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ORIGINAL ARTICLE

The pathogenicity-associated regulators participating in the regulatory cascade for RaxSTAB and RaxX in *Xanthomonas oryzae* pv. *oryzae*

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Abstract

The RaxX sulfopeptide, secreted via a type I secretion system, is crucial for activating XA21-mediated innate immunity in resistant rice lines bearing the XA21 receptor kinase. Certain pathogenicity-associated regulators that control the expression of the *raxSTAB-raxX* gene cluster have been functionally characterized, but the comprehensive regulatory cascade of RaxSTAB and RaxX in *Xanthomonas oryzae* pv. *oryzae* (Xoo) remains incompletely understood. Our investigation revealed that pathogenicity-associated regulators, including HrpG, HrpX, VemR, PhoR, and Clp, form a regulatory cascade governing the expression of the *raxSTAB-raxX* gene cluster. HrpG regulates the *raxSTAB-raxX* gene cluster transcription through the key regulator HrpX. VemR also participates in the transcription of the *raxSTAB-raxX*. The histidine kinase PhoR positively modulates *raxSTAB-raxX* expression, while the global regulator Clp directly binds the *raxX* promoter region to promote its transcription. These findings shed light on the intricate regulatory cascade of *rax*-related genes in Xoo, emphasizing the complex roles of pathogenicity-associated regulators within the pathogenic regulatory system.

KEYWORDS
pathogenicity-associated regulators, RaxX, regulatory cascade, type I secretion system, *Xanthomonas oryzae* pv. *oryzae*

1 | INTRODUCTION

It is fascinating how pathogenic bacteria can adapt to various environmental niches, especially when transitioning between free-living and virulent states. Their ability to successfully infect susceptible host plants hinges on intricate transcriptional reprogramming. Pathogenicity-associated regulators, like two-component systems (TCSs) and transcriptional regulators (TRs), play crucial roles in sensing and responding to host environmental cues (Mole et al., 2007; Qian et al., 2008). The intricate pathogenicity regulatory cascades formed by TCSs and TRs empower bacteria to overcome host immunity and survive throughout infection (Buttner & Bonas, 2010).

Xanthomonas oryzae pv. *oryzae* (Xoo) is a notorious gram-negative phytopathogen that causes bacterial blight disease and severe yield loss in rice crops worldwide (Liu et al., 2014; Mew et al., 1993). Xoo secretes a small protein called RaxX (also named AvrXa21) that resembles the PSY telopeptide hormone found in plants (Pruitt et al., 2017). Rice cultivars that carry the XA21 immune receptor can trigger a strong defence response against Xoo strains that produce the sulphated RaxX peptide (Ercoli et al., 2022; Wang et al., 1996).

The *raxSTAB* gene cluster participates in the maturation and/or transport of the RaxX. The *raxST* gene encodes a sulphotransferase, *raxB* encodes a peptidase-containing transporter, and *raxA* encodes a periplasmic adaptor protein (Ronald, 2014). The *raxC* gene encodes an outer membrane protein (da Silva et al., 2004). The RaxA, RaxB, and RaxC proteins comprise the type I secretion system (T1SS) structure, which is crucial for the maturation and secretion of the RaxX precursor (da Silva et al., 2004). All the above proteins closely associated with RaxX activity post-translationally modify the precursor of RaxX through sulphation and endoproteolytic cleavage, and the matured RaxX is secreted to the extracellular space (Han et al., 2012; Luu et al., 2019; Pruitt et al., 2015). Despite the importance of the RaxX biosynthesis pathway and the Xoo–host interaction, the regulatory factors that govern *rax*-related genes are still not fully understood.

The TCSs and TRs of phytopathogens are important in regulating target gene transcription under various situations, including in vitro and in vivo environments (Buttner & Bonas, 2010; Mole et al., 2007; Wang & Qian, 2019). Many studies have reported the transcription of the *raxSTAB-raxX* cluster regulated by TCSs and TRs over the past 20 years (Ercoli et al., 2022). It was reported that the RaxR-RaxH TCS regulates the *raxSTAB* operon expression. RaxR also regulates the PhoP-PhoQ TCS, and these two TCSs participate in the RaxX activity in Xoo (Burdman et al., 2004; Lee et al., 2008). Recent studies revealed that the HrpX protein, a broad pathogenicity regulon, is a key TR that directly regulates *raxST* and *raxX* transcription by binding the plant-inducible promoter (PIP) box in the promoter region for each gene (Joe et al., 2021). In addition, the master TR Sar, located upstream of HrpX, also positively regulates the transcription of *raxST* and *raxX* by binding their respective promoter regions (Shao et al., 2023). Therefore, the pathogenicity-related regulators might directly or indirectly control the transcription of *raxST* and/or *raxX*. Our previous RNA sequencing data showed that some regulators participate in *rax*-related gene transcription (Zheng et al., 2021). Despite the identification of several *rax*-associated regulators in Xoo, previous research has focused on them individually. Given the complexity of regulatory cascades, the relationships among regulators that control *raxSTAB-raxX* transcription in Xoo are still poorly understood, and the regulatory cascade of the *rax* cluster remains to be fully elucidated.

In this study, we elucidate the role of pathogenicity-related regulators HrpG, HrpX, PhoR, VemR, and Clp in forming the regulatory cascade for *raxSTAB* or *raxX* expression in Xoo, promoting understanding of the complex regulatory cascade of the RaxSTAB and RaxX from a global perspective.

2 | RESULTS

2.1 | HrpG regulates *raxSTAB* and *raxX* transcription through HrpX

The *raxSTAB-raxX* consists of a gene cluster in the genome of PXO99^{A} (Figure 1a). The *raxST*, *raxA*, and *raxB* genes are arranged in an operon (da Silva et al., 2004; Shao et al., 2023). According to our prior RNA-sequencing data, it appeared that *raxX*, *raxST*, *raxA*, and *raxB* might be under potential regulation by seven, five, four, and four of the 10 pathogenicity-associated regulators, respectively (Table S3) (Zheng et al., 2021). However, the precise relationship between these regulators (HrpG, HrpX, VemR, Clp, PhoR, and ColS) and the *rax* cluster remains unclear. A recent study showed that HrpX, a type III secretion system (T3SS) regulator, is a key regulator required to transcribe *raxX* and *raxSTAB* (Joe et al., 2021). HrpG, a response regulator, is upstream of HrpX and directly binds the promoter region of *hrpX* to promote its transcription in *Xanthomonas* species (Teper et al., 2021; Wengelnik et al., 1996). Based on all the above reports, we deduced that some pathogenicity-associated regulators form complicated regulatory cascades for *raxSTAB* and *raxX* expression.

