INTRODUCTION

The plant pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo), a gram-negative bacterium belonging to the class gamma-proteobacteria (Timilsina et al., 2020), is an important bacterial pathogen that severely affects rice yields worldwide by causing leaf blight disease (Jiang et al., 2020). Due to its economic or scientific importance, plant bacteriologists selected *X. oryzae* pv. *oryzae* as one of the "top 10" bacterial plant pathogens (Mansfield et al., 2012). To adapt to the multiple changing environmental factors and survive in the host plant, the physiological metabolism of bacteria needs to be precisely regulated (Li et al., 2020; Yang et al., 2020; Zheng et al., 2021). Numerous virulence-associated factors facilitate bacterial proliferation in the host by suppressing the plant immunity and promoting symptom development (Buttner & Bonas, 2010; Timilsina et al., 2020), including extracellular polysaccharide (EPS) (Xue et al., 2018), lipopolysaccharide (LPS) (Petrocelli et al., 2012), secretion systems and effectors (Alvarez-Martinez et al., 2021; Timilsina et al., 2020). Among all the virulence-associated factors, the secretion systems are the most important (Alvarez-Martinez et al., 2021). In gram-negative bacteria, at least six secretion systems have been reported, from the type I secretion system to the type VI secretion system.

Transcriptional regulator Sar regulates the multiple secretion systems in *Xanthomonas oryzae*

Yanan Shao¹ | Guiyu Tang¹ | Yuanyuan Huang¹ | Wenli Ke¹ | Shasha Wang¹ | Dehong Zheng¹,² | Lifang Ruan¹,³

¹State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, China
²National Demonstration Center for Experimental Plant Science Education, College of Agriculture, Guangxi University, Nanning, China
³College of Resources and Environment, Tibet Agriculture & Animal Husbandry University, Linzhi, China

Abstract

*Xanthomonas oryzae* pv. *oryzae* (Xoo) is a notorious plant pathogen that causes leaf blight of rice cultivars. The pathogenic bacteria possess numerous transcriptional regulators to regulate various biological processes, such as pathogenicity in the host plant. Our previous study identified a new master regulator PXO_RS20790 that is involved in pathogenicity for Xoo against the host rice. However, the molecular functions of PXO_RS20790 are still unclear. Here, we demonstrate that transcriptional regulator Sar (PXO_RS20790) regulates multiple secretion systems. The RNA-sequencing analysis, bacterial one-hybrid assay, and electrophoretic mobility shift assay revealed that Sar enables binding of the promoters of the T1SS-related genes, the avirulence gene, raxX, and positively regulates these genes’ expression. Meanwhile, we found that Sar positively regulated the T6SS-1 clusters but did not regulate the T6SS-2 clusters. Furthermore, we revealed that only T6SS-2 is involved in interbacterial competition. We also indicated that Sar could bind the promoters of the T3SS regulators, hrgP and hrgX, to activate these two genes’ transcription. Our findings revealed that Sar is a crucial regulator of multiple secretion systems and virulence.

KEYWORDS

RaxX, regulation, secretion system, transcriptional regulator, Xanthomonas oryzae

1 | INTRODUCTION

The plant pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo), a gram-negative bacterium belonging to the class gamma-proteobacteria (Timilsina et al., 2020), is an important bacterial pathogen that severely affects rice yields worldwide by causing leaf blight disease (Jiang et al., 2020). Due to its economic or scientific importance, plant bacteriologists selected *X. oryzae* pv. *oryzae* as one of the "top 10" bacterial plant pathogens (Mansfield et al., 2012). To adapt to the multiple changing environmental factors and survive in the host plant, the physiological metabolism of bacteria needs to be precisely regulated (Li et al., 2020; Yang et al., 2020; Zheng et al., 2021). Numerous virulence-associated factors facilitate bacterial proliferation in the host by suppressing the plant immunity and promoting symptom development (Buttner & Bonas, 2010; Timilsina et al., 2020), including extracellular polysaccharide (EPS) (Xue et al., 2018), lipopolysaccharide (LPS) (Petrocelli et al., 2012), secretion systems and effectors (Alvarez-Martinez et al., 2021; Timilsina et al., 2020). Among all the virulence-associated factors, the secretion systems are the most important (Alvarez-Martinez et al., 2021). In gram-negative bacteria, at least six secretion systems have been reported, from the type I secretion system to the type VI secretion system.
system (T1SS to T6SS), and these secretion systems are vital to pathogenenic bacterial survival in complex environments, especially in the host plant (Alvarez-Martinez et al., 2021; Costa et al., 2015).

In Xanthomonas, the T1SS consists of three genes, raxA, raxB, and raxC, which encode a membrane protein, a peptidase-containing ABC transporter, and an outer membrane protein, respectively (da Silva et al., 2004; Han et al., 2012; Liu et al., 2019). The first gene in the raxSTAB operon, raxST, encodes a sulfotransferase-like protein (Han et al., 2012). A sulfated RaxX (AvrXa21) secreted by the T1SS is required to activate Xa21-mediated recognition and immunity (Ercoli et al., 2022; Han et al., 2012; Liu et al., 2019; da Silva et al., 2004). It has been reported that the RaxR-RaxH two-component system (TCS) regulates the expression of eight rax genes (Lee et al., 2006, 2008). Meanwhile, the PhoP-PhoQ TCS is negatively regulated by the RaxR-RaxH TCS and is also required for AvrXa21 activity (Lee et al., 2008). A study revealed a key T3SS-related response regulator HrpX directly regulating the expression of raxSTAB-raxX; moreover, the HrpX-binding plant-inducible promoter (PIP) was found in the promoter region of raxSTAB and raxX, respectively (Joe et al., 2021). It is worth noting that our previous RNA-seq data showed that some pathogenicity-related regulators were participating in raxSTAB and/or raxX expression, such as HrpG, HrpX, VemR, and PXO_RS20790 (Zheng et al., 2021). However, the regulation of raxX and raxSTAB cluster expressions remains elusive.

The T6SS is a bacterial contact-dependent contractile nanomachine used to inject proteinaceous protein effectors into eukaryotic or prokaryotic cells to manipulate the host and direct killing of competitors in complex communities (Wang et al., 2019; Yu et al., 2021). In Pseudomonas aeruginosa, the T6SS has displayed a significant role in interspecies competition, transportation of metal ions, and diverse stresses (Basler et al., 2013; Han et al., 2019; Hood et al., 2010; Lin et al., 2015, 2017). In plant pathogens such as Xanthomonas citri, the T6SS was required to resist Dictyostelium discoideum predation (Bayer-Santos et al., 2018). In addition, the T6SS of Xanthomonas species is also involved in pathogenicity (Montenegro Benavides et al., 2021). Some Xanthomonas strains have two phylogenetically distinct T6SS clusters, T6SS-1 and T6SS-2 (Bayer-Santos et al., 2019). A recent study reported that T6SS-2 plays a vital role in interspecies competition in X. oryzae pv. oryzicola (Zhu et al., 2020). However, the biological functions and regulations of T6SSs have not been elucidated in most Xanthomonas species.

