ORIGINAL ARTICLE

Molecular Plant Pathology  **WILEY**

# HpaP divergently regulates the expression of *hrp* genes in *Xanthomonas oryzae* pathovars *oryzae* and *oryzicola*

Rui-Fang Li[1] | Pei-Dong Ren[2] | Da-Pei Zhang[2] | Ping Cui[2] | Gui-Ning Zhu[1] | Xiao-Yong Xian[1] | Ji-Liang Tang[2] | Guang-Tao Lu[2]

[1]Plant Protection Research Institute, Guangxi Academy of Agricultural Science, Key Laboratory of Green Prevention and Control on Fruits and Vegetables in South China Ministry of Agriculture and Rural Affairs, Guangxi Key Laboratory of Biology for Crop Diseases and Insect Pests, Nanning, Guangxi, China

[2]State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, College of Life Science and Technology, Guangxi University, Nanning, China

**Correspondence**

Rui-Fang Li, Plant Protection Research Institute, Guangxi Academy of Agricultural Science, 174 Daxue Road, Nanning, Guangxi 530007, China.
Email: ruifangli@gxaas.net

Guang-Tao Lu, College of Life Science and Technology, Guangxi University, 100 Daxue Road, Nanning, Guangxi 530004, China.
Email: lugt@gxu.edu.cn

**Funding information**

National Natural Science Foundation of China, Grant/Award Number: 32160617; State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Grant/Award Number: SKLCUSA-a201903

## Abstract

The bacterial pathogens *Xanthomonas oryzae* pathovars *oryzae* (Xoo) and *oryzicola* (Xoc) cause leaf blight and leaf streak diseases on rice, respectively. Pathogenesis is largely defined by the virulence genes harboured in the pathogen genome. Recently, we demonstrated that the protein HpaP of the crucifer pathogen *Xanthomonas campestris* pv. *campestris* is an enzyme with both ATPase and phosphatase activities, and is involved in regulating the synthesis of virulence factors and the induction of the hypersensitive response (HR). In this study, we investigated the role of HpaP homologues in Xoo and Xoc. We showed that HpaP is required for full virulence of Xoo and Xoc. Deletion of *hpaP* in Xoo and Xoc led to a reduction in virulence and alteration in the production of virulence factors, including extracellular polysaccharide and cell motility. Comparative transcriptomics and reverse transcription-quantitative PCR assays revealed that in XVM2 medium, a mimic medium of the plant environment, the expression levels of *hrp* genes (for HR and pathogenicity) were enhanced in the Xoo *hpaP* deletion mutant compared to the wild type. By contrast, in the same growth conditions, *hrp* gene expression was decreased in the Xoc *hpaP* deletion mutant compared to the wild type. However, an opposite expression pattern was observed when the pathogens grew in planta, where the expression of *hrp* genes was reduced in the Xoo *hpaP* mutant but increased in the Xoc *hpaP* mutant. These findings indicate that HpaP plays a divergent role in Xoo and Xoc, which may lead to the different infection strategies employed by these two pathogens.

**KEYWORDS**
divergent regulation, HpaP, *hrp* genes, transcriptome, *Xanthomonas oryzae*

## 1 | INTRODUCTION

The genus *Xanthomonas* comprises an important ubiquitous group of gram-negative phytopathogenic bacteria that cause severe diseases in many agronomically important crops throughout the world. *Xanthomonas* species and pathovars within a species show host specificity and infect approximately 124 monocotyledonous and 268 dicotyledonous plants. Many of these pathogens exhibit tissue specificity, invading either host vasculature or the spaces between mesophyll cells of their hosts (Büttner & Bonas, 2010;

---

Mansfield et al., 2012). Among the most prominent of these pathogens is the species *Xanthomonas oryzae*, the causative agent of two distinct and severe diseases of rice (*Oryza sativa*) and some wild relatives. *X. oryzae* has two pathovars, *oryzae* and *oryzicola*; the former (Xoo) invades through the vascular system, causing bacterial leaf blight disease, while the latter (Xoc) colonizes the intercellular spaces of the parenchyma tissues, resulting in bacterial leaf streak disease (Mansfield et al., 2012; Niño-Liu et al., 2006). Bacterial leaf blight is one of the most serious diseases of rice, leading to 20%–50% yield losses. Bacterial leaf streak is emerging in importance: in the past two decades, the disease has expanded rapidly in some regions and became more devastating than bacterial leaf blight (Niño-Liu et al., 2006).