Some experiments were performed to detect the relationship of this regulation between HrpG and HrpX. A reverse transcription-quantitative PCR (RT-qPCR) assay showed that the deletion of *hrpG* or *hrpX* resulted in significantly decreased mRNA levels of *raxST*, *raxA*, *raxB*, and *raxX* compared to those of in the wild-type strain PXO99^{A} (Figure 1b). Moreover, a β-glucuronidase (GUS) assay found that both *raxST* and *raxX* promoter GUS activities of the *hrpG* or *hrpX* mutants were significantly decreased compared to those of the PXO99^{A} strain (Figure 1c). To further verify HrpG and HrpX positively regulated RaxX protein expression, the hexahistidine-tagged DNA was knocked in the *raxX* 3′ ends in the PXO99^{A}, *hrpG* mutant, and *hrpX* mutant strain separately. A western blotting assay showed that the expression of RaxX was abolished in both the *hrpG* and *hrpX* mutants when cultured in nutrient broth (NB) or XOM2-defined (for *hrp* induction) media (Figure 1d,e). Therefore, both HrpG and HrpX positively regulate *raxSTAB* and *raxX* expression.

Previous studies indicated that mutation of the PIP box in the *raxST* and *raxX* promoter region almost abolished the expression of these two genes in Xoo strain PXO99^{A} (Joe et al., 2021). However, it remained uncertain whether HrpG directly participated in regulating the expression of *raxSTAB* and *raxX*. Therefore, an electrophoretic mobility shift assay (EMSA) was performed to examine this regulation. The promoter regions of *raxST* and *raxX* were amplified and used as DNA in an EMSA to examine whether HrpG directly regulates the transcription of *raxST* and *raxX*. The EMSA results showed that the HrpG protein could not interact with these two promoters in vitro (Figure 1f). Taken together, all the above results indicated that HrpG regulates *raxSTAB-raxX* expression through the key regulator HrpX.

2.2 | VemR positively regulates *raxST* and *raxX* expression

We carried out a series of experiments to determine the mechanism by which VemR regulates the expression of *rax*-related genes. First, we used the RT-qPCR and GUS assays to test the transcriptional level differences of the *raxSTAB-raxX* gene cluster between

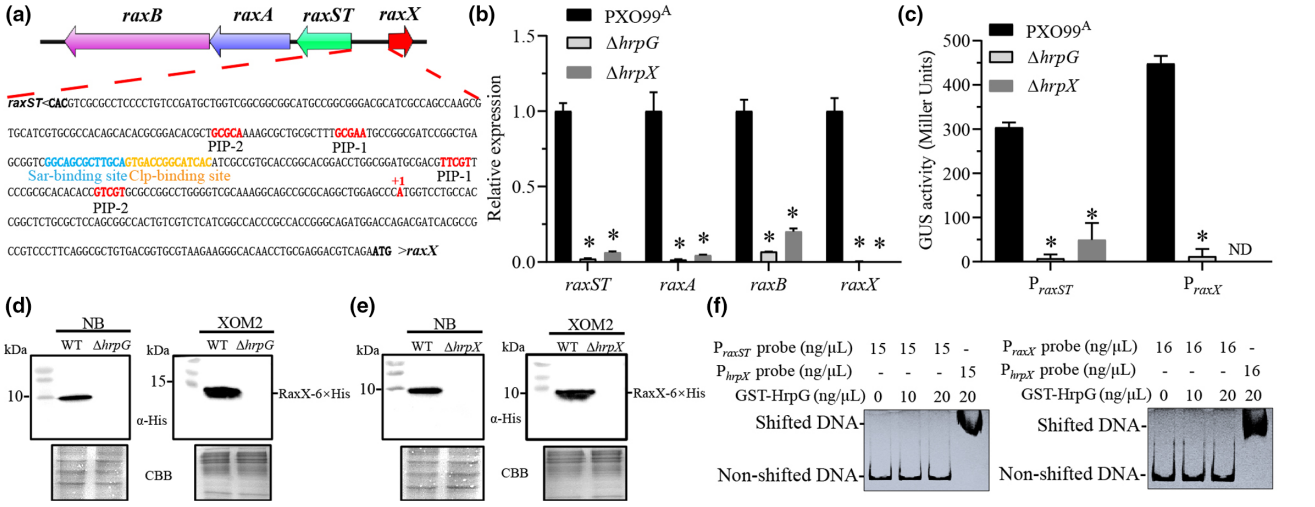

FIGURE 1 HrpG regulates the expression of the *rax* gene cluster through HrpX. (a) The transcription control region of *raxSTAB-raxX* exhibits divergent transcription. The sequence spans from the initiation codon of *raxST* to the initiation codon of *raxX*. The plant-inducible promoter (PIP) box sequences (HrpX binding site), Sar-binding site, Clp-binding site, and the *raxX* transcription initiation site are indicated. (b and c) *raxX* and *raxST* transcription requires HrpG and HrpX. Strains were grown in XOM2 medium to the logarithmic phase. (b) RNA was extracted from the wild-type (PXO99^{A}), *hrpG*, and *hrpX* mutant strains. The expression of the *rax*-related genes was analysed by reverse transcription-quantitative PCR and normalized to the 16S rDNA reference gene expression. (c) The promoter activities of *raxST* and *raxX* in the wild-type strain, *hrpG* and *hrpX* mutant strains by the β-glucuronidase (GUS) assay. ND, not detected. Error bars represent the standard deviation of three technical replicates. Student's *t* test ($p < 0.05$) was used to separate the significantly different means (denoted by *) relative to the wild-type strain. (d and e) Quantitative analysis of RaxX protein at the post-transcriptional level of the wild-type strain (WT), *hrpG* (d), and *hrpX* (e) mutant strains after culturing in nutrient broth (NB) or XOM2 medium. The result of Coomassie brilliant blue (CBB) staining on the lower panel showed that an approximately equal amount of total bacterial protein was loaded on each lane. (f) No interactions were detected between the HrpG protein and the promoter's DNA of *raxST* and *raxX* by the electrophoretic mobility shift assays. The *hrpX* promoter is a positive control. Similar results were observed in at least two independent experiments.

the PXO99^{A} and the *vemR* mutant strain. The expression of all four *rax* genes (*raxST*, *raxA*, *raxB* and *raxX*) was significantly reduced in the *vemR* mutant strain (Figure 2a). The GUS assay further confirmed that *raxST* and *raxX* promoter activities were significantly impaired in the *vemR* mutant strain compared with those in the PXO99^{A} strain (Figure 2b). Second, we tested the differences in the RaxX protein levels in the wild-type strain PXO99^{A} and the *vemR* mutant strain after culturing in NB and XOM2 medium. As shown in Figure 2c, a western blotting assay verified that the RaxX protein level was reduced in the *vemR* mutant strain compared with the PXO99^{A} strain. These findings suggest that the VemR protein is crucial for promoting the transcription of the *rax*-related genes.