Among all these secretion systems, T3SS plays a crucial role in both animal and plant pathogens of successful infection in the host (Deng et al., 2017; McCann & Gutman, 2008). T3SS is encoded by the hrp gene clusters, containing multiple transcription elements consisting of more than 20 genes (Buttner & Bonas, 2002). In X. oryzae, the activation of hrp gene clusters in plants or on the basic medium XOM2 (X. oryzae pv. oryzicola hrp-inducing medium 2) requires transcriptional regulators (TRs) HrpG and HrpX (Tsuge et al., 2002). They regulate T3SS by regulating the expression of hrp gene clusters (Guo et al., 2011). HrpG is an OmpR family response regulator that belongs to the two-component system and activates the expression of hpx, which encodes an AraC-type regulator (Teper et al., 2021). HrpX binds to the PIP box region upstream of the T3SS gene clusters to regulate the expression of T3SS-related genes (Koebnik et al., 2006). Although a few studies have been reported to identify regulators that could directly activate hrpG and/or hpx transcription, many TRs have not been identified (An et al., 2011; Huang et al., 2009).

The GntR TR family is a broad and abundant TR family in prokaryotes (Hoskisson & Rigali, 2009). Among bacteria, members of the GntR regulate various biological processes, including oxidative stress, metabolic pathways, motility and adhesion, and pathogenicity (Su et al., 2016; Zhou et al., 2017). However, many TRs in bacterial species belonging to the genus Xanthomonas have never been investigated. In the present study, we report that a versatile TR Sar regulates the expression of T1SS, T3SS, and T6SS.

2 | RESULTS

2.1 | PXO_RS20790 of X. oryzae is involved in oxidative stress

Our previous study identified a novel TR PXO_RS20790, a new master pathogenicity-associated regulator in almost all regulators in the PXO99A genome (Zheng et al., 2021) (Figure S1a,b). The PXO_RS20790 gene (named sar in this study) is annotated to encode a GntR-family TR with an N-terminal DNA-binding domain and a C-terminal UTRA domain (Figure 1a). Several pathogenicity-related phenotypes were investigated to explore the factors that resulted in the virulence attenuation of the PXO_RS20790 deletion mutant. First, in vitro growth assays were performed for comparisons between wild-type and PXO_RS20790 mutant strains. The results showed that no significant difference was found between PXO_RS20790 mutant and WT strain growth in nutrient broth (NB) or XOM2 medium (Figure 1b,c). In addition, the result also showed that the PXO_RS20790 mutant had no significant influence on other pathogenicity-related phenotypes, including exopolysaccharides (EPS) (Dharmapuri & Sonti, 1999; Kim et al., 2009), swarming motility (Qi et al., 2020), and extracellular cellulase (Tayi et al., 2018) (Figure S1c). However, the PXO_RS20790 deletion mutant was more sensitive to hydrogen peroxide (H2O2), which, as one of the reactive oxygen species (ROS), compared with that in wild-type strain PXO99A, and a complemented strain showed that H2O2 resistance could be restored to the wild type (Figure 1d). These results indicate that PXO_RS20790 is involved in the pathogenicity and oxidative stress of X. oryzae pv. oryzae.

2.2 | Detecting PXO_RS20790 regulons by bacterial one-hybrid

Our previous RNA-Seq analysis revealed that 428 genes were differentially expressed in the PXO_RS20790 mutant compared with those in PXO99A (Zheng et al., 2021). These observations showed that PXO_RS20790 directly or indirectly regulates expression of numerous genes, especially secretion system-associated genes.
To further identify genes or operons regulated directly by PXO_RS20790 in Xoo PXO99 A, we identified PXO_RS20790-binding promoters by the bacterial one-hybrid system. We constructed a promoter sublibrary containing 396 operons that contain the PXO_RS20790-regulated operons. The full length of PXO_RS20790 was used as bait to screen the promoters in these 396 operons. The results showed that PXO_RS20790 interacted with 74 promoters (Figure S2 and Table S3). The 74 targets could be divided into 12 classes according to the function of these genes (Figure 2a). It is noteworthy that PXO_RS20790 interacted with the promoter of secretion system-related genes, including T1SS, T3SS, and T6SS (Figure 2a).

Based on the bacterial one-hybrid results, a consensus PXO_RS20790-binding DNA motif (WSKKCMDGCGCHRSN) was predicted from the putative promoter regions of the 74 Sar-regulated operons (Figure 2b). Furthermore, we employed an electrophoretic mobility shift assay (EMSA) to verify this interaction. The EMSA verified physical binding between PXO_RS20790 and a synthetic double-stranded DNA consensus motif (WSKKCMDGCGCHRSN). The binding almost disappeared when this DNA motif’s nucleotides were mutated (Figure 2c). Next, a synthetic double-stranded DNA motif GGCGTCGAGCGCCAGGC was examined based on the conserved motif of PXO_RS20790. We found that PXO_RS20790 interacted with this motif but could not bind the mutated motif (Figure S3a). Taken together, these findings demonstrated that PXO_RS20790 acts as a TR for the expression of secretion systems. Therefore, PXO_RS20790 was named Sar, which stands for the secretion-associated TR.

2.3 | Sar positively regulates the expression of T1SS-related genes and raxX

The rice receptor-like kinase Xa21 provides immunity against Xoo strains that carry raxX (AvrXa21). The T1SS-related rax genes of Xoo are also required for RaxX activity (Ercoli et al., 2022). Sar could be involved in activating the transcription of the raxSTAB gene cluster according to our previous RNA-Seq data and the bacterial one-hybrid assay (Zheng et al., 2021) (Figure 2a). A previous study showed that raxST, raxA, and raxB probably constitute an operon (da Silva et al., 2004). Reverse transcription PCR (RT)-PCR results also indicated that raxST, raxA, and raxB are co-transcribed (Figure S4). First, a quantitative real-time reverse transcription PCR (RT-qPCR) assay was performed to determine whether Sar has any regulatory relations with the T1SS rax cluster (Figure 3a). The analysis indicated that sar deletion resulted
were observed in two independent experiments according to the conserved Sar binding motifs in (b). Similar results were obtained for wild-type (top) and mutated (bottom) DNA probes in an EMSA to examine whether Sar directly regulates rax gene expression. The shifted 6-carboxy-fluorescein (FAM)-labelled DNA bands were observed when raxST or raxX promoter DNA was incubated with increasing amounts of Sar protein, and the shifted DNA progressively disappeared when the nonlabelled DNA was added to the competitive assay (Figure 3d,e). The Sar binding motif was found in raxST and raxX promoter regions according to the EMSA (Figure S3b). Deletion of the Sar-binding motif in the raxST promoter region significantly reduced the mRNA level of raxSTAB compared to the WT strain (Figure 3f). The results confirmed that Sar could control rax-related gene transcription by binding to these two promoters.