Although Xoc and Xoo are closely related pathogens (Ochiai & Kaku, 1999; Vauterin et al., 1995), they infect rice in distinct ways and cause different symptoms. Xoo infects rice either through water pores (hydathodes) at the leaf tip and margins or through wounds in the leaves or roots, and colonizes the vascular tissues and enters the xylem vessels. Once in the xylem, the pathogen continues to grow and moves through longitudinal and commissural veins to generate tannish-grey to white lesions (Ou, 1985). Xoc, by contrast, enters rice leaves mainly through leaf stomata or wounds, and multiplies in the substomatal cavity and then colonizes the intercellular spaces (i.e., the apoplast) of the mesophyll, causing water-soaked interveinal lesions that develop into translucent streaks (Mansfield et al., 2012; Niño-Liu et al., 2006). Although Xoc may infect plant through wounds, it is limited to the mesophyll apoplast and does not invade the xylem (Ou, 1985).

In past decades, a large number of Xoo pathogenesis-related genes have been identified and characterized. Multiple factors have been shown to contribute to the virulence of Xoo; these include the production of extracellular polysaccharide (EPS), adhesion, biofilm formation, cell motility, diffusible signal factor (DSF)-dependent cell–cell signalling, the production of degradative extracellular enzymes such as pectate lyases, cellulases, xylanases, and proteases secreted by the type II secretion system (T2SS), and the effectors secreted by the type III secretion system (T3SS) (Büttner & Bonas, 2010; White, 2016).

Molecular pathogenic (virulence) mechanisms of Xoc are, in contrast, less explored than those of Xoo. Genomic studies have revealed that many pathogenicity and virulence genes are highly conserved between these two pathogens. Both pathovars contain similar genes for EPS synthesis, extracellular enzymes, the T3SS, the DSF-dependent cell–cell signal system, and two-component signal transduction systems (Lu et al., 2008; Timilsina et al., 2020), suggesting they might share common virulence mechanisms. To date, the molecular basis of the differences in tissue specificity and symptom development between the two diseases is still obscure. The T3SS plays an important role in the interactions between bacterial pathogens and host plants by injecting the T3SS-secreted effectors (T3Es) into plant cells. Differences in the complement and/or expression of T3Es between the two pathovars of *X. oryzae* could presumably contribute to the differences in tissue specificity and symptomology.

Knowledge of the T3SS in xanthomonads arises mostly from the investigation of the species *Xanthomonas campestris*. The T3SS apparatus is encoded by more than 20 *hrp* (hypersensitive response and pathogenicity) genes, which are mainly organized within six operons named *hrpA* to *hrpF*. Mutation of *hrp* genes leads to the loss of the bacterial ability to cause full virulence and hypersensitive response (HR) induction (Lindgren, 1997). The activation of *hrp* genes, as well as some genes that encode T3Es, is mainly controlled by two key regulators, HrpG (an OmpR family regulator) and HrpX (an AraC-type transcriptional activator) (Wengelnik et al., 1996; Wengelnik & Bonas, 1996).

Several other regulatory proteins have been shown to control the expression of *hrp* genes in *X. oryzae* species. Trh (transcriptional regulator for *hrp*), a member of the GntR regulator family, directly or indirectly activates *hrpG* transcription (Tsuge et al., 2006). KdgR, a transcriptional regulator of the IclR family, involved in pectin catabolism, seems to directly repress the expression of *hrp* genes via HrpG and HrpX (Lu et al., 2011). RsmA, a posttranscriptional regulator of the RNA-binding protein family, has been shown to activate the expression of *hrp* genes at both transcriptional and posttranscriptional levels by directly binding to the untranslated region of *hrpG* (Andrade et al., 2014). GamR, a LysR-type transcriptional regulator that regulates the galactose metabolism, positively regulates the expression of *hrp* genes by binding to the upstream region of *hrpG* (Rashid et al., 2016). XylR, a repressor of xylan and xylose utilization, belonging to the LacI family, appears to negatively regulate the expression of *hrp* genes via HrpX accumulation (Ikawa et al., 2018). In addition, three histone-like nucleoid-structuring (H-NS) proteins, XrvA, XrvB and XrvC, have been demonstrated to affect the expression of *hrp* genes (Feng et al., 2009; Kametani-Ikawa et al., 2011; Liu et al., 2016). A two-component signal transduction system, PhoP/PhoQ, has also been reported to be involved in the transcriptional regulation of *hrpG* (Lee et al., 2008). These findings reveal the presence of a complex, sophisticated regulatory network that regulates the expression of the T3SS in *X. oryzae*.