VemR is an orphan response regulator with only a REC domain (Figure 2d), which prevents it from directly binding to the *raxST* or *raxX* promoter regions to regulate the transcription of these genes. A recent study confirmed that the VemR homologue physically interacts with the HrpG homologue to enhance *hrpX* transcription in *Xanthomonas campestris* pv. *campestris* (Xcc) (Li et al., 2023). We found that the mRNA level of *hrpX* was decreased in the *vemR* mutant compared to the wild-type strain (Table S4). We deduced that HrpG may interact with VemR for *hrpX* transcription in Xoo. However, no interaction was found between HrpG and VemR proteins (Figure 2e).

2.3 | PhoR positively regulates *rax*-related gene expression

We found that two histidine kinases, ColS and PhoR, potentially regulate *raxX* or *raxST* expression from the RNA-seq data (Table S3). The RT-qPCR and GUS results showed that the histidine kinase ColS may not regulate the *raxX-raxSTAB* cluster expression (Figure S1a,b). PhoR is a histidine kinase essential for bacteria to survive in a phosphate-starvation environment (Zheng et al., 2018). The RT-qPCR assay revealed a significant decrease in the mRNA levels of *raxST* and *raxX* in the *phoR* mutant strain compared to the wild-type strain PXO99^{A} (Figure 3a). The GUS assay further showed that *raxST* and *raxX* promoter GUS activities were decreased in the *phoR* mutant strain in contrast with the PXO99^{A} strain (Figure 3b). A western blotting assay evaluated the differences in RaxX protein levels between the PXO99^{A} and *phoR* mutant strains. Both strains, each carrying a 6×His tag at the 3′-end of *raxX*, were cultured in XOM2 medium before the assay. The result showed that the signal of RaxX-6×His in the *phoR* mutant strain was decreased compared with the PXO99^{A} strain (Figure 3c). All the above results demonstrated that the histidine kinase PhoR positively regulates *raxST* and *raxX* expression.

However, PhoR is not a transcriptional regulator that cannot directly bind to the promoter region of these two genes. PhoB is a cognate response regulator of PhoR and is required to express target

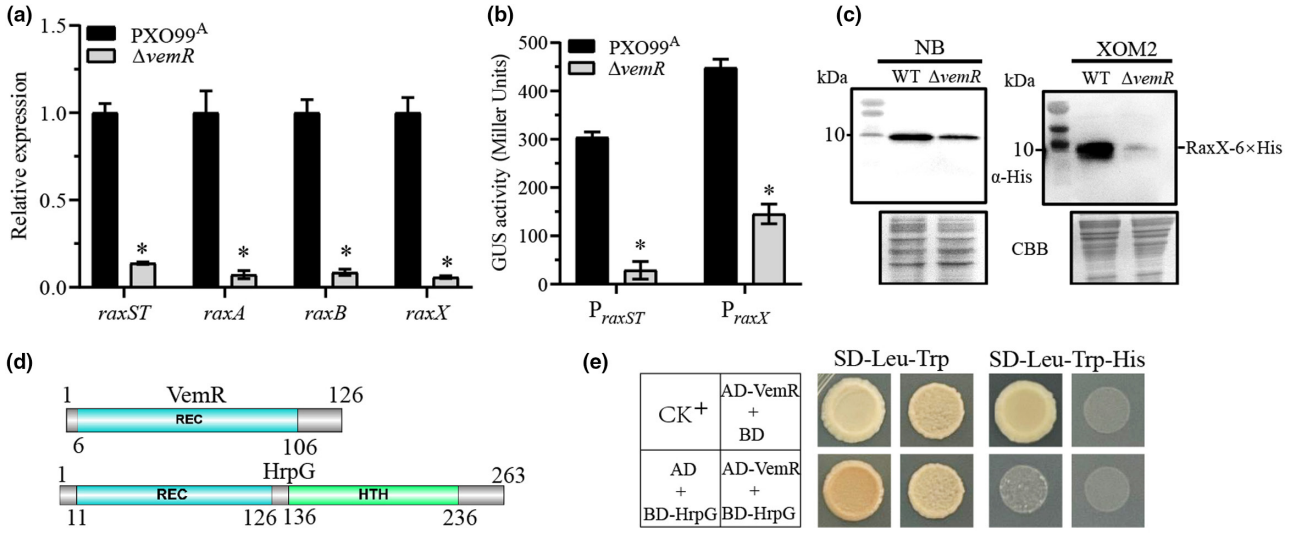

FIGURE 2 VemR positively regulates the expression of the *raxSTAB-raxX* gene cluster independently of HrpG. (a) Relative expression of *raxST*, *raxA*, *raxB*, and *raxX* in the *vemR* mutant strain compared to those in the wild-type strain PXO99^A by the reverse transcription-quantitative PCR assay. The 16S rDNA gene is used as an internal control. (b) The promoter activity of *raxST* and *raxX* in the *vemR* mutant strain compared to those in the wild-type PXO99^A strain by the β-glucuronidase (GUS) assay. In (a) and (b), error bars indicate the standard deviation of three technical replicates. Student's *t* test ($p < 0.05$) was used to separate the significantly different means (denoted by *) relative to the wild-type strain PXO99^A. (c) RaxX protein quantitative analysis at the post-transcriptional level of the wild-type strain (WT) and *vemR* mutant strain by western blotting analysis after culturing in nutrient broth (NB) or XOM2 medium. The Coomassie brilliant blue (CBB) results represent the loading control of the total protein. (d) Schematic view of the putative secondary structures of VemR and HrpG according to the Pfam database. VemR has a phosphoacceptor receiver (REC) domain only. (e) Detection of the interaction between the VemR and HrpG proteins by the yeast two-hybrid assay. The pGBKT7-53 and pGADT7-T pair is the positive control (CK$^+$). Similar results were observed in two independent experiments.

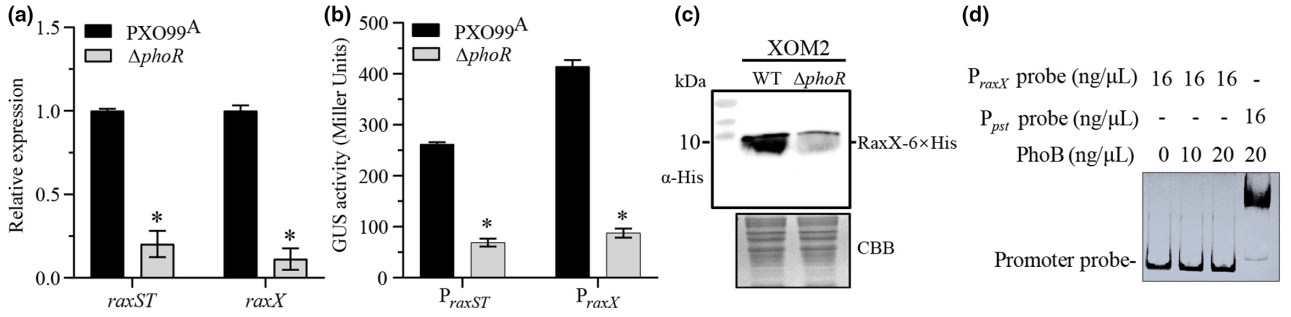

FIGURE 3 PhoR positively regulates the *rax* gene cluster expression. (a) Relative expression of *raxST* and *raxX* in the *phoR* mutant strain compared to those in the wild-type strain PXO99^A by the reverse transcription-quantitative PCR assay. The 16S rDNA gene was used as an internal control. (b) The promoter activity of *raxST* and *raxX* in the *phoR* mutant strain compared to those in the wild-type strain PXO99^A by the β-glucuronidase (GUS) assay. Error bars indicate the standard deviation of three technical replicates. Student's *t* test ($p < 0.05$) was used to separate the significantly different means (denoted by *) relative to the wild-type PXO99^A strain. (c) Quantitative analysis of RaxX protein was performed in both wild-type (WT) and *phoR* mutant strains after culturing in the *hrp*-inducing XOM2 medium. The Coomassie brilliant blue (CBB) results represent the loading control of the total protein. (d) PhoB did not interact with the promoter of *raxX* by the electrophoretic mobility shift assay. The shifted band represents the interaction between the PhoB and the *pst* promoter. Similar results were observed in two independent experiments.

genes (Zheng et al., 2018). Therefore, we hypothesized that PhoR regulates *raxST* and *raxX* transcription through the PhoB protein. An EMSA was performed to test the interaction of PhoB protein and *raxX* promoter DNA. The result showed that the PhoB protein could not interact with the *raxX* promoter DNA (Figure 3d). Taken together, PhoR positively regulates *raxST* and *raxX* expression but not through PhoB.

2.4 | The global transcriptional regulator Clp directly regulates *raxX* expression but not the *raxSTAB* gene cluster

The global regulator Clp, a c-di-GMP effector, plays a vital role in chemotaxis, signal transduction, and the expression of virulence factors (Lee et al., 2003; Qian et al., 2013; Tao et al., 2010). Our previous

RNA-seq data showed that Clp potentially regulates *raxX* but not the *raxSTAB* gene cluster (Table S3) (Zheng et al., 2021). The RT-qPCR results showed that only the *raxX* mRNA level was significantly downregulated in the *clp* mutant strain compared with the wild-type strain PXO99^{A} (Figure 4a). The GUS assay confirmed that the *raxX* promoter activity of the *clp* mutant strain was lower than the wild-type strain PXO99^{A} (Figure 4b). Moreover, the western blotting assay showed that the RaxX protein level was decreased in the *clp* mutant strain compared with the wild-type strain PXO99^{A} (Figure 4c).

To determine whether Clp directly regulates the transcription of *raxX*, the interaction between the Clp protein and the promoter DNA of *raxX* was investigated. The EMSA result showed that the Clp protein interacted with the *raxX* promoter DNA (Figure 4d). Many studies have shown that Clp homologues have a conserved DNA-binding motif (5′-GTGN$_8$CAC-3′). We found the Clp-binding site (5′-GTGACCGCCATCAC-3′) in the promoter region of *raxX* (Figure 1a). We used AlphaFold 3 to predict and evaluate the interaction between the Clp dimer and the promoter double-stranded (ds) DNA of the potential binding sequence (Abramson et al., 2024). The prediction results showed that the interface-predicted template modelling (ipTM) score is higher than 0.8, representing a confident high-quality prediction of the interaction between Clp and this motif (Figure 4e). However, mutation of the potential binding site of the Clp impaired this interaction (Figure 4e). Taken together, all the above results indicate that Clp directly interacts with the *raxX* promoter to facilitate its transcription.

3 | DISCUSSION

In this study, we demonstrated that pathogenicity-associated regulators HrpG, HrpX, VemR, PhoR, and Clp, participated in regulating the expression of the *raxSTAB-raxX* gene cluster through regulatory cascades (Figure 5). We found that HrpG and HrpX are the key executors in regulating the expression of the *raxSTAB* operon and *raxX* (Figure 1). However, the HrpG protein does not directly regulate the *raxSTAB-raxX* transcription but instead acts through HrpX. We found that the global transcriptional regulator Clp directly activates *raxX* transcription. Moreover, we identified two novel TCS-related proteins, the histidine kinase PhoR and the orphan response regulator VemR, that are involved in the transcription of the *raxSTAB-raxX* cluster (Figure 5).

A recent study revealed that HrpX is a key regulator for the *raxX-raxSTAB* gene cluster transcription by binding the promoter region of the PIP-box of the *raxST* and *raxX* (Joe et al., 2021). Our previous RNA-sequencing data found that the *rax*-related genes were severely downregulated in both *hrpG* and *hrpX* deletion mutants compared with those in the wild-type PXO99^{A} strain (Table S3) (Zheng et al., 2021). We also found that the Xoo HrpG and HrpX regulons contain 278 and 167 target genes, respectively. Approximately 97.6% (164/167) of the target genes of HrpX were found to be regulated by HrpG (Zheng et al., 2021). Although 164 genes (including *raxX* and *raxSTAB*) were differentially expressed in both HrpG and HrpX (Zheng et al., 2021), only HrpX but not HrpG directly regulates

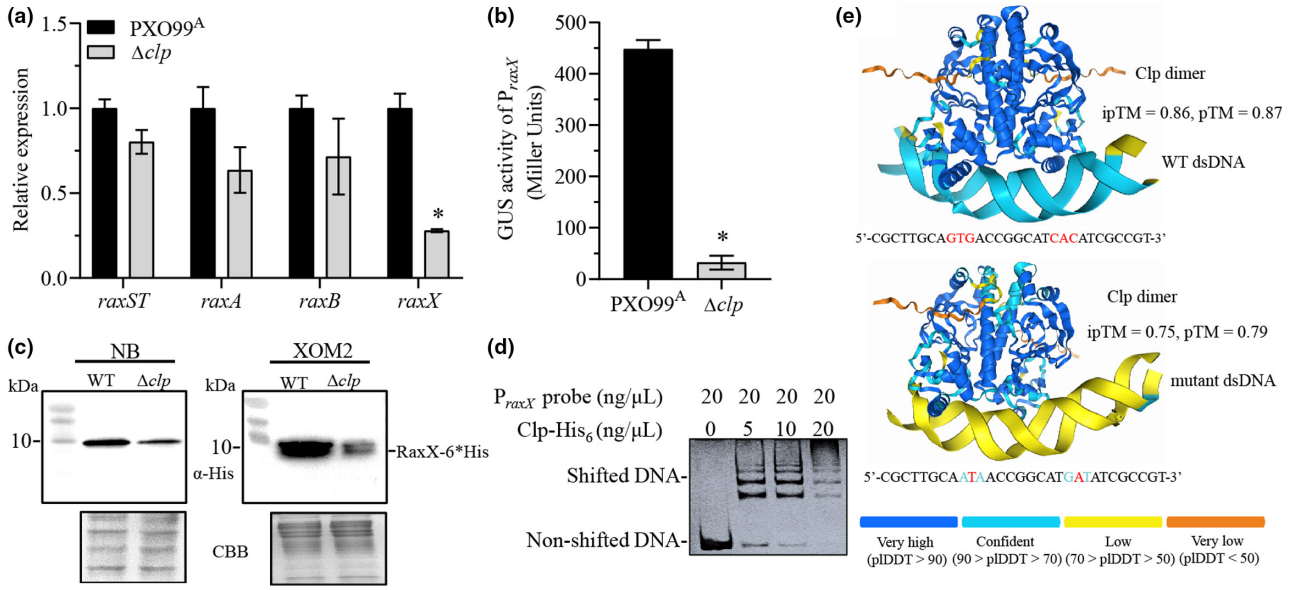

FIGURE 4 Clp directly regulates *raxX* expression. (a) Relative expression of *raxST*, *raxA*, *raxB*, and *raxX* in the wild-type (PXO99^{A}) and *clp* mutant strains. The 16S rDNA gene is used as an internal control. (b) The promoter's activity of *raxX* in the wild-type and *clp* mutant strains. Student's *t* test ($p < 0.05$) was used to separate the significantly different means (denoted by *) relative to the wild-type strain. (c) Quantitative analysis of RaxX protein was performed in both wild-type (WT) and *clp* mutant strains after culturing in nutrient broth (NB) and the *hrp*-inducing XOM2 medium. (d) The direct interaction between the Clp protein and the *raxX* promoter DNA was detected using an electrophoretic mobility shift assay. Shifted bands indicate the *raxX* promoter DNA interacts with the Clp protein. Similar results were observed in two independent experiments. (e) The predicted structure of the Clp dimer and the wild-type or mutant Clp-binding motif of the *raxX* promoter complex were predicted using AlphaFold 3. The colour represents the pLDDT score and the prediction accuracy. ipTM, interface-predicted template modelling score.

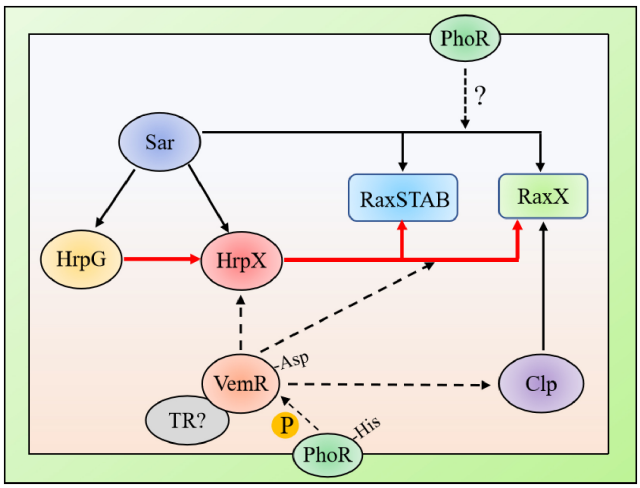

FIGURE 5 A schematic representation of the regulatory cascade involving pathogenicity-associated regulators and the *raxX-raxSTAB* cluster. In the regulatory cascade, the regulators Sar, HrpX, and Clp directly regulate the *raxX* and/or *raxSTAB* expression. HrpG regulates the *raxX-raxSTAB* cluster through the key regulator HrpX. The global transcriptional regulator Clp only regulates *raxX* expression by directly interacting with the *raxX* promoter. The histidine kinase PhoR may regulate the expression of the *rax* cluster, potentially through VemR. However, the orphan response regulator VemR governs the *rax* cluster through an unknown pathway.

the *raxX-raxSTAB* transcription (Figure 1f) (Joe et al., 2021). Recent research has shown that HrpG uses the consensus-binding motif 5′-ATTYYVWT-3′ in its target genes in *X. campestris* (Zhang et al., 2020). However, we did not find this consensus-binding motif in the *raxST* or *raxX* promoters in Xoo. Therefore, we believe that HrpG regulates *rax*-related gene expression through HrpX.

The Clp protein from *Xanthomonas* spp. is a cyclic AMP receptor protein (CRP) superfamily member and participates in the yield of extracellular polysaccharides (EPS), type II secretion system extracellular enzymes, and pathogenicity (Buttner & Bonas, 2010). In this study, we found a new function of the Clp regulator protein, namely involvement in RaxX expression (Figure 4). In *X. campestris*, the Clp homologue uses a conserved DNA-binding motif 5′-GYSN$_8$SRC-3′ in the promoter region of its target genes according to previous studies (Ge & He, 2008; Hsiao et al., 2005, 2009; Liu et al., 2019). We found a putative Clp-binding site (5′-GTGACCGGCATCAC-3′) in the *raxX* promoter region (Figure 1a). AlphaFold 3 is a powerful artificial intelligence program for predicting biomolecular interactions (Abramson et al., 2024). The Clp dimer and the putative Clp-binding motif were predicted to have a strong interaction, indicated by high pLDDT and ipTM scores. However, this interaction would be impaired by a mutation in the binding motif (Figure 4e). We found that Clp regulated *raxX* transcription but not *raxSTAB* (Figure 1a, Table S3). We also found that the Sar-binding site is upstream of the Clp-binding site (Figure 1a). We deduced that Sar occupies this region, preventing Clp from recruiting RNA polymerase for *raxSTAB* transcription.

Previous studies revealed that VemR homologues are involved in the virulence, cell motility, and EPS production of *Xanthomonas* species (Li et al., 2020, 2023; Wu et al., 2019; Zheng et al., 2021). Recently, Li et al. (2023) confirmed the VemR homologue in Xcc (VemR$_{Xcc}$, coverage 95%, identity 100%) physically interacts with the HrpG homologue (HrpG$_{Xcc}$, coverage 96%, identity 77%) for enhancing *hrpX* transcription. However, VemR is an orphan response regulator that only has a REC domain (Figure 2d), which could not directly bind to the *raxST* or *raxX* promoter region for these genes' transcription. Moreover, the mRNA level of *raxX-raxSTAB* was significantly decreased in the *vemR* mutant strain compared to PXO99^A after culturing in the XOM2 defined medium (Figure 2a, Table S3). Therefore, from the regulatory relationship of the pathogenicity-related regulators, we speculate that VemR potentially interacts with other transcriptional regulators, such as HrpG, Sar, PhoR, HpaR1, and Zur, for the *raxX-raxSTAB* transcription in Xoo (Table S4). However, yeast two-hybrid assays failed to detect an interaction between the VemR and HrpG, HpaR1 or Zur proteins (Figures 2d and S2). In contrast, bacterial two-hybrid assays showed that VemR interacted with PhoR, but did not interact with Sar or HrpG (Figures 2d and S3). Consequently, we deduced that VemR regulates the *raxSTAB-raxX* gene cluster expression maybe through the other transcriptional regulators. Future research should aim to identify the transcriptional regulator(s) that interact with VemR to elucidate this regulatory mechanism in Xoo.

Previous studies showed that the PhoP-PhoQ and RaxR-RaxH TCSs are involved in *rax*-related gene expression (Burdman et al., 2004; Lee et al., 2008). Our previous research showed that the *raxH* (*PXO_RS06055*) deletion mutant did have impaired pathogenicity compared to the wild-type strain (Zheng et al., 2021). Therefore, we did not focus on the RaxH-RaxR TCS. Future studies should verify the relationship between RaxH-RaxR and those newly identified regulators in Xoo. Besides the RaxR-RaxH and PhoP-PhoQ TCSs, we identified a new TCS, the histidine kinase PhoR, that is involved in the expression of the *rax* gene cluster (Figure 3). PhoR is a key regulator for regulating phosphate homeostasis in bacteria (Lamarche et al., 2008; Zheng et al., 2018). We found that the PhoR regulon contains 911 target genes involved in nutrient transport and metabolism in Xoo (Zheng et al., 2021). In most cases, TCS genes are co-transcribed as an operon in the genome. However, several examples of orphan sensor kinases and response regulators have been described in the literature, such as StoS and VemR in *X. oryzae* (Cai et al., 2022; Zheng et al., 2016), and other TCSs in animal-pathogenic bacteria (Elsen et al., 2024; Fernandez-Ciruelos et al., 2023). Many studies demonstrated that a non-cognate sensor kinase phosphorylates the response regulator VemR in the absence of the cognate kinase in *Xanthomonas* species (Cai et al., 2022; Li et al., 2020; Lin et al., 2022). Additionally, 340 target genes are co-regulated by VemR and PhoR in Xoo (Zheng et al., 2021). We deduced that VemR might be phosphorylated by PhoR, thereby regulating the expression of the *rax*-related genes. Future work should focus in depth on the mechanism of this regulation.

The western blotting assay revealed that the RaxX protein was expressed in Xoo after cultivation in rich (NB) and minimal (XOM2) medium conditions. Interestingly, we deduced that the RaxX protein

expressed in the rich medium may have an additional function in the Xoo cells. We evaluated some bacterial phenotypes (EPS, motility and iron-depleted condition) of the *raxX* mutant. The results showed that the *raxX* deletion mutant did not affect the yield of EPS, the swarming motility or the growth in iron-limited conditions compared to the wild-type strain (Figure S4). Future research should focus on determining the function of the RaxX protein in bacterial cells or interbacterial interaction beyond its function in the Xoo–rice interaction.

In conclusion, the present study identified four novel pathogenicity-related regulators, HrpG, VemR, PhoR, and Clp, participating in the *raxSTAB* and/or *raxX* gene cluster expression. The HrpG and HrpX are the key regulators for regulating the *rax* cluster transcription. The global regulator Clp directly regulates *raxX* transcription by interacting with the *raxX* promoter. Two novel TCSs, VemR and PhoR, also participated in the regulatory cascade for *raxSTAB* and *raxX* expression from an unknown pathway. These findings enhance our understanding of the intricate regulatory cascade of RaxSTAB and RaxX from a broader perspective.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, primers, and culture conditions

The gene-deleted mutants of Xoo PXO99^A and plasmids used in the study are listed in Table S1. Primers used in this study are listed in Table S2. Xoo strains were cultivated in the NB medium (sucrose, 10 g/L; polypeptone, 5 g/L; beef extract, 3 g/L; yeast extract 1 g/L; pH 7.0) or NB agar and *hrp*-inducing medium XOM2 (D-xylose, 0.18%; sodium L-(+)-glutamate, 10 mM; D,L-methionine, 670 μM; KH_2PO_4, 14.7 mM; 5 mM $MgCl_2$; $MnSO_4$, 40 μM; and Fe_{III}-EDTA, 240 μM; pH 6.5–7.0) at 28°C. *Escherichia coli* strains were grown in Luria Bertani (LB) broth or on LB agar at 37°C. The antibiotics were added at the following final concentrations: spectinomycin, 100 μg/mL; ampicillin, 100 μg/mL; kanamycin, 50 μg/mL; chloramphenicol, 34 μg/mL; streptomycin, 12.5 μg/mL; and tetracycline, 12.5 μg/mL.

4.2 | Genetic manipulation

The insertion of the 6×His tag gene upstream of the termination codon of *raxX* 3′ ends in Xoo was carried out by allelic homologous recombination as described previously (Shao et al., 2023). For the *raxX* in-frame deletion assay, the 506 bp upstream and 503 bp downstream homologous DNA fragments were ligated to flanks of the *raxX* gene by PCR and cloned into pK18mobsacB to generate pK18-*raxX*. The recombinant pK18-*raxX* and pK18-*raxX*-His6 (Shao et al., 2023) plasmids were electroporated into different Xoo strains to generate the first cross-over mutant separately, followed by a second cross-over exchange after culturing on NA medium containing 10% sucrose. PCR and Sanger sequencing were conducted to further confirm the presence of the *raxX* mutant or *raxX:His*$_6$ fused gene in different Xoo strains.

4.3 | RT-qPCR

Xoo strains were cultured in XOM2 medium to logarithmic phase (OD_{600} value at 0.5, for the *phoR* deletion mutant at 0.3). The total RNAs from Xoo were extracted with an EasyPure RNA kit (TransGen Biotech), and reverse transcription was performed using a cDNA synthesis kit (Takara Bio) according to the manufacturer's instructions. The mRNA level was quantified by RT-qPCR using SYBR Green master mix (Vazyme Biotech) in a QuantStudio ViiA7 system (Applied Biosystems). Relative quantification of gene expression was carried out using the 16S rDNA gene as an internal control.

4.4 | β-Glucuronidase assay

The promoter DNA fragments upstream of *raxST* and *raxX* open reading frame (ORF) were amplified and cloned into pHG3 (Zou et al., 2021) to generate the reporter plasmids pHG3-P_{raxST} and pHG3-P_{raxX}, respectively. The recombinant vector was electroporated into PXO99^A and mutant strains. The GUS assay was performed as previously described (Shao et al., 2023). Briefly, Xoo strains were grown in XOM2 medium until reaching an OD_{600} of 0.5 (for the *phoR* mutant at 0.3). Subsequently, the cell pellets were harvested and subjected to sonication to obtain the supernatant. GUS activity was determined by measuring the absorbance at absorbance at 415 nm using *p*-nitrophenyl-β-D-glucuronide (PNPG) as the substrate.

4.5 | SDS-PAGE and western blotting

Xoo strains with the 6×His-tagged *raxX* gene were cultivated in NB or XOM2 medium until they reached the logarithmic phase (for NB medium, the OD_{600} value at 0.8; for the XOM2 medium, the OD_{600} value at 0.5, for the *phoR* deletion mutant at 0.3). Equal amounts of cells were harvested, and the pellets were resuspended in phosphate-buffered saline (PBS) for ultrasonic disruption. Protein samples from the supernatant were separated by 10% (wt/vol) SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, the primary antibody used was a mouse anti-His tag monoclonal antibody (Proteintech); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was used as the secondary antibody (Proteintech). The Pierce-enhanced chemiluminescence (Thermo Fisher Scientific) was used as the HRP substrate, allowing visualization of the signal.

4.6 | Electrophoretic mobility shift assay

The Clp and HrpG ORFs were cloned into the pET-28a and pGEX-6P-1 vectors to express hexahistidine-tagged Clp (Clp-6×His) or

PhoB (Zheng et al., 2018), and glutathione S-transferase (GST)-tagged HrpG. According to the manufacturer's instructions, Clp-6×His expressed in *Escherichia coli* Rosetta (DE3) was purified using a Ni-NTA affinity column (Beijing CoWin Biotech), and HrpG-GST expressed in *E. coli* BL21 (DE3) was purified using GSTSep glutathione agarose resin (Yeasen Biotech). The purified proteins were then dialysed in 20 mM Tris–HCl buffer (pH 8.0) supplemented with 5% glycerol and 150 mM NaCl to remove imidazole. The promoter's DNA upstream from the *raxX*, *raxST*, *pst* operon or *hrpX* ORF (primers are listed in Table S2) was amplified and purified using a gel extraction kit (Omega Bio-Tek). DNA probes were incubated with tested proteins in the EMSA buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mg/mL bovine serum albumin (BSA), and 5% glycerol. The regulator protein was incubated with the promoter DNA at 28°C for 45 min. After the incubation, the samples were loaded onto a 5% native polyacrylamide gel and run in a 0.5×Tris-borate buffer at 120 V for 45 min. After the electrophoresis, the gel was stained with ethidium bromide and then visualized under UV light to display the image and observe the DNA bands.

4.7 | Bacterial two-hybrid assay and yeast two-hybrid assay

The BacterioMatch II two-hybrid system (Stratagene) was employed to detect protein–protein interactions in vivo. The full-length *sar*, *hrpG*, *vemR*, and *phoR* were amplified by PCR using the total DNA of Xoo strain PXO99^{A} as the template and the corresponding set of oligonucleotide primers (listed in Table S2). The *vemR* gene was cloned into pBT to generate the pBT-*vemR* plasmid, and the *sar*, *hrpG*, and *phoR* genes were cloned into pTRG to generate the pTRG-*sar*, pTRG-*hrpG*, and pTRG-*phoR* plasmids, respectively (Table S1). Plasmid pairs were used to co-transform the reporter strain *E. coli* XL1-Blue MRF′. The bacterial two-hybrid assay was conducted following the protocols provided by the manufacturer.

The Matchmaker GAL4 two-hybrid system (Clontech) was used for yeast two-hybrid assay to detect the interaction between VemR and the proteins HrpG, HpaR1 or Zur. The HrpG, HpaR1 or Zur coding regions were amplified and cloned into the bait vector pGBKT7, and the VemR coding region was cloned into the prey vector pGADT7. The pGBKT7-*hrpG* and pGADT7-*vemR* plasmids were separately transformed into competent *Saccharomyces cerevisiae* AH109 and Y187. Pairwise yeast mating was conducted on 2×yeast extract peptone dextrose (YPD) liquid medium at 70 rpm and 28°C for 48 h. The protein–protein interactions were detected on selective minimal synthetic dropout media (SD/−Leu/−Trp and SD/−Leu/−Trp/−His). The co-expression of pGBKT7-P53 and pGADT7-T served as a positive interaction control, while the co-expression of pGADT7-*vemR* and pGBKT7, as well as pGADT7 and pGBKT7-*TR*, served as negative controls.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

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

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REFERENCES

Abramson, J., Adler, J., Dunger, J., Evans, R., Green, T., Pritzel, A. et al. (2024) Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature*, 630, 493–500.

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. (2010) Regulation and secretion of *Xanthomonas* virulence factors. *FEMS Microbiology Reviews*, 34, 107–133.

Cai, L., Ma, W., Zou, L., Xu, X., Xu, Z., Deng, C. et al. (2022) *Xanthomonas oryzae* pv. *oryzicola* response regulator VemR is co-opted by the sensor kinase CheA for phosphorylation of multiple pathogenicity-related targets. *Frontiers in Microbiology*, 13, 928551.

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.

Elsen, S., Simon, V. & Attree, I. (2024) Cross-regulation and cross-talk of conserved and accessory two-component regulatory systems orchestrate *Pseudomonas* copper resistance. *PLoS Genetics*, 20, e1011325.

Ercoli, M.F., Luu, D.D., Rim, E.Y., Shigenaga, A., Teixeira de Araujo, A., Jr., Chern, 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.

Fernandez-Ciruelos, B., Potmis, T., Solomin, V. & Wells, J.M. (2023) Cross-talk between QseBC and PmrAB two-component systems is crucial for regulation of motility and colistin resistance in enteropathogenic *Escherichia coli*. *PLoS Pathogens*, 19, e1011345.

Ge, C. & He, C. (2008) Regulation of the type II secretion structural gene *xpsE* in *Xanthomonas campestris* pathovar *campestris* by the global transcription regulator Clp. *Current Microbiology*, 56, 122–127.

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.

Hsiao, Y.M., Fang, M.C., Sun, P.F. & Tseng, Y.H. (2009) Clp and RpfF up-regulate transcription of *pelA1* gene encoding the major pectate lyase in *Xanthomonas campestris* pv. *campestris*. *Journal of Agricultural and Food Chemistry*, 57, 6207–6215.

Hsiao, Y.M., Liao, H.Y., Lee, M.C., Yang, T.C. & Tseng, Y.H. (2005) Clp up-regulates transcription of *engA* gene encoding a virulence factor in *Xanthomonas campestris* by direct binding to the upstream tandem Clp sites. *FEBS Letters*, 579, 3525–3533.

Joe, A., Stewart, V. & Ronald, P.C. (2021) The HrpX protein activates synthesis of the RaxX sulfopeptide, required for activation of XA21-mediated immunity to *Xanthomonas oryzae* pv. *oryzae*. *Molecular Plant–Microbe Interactions*, 34, 1307–1315.

Lamarche, M.G., Wanner, B.L., Crepin, S. & Harel, J. (2008) The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiology Reviews*, 32, 461–473.

Lee, M.C., Weng, S.F. & Tseng, Y.H. (2003) Flagellin gene *fliC* of *Xanthomonas campestris* is upregulated by transcription factor Clp. *Biochemical and Biophysical Research Communications*, 307, 647–652.

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, *hrpG* expression, and virulence. *Journal of Bacteriology*, 190, 2183–2197.

Li, R.F., Peng, J.L., Liu, Q.Q., Chang, Z., Huang, Y.X., Tang, J.L. et al. (2023) *Xanthomonas campestris* VemR enhances the transcription of the T3SS key regulator HrpX via physical interaction with HrpG. *Molecular Plant Pathology*, 24, 232–247.

Li, R.F., Wang, X.X., Wu, L., Huang, L., Qin, Q.J., Yao, J.L. et al. (2020) *Xanthomonas campestris* sensor kinase HpaS co-opts the orphan response regulator VemR to form a branched two-component system that regulates motility. *Molecular Plant Pathology*, 21, 360–375.

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, G.F., Su, H.Z., Sun, H.Y., Lu, G.T. & Tang, J.L. (2019) Competitive control of endoglucanase gene *engXCA* expression in the plant pathogen *Xanthomonas campestris* by the global transcriptional regulators HpaR1 and Clp. *Molecular Plant Pathology*, 20, 51–68.

Liu, W.D., Liu, J.L., Triplett, L., Leach, J.E. & Wang, G.L. (2014) Novel insights into rice innate immunity against bacterial and fungal pathogens. *Annual Review of Phytopathology*, 52, 213–241.

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.

Mew, T.W., Alvarez, A.M., Leach, J.E. & Swings, J. (1993) Focus on bacterial-blight of rice. *Plant Disease*, 77, 5–12.

Mole, B.M., Baltrus, D.A., Dangl, J.L. & Grant, S.R. (2007) Global virulence regulation networks in phytopathogenic bacteria. *Trends in Microbiology*, 15, 363–371.

Pruitt, R.N., Joe, A., Zhang, W., Feng, W., Stewart, V., Schwessinger, B. et al. (2017) A microbially derived tyrosine-sulfated peptide mimics a plant peptide hormone. *New Phytologist*, 215, 725–736.

Pruitt, 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.

Qian, G., Liu, C., Wu, G., Yin, F., Zhao, Y., Zhou, Y. et al. (2013) AsnB, regulated by diffusible signal factor and global regulator Clp, is involved in aspartate metabolism, resistance to oxidative stress and virulence in *Xanthomonas oryzae* pv. *oryzicola*. *Molecular Plant Pathology*, 14, 145–157.

Qian, W., Han, Z.J. & He, C. (2008) Two-component signal transduction systems of *Xanthomonas* spp.: a lesson from genomics. *Molecular Plant–Microbe Interactions*, 21, 151–161.

Ronald, P.C. (2014) The role of RaxST, a prokaryotic sulfotransferase, and RaxABC, a putative type I secretion system, in activation of the rice XA21-mediated immune response. *Scientifica (Cairo)*, 2014, 532816.

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.

Tao, F., He, Y.W., Wu, D.H., Swarup, S. & Zhang, L.H. (2010) The cyclic nucleotide monophosphate domain of *Xanthomonas campestris* global regulator Clp defines a new class of cyclic di-GMP effectors. *Journal of Bacteriology*, 192, 1020–1029.

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.

Wang, F.F. & Qian, W. (2019) The roles of histidine kinases in sensing host plant and cell-cell communication signal in a phytopathogenic bacterium. *Philosophical Transactions of the Royal Society of London Series B Bioloical Sciences*, 374, 20180311.

Wang, G.L., Song, W.Y., Ruan, D.L., Sideris, S. & Ronald, P.C. (1996) The cloned gene, *Xa21*, confers resistance to multiple *Xanthomonas oryzae* pv. *oryzae* isolates in transgenic plants. *Molecular Plant–Microbe Interactions*, 9, 850–855.

Wengelnik, K., Van den Ackerveken, G. & Bonas, U. (1996) HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. *Molecular Plant–Microbe Interactions*, 9, 704–712.

Wu, W., Zhao, Z., Luo, X., Fan, X., Zhuo, T., Hu, X. et al. (2019) Response regulator VemR regulates the transcription of flagellar rod gene *flgG* by interacting with σ_{54} factor RpoN2 in *Xanthomonas citri* ssp. *citri*. *Molecular Plant Pathology*, 20, 372–381.

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., 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.

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., Yao, X., Duan, M., Luo, Y., Liu, B., Qi, P. et al. (2016) Two overlapping two-component systems in *Xanthomonas oryzae* pv. *oryzae* contribute to full fitness in rice by regulating virulence factors expression. *Scientific Reports*, 6, 22768.

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.

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