To further verify Sar positively regulated RaxX protein expression, the 6×His C-terminal tag was knocked in the raxx gene in the WT strain and sar mutant strain. These two strains were cultured in NB and XOM2 medium. The western blotting assay showed that raxX translated proteins. The expression of RaxX was decreased in the Δsar mutant in hrp-inducing XOM2 medium compared with that in the WT strain (Figure 3g). Taken together, the results indicate that Sar binds to the promoter region of T1SS and raxX directly and positively controls T1SS and raxX expression.

2.4 Sar regulates the expression of T6SS-1 related genes but not the T6SS-2

Gram-negative pathogens have a multifunctional type VI secretion system (T6SS), affecting interbacterial competition, virulence to the eukaryotic host, and metal ions uptake (Bernal et al., 2018; Lin et al., 2017; Sana et al., 2016). There are two phylogenetically distinct T6SS gene clusters, T6SS-1 and T6SS-2, in the genome of Xoo PXO99A. However, the function and regulation of T6SSs in Xanthomonas species are poorly understood. According to the bacterial one-hybrid results, Sar could bind the six promoter regions of T6SS operons (Figures 2a and 4a, and Table S3). We found that T6SS-1 gene clusters were activated on the plant-mimicking XOM2 medium; in contrast, T6SS-2 related gene expression was induced under nutrient-rich conditions (NB medium) (Figure S5a,b). Therefore, we deduced that T6SS-1 and T6SS-2 have different functions.

Subsequently, the RT-qPCR assay was performed to verify the regulation relationship between Sar and T6SS-related genes. The WT and sar mutant strains were cultivated in XOM2 medium, and the mRNA levels of PXO_00245, PXO_00263, PXO_00264 (hcp1), and PXO_00265 in the sar mutant were significantly decreased compared with those in the WT strain (Figure 4b). Next, we measured the T6SS-2-related gene expression of Xoo strains in NB medium. The mRNA levels of PXO_02045, PXO_02046, and PXO_02047 (hcp2) in the sar mutant were increased slightly compared to those in the WT strain (Figure 4c). Thus, Sar may regulate T6SS-1-related gene expression, but not T6SS-2. The promoter regions of PXO_00244 and PXO_00266 were amplified and used as

![Figure 2](image-url) The analysis of the PXO_RS20790 regulon by bacterial one-hybrid assay. (a) Functional categories of the genes with promoters that were putatively bound by PXO_RS20790. Details of the genes are given in Table S3. (b) Predicted consensus PXO_RS20790-binding DNA motifs based on bacterial one-hybrid data and MEME suite analysis. (c) Verification of PXO_RS20790-binding DNA motif by electrophoretic mobility shift assay (EMSA). Two oligonucleotides were synthesized and incubated with the PXO_RS20790 protein in the EMSA, which was used to measure the interaction between the PXO_RS20790 protein and the oligonucleotide wild-type (top) and mutated (bottom) DNA probes according to the conserved Sar binding motifs in (b). Similar results were observed in two independent experiments.
FAM-labelled DNA in an EMSA to examine whether Sar directly regulates transcription of T6SS-1 operons. The results showed that the Sar protein bound to these two promoters (Figure 4d). Therefore, the Sar protein regulates T6SS-1 expression but does not regulate T6SS-2.

Hcp protein is a vital component of the T6SS tube that is required for the secretion of T6SS effectors (Silverman et al., 2013). Therefore, hcp deletion mutants were obtained in Xoo PXO99A and named Δhcp1 (the mutant in T6SS-1), Δhcp2 (the mutant in T6SS-2), and Δhcp1Δhcp2. Plant inoculation revealed that neither hcp1 nor hcp2 was required for the full virulence of the Xoo PXO99A strain (Figure S5c).

An antagonism assay was performed to identify whether T6SS-1 or T6SS-2 participated in interbacterial killing (Silverman et al., 2013). To determine if the Xoo strains (WT, Δsar, Δhcp1, Δhcp2, and Δhcp1Δhcp2) can target other bacteria, we tested them...
with two different strains of Xanthomonas campestris pv. campestris 8004 (Xcc 8004) and Escherichia coli XL1-Blue for interspecies and intergeneric competition, respectively. Our results showed that the Δhcp1 (T6SS-1) of Xoo PXO99A and the Δsar strain did not antagonize these Xcc or E. coli; however, the Δhcp2 (T6SS-2) did not antagonize Xcc 8004 but did antagonize E. coli (Figure 4e).

The bacterial CFU assay was also performed between Xoo strains and the competitor E. coli. The results revealed that the bacterial population of Δhcp2 was significantly reduced compared with that of the WT strain, but this mutant strain could promote competitor E. coli survival (Figure 4f,g). The results indicate that the T6SS-2 in Xoo PXO99A is involved in competition with E. coli, consistent with the recent study in X. oryzae pv. oryzicola (Zhu et al., 2020). Taken together, these results demonstrate that Sar positively regulates T6SS-1 gene expression, but does not regulate T6SS-2 gene expression.

FIGURE 4 Sar is not required for interbacterial competition. (a) The genomic organization of the T6SS-1 and T6SS-2 gene clusters, which are probably regulated by Sar in PXO99A. (b,c) Relative expression of T6SS-related genes in PXO99A and Δsar detected by reverse transcription-quantitative PCR. Significance was tested by Student’s t test (*p < 0.05). Error bars indicate the standard deviation of three technical replicates. (d) The direct interaction between the Sar protein and PXO_00244 and PXO_00266 DNA tagged with 6-carboxy-fluorescein (FAM) was detected by electrophoretic mobility shift assay (EMSA). The final concentrations of Sar and DNA in the 20-μl reaction system are shown above each lane. Shifted bands indicate the probe DNA interacts with the Sar protein. The unlabelled promoter DNA was added to the incubation system to compete with the interaction between Sar and the FAM-labelled probe. P00244 and P00266 are the abbreviations for the promoter DNA of PXO_00244 and PXO_00266, respectively. (e) Hcp2 (T6SS-2) is vital to the competition for Xoo PXO99A. Different Xanthomonas oryzae pv. oryzae (Xoo) cells compete with Xanthomonas campestris pv. campestris (Xcc) 8004 and Escherichia coli XL1-Blue. The images show Xcc 8004 (left) and E. coli XL1-Blue (right) with a serial 10-fold dilution spotted on nutrient broth (NB) agar with rifampicin plates and Luria-Bertani (LB) agar with kanamycin plates, respectively. (f,g) The bacterial CFU for Xoo strains (f) and E. coli (g). Xoo strains possess a pHM1 cosmid (spectinomycin resistance) and the E. coli XL1-Blue strain has a kanamycin resistance gene. The different Xoo–E. coli strains were co-cultured strains with serial 10-fold dilutions. Then, the diluted samples were spread on spectinomycin NB agar plates and kanamycin LB agar plates, respectively. The bacterial populations of Xoo (f) and E. coli (g) were counted. Similar results were observed in two independent experiments.
2.5 | Sar is involved in activation of the expression of T3SS through HrpG and HrpX