In previous work we identified HpaP (for HR and pathogenicity-associated phosphatase), a dual enzyme with both ATPase and phosphatase activity, that regulates virulence and HR induction in *X. campestris* pv. *campestris* (Xcc), the causal agent of black rot disease of cruciferous plants. Here, we examine the role of the homologues of this protein in Xoc and Xoo. We show that HpaP is conserved in *Xanthomonas* spp. and is required for full virulence in Xoc and Xoo. However, HpaP has opposite effects on the expression of the T3SS in Xoo and Xoc, implying a significantly different role for HpaP in these two related pathogens.

## 2 | RESULTS

### 2.1 | The *hpaP* from Xoo or Xoc restores the phenotype of the Xcc *hpaP* deletion mutant to wild type

The *hpaP* gene in Xcc is 873 bp long and encodes a protein comprising 290 amino acids (Cui et al., 2018). A BLAST search revealed that most sequenced *Xanthomonas* species and pathovars have a protein with more than 90% amino acid sequence identity to the Xcc HpaP,
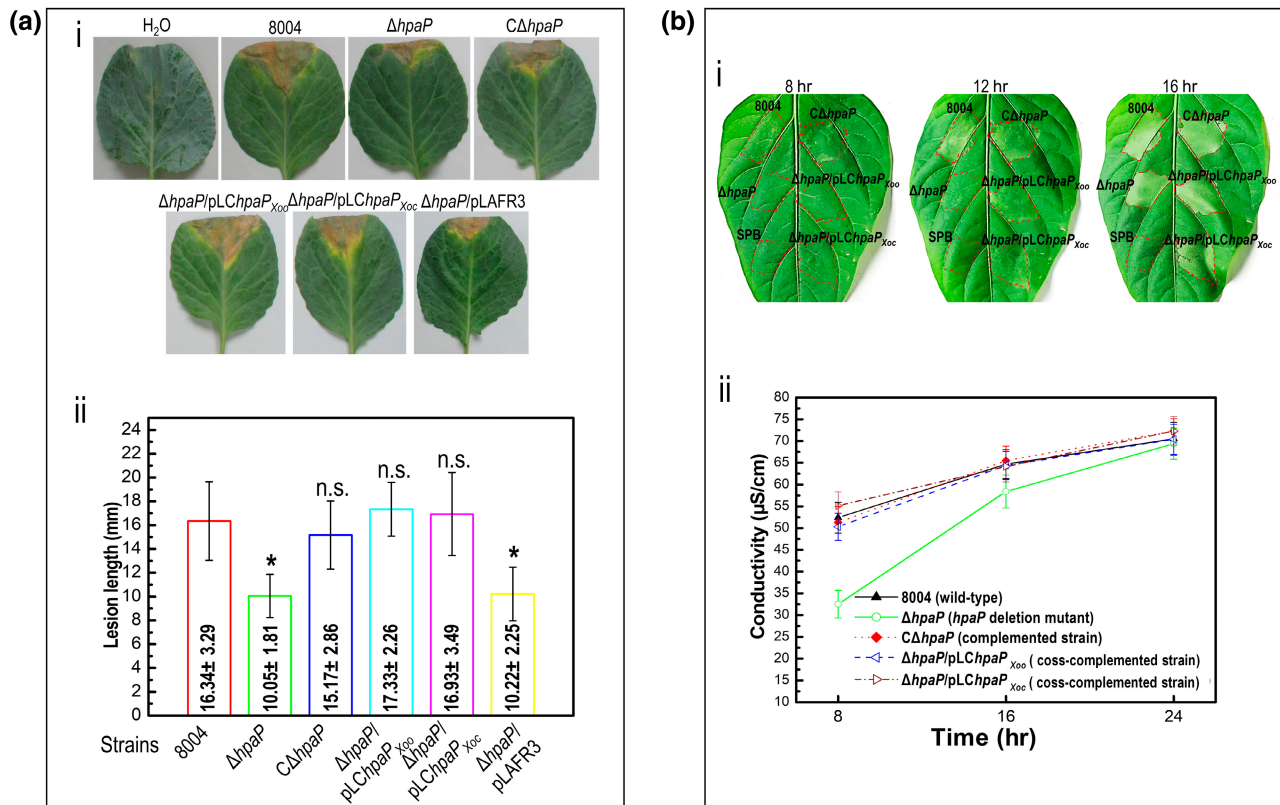
**FIGURE 1** The *hpaP* gene from *Xanthomonas oryzae* restores phenotypes of the *Xanthomonas campestris* pv. *campestris* (Xcc) *hpaP* deletion mutant. (a) Virulence test of Xcc strains in host plants. The Xcc wild-type strain 8004, *hpaP* deletion mutant Δ*hpaP*, complemented strain CΔ*hpaP*, and cross-complemented strains Δ*hpaP*/pLC*hpaP*$_{Xoc}$ and Δ*hpaP*/pLC*hpaP*$_{Xoo}$, and control strain Δ*hpaP*/pLAFR3 (Δ*hpaP* mutant with empty vector) were cultured in NYG medium overnight and then adjusted to $10^6$ cfu/ml in sterile ultrapure water. Thirty-day-old cabbages (*Brassica oleracea* 'Jingfeng No. 1') were inoculated with bacterial suspensions of the Xcc strains by the leaf-clipping method. (i) Cabbage leaves infected with Xcc strains showing symptoms as lesions 10 days postinoculation (dpi). (ii) Lesion lengths were scored 10 dpi. Values given are the mean ± *SD* from 15 inoculated leaves in an experiment. Significance was determined by analysis of variance and Dunnett's post hoc test for comparison to the wild type. *$p < 0.05$; n.s., not significant. The experiment was repeated three times and similar results were obtained in two other independent experiments. (b) Hypersensitive response (HR) test of Xcc strains in nonhost plants. Bacterial cells from overnight culture were resuspended in 10 mM sodium phosphate buffer to a density of $10^7$ cfu/ml. Approximately 5 μl of bacterial resuspension was infiltrated into pepper (*Capsicum annuum* 'ECW-10R') leaf mesophyll tissue. (i) HR symptoms photographed at 8, 12 and 16 h postinoculation (hpi). Three replications were performed in each experiment, and each experiment was repeated three times. The results presented are from a representative experiment and similar results were obtained in all other independent experiments. (ii) Electrolyte leakage from pepper leaves inoculated with Xcc strains. The conductivity of the infiltrated spots was measured at 8, 16 and 24 hpi, with four 0.4 cm$^2$ leaf discs collected from the infiltrated area for each sample. Three samples were taken for each measurement in each experiment. Data are shown as the mean ± *SD* of three replicates from a representative experiment, and similar results were obtained in two other independent experiments.

and that the Xoo and Xoc HpaP homologues share 99.3% identical residues with each other, and 92.8% and 93.4% identical residues with the Xcc HpaP, respectively (Figure S1). Studies of the sequences of HpaP proteins from 24 sequenced *Xanthomonas* species or pathovars by nearest-neighbour analysis showed that the HpaP proteins are highly conserved within the *Xanthomonas* genus, although they seem to fall into two distinct clades/families (Figure S1).

To verify whether the HpaP homologues in the species *X. oryzae* play similar roles to the previously characterized Xcc HpaP, the *hpaP* coding sequences of Xoo and Xoc were PCR-amplified with the corresponding primer sets (Table S1) and cloned into the vector pLAFR3 (Table S2), resulting in recombinant plasmids pLC*hpaP*$_{Xoo}$ and pLC*hpaP*$_{Xoc}$, respectively (Table S2). The recombinant plasmids

were then introduced into the Xcc *hpaP* deletion mutant strain Δ*hpaP* to generate the cross-complemented strains Δ*hpaP*/pLC*hpaP*$_{Xoo}$ and Δ*hpaP*/pLC*hpaP*$_{Xoc}$ (Table S2). These cross-complemented strains and the Xcc *hpaP* deletion mutant Δ*hpaP* were tested for virulence in the host plant Chinese radish using a leaf clipping assay. Consistent with our previous study (Cui et al., 2018), Δ*hpaP* induced a significantly shorter lesion length compared with the wild type (Figure 1a), while both of the cross-complemented strains induced wild-type disease. In a further set of experiments, the ability of Xcc strains to induce the HR in the leaves of nonhost plant pepper cultivar ECW-10R was examined. The results revealed that the cross-complemented strains elicited similar HR symptoms to those seen with the wild-type strain (Figure 1b). Taken together,

these data showed that the HpaP proteins from Xoo and Xoc are functional orthologues of the Xcc protein and can act in Xcc to induce typical black rot symptoms in the host plant and an HR in the nonhost plant.

## 2.2 | *hpaP* in Xoo and Xoc is essential for the full bacterial virulence

The above data revealed that the HpaP homologue from Xoo or Xoc could substitute for the function of HpaP in Xcc. To explore the detailed function of HpaP within each *X. oryzae* pathovar, in-frame deletion mutants of *hpaP* were constructed from the Xoo strain PXO99[A] and the Xoc strain GX01 using the suicide vector pK18*mobsacB*. The derived mutant strains were designated $\Delta hpaP_{Xoo}$ and $\Delta hpaP_{Xoc}$, respectively (Table S2). Simultaneously, a complemented strain for each of the mutants was also constructed by introducing the recombinant plasmids pLC*hpaP*$_{Xoo}$ and pLC*hpaP*$_{Xoc}$ into the mutants $\Delta hpaP_{Xoo}$ and $\Delta hpaP_{Xoc}$, respectively. The resulting complemented strains were named as C$\Delta hpaP_{Xoo}$ and C$\Delta hpaP_{Xoc}$ (Table S2). The bacterial cells of Xoo strains (PXO99[A], $\Delta hpaP_{Xoo}$ and C$\Delta hpaP_{Xoo}$) and Xoc strains (GX01, $\Delta hpaP_{Xoc}$ and C$\Delta hpaP_{Xoc}$) were inoculated into the leaves of the susceptible rice plant using leaf clipping and infiltration methods, respectively. The results revealed that both mutants caused less disease symptoms and significantly shorter lesion length compared with the wild type, whilst the complemented strains could induce wild-type disease (Figure 2), indicating both *hpaP* genes in Xoo and Xoc are required for full virulence.

To explore if these HpaP orthologues manipulate specific functions that are known to be required for virulence in Xoo, we examined EPS production, extracellular enzymes (cellulase in Xoo and protease in Xoc), and cell motility. The results showed that the Xoo *hpaP* mutant strain $\Delta hpaP_{Xoo}$ and Xoc *hpaP* mutant strain $\Delta hpaP_{Xoc}$ displayed moderately decreased EPS production (Figure 3a), and swimming and swarming motility (Figure 3b), but not the activity of extracellular enzymes (Figure S2). However, these phenotypes of each complemented strain were similar to the wild type under the test conditions. To clarify that mutation in HpaP did not influence the growth of Xoo and Xoc, the growth characteristics of the Xoo strains or Xoc strains in nutrient broth medium were assessed. The results revealed that both the $\Delta hpaP_{Xoo}$ mutant and the $\Delta hpaP_{Xoc}$ mutant displayed wild-type growth properties (Figure S3).

## 2.3 | HpaP has different effects on HR induction by Xoo and Xoc

The influence of HpaP on the virulence of Xoo and Xoc prompted us to examine the role of this protein in the induction of the HR in a nonhost plant. Accordingly, the same Xoo and Xoc strains used above were introduced into the leaves of the nonhost plant *Nicotiana benthamiana*. The results revealed that the Xoo *hpaP* mutant strain

$\Delta hpaP_{Xoo}$ elicited similar HR symptoms to those seen with the wild-type strain PXO99[A] (Figure 4ai), implying that the HpaP had no obvious impacts on the HR induction in Xoo. In contrast, the Xoc *hpaP* mutant strain $\Delta hpaP_{Xoc}$ induced a delayed and weakened HR compared to the wild-type strain GX01 (Figure 4bi), while the complemented strain C$\Delta hpaP_{Xoc}$ could stimulate wild-type HR symptoms, indicating that the HpaP in Xoc, similar to its counterpart in Xcc (Cui et al., 2018), was required for the bacterial ability to induce HR.

An electrolyte leakage assay was used to estimate the HR induction quantitatively. Here, leaf tissues within the infiltration areas were collected at three time points (24, 36 and 48 h) postinoculation of the Xoo or Xoc strains. The results showed that the Xoo *hpaP* mