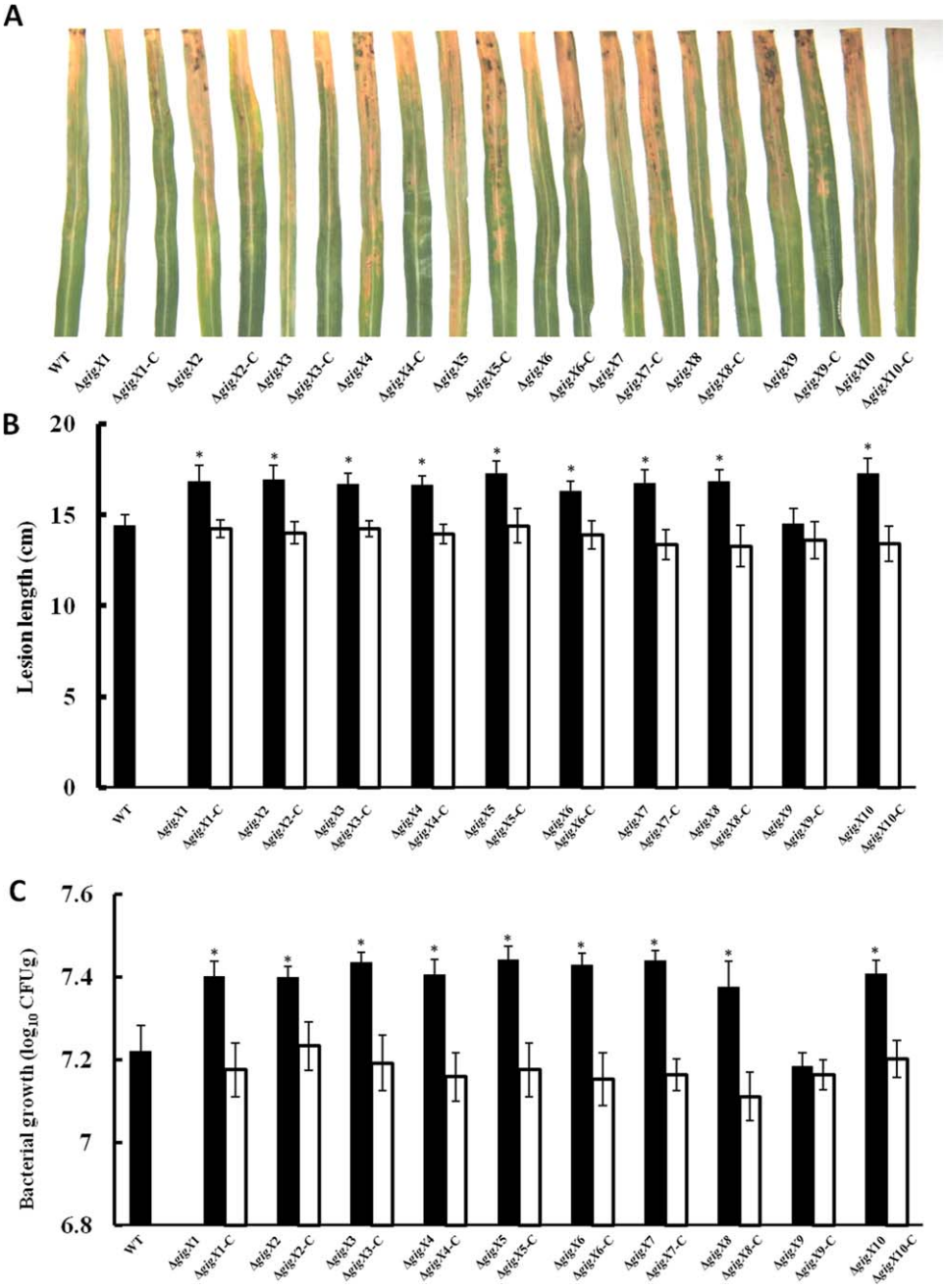

Fig. 4 Virulence on susceptible rice cultivar IR24 of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains. (A) Disease symptoms were recorded at 14 days after inoculation by photography. *Xoo* strains were inoculated by leaf clipping after bacterial growth had reached an optical density at 600 nm (OD_{600}) 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) Bacterial population was detected at 14 days after inoculation. Three leaves were mixed as one sample for each strain. The experiments were repeated three times, independently. Error bars represent standard deviations, and asterisk indicates $P < 0.05$ (Student's *t*-test). CFU, colony-forming unit; WT, wild-type.

DISCUSSION

To elucidate the genetic determinants and biological roles of flagellin glycosylation in *Xoo*, we identified the structure of *gigX*, a 10-gene-containing GI responsible for flagellin glycosylation, characterized its regulation of expression by the σ^{54} factor RpoN2 and transcriptional activator FleQ, and analysed the functions in flagellar motility and virulence through bioinformatics and genetic analysis. We demonstrated that the RpoN2/FleQ-directed *gigX* cluster plays a crucial role in glycan biosynthesis, nine genes of which (except the *gigX*9-encoding methyltransferase) were required for flagellin glycosylation. To date, only a few flagellin glycosylation pathways have been elucidated in pathogenic bacteria, including the Wbp and Vio pathways of *P. aeruginosa* and *P. syringae*, respectively (Larkin and Imperiali, 2009; Larkin *et al.*, 2010; Yamamoto *et al.*, 2011). To our immediate knowledge, this is the first report to identify a tandem of genes that were co-transcribed under the direction of RpoN2 and FleQ, and to determine the flagellin glycosylation process in *Xoo*.

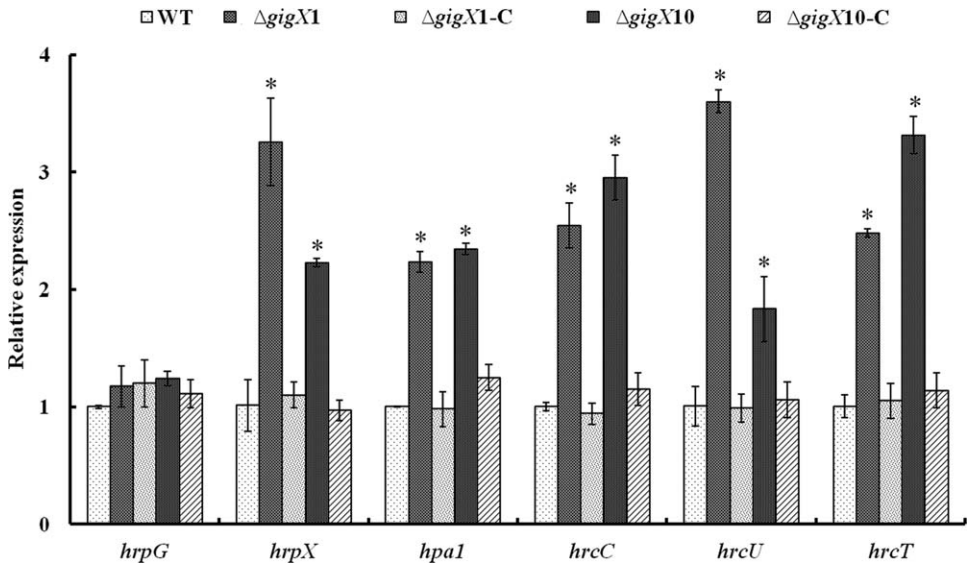

Fig. 5 Transcriptional levels of type III secretion system (T3SS)-related genes in *Xanthomonas oryzae* pv. *oryzae* strains. The relative expression of T3SS-related genes was detected by qRT-PCR in the wild-type, Δ*gigX1* and Δ*gigX10*, and the fold changes of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bars represent standard deviations from three biological repeats, and asterisk indicates $P < 0.05$ by Student's *t*-test.

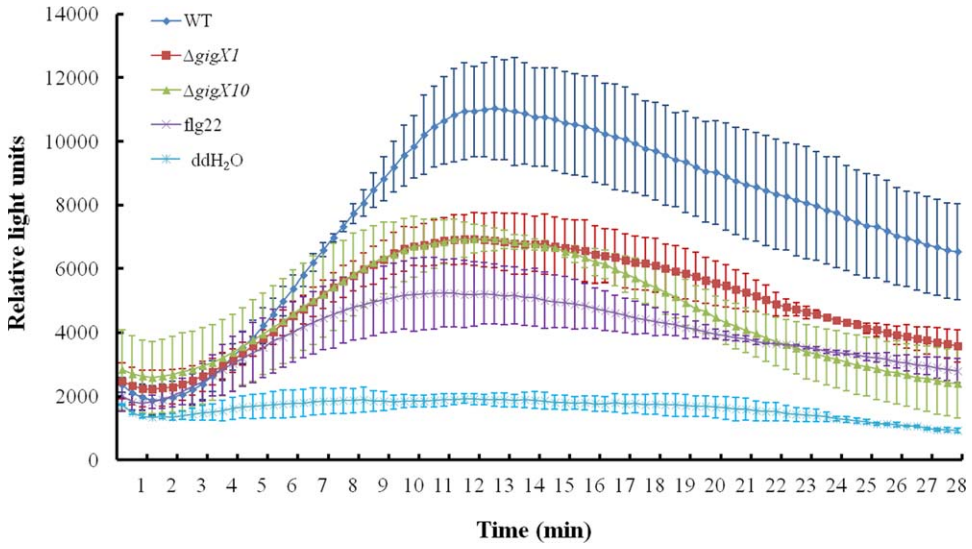

Fig. 6 H_2O_2 generation in flagellin-treated rice leaves. Leaf discs (4 mm in diameter) of rice susceptible cultivar IR24 were treated with 100 nM flagellin from the wild-type, Δ*gigX*1 or Δ*gigX*10; 100 nM flg22 was used as a positive control. The experiments were repeated at least three times, independently. The error bars represent standard deviations.

Our demonstration of the flagellin glycosylation pathway (designed as the GigX pathway) encoded by the *gigX* cluster in *Xoo* is supported by the following experimental evidence. First, the structure and predicted function of the *gigX* genes were quite similar to those of *P. aeruginosa* and *P. syringae* (Table 1). In *P. aeruginosa*, the Wbp pathway was encoded by 14 GI genes encoding the sugar biosynthetic and transfer enzymes, including glycosyltransferase, acetyltransferase, methyltransferase, 3-oxoacyl-ACP synthase and reductase, and nucleotide sugar transaminase (Arora *et al.*, 2001). In *P. syringae*, two GIs were identified, in which two genes from the smaller GI encoded glycosyltransferase, whereas seven genes from the larger GI showed high homology with the *P. aeruginosa* GI genes and encoded the Vio pathway (Taguchi *et al.*, 2006; Yamamoto *et al.*, 2011). Second, the genetic analysis of *gigX* through gene deletion mutagenesis and glycosylation detection of flagellins extracted from the wild-type and mutant strains confirmed that all *gigX* genes were necessary for the post-translational modification of flagellin with glycosylation, acetylation and methylation (Fig. 2), suggesting that flagellin glycosylation requires glycosyltransferase, acetyltransferase, 3-oxoacyl-ACP synthase and reductase, and nucleotide sugar transaminase, etc. The biochemical activities and modes of action of each of the *gigX*-encoded enzymes and proteins in the process of glycan biosynthesis and transfer will be further investigated. The identification of flagellin glycans is also required for comprehensive elucidation of the GigX pathway in *Xoo*.

The effect of flagellin glycosylation on swimming or swarming motility varied in *P. syringae* and *P. aeruginosa* in each non-identical experimental system (Arora *et al.*, 2005; Taguchi *et al.*,

2006, 2008). For example, flagellin glycosylation affected surface swarming on 0.5% semisolid agar plates and swimming motility in a highly viscous liquid medium, but did not affect surface swimming on 0.3% agar plates in *P. syringae*, whereas flagellin glycosylation did not influence surface swimming on 0.325% agar plates in *P. aeruginosa*. We observed that swimming motility on 0.25% agar plates was not affected in most Δ*gigX* mutants, except Δ*gigX*6 (Fig. 3B), and swarming motility on 0.5% agar plates was not altered in all *gigX* mutants (Fig. 3C), indicating no effects of flagellin glycosylation on flagellar motility in *Xoo*. Combined with other investigations, this study reveals the functional differences of flagellin glycosylation in the regulation of bacterial motility.

Glycosylated flagellins have been identified in other pathogenic bacteria; however, the knowledge of their biological role in the regulation of pathogen virulence and host innate immune responses remains limited. In general, flagellin glycosylation positively promotes bacterial virulence expression and host innate susceptibility. In this study, however, we observed that all Δ*gigX* mutants, except Δ*gigX*9, showed significantly enhanced virulence and bacterial growth *in planta* (Fig. 4), and *gigX*1 and *gigX*10, the first and last genes of the *gigX* cluster, encoding nucleotide sugar transaminase and glycosyltransferase, respectively, both displayed a negative effect on T3SS-related *hrp* gene expression (Fig. 5). In addition, *in trans* expression of each gene in the relevant mutants restored the virulent phenotype to near wild-type levels (Figs 4 and 5). Therefore, this is the first study to report that *gigX*-mediated glycosylation modification of flagellin negatively affects virulence in rice. Although more detailed mechanisms underlying such a novel finding need to be further explored, the possible explanations are proposed as follows. First, glycosylated flagellin may be recognized by the host surveillance system to elicit a strong PTI, thus resulting in attenuated virulence and *in planta* growth of *Xoo* in rice. This is evidenced, in part, by our current observation that glycosylated flagellin elicits higher H_2O_2 levels than unglycosylated flagellin in leaves of rice IR24 (Fig. 6). This is in contrast with the current view that flagellin glycosylation functions to evade the plant surveillance system (Hanuszkiewicz *et al.*, 2014; Khodai-Kalaki *et al.*, 2015; Taguchi *et al.*, 2006, 2008, 2009, 2010). Second, the bacterial glycan composition itself, other than flagellin, which is generated through the GigX pathway, may act directly as a PAMP for PTI induction in rice. Finally, other components, such as O-antigens, lipopolysaccharide and pili, which are targeted and modified by the GigX system, are novel virulence factors which may affect or facilitate bacterial pathogenesis in rice. Further experiments are required to confirm the plausible hypotheses and to identify the differences in response to glycosylated or unglycosylated flagellins in different rice cultivars. Future investigations should address two important questions. Is this case a ubiquitous phenomenon or rather an exception with regard to the role of flagellin glycosylation? Why has *Xoo* evolved such a complicated GigX system to glycosylate flagellins, which is always effectively recognized to elicit a strong PTI in rice?

*gigX*6, encoding a putative acetyltransferase GigX6, is required for flagellin glycosylation (Li *et al.*, 2015), EPS production and biofilm formation (Table 1, Figs 2A, S2 and S3), and GigX6 inhibition affects swimming motility (Fig. 3B). These observations indicate that acetyltransferase might play a critical role in the regulation of flagellin glycosylation, flagellar motility, EPS production and biofilm formation. In *P. syringae*, acetyltransferase VioB is essential for flagellin glycosylation, and *vioB* mutation strongly impairs swarming motility and weakly reduces swimming motility (Nguyen *et al.*, 2009; Yamamoto *et al.*, 2011). In *Xanthomonas* spp., acetyltransferases are specifically involved in the EPS biogenesis pathway (Katzen *et al.*, 1998; Kim *et al.*, 2009). Further studies are needed to reveal the molecular mechanism by which GigX6-mediated acetylation affects motility, EPS production and biofilm formation in *Xoo*.

*gigX*9 encodes a putative methyltransferase (Table 1). The position of the flagellin band in SDS-PAGE analysis is not drastically changed in Δ*gigX*9 compared with the wild-type, but the value of each mass spectral peak of flagellin glycans tends to be smaller than those of the wild-type (Fig. 2B), suggesting that the flagellin glycan may be unmethylated. Similar results have also been reported in the methyltransferase gene *vioM* in the flagellin GI of *P. syringae* pv. *tabaci* 6605 (Yamamoto *et al.*, 2011). However, the relationship of flagellin methylation to bacterial virulence remains to be elucidated. We found that *gigX9*-mediated methylation is not required for glycosylated flagellin to affect virulence on rice in *Xoo*. This finding helps us to better understand the regulatory role of flagellin methylation in bacterial virulence.

Xcc, the crucifer black rot pathogen taxonomically close to *Xoo*, has a five gene-containing GI of flagellin including the glycosyltransferase gene *fgt*, and Δ*fgt* shows significantly reduced motility and virulence on cabbage (Ichinose *et al.*, 2013). Sequence alignment analysis reveals that *Xoo* GigX10 and *Xcc* Fgt show 46% amino acid identity (Fig. S4, see Supporting Information). The molecular mass of *Xoo* flagellin glycan is 1 kDa larger than that of *Xcc*. In addition, GigX10 and Fgt displayed different roles in virulence on host plants. Thus, significant differences in terms of glycan composition and regulation of the virulence phenotype of flagellins might exist between the two bacterial pathogens.

Overall, the current study provides novel insights into the post-translational modification with glycosylation, acetylation and methylation of flagellin, and implicates its regulatory role in motility, EPS production, biofilm formation and virulence in *Xoo*.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Xoo* strains were grown in M210 liquid medium or in peptone sucrose

Table 2 Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics*	Reference or source
Escherichia coli		
DH5α	*supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
Xanthomonas oryzae **pv.** *oryzae*		
PXO99^{A}	Wild-type strain, Philippine race 6	Laboratory collection
Δ*gigX*1	*gigX*1 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*2	*gigX*2 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*3	*gigX*3 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*4	*gigX*4 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*5	*gigX*5 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*6	*gigX*6 gene deletion mutant derived from PXO99^{A}	Our laboratory
Δ*gigX*7	*gigX*7 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*8	*gigX*8 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*9	*gigX*9 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*10	*gigX*10 gene deletion mutant derived from PXO99^{A}	This study
Δ*gigX*1-C	Δ*gigX*1 containing plasmid pBBR-*gigX*1, Ap^{r}	This study
Δ*gigX*2-C	Δ*gigX*2 containing plasmid pBBR-*gigX*2, Ap^{r}	This study
Δ*gigX*3-C	Δ*gigX*3 containing plasmid pBBR-*gigX*3, Ap^{r}	This study
Δ*gigX*4-C	Δ*gigX*4 containing plasmid pBBR-*gigX*4, Ap^{r}	This study
Δ*gigX*5-C	Δ*gigX*5 containing plasmid pBBR-*gigX*5, Ap^{r}	This study
Δ*gigX*6-C	Δ*gigX*6 containing plasmid pBBR-*gigX*6, Ap^{r}	Our laboratory
Δ*gigX*7-C	Δ*gigX*7 containing plasmid pBBR-*gigX*7, Ap^{r}	This study
Δ*gigX*8-C	Δ*gigX*8 containing plasmid pBBR-*gigX*8, Ap^{r}	This study
Δ*gigX*9-C	Δ*gigX*9 containing plasmid pBBR-*gigX*9, Ap^{r}	This study
Δ*gigX*10-C	Δ*gigX*10 containing plasmid pBBR-*gigX*10, Ap^{r}	This study
Δ*fleQ*	*fleQ* gene deletion mutant derived from PXO99^{A}, Gm^{r}	Our laboratory
Δ*fleQ-C*	Δ*fleQ* containing plasmid pHM1-*fleQ*, Sp^{r}	Our laboratory
Δ*rpoN2*	*rpoN2* gene deletion mutant derived from PXO99^{A}, Gm^{r}	Our laboratory
Δ*rpoN2-C*	Δ*rpoN2* containing plasmid pHM1-*rpoN2*, Sp^{r}	Our laboratory
Δ*fliC*	*fliC* gene deletion mutant derived from PXO99^{A}, Gm^{r}	Our laboratory
Plasmid		
pKMS1	Suicidal vector carrying *sacB* gene for non-marker mutagenesis, Km^{r}	Li *et al.* (2011)
pKM-*gigX*1	pKMS1 derivative carrying a *gigX*1 mutation, Km^{r}	This study
pKM-*gigX*2	pKMS1 derivative carrying a *gigX*2 mutation, Km^{r}	This study
pKM-*gigX*3	pKMS1 derivative carrying a *gigX*3 mutation, Km^{r}	This study
pKM-*gigX*4	pKMS1 derivative carrying a *gigX*4 mutation, Km^{r}	This study
pKM-*gigX*5	pKMS1 derivative carrying a *gigX5* mutation, Km^{r}	This study
pKM-*gigX*7	pKMS1 derivative carrying a *gigX*7 mutation, Km^{r}	This study
pKM-*gigX*8	pKMS1 derivative carrying a *gigX*8 mutation, Km^{r}	This study
pK18mobsacB	Suicidal vector carrying *sacB* gene for mutagenesis, Km^{r}	Schafer *et al.* (1994)
pK18-*gigX*6	pK18mobSacB derivative carrying a *gigX*6 mutation, Km^{r}, Gmr	Our laboratory
pK18-*gigX*9	pK18mobSacB derivative carrying a *gigX*9 mutation, Km^{r}, Gmr	This study
pK18-*gigX*10	pK18mobSacB derivative carrying a *gigX*10 mutation, Km^{r}, Gmr	This study
pBBR1MCS-4	Broad-host-range expression vector, Ap^{r}	Kovach *et al.* (1995)
pBBR-*gigX*1	pBBR1MCS-4 carrying the full length of *gigX*1, Ap^{r}	This study
pBBR-*gigX2*	pBBR1MCS-4 carrying the full length of *gigX*2, Ap^{r}	This study
pBBR-*gigX*3	pBBR1MCS-4 carrying the full length of *gigX*3, Ap^{r}	This study
pBBR-*gigX*4	pBBR1MCS-4 carrying the full length of *gigX*4, Ap^{r}	This study
pBBR-*gigX*5	pBBR1MCS-4 carrying the full length of *gigX*5, Ap^{r}	This study
pBBR-*gigX*6	pBBR1MCS-4 carrying the full length of *gigX*6, Ap^{r}	Our laboratory
pBBR-*gigX*7	pBBR1MCS-4 carrying the full length of *gigX*7, Ap^{r}	This study
pBBR-*gigX*8	pBBR1MCS-4 carrying the full length of *gigX*8, Ap^{r}	This study
pBBR-*gigX*9	pBBR1MCS-4 carrying the full length of *gigX*9, Ap^{r}	This study
pBBR-*gigX*10	pBBR1MCS-4 carrying the full length of *gigX*10, Ap^{r}	This study
pHM1	Broad-host-range expression vector, Sp^{r}	Hopkins *et al.* (1992)
pHM1-P	pHM1 carrying the *gigX* promoter and a promoterless *lacZ* gene, Sp^{r}	This study

*Ap^{r}, Km^{r}, Sp^{r} and Gm^{r} indicate resistance to ampicillin, kanamycin, streptomycin and gentamicin, respectively.

agar (PSA) solid medium at 28 °C, and *Escherichia coli* strains were maintained in Luria–Bertani medium at 37 °C. The antibiotics ampicillin (Ap), gentamicin (Gm), streptomycin (Sp) and kanamycin (Km) were used at 100, 50, 50 and 50 μg/mL, respectively.

Bioinformatics analysis of the *gigX* cluster

The gene location, size of products and annotation of the *gigX* genes in *Xoo* wild-type strain PXO99^A, and the homologous sequences of *gigX* genes in *P. aeruginosa* strain PAK and *P. syringae* pv. *tabaci* strain 6605, were obtained from the National Center for Biotechnology Information (NCBI) website. Relevant sequence alignment was performed using DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA).

Generation of *gigX1*-p::*lacZ* and assay for β-galactosidase activity

A DNA fragment (350 bp) containing the predicted −12/–24 region of the *gigX* promoter was amplified from PXO99^A genomic DNA using specific primers (*gigXp*F/R), and ligated into the modified pHM1 vector containing a promoterless *lacZ* gene. The recombinant pHM1-P was introduced into PXO99^A, Δ*rpoN2*, Δ*fleQ* and their complementary strains. The strains transformed with pHM1-P were selected by resistance to streptomycin. These strains were grown as described previously to measure the β-galactosidase activity in cellular extracts using the β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA) (Zhan *et al.*, 2008). Assays were performed with three biological replicates. The primer sequences are listed in Table S1 (see Supporting Information).

RNA isolation and qRT-PCR analysis

Bacterial strains were cultured in M210 medium until an optical density at 600 nm (OD_{600}) of 0.6 was attained. Cells were then collected by centrifugation at 12 000 ***g***. To induce the expression of T3SS-related genes, *Xoo* strains were subcultured in XOM2 medium at 28 °C for 12 h and harvested by centrifugation at 12 000 ***g*** for 5 min (Yang *et al.*, 2015). Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase, and the cDNA fragments were synthesized using Superscript III reverse transcriptase (Invitrogen) with random primers. The *gyrB* gene was used as a reference gene for qRT-PCR assays. The qRT-PCR analysis was performed as described previously (Yu *et al.*, 2014) with primers designed using Primer Premier 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA) (Table S1). Three biological replicates and triplicate PCR were tested for every sample.

Construction of gene deletion mutants and complementation strains

The gene deletion mutants of *gigX* derived from PXO99^A were generated by homologous recombination using the suicide vector pKMS1, as reported previously (Li *et al.*, 2011, 2015). In brief, the left and right arms of the gene were amplified by PCR from *Xoo* genomic DNA with the relevant F/R primers (Table S1). These fragments were ligated into suicide vector pKMS1, resulting in plasmid pKM-Y (Y represents the name of the *gigX* gene). Then, pKM-Y was introduced into PXO99^A by electroporation. The transformant was first selected on NAN medium (1% tryptone, 0.1% yeast extract, 0.3% peptone and 1.5% agar) containing Km, followed by continuous transfer culture in NBN liquid medium (1% sucrose) five times. The candidates were screened on NAS medium (10% sucrose). The gene deletion mutant that could grow on NAS, but was sensitive to Km, was validated by PCR analysis. To generate the complementary strain, the coding region and promoter of the gene were amplified with the relevant F/R primers (Table S1) and inserted into vector pBBR1MCS-4, and the recombinant plasmids were electroporated into the relevant mutants for complementation analysis. Finally, these complementary strains were further confirmed by PCR analysis.

Extraction and purification of flagellin proteins

Purification of flagellin proteins was performed as described previously with some modifications (Taguchi *et al.*, 2006). Briefly, *Xoo* wild-type and mutants were inoculated in M210 for 48 h at 28 °C, and collected by centrifugation at 12 000 ***g*** for 5 min. The cells were resuspended in 1/3 vol MMMF medium (50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, 10 mM mannitol and 10 mM fructose, pH 5.7) for 24 h at 23 °C. Then, the cells were harvested by centrifugation and resuspended in 50 mM sodium phosphate medium (pH 7.0), and violently shaken for 3 min. After centrifugation at 7000 ***g*** for 10 min, the supernatant was filtered through a 0.45-μm pore size filter and centrifuged at 100 000 ***g*** for 1 h; pellets of purified flagellin were obtained and suspended in sterilized double-distilled water (ddH_2O) at a final concentration of 100 μg/mL.

Western blotting and glycosylation detection of flagellin

Purified flagellin proteins (10 μL) from *Xoo* wild-type and mutant strains were separated by SDS-PAGE at 100 V for 1.5 h, and electroblotted onto a poly(vinylidene difluoride) (PVDF) membrane at 20 V for 1 h. Then, the flagellin proteins were detected by an anti-flagellin first antibody and goat anti-rabbit second antibody (Beijing Protein Innovation, Beijing, China) conjugated with horseradish peroxidase (Tiangen Biotech, Beijing, China). For glycoprotein analysis, the purified flagellin proteins (10 μL) were detected by a GelCode glycoprotein detection kit (Pierce Biotechnology, Rockford, IL, USA) after SDS-PAGE.

MS analysis of flagellin

Purified flagellin proteins from *Xoo* strains were subjected to MS as described previously (Taguchi *et al.*, 2006). Flagellins were dialysed in water with 0.1% (v/v) trifluoroacetic acid (TFA), and added to an equal volume of mixture containing a saturated solution of sinapinic acid in 33% acetonitrile–water with 0.1% TFA (v/v). The mixtures were deposited on a stainless steel target plate, and the mass spectra of flagellins were analysed by a Biflex III MALDI-TOF mass spectrometer (Bruker, Ibaraki, Japan) in a linear, positive mode with a mass accuracy of 0.2%.

Electron microscopy visualization of flagellum

Xoo strains were grown on PSA medium at 28 °C for 48 h, and suspended in ddH_2O. Then, the suspension was deposited onto grids coated with Formvar (Standard Technology, Ormond Beach, FL, USA), stained with 2%

uranyl acetate for 30 s and dried for 10 min at room temperature. The flagella were observed using a transmission electron microscope (H-7500, Hitachi, Tokyo, Japan).

Flagellar motility assay

Xoo strains were grown in M210 medium until OD_{600} of 0.8 was reached. The cells were harvested by centrifugation at 12 000 ***g*** for 5 min and resuspended in ddH_2O. For swimming and swarming motility assays, 2 μL of bacterial suspension were inoculated onto semisolid medium plates (0.03% peptone, 0.03% yeast extract and 0.25% agar) and SWM plates (0.5% peptone, 0.3% yeast extract and 0.5% agar), respectively, and incubated at 28 °C for 2 and 4 days; the diameters of the bacterial swimming and swarming zones were measured. The experiments were repeated three times, independently.

Pathogenicity assay

The *Xoo* wild-type and mutant strains were grown in M210 medium at 28 °C for 48 h, collected by centrifugation at 7 000 ***g*** for 15 min and resuspended in ddH_2O (OD_{600} = 0.8). The virulence of these strains was detected on susceptible rice (*Oryza sativa* L. ssp. *indica* IR24) by leaf clipping, and the lesion lengths of 10 leaves were recorded at 14 days after inoculation for every strain. The population of bacteria on inoculated leaves was also detected. Three inoculated leaves were ground in ddH_2O with a mortar and grinding rod, and the mixture was diluted to optical concentration, and spread onto PSA plates. The colonies of bacteria were counted after incubation at 28 °C for 72 h. The experiments were repeated three times, independently.

In vitro growth rate measurement

Xoo strains were grown in M210 medium until OD_{600} = 0.8. For bacterial growth assay, 200 μL of bacteria were inoculated in 100 mL of M210 liquid medium and cultured at 28 °C at 200 rpm. Then, the growth curves of these strains were obtained by testing OD_{600} every 6 h. The experiments were repeated three times, independently.

Exopolysaccharide production assay

To estimate exopolysaccharide production, bacterial strains were grown in M210 medium at 28 °C at OD_{600} = 2.5, and the supernatants were collected by centrifugation at 7000 ***g*** for 15 min. Two volumes of ethanol were added to the supernatants, and the mixture was held at −20 °C for 2 h. EPS was obtained by centrifugation at 12 000 ***g*** for 10 min and dried at 55 °C for 48 h. Finally, the dry weights were recorded as an estimate of EPS (Yang *et al.*, 2014).

Biofilm formation assay

The biofilm formation assay was performed as described previously (Yang *et al.*, 2014). Briefly, bacteria were grown in M210 at 200 rpm to OD_{600} = 0.5, and then 5 mL were transferred to tubes and standing inoculated at 28 °C for 72 h. Bacterial pellicles were stained with 0.1% crystal violet for 15 min at room temperature. The tubes were rinsed gently with water and photographed. Finally, the biofilm was dissolved with ethanol, and the absorbance at 490 nm was recorded. The experiments were repeated three times, independently.

Assay for H_2O_2 production in flagellin-treated rice leaves

H_2O_2 production assay was performed as described previously (Ding *et al.*, 2012). In brief, leaf discs (4 mm in diameter) were excised from 6-week-old rice plants (*Oryza sativa* L. ssp. *indica* IR24). After incubation in ddH_2O for 12 h, three leaf discs were treated with a mixture containing 100 nM of flagellin, 100 μL luminal solution (Thermo Scientific, Rockford, IL, USA), 10 nM horseradish peroxidase (Sigma-Aldrich, St Louis, MO, USA) and 200 μL ddH_2O. The flagellins were extracted and purified from the wild-type, Δ*gigX1* and Δ*gigX10* as described above; 100 nM of flg22, a 22-amino-acid containing peptide (QRLSTGSRINSAKDDAAGLQIA) that was artificially synthesized (BGI, Shenzhen, China), was used as a positive control. Luminescence was recorded every 20 s for 28 min on a Glomas 20/20 luminometer (Promega). The experiments were repeated at least three times, independently.

Statistical analysis

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

ACKNOWLEDGEMENTS

This work was supported by grants from the National Basic Research Program, China (2011CB100700) and National High-Technology Research Program, China (2012AA101504) for C.H.

REFERENCES

Arora, S.K., Bangera, M., Lory, S. and Ramphal, R. (2001) A genomic island in *Pseudomonas aeruginosa* carries the determinants of flagellin glycosylation. *Proc. Natl. Acad. Sci. USA*, **98**, 9342–9347.

Arora, S.K., Neely, A.N., Blair, B., Lory, S. and Ramphal, R. (2005) Role of motility and flagellin glycosylation in the pathogenesis of *Pseudomonas aeruginosa* burn wound infections. *Infect. Immun.* **73**, 4395–4398.

Asakura, H., Churin, Y., Bauer, B., Boettcher, J.P., Bartfeld, S., Hashii, N., Kawasaki, N., Mollenkopf, H.J., Jungblut, P.R., Brinkmann, V. and Meyer, T.F. (2010) *Helicobacter pylori* HP0518 affects flagellin glycosylation to alter bacterial motility. *Mol. Microbiol.* **78**, 1130–1144.

Chaban, B., Voisin, S., Kelly, J., Logan, S.M. and Jarrell, K.F. (2006) Identification of genes involved in the biosynthesis and attachment of *Methanococcus voltae* N-linked glycans: insight into N-linked glycosylation pathways in Archaea. *Mol. Microbiol.* **61**, 259–268.

Das, A., Rangaraj, N. and Sonti, R.V. (2009) Multiple adhesin-like functions of *Xanthomonas oryzae* pv. *oryzae* are involved in promoting leaf attachment, entry, and virulence on rice. *Mol. Plant–Microbe Interact*, **22**, 73–85.

Ding, B., Bellizzi Mdel, R., Ning, Y., Meyers, B.C. and Wang, G.L. (2012) HDT701, a histone H4 deacetylase, negatively regulates plant innate immunity by modulating histone H4 acetylation of defense-related genes in rice. *Plant Cell*, **24**, 3783–3794.

Furutani, A., Tsuge, S., Oku, T., Tsuno, K., Inoue, Y., Ochiai, H., Kaku, H. and Kubo, Y. (2003) Hpa1 secretion via type III secretion system in *Xanthomonas oryzae* pv. *oryzae*. *J. Gen. Plant Pathol.* **69**, 271–275.

Guerry, P., Ewing, C.P., Schirm, M., Lorenzo, M., Kelly, J., Pattarini, D., Majam, G., Thibault, P. and Logan, S. (2006) Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence. *Mol. Microbiol.* **60**, 299–311.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557–580.

Hanuszkiewicz, A., Pittock, P., Humphries, F., Moll, H., Rosales, A.R., Molinaro, A., Moynagh, P.N., Lajoie, G.A. and Valvano, M.A. (2014) Identification of the flagellin glycosylation system in *Burkholderia cenocepacia* and the contribution of glycosylated flagellin to evasion of human innate immune responses. *J. Biol. Chem.* **289**, 19 231–19 244.

Hirai, H., Takai, R., Kondo, M., Furukawa, T., Hishiki, T., Takayama, S. and Che, F.S. (2014) Glycan moiety of flagellin in *Acidovorax avenae* K1 prevents the recognition by rice that causes the induction of immune responses. *Plant Signal. Behav.* **9**, e972782.

Hopkins, C.M., White, F.F., Choi, S.H., Guo, A. and Leach, J.E. (1992) Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant–Microbe Interact*, **5**, 451–459.

Ichinose, Y., Taguchi, F., Yamamoto, M., Ohnishi-Kameyama, M., Atsumi, T., Iwaki, M., Manabe, H., Kumagai, M., Nguyen, Q.T., Nguyen, C.L., Inagaki, Y., Ono, H., Chiku, K., Ishii, T. and Yoshida, M. (2013) Flagellin glycosylation is ubiquitous in a broad range of phytopathogenic bacteria. *J. Gen. Plant Pathol.* **79**, 359–365.

Jones, J.D. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.

Katzen, F., Ferreiro, D.U., Oddo, C.G., Ielmini, M.V., Becker, A., Puhler, A. and Ielpi, L. (1998) *Xanthomonas campestris* pv. *campestris* gum mutants: effects on xanthan biosynthesis and plant virulence. *J. Bacteriol.* **180**, 1607–1617.

Khodai-Kalaki, M., Andrade, A., Fathy Mohamed, Y. and Valvano, M.A. (2015) *Burkholderia cenocepacia* lipopolysaccharide modification and flagellin glycosylation affect virulence but not innate immune recognition in plants. *mBio*, **6**, e00679-15.

Kim, S.Y., Kim, J.G., Lee, B.M. and Cho, J.Y. (2009) Mutational analysis of the gum gene cluster required for xanthan biosynthesis in *Xanthomonas oryzae* pv *oryzae*. *Biotechnol. Lett.* **31**, 265–270.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd. and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*, **166**, 175–176.

Larkin, A. and Imperiali, B. (2009) Biosynthesis of UDP-GlcNAc(3NAc)A by WbpB, WbpE, and WbpD: enzymes in the Wbp pathway responsible for O-antigen assembly in *Pseudomonas aeruginosa* PAO1. *Biochemistry*, **48**, 5446–5455.

Larkin, A., Olivier, N.B. and Imperiali, B. (2010) Structural analysis of WbpE from *Pseudomonas aeruginosa* PAO1: a nucleotide sugar aminotransferase involved in O-antigen assembly. *Biochemistry*, **49**, 7227–7237.

Lithgow, K.V., Scott, N.E., Iwashkiw, J.A., Thomson, E.L., Foster, L.J., Feldman, M.F. and Dennis, J.J. (2014) A general protein O-glycosylation system within the *Burkholderia cepacia* complex is involved in motility and virulence. *Mol. Microbiol.* **92**, 116–137.

Li, H.Y., Yu, C., Chen, H.M., Tian, F. and He, C.Y. (2015) PXO_00987, a putative acetyltransferase, is required for flagellin glycosylation, and regulates flagellar motility, exopolysaccharide production, and biofilm formation in *Xanthomonas oryzae* pv. *oryzae*. *Microb. Pathog.* **85**, 50–57.

Li, Y.R., Zou, H.S., Che, Y.Z., Cui, Y.P., Guo, W., Zou, L.F., Chatterjee, S., Biddle, E.M., Yang, C.H. and Chen, G.Y. (2011) A novel regulatory role of HrpD6 in regulating hrp-hrc-hpa genes in *Xanthomonas oryzae* pv. *oryzicola*. *Mol. Plant–Microbe Interact*, **24**, 1086–1101.

Logan, S.M. (2006) Flagellar glycosylation – a new component of the motility repertoire? *Microbiology*, **152**, 1249–1262.

Merino, S. and Tomas, J.M. (2014) Gram-negative flagella glycosylation. *Int. J. Mol. Sci.* **15**, 2840–2857.

Nguyen, L.C., Yamamoto, M., Ohnishi-Kameyama, M., Andi, S., Taguchi, F., Iwaki, M., Yoshida, M., Ishii, T., Konishi, T., Tsunemi, K. and Ichinose, Y. (2009) Genetic analysis of genes involved in synthesis of modified 4-amino-4,6-dideoxyglucose in flagellin of *Pseudomonas syringae* pv. *tabaci*. *Mol. Genet. Genomics*, **282**, 595–605.

Nino-Liu, D.O., Ronald, P.C. and Bogdanove, A.J. (2006) *Xanthomonas oryzae* pathovars: model pathogens of a model crop. *Mol. Plant Pathol.* **7**, 303–324.

Nothaft, H. and Szymanski, C.M. (2010) Protein glycosylation in bacteria: sweeter than ever. *Nat. Rev. Microbiol.* **8**, 765–778.

Prouty, M.G., Correa, N.E. and Klose, K.E. (2001) The novel sigma54- and sigma28-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. *Mol. Microbiol.* **39**, 1595–1609.

Salzberg, S.L., Sommer, D.D., Schatz, M.C., Phillippy, A.M., Rabinowicz, P.D., Tsuge, S., Furutani, A., Ochiai, H., Delcher, A.L., Kelley, D., Madupu, R., Puiu, D., Radune, D., Shumway, M., Trapnell, C., Aparna, G., Jha, G., Pandey, A., Patil, P.B., Ishihara, H., Meyer, D.F., Szurek, B., Verdier, V., Koebnik, R., Dow, J.M., Ryan, R.P., Hirata, H., Tsuyumu, S., Won Lee, S., Seo, Y.S., Sriariyanum, M., Ronald, P.C., Sonti, R.V., Van Sluys, M.A., Leach, J.E., White, F.F. and Bogdanove, A.J. (2008) Genome sequence and rapid evolution of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* PXO99^{A}. *BMC Genomics*, **9**, 204.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. and Puhler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene*, **145**, 69–73.

Scott, A.E., Twine, S.M., Fulton, K.M., Titball, R.W., Essex-Lopresti, A.E., Atkins, T.P. and Prior, J.L. (2011) Flagellar glycosylation in *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *J. Bacteriol.* **193**, 3577–3587.

Song, C.F. and Yang, B. (2010) Mutagenesis of 18 type III effectors reveals virulence function of XopZ(PXO99) in *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant–Microbe Interact*, **23**, 893–902.

Taguchi, F., Takeuchi, K., Katoh, E., Murata, K., Suzuki, T., Marutani, M., Kawasaki, T., Eguchi, M., Katoh, S., Kaku, H., Yasuda, C., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2006) Identification of glycosylation genes and glycosylated amino acids of flagellin in *Pseudomonas syringae* pv. *tabaci*. *Cell. Microbiol.* **8**, 923–938.

Taguchi, F., Shibata, S., Suzuki, T., Ogawa, Y., Aizawa, S., Takeuchi, K. and Ichinose, Y. (2008) Effects of glycosylation on swimming ability and flagellar polymorphic transformation in *Pseudomonas syringae* pv. *tabaci* 6605. *J. Bacteriol.* **190**, 764–768.

Taguchi, F., Suzuki, T., Takeuchi, K., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2009) Glycosylation of flagellin from *Pseudomonas syringae* pv. *tabaci* 6605 contributes to evasion of host tobacco plant surveillance system. *Physiol. Mol. Plant Pathol.* **74**, 11–17.

Taguchi, F., Yamamoto, M., Ohnishi-Kameyama, M., Iwaki, M., Yoshida, M., Ishii, T., Konishi, T. and Ichinose, Y. (2010) Defects in flagellin glycosylation affect the virulence of *Pseudomonas syringae* pv. *tabaci* 6605. *Microbiology*, **156**, 72–80.

Takeuchi, K. (2008) Studies on flagellin glycosylation and pathogenicity of *Pseudomonas syringae*. *J. Gen. Plant Pathol.* **74**, 461–462.

Tian, F., Yu, C., Li, H.Y., Wu, X.L., Li, B., Chen, H.M., Wu, M.S. and He, C.Y. (2015) Alternative sigma factor RpoN2 is required for flagellar motility and full virulence of *Xanthomonas oryzae* pv. *oryzae*. *Microbiol. Res.* **170**, 177–183.

Tsuge, S., Nakayama, T., Terashima, S., Ochiai, H., Furutani, A., Oku, T., Tsuno, K., Kubo, Y. and Kaku, H. (2006) Gene involved in transcriptional activation of the hrp regulatory gene hrpG in *Xanthomonas oryzae* pv. *oryzae*. *J. Bacteriol.* **188**, 4158–4162.

White, F.F. and Yang, B. (2009) Host and pathogen factors controlling the rice–*Xanthomonas oryzae* interaction. *Plant Physiol.* **150**, 1677–1686.

Yamamoto, M., Ohnishi-Kameyama, M., Nguyen, C.L., Taguchi, F., Chiku, K., Ishii, T., Ono, H., Yoshida, M. and Ichinose, Y. (2011) Identification of genes involved in the glycosylation of modified viosamine of flagellins in *Pseudomonas syringae* by mass spectrometry. *Genes*, **2**, 788–803.

Yang, F., Tian, F., Li, X.T., Fan, S.S., Chen, H.M., Wu, M.S., Yang, C.H. and He, C.Y. (2014) The degenerate EAL-GGDEF domain protein Filp functions as a cyclic di-GMP receptor and specifically interacts with the PilZ-domain protein PXO_02715 to regulate virulence in *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant–Microbe Interact*, **27**, 578–589.

Yang, F.H., Tian, F., Chen, H.M., Hutchins, W., Yang, C.H. and He, C.Y. (2015) The *Xanthomonas oryzae* pv. *oryzae* PilZ domain proteins function differentially in cyclic di-GMP binding and regulation of virulence and motility. *Appl. Environ. Microbiol.* **81**, 4358–4367.

Yu, C., Chen, H.M., Tian, F., Leach, J.E. and He, C.Y. (2014) Differentially-expressed genes in rice infected by *Xanthomonas oryzae* pv. *oryzae* relative to a flagellin-deficient mutant reveal potential functions of flagellin in host–pathogen interactions. *Rice*, **7**, 20.

Zhan, L.J., Han, Y.P., Yang, L., Geng, J., Li, Y.L., Gao, H., Guo, Z.B., Fan, W., Li, G., Zhang, L.F., Qin, C., Zhou, D.S. and Yang, R.F. (2008) The cyclic AMP receptor protein, CRP, is required for both virulence and expression of the minimal CRP regulon in *Yersinia pestis* biovar microtus. *Infect. Immun.* **76**, 5028–5037.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Bacterial growth *in vitro* of *Xanthomonas oryzae* pv. *oryzae* strains. Bacterial growth in M210 medium was determined by measuring the optical density at 600 nm (OD_{600}) at 6-h intervals. Experiments were performed with three biological replicates.

Fig. S2 Exopolysaccharide production of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains. Extracellular polysaccharide (EPS) secretion in *Xoo* strains was detected by examination of the colony and quantification of EPS production. Values represent the average of three independent experiments, and the error bars indicate the standard deviations.

Fig. S3 Biofilm formation of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains. Biofilm formation in *Xoo* strains was quantified. The values represent the average of three independent experiments, and the error bars indicate the standard deviations.

Fig. S4 Sequence alignment analysis of glycosyltransferase from *Xanthomonas oryzae* pv. *oryzae* PXO99^{A} and *Xanthomonas campestris* pv. *campestris* ATCC33913. The sequences were downloaded from the National Center for Biotechnology Information (NCBI) website, and sequence alignment was performed using DNAMAN software. The shading indicates identical amino acids.

Table S1 Primers used in this study