In *X. campestris* pv. *vesicatoria*, HrpX has been shown to regulate hrp gene transcription by directly binding to the Pip box in the promoters of *hrp* operons (Koebnik et al., 2006). From the transcriptome of Sar, it was found that Sar positively regulates *hrp- hrc- hpa* related genes (Zheng et al., 2021). The RT-qPCR assay confirmed the RNA-Seq data. The result showed that the mRNA levels of *hrpG*, *hrpX*, and T3SS-related genes, including *sctR*, *hrcS*, and *hpaB* in the Δsar mutant strain, were significantly lower than that of the WT strain (Figure 5a). These results indicate that Sar promotes the transcription of *hrpG* and *hrpX*.

The bacterial one-hybrid showed that Sar could physically interact with the promoter regions of *hrpG* and *hrpX* (Figure S2e 5–3, 5–4 and Table S3). To confirm whether Sar regulates *hrpG* and *hrpX* transcription directly, the promoter regions of *hrpG* and *hrpX* were amplified and used as DNA probes in the EMSA assay. As shown in Figure 5b, the shifted FAM-labelled DNA bands were observed when *hrpG* or *hrpX* promoter DNA was incubated with increasing amounts of Sar protein, and the shifted DNA progressively disappeared when the nonlabelled DNA was added to the competitive assay. These results indicate that Sar specifically interacts with *hrpG* and *hrpX* promoters, and directly controls the transcription of these two genes.

A recent study showed that the HrpG-binding motif is [ATT(C/T) (C/T)(G/C/A)(T/A)T] in *Xcc* 8004 (Zhang et al., 2020). The HrpG protein of Xoo PXO99A shared 77% identity with HrpG_{Xcc}. We found the HrpG-binding motif (ATTCCATT) in the promoter region of *hrpX* (Figure 5c). We also found the putative Sar-binding site in the promoter regions of *hrpG* and *hrpX* (Figure 5c). Meanwhile, the EMSA confirmed that Sar could bind these two DNA motifs (Figure S3c). Mutation of the Sar-binding motif in the *hrpX* promoter significantly reduced the mRNA level of *hrpX* and T3SS-related genes compared to the wild-type strain (Figure 5d). Taken together, Sar could activate both *hrpG* and *hrpX* transcription.

3 | DISCUSSION

TRs are vital to bacteria survival when they encounter biotic or abiotic stresses. For pathogenic *X. oryzae*, some TRs are required for regulating virulence-related gene expression (Zheng et al., 2021). In this study, we identified the GntR-family TR Sar as a multiple secretion systems regulator in *Xanthomonas*. We verified that deletion of sar in Xoo resulted in the reduction of type I secretion and type III secretion gene expression. We also showed that Sar positively regulates T6SS-1, but does not regulate T6SS-2 gene expression, and T6SS-2 is vital to intergeneric competition for Xoo. Further analysis discovered that Sar directly promotes secretion system-associated gene expression. Our results revealed that Sar is a master pathogenicity-associated regulator regulating T1SS, T3SS, and T6SS associated gene expression (Figure 6).
of RaxX sulfopeptide has required the transcription of the raxST gene (et al., 2021; Lee et al., 2008). HrpX is a positive key regulator that regulate the HrpG/HrpX regulon.

The production of RaxX is required for the XA21-dependent immune response (Luu et al., 2019; Pruitt et al., 2015). The production of RaxX requires the transcription of the raxST gene. Both PhoPQ and RaxRH TCSs are required for response (Luu et al., 2019; Pruitt et al., 2015). The production of RaxX is required for the XA21-dependent immune response (Burdman et al., 2004; Joe et al., 2021). We believe that Sar is one of the switches for regulating the transcription of raxST.

In X. oryzae, the avirulence protein RaxX is sulfated by RaxST and exported through T1SS composed of RaxA, RaxB, and RaxC. The sulfated RaxX is required for the XA21-dependent immune response (Luu et al., 2019; Pruitt et al., 2015). The production of RaxX is required for the XA21-dependent immune response (Burdman et al., 2004; Joe et al., 2021; Lee et al., 2008). HrpX is a positive key regulator that can bind the PIP box of the raxST gene and RaxX expression (Joe et al., 2021). Although HrpX regulates the RaxX biosynthetic pathway, the regulators that control raxSTAB-raxX gene expression are still elusive. We found that raxST, raxA, and raxB are co-transcribed in the same operon (Figure S4). We also found that the transcription levels of raxSTAB-raxX were significantly decreased in the sar mutant compared to that of the wild-type strain (Figure 3b,c). Further analysis showed that Sar could bind the promoter region of raxST and raxX (Figure 3d,e). We found that Sar is a positive TR located upstream of HrpX for its transcription. The expression of raxSTAB and raxX is required for both Sar and HrpX according to our previous RNA-Seq data (Figures 2 and S) (Zheng et al., 2021). We believe that Sar is one of the switches for regulating the expression of rax-related genes, but not the most crucial one. HrpX is the critical regulator that controls rax-related gene transcription (Joe et al., 2021). Together, we found a novel TR that could bind to the promoter region of raxST and raxX for their expression. However, the regulation of rax-related genes is complicated, therefore future work should focus on the regulatory network between regulators and rax-related genes.

Gram-negative bacteria utilize the T6SS as a versatile weapon for survival in many competitions, such as interbacterial killing, eukaryotic toxicity, and metal ion uptake (Bayer-Santos et al., 2018; Yu et al., 2021). There are two T6SS clusters in the genome of Xoo PXO99A, named T6SS-1 and T6SS-2. Our bacterial one-hybrid data showed that Sar could interact with six predicted promoters in the T6SS-1 and T6SS-2 clusters (Figure 2a and Table S3). Further analysis confirmed this interaction (Figure 4c). We found that Sar positively regulates T6SS-1, but does not regulate T6SS-2 (Figure 4a,b). Hcp is an essential component of the T6SS tube and chaperon for effectors’ secretion. Neither Hcp1 nor Hcp2 were required for the pathogenicity for Xoo against host rice cultivar IR24 (Figure S5c). We also found that T6SS-2 (Hcp2), but not T6SS-1 (Hcp1), was required for competition with E. coli (Figure 4d), which is consistent with a recent report in X. oryzae pv. oryzicola (Zhu et al., 2020). Moreover, the sar and hpc1 mutant strains showed similar phenotypes according to interspecies and intergeneric competition assays (Figure 4e-g). The results showed that Sar is not responsible for interbacterial competition like T6SS-1. More experiments should be performed to explore the Sar regulating T6SS-1 related phenotype except for interbacterial competition. The present experimental design could not result in a different phenotype due to the weak differential expression of the T6SS-2 (less than 2-fold) between the Sar mutant and the WT. An experiment with higher resolution should be designed to identify the difference regarding interbacterial competition, for example exploring the interbacterial competition ability in the infection process.

The T3SS is vital to gram-negative bacterial pathogens. In Xanthomonas bacteria, the virulence relies on the secretion and translocation of effectors of the T3SS controlled by two master TRs, HrpG and HrpX (Teper et al., 2021). Disruption of these two regulators in Xanthomonas spp. completely abolishes pathogenicity on the host plants (Kamdar et al., 1993; Wengelnik et al., 1996). Many studies focus on the regulatory networks that regulate and are regulated by the HrpG/HrpX regulon (Buttner & Bonas, 2010; Teper et al., 2021). The expression and activity of HrpG and HrpX requires regulatory factors, such as TRs (Pandey et al., 2016; Rashid et al., 2016; Zhou et al., 2017), and TCSs (Li et al., 2014; Lin et al., 2022; Subramoni et al., 2012). In this study, we found a new TR, Sar, that directly binds to the promoter region of HrpG and HrpX for their expression (Figure 5). In the promoter region of hrpX, the Sar binding site (ATCGTGTGCGCCAGC) is downstream of the HrpG binding site (ATTCCATT) and these two binding sites are separated by only 16 base pairs (Figure 5c). Sar protein may have a physical interaction with HrpG protein (data not shown). We think that HrpX is the direct executor, while Sar is a small switch upstream of HrpX, but not a critical switch. We found that Sar protein is conserved in Xanthomonas spp. (Zheng et al., 2021) and other genera (Figure S6).

This study advances our understanding of the fine-tuned regulation of pathogenicity regulatory networks in Xanthomonas. Future work should figure out the relationship of the different regulators that regulate the HrpG/HrpX regulon.
4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. E. coli strains were grown aerobically at 37°C in Luria-Bertani (LB) medium. Xoo strains were routinely cultivated at 28°C in NB medium (polypeptone 5 g/L, yeast extract 1 g/L, sucrose 20 g/L, beef extract 3 g/L, pH 7.0) or minimal XOM2 medium (D-xylose 1.8 g/L, methionine 0.099 g/L, sodium glutamate 1.87 g/L, KH₂PO₄ 2 g/L, MnSO₄ 0.006 g/L, MgCl₂ 0.2 g/L, pH 6.5–7.0). If necessary, antibiotics were added at the following concentrations: Xoo, kanamycin (50 μg/ml), spectinomycin (100 μg/ml); for X. campestris pv. campestris, rifampicin (25 μg/ml); for E. coli, spectinomycin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (34 μg/ml), streptomycin (8 μg/ml), and tetracycline (12.5 μg/ml).

4.2 | Bacterial genetic manipulation

According to previous studies, the suicide vector pK18mobSacB was used to construct the in-frame deletion mutants based on the homologous double-crossover method (Zheng et al., 2018). pHM1, pET-28a, pTRG, or pBXcmT were used to construct recombinant vectors for genetic complementation or overexpression of the genes, which were then electroporated into the corresponding bacterial strain. All primer sequences used in this study are listed in Table S2.

4.3 | Virulence assay

The Xoo strains were cultured in NB medium overnight and washed twice by ddH₂O, then the OD₆₀₀ was adjusted to 0.8. The 4- to 5-week-old rice plants (susceptible rice cultivar IR24) were inoculated with different Xoo strains by the leaf-clipping method (Yang & Bogdanove, 2013). Disease symptoms were measured 14 days post-inoculation. The experiments were repeated at least three times.

4.4 | RT-qPCR

Xoo strains were cultured in XOM2 medium to the logarithmic phase (OD₆₀₀ at 0.5). According to the manufacturer’s protocol, the RNA of different cell pellets was extracted using an EasyPure RNA Kit (TransGen Biotech). Afterwards, the crude RNA was digested with DNase I (RNase-free) followed by a PCR procedure to ensure that the RNA samples were DNA-free. Then, the purified RNA was reverse transcribed to cDNA using a cDNA synthesis kit (Takara Bio) based on the manufacturer’s protocol. Relative quantification of gene expression was performed using the 16S rRNA gene as an internal control.

4.5 | Bacterial one-hybrid

The open reading frame of sar was used to construct the pTRG-sar plasmid for the bacterial one-hybrid assay. Then, pTRG-sar and 396 pBXcmT-promoter plasmids were co-transformed into E. coli XR (XL1-Blue MRF). Subsequently, 396 clones were cultured in both nonselective and selective screening media to detect the interaction of Sar with the 396 promoters. The promoter IDs are listed in Table S1.

4.6 | EMSA

The sar open reading frame was cloned into the pET-28a vector to express hexahistidine-tagged Sar. According to the manufacturer’s instructions, Sar expressed in E. coli Rosetta (DE3) was purified using a Ni-NTA affinity column (Beijing CoWin Biotech). The purified protein was then dialysed in 20 mM Tris–HCl (pH 8.0) buffer supplemented with 5% glycerol and 150 mM NaCl to remove the residual imidazole. The promoter DNA probes were amplified and purified with a Gel Extraction Kit (Omega Bio-tek). FAM-labelled DNA probes were incubated with Sar protein in an improved EMSA buffer [20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mg of bovine serum albumin per ml and 5% glycerol] at 28°C for 45 min (Huang et al., 2008). For the competitive assay, different amounts of unlabelled probe were added to the EMSA system. Samples were then loaded on a 5% polyacrylamide gel prepared and run in 0.5× Tris-Borate buffer at 120 V for 45 min. The positions of the labelled probes in the polyacrylamide gel were visualized by a Typhoon Scanner (GE Healthcare). For the unlabelled EMSA, the gel was stained with ethidium bromide after electrophoresis, followed by image display under ultraviolet light.

4.7 | GUS assay

The promoters of raxST and raxX were cloned in pHG3 plasmid (Zou et al., 2021) and transformed into WT strain PXO99Δ and the Δsar mutant strain. The activity of β-glucuronidase (GUS) was measured as described (Jefferson et al., 1986). Briefly, Xoo strains were cultured in XOM2 medium until the OD₆₀₀ was 0.5. Cell pellets were collected and sonicated to obtain the supernatant. The absorbance of p-nitrophenol was measured at 415 nm using 4-nitrophenyl β-D-glucopyranoside (PNPG) as a substrate.

4.8 | Interbacterial competition assay

Xoo strains (PXO99Δ, Δsar, Δhcp1, Δhcp2, Δhcp1Δhcp2), Xcc 8004 (resistant to rifampicin), and E. coli XL1-Blue (resistant to kanamycin) were cultivated to log-phase and resuspended to OD₆₀₀ = 0.8. Xoo cells were then mixed with Xcc 8004 or E. coli XL1-Blue in a ratio of 10:1. A total of 5 μl of the above mixtures was spotted on NB agar plates and incubated at 28°C for 48 h. The spots were excised.
suspended in sterile water, and 5-μl serial 10-fold dilutions were spotted on rifampicin NB agar plates and incubated at 28°C for 60 h or on kanamycin LB agar plates and incubated at 37°C for 48 h.

4.9 Western blot

Western blot was performed using standard protocols with anti-His mouse monoclonal antibodies (Proteintech) and a goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) secondary antibody (Proteintech), and visualized by a chemiluminescent detector system using Pierce enhanced chemiluminescence (Thermo Fisher Scientific) as the HRP substrate.

ACKNOWLEDGEMENTS

We especially thank Dr Gongyou Chen (Shanghai Jiao Tong University) for providing the pHG3 plasmid. This work was supported by the National Natural Science Foundation of China (31970123), the sixth batch of flexible talent introduction projects of Tibet Agriculture & Animal Husbandry College (NYRXRC-2022-03), and the open funds of the State Key Laboratory of Agricultural Microbiology to D.H.Z. The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Dehong Zheng https://orcid.org/0000-0001-6339-2038
Lifang Ruan https://orcid.org/0000-0001-9307-9579

REFERENCES

Alvarez-Martinez, C.E., Sgro, G.G., Araujo, G.G., Paiva, M.R.N., Matsuyama, B.Y., Guzzo, C.R. et al. (2021) Secrete or perish: the role of secretion systems in Xanthomonas biology. Computational and Structural Biotechnology Journal, 19, 279–302.

An, S.Q., Lu, G.T., Su, H.Z., Li, R.F., He, Y.Q., Jiang, B.L. et al. (2011) Systematic mutagenesis of all predicted gntR genes in Xanthomonas campestris pv. campestris reveals a GntR family transcriptional regulator controlling hypersensitive response and virulence. Molecular Plant-Microbe Interactions, 24, 1027–1039.

Basler, M., Ho, B.T. & Mekalanos, J.J. (2013) Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. Cell, 152, 884–894.

Bayer-Santos, E., Lima, L.D., Ceseti, L.D., Ratagami, C.Y., de Santana, E.S., da Silva, A.M. et al. (2018) Xanthomonas citri T6SS mediates resistance to Dickeyostelium predation and is regulated by an ECF sigma factor and cognate Ser/Thr kinase. Environmental Microbiology, 20, 1562–1575.

Bayer-Santos, E., Ceseti, L.M., Farah, C.S. & Alvarez-Martinez, C.E. (2019) Distribution, function and regulation of type 6 secretion systems of Xanthomonadales. Frontiers in Microbiology, 10, 1635.

Bernal, P., Llamas, M.A. & Filloux, A. (2018) Type VI secretion systems in plant-associated bacteria. Environmental Microbiology, 20, 1–15.

Burdman, S., Shen, Y., Lee, S.W., Xue, Q. & Ronald, P. (2004) RaxH/RaxR: a two-component regulatory system in Xanthomonas oryzae pv. oryzae required for AvrXa21 activity. Molecular Plant-Microbe Interactions, 17, 602–612.

Buttner, D. & Bonas, U. (2002) Getting across–bacterial type III effectors on their way to the plant cell. The EMBO Journal, 21, 5313–5322.

Buttner, D. & Bonas, U. (2010) Regulation and secretion of Xanthomonas virulence factors. FEMS Microbiology Reviews, 34, 107–133.

Costa, T.R., Felisberto-Rodrigues, C., Meir, A., Prevost, M.S., Redzej, A., Trokter, M. et al. (2015) Secretion systems in gram-negative bacteria: structural and mechanistic insights. Nature Reviews Microbiology, 13, 343–359.

da Silva, F.G., Shen, Y., Dardick, C., Burdman, S., Yadav, R.C., de Leon, A.L. et al. (2004) Bacterial genes involved in type I secretion and sulfation are required to elicit the rice Xa21-mediated innate immune response. Molecular Plant-Microbe Interactions, 17, 593–601.

Deng, W., Marshall, N.C., Rowland, J.L., McCoy, J.M., Worrall, L.J., Santos, A.S. et al. (2017) Assembly, structure, function and regulation of type III secretion systems. Nature Reviews Microbiology, 15, 323–337.

Dharmapuri, S. & Sonti, R.V. (1999) A transposon insertion in the gumG homologue of Xanthomonas oryzae pv. oryzae causes loss of extracellular polysaccharide production and virulence. FEMS Microbiology Letters, 179, 53–59.

Ercoli, M.F., Luu, D.D., Rim, E.Y., Shigenaga, A., Teixeira de Araujo, A. Jr., Chen, M. et al. (2022) Plant immunity: rice Xa21-mediated resistance to bacterial infection. Proceedings of the National Academy of Sciences of the United States of America, 119, e2121568119.

Guo, Y., Figueiredo, F., Jones, J. & Wang, N. (2011) HrpG and HrpX play global roles in coordinating different virulence traits of Xanthomonas axonopodis pv. citri. Molecular Plant-Microbe Interactions, 24, 649–661.

Han, S.W., Lee, S.W., Bahar, O., Schwessinger, B., Robinson, M.R., Shaw, J.B. et al. (2012) Tyrosine sulfation in a gram-negative bacterium. Nature Communications, 3, 1153.

Han, Y.Y., Wang, T.T., Chen, G.K., Pu, Q.Q., Liu, Q., Zhang, Y.N. et al. (2019) A Pseudomonas aeruginosa type VI secretion system regulated by CueR facilitates copper acquisition. PLoS Pathogens, 15, e1008198.

Hood, R.D., Singh, P., Hsu, F.S., Guvener, T., Carl, M.A., Trinidad, R.R.S. et al. (2010) A type VI secretion system of Pseudomonas aeruginosa targets, a toxin to bacteria. Cell Host & Microbe, 7, 25–37.

Hoskisson, P.A. & Rigali, S. (2009) Chapter 1: variation in form and function the helix-turn-helix regulators of the GntR superfamily. Advances in Applied Microbiology, 69, 1–22.

Huang, D.L., Tang, D.J., Liao, Q., Li, H.C., Chen, Q., He, Y.Q. et al. (2008) The Zur of Xanthomonas campestris functions as a repressor and an activator of putative zinc homeostasis genes via recognizing two distinct sequences within its target promoters. Nucleic Acids Research, 36, 4295–4309.

Huang, D.L., Tang, D.J., Liao, Q., Li, X.Q., He, Y.Q., Feng, J.X. et al. (2009) The Zur of Xanthomonas campestris is involved in hypersensitive response and positively regulates the expression of the hrp cluster via hrpX but not hrpG. Molecular Plant-Microbe Interactions, 22, 321–329.

Jefferson, R.A., Burgess, S.M. & Hirsh, D. (1986) Beta-glucuronidase from Escherichia coli as a gene-fusion marker. Proceedings of the National Academy of Sciences of the United States of America, 83, 8447–8451.

Jiang, N., Yan, J., Liang, Y., Shi, Y., He, Z., Wu, Y. et al. (2020) Resistance genes and their interactions with bacterial blight/leaf streak pathogens (Xanthomonas oryzae) in Rice (Oryza sativa L.) an updated review. Rice (N Y), 13, 3.

Joe, A., Stewart, V. & Ronald, P.C. (2021) The HrpX protein activates synthesis of the RaxX sulfoprotein, required for activation of Xa21-mediated immunity to Xanthomonas oryzae pv. oryzae. Molecular Plant-Microbe Interactions, 34, 1307–1315.

Kamdar, H.V., Kamoun, S. & Kado, C.I. (1993) Restoration of pathogenicity of avirulent Xanthomonas oryzae pv. oryzae and X. campestris pathovars by reciprocal complementation with the hrpXo and hrpXc genes.
genes and identification of HrpX function by sequence analyses. *Journal of Bacteriology*, 175, 2017–2025.
Kim, S.Y., Kim, J.G., Lee, B.M. & Cho, J.Y. (2009) Mutational analysis of the gum gene cluster required for xanthan biosynthesis in *Xanthomonas oryzae pv. oryzae*. *Biotechnology Letters*, 31, 265–270.
Koebnik, R., Kruger, A., Thieme, F., Urban, A. & Bonas, U. (2006) Specific binding of the *Xanthomonas campestris pv. vesicatoria* AraC-type transcriptional activator HrpX to plant-inducible promoter boxes. *Journal of Bacteriology*, 188, 7652–7660.
Lee, S.W., Han, S.W., Bartley, L.E. & Ronald, P.C. (2006) Unique characteristics of *Xanthomonas oryzae pv. oryzae* AvrXa21 and implications for plant innate immunity. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 18395–18400.
Lee, S.W., Jeong, K.S., Han, S.W., Lee, S.E., Phee, B.K., Hahn, T.R. et al. (2008) The *Xanthomonas oryzae pv. oryzae* PhoPQ two-component system is required for AvrXa21 activity, hrgP expression, and virulence. *Journal of Bacteriology*, 190, 2183–2197.
Li, R.F., Lu, G.T., Li, L., Su, H.Z., Feng, G.F., Chen, Y. et al. (2014) Identification of a putative cognate sensor kinase for the two-component response regulator HrpG, a key regulator controlling the expression of the hrg genes in *Xanthomonas campestris pv. campestris*. *Environmental Microbiology*, 16, 2053–2071.
Li, R.F., Wang, X.X., Wu, L., Huang, L., Qin, Q.J., Yao, J.L. et al. (2020) *Xanthomonas campestris* sensor kinase Hpa5 co-opts the orphan response regulator VemR to form a branched two-component system that regulates motility. *Molecular Plant Pathology*, 21, 360–375.
Lin, J.S., Cheng, J.L., Chen, K.Q., Guo, C.H., Zhang, W.P., Yang, X. et al. (2015) The icmF3 locus is involved in multiple adaptation- and virulence-related characteristics in *Pseudomonas aeruginosa* PA01. *Frontiers in Cellular and Infection Microbiology*, 5, 70.
Lin, J.S., Zhang, W.P., Cheng, J.L., Yang, X., Zhu, K.X., Wang, Y. et al. (2017) A *Pseudomonas* T6SS effectors can recruit PQS-containing outer membrane vesicles for iron acquisition. *Nature Communications*, 8, 14888.
Lin, M., Wu, K., Zhan, Z., Mi, D., Xia, Y., Niu, X. et al. (2022) The RavA/VemR two-component system plays vital regulatory roles in the motility and virulence of *Xanthomonas campestris*. *Molecular Plant Pathology*, 23, 355–369.
Liu, F., McDonald, M., Schwessinger, B., Joe, A., Pruitt, R., Erickson, T. et al. (2019) Variation and inheritance of the *Xanthomonas rax-raxSTAB* gene cluster required for activation of Ag21-mediated immunity. *Molecular Plant Pathology*, 20, 656–672.
Luu, D.D., Joe, A., Chen, Y., Parys, K., Bahar, O., Pruitt, R. et al. (2019) Biosynthesis and secretion of the microbial sulfated peptide RaxX and binding to the rice Xa21 immune receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 116, 8525–8534.
Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriendiyanum, M., Ronald, P. et al. (2012) Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*, 13, 614–629.
McCann, H.C. & Guttmann, D.S. (2008) Evolution of the type III secretion system and its effectors in plant–microbe interactions. *New Phytologist*, 177, 33–47.
Montenegro Benavides, N.A., Alvarez, B.A., Arrieta-Ortiz, M.L., Rodriguez, R.L., Botero, D., Tabima, J.F. et al. (2021) The type VI secretion system of *Xanthomonas phaseoli pv. manihotis* is involved in virulence and in vitro motility. * BMC Microbiology*, 21, 14.
Pandey, S.S., Patnana, P.K., Komatsu, S.K., Tomar, A. & Chatterjee, S. (2016) Co-regulation of iron metabolism and virulence associated functions by iron and XilR, a novel iron binding transcription factor, in the plant pathogen *Xanthomonas*. *PloS Pathogens*, 12, e1006019.
Petrocelli, S., Tondo, M.L., Daurelio, L.D. & Orellano, E.G. (2012) Modifications of *Xanthomonas axonopodis pv. citri* lipopolysaccharide affect the basal response and the virulence process during citrus canker. *PloS One*, 7, e40051.
Pruiitt, R.N., Schwessinger, B., Joe, A., Thomas, N., Liu, F., Albert, M. et al. (2015) The rice immune receptor XA21 recognizes a tyrosine-sulfated protein from a gram-negative bacterium. *Science Advances*, 1, e1500245.
Qi, Y.H., Huang, L., Liu, G.F., Leng, M. & Lu, G.T. (2020) PiILG and PiILH antagonistically control flagellum-dependent and pili-dependent motility in the phytopathogen *Xanthomonas campestris pv. campestris*. *BMC Microbiology*, 20, 37.
Rashid, M.M., Ikawa, Y. & Tsuge, S. (2016) GamR, the LysR-type galactose metabolism regulator, regulates hrg gene expression through transcriptional activation of two key hrg regulators, HrpG and HrpX, in *Xanthomonas oryzae pv. oryzae*. *Applied and Environmental Microbiology*, 82, 3947–3958.
Sana, T.G., Flaughnatti, N., Lugo, K.A., Lam, L.H., Jacobson, A., Baylot, V. et al. (2016) Salmonella typhimurium utilizes a T6SS-mediated antibacterial weapon to establish in the host gut. *Proceedings of the National Academy of Sciences of the United States of America*, 113, E5044–E5051.
Silverman, J.M., Aghello, D.M., Zheng, H., Andrews, B.T., Li, M., Catalano, C.E. et al. (2013) Haemolysin coregulated protein is an exported receptor and chaperone of type VI secretion substrates. *Molecular Cell*, 51, 584–593.
Su, H.Z., Wu, L., Qi, Y.H., Liu, G.F., Lu, G.T. & Tang, J.L. (2016) Characterization of the GntR family regulator HpaR1 of the crucifer black rot pathogen *Xanthomonas campestris* pathovar campestris. *Scientific Reports*, 6, 19862.
Subramoni, S., Pandey, A., Vishnu Priya, M.R., Patel, H.K. & Sonti, R.V. (2012) The ColRS system of *Xanthomonas oryzae pv. oryzae* is required for virulence and growth in iron-limiting conditions. *Molecular Plant Pathology*, 13, 690–703.
Taiy, L., Kumar, S., Nathawat, R., Haque, A.S., Maku, R.V., Patel, H.K. et al. (2018) A mutation in an exoglucanase of *Xanthomonas oryzae pv. oryzae*, which confers an endo mode of activity, affects bacterial virulence, but not the induction of immune responses, in rice. *Molecular Plant Pathology*, 19, 1364–1376.
Teper, D., Pandey, S.S. & Wang, N. (2021) The HrpG/HrpX regulon of xanthomonads – An insight to the complexity of regulation of virulence traits in phytopathogenic bacteria. *Microorganisms*, 9, 187.
Timilsina, S., Potnis, N., Newberry, E.A., Liyanapathirane, P., Iruegas-Bocardo, F., White, F.F. et al. (2020) *Xanthomonas* diversity, virulence and plant-pathogen interactions. *Nature Reviews Microbiology*, 18, 415–427.
Tsuge, S., Furutani, A., Fukunaka, R., Oku, T., Tsuno, K., Ochiai, H. et al. (2002) Expression of *Xanthomonas oryzae pv. oryzae* hrg genes in *XOM2*, a novel synthetic medium. *Journal of General Plant Pathology*, 68, 363–371.
Wang, J., Broman, M. & Basler, M. (2019) Assembly and subcellular localization of bacterial type VI secretion systems. *Annual Review of Microbiology*, 73, 621–638.
Wengelnik, K., Van den Ackerveken, G. & Bonas, U. (1996) HrpG, a key hrg regulatory protein of *Xanthomonas campestris pv. vesicatoria* is homologous to two-component response regulators. *Molecular Plant-Microbe Interactions*, 9, 704–712.
Xue, D., Tian, F., Yang, F., Chen, H., Yuan, X., Yang, C.H. et al. (2018) Phosphodiesterase EdpX1 promotes *Xanthomonas oryzae pv. oryzae* virulence, exopolysaccharide production, and biofilm formation. *Applied and Environmental Microbiology*, 84, e01717-18.
Yang, B. & Bogdanove, A. (2013) Inoculation and virulence assay for bacterial blight and bacterial leaf streak of rice. Methods in *Molecular Biology*, 956, 249–255.
Yang, P., Li, F.J., Huang, S.W., Luo, M., Lin, W., Yuan, G.Q. et al. (2020) Physiological and transcriptional response of *Xanthomonas oryzae pv. oryzae* to Berberine, an emerging chemical control. *Phytopathology*, 110, 1027–1038.
Yue, J.W., Xue, P., Fu, Y. & Yang, L. (2021) T6SS mediated stress responses for bacterial environmental survival and host adaptation. *International Journal of Molecular Sciences*, 22, 478.
Zhang, H.Y., Wei, J.W., Qian, W. & Deng, C.Y. (2020) Analysis of HrpG regulons and HrpG-interacting proteins by ChIP-seq and affinity proteomics in *Xanthomonas campestris*. *Molecular Plant Pathology*, 21, 388–400.

Zheng, D., Xue, B., Shao, Y., Yu, H., Yao, X. & Ruan, L. (2018) Activation of PhoBR under phosphate-rich conditions reduces the virulence of *Xanthomonas oryzae* pv. *oryzae*. *Molecular Plant Pathology*, 19, 2066–2076.

Zheng, D., Wang, H., Zhong, H., Ke, W., Hu, H., Sun, M. et al. (2021) Elucidation of the pathogenicity-associated regulatory network in *Xanthomonas oryzae* pv. *oryzae*. *mSystems*, 6, e00789-20.

Zhou, X., Yan, Q. & Wang, N. (2017) Deciphering the regulon of a GntR family regulator via transcriptome and ChIP-exo analyses and its contribution to virulence in *Xanthomonas citri*. *Molecular Plant Pathology*, 18, 249–262.

Zhu, P.C., Li, Y.M., Yang, X., Zou, H.F., Zhu, X.L., Niu, X.N. et al. (2020) Type VI secretion system is not required for virulence on rice but for inter-bacterial competition in *Xanthomonas oryzae* pv. *oryzicola*. *Research in Microbiology*, 171, 64–73.

Zou, L., Zhang, C., Li, Y., Yang, X., Wang, Y., Yan, Y. et al. (2021) An improved, versatile and efficient modular plasmid assembly system for expression analyses of genes in *Xanthomonas oryzae*. *Molecular Plant Pathology*, 22, 480–492.

**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

---

**How to cite this article:** Shao, Y., Tang, G., Huang, Y., Ke, W., Wang, S. & Zheng, D. et al. (2023) Transcriptional regulator Sar regulates the multiple secretion systems in *Xanthomonas oryzae*. *Molecular Plant Pathology*, 24, 16–27. Available from: https://doi.org/10.1111/mpp.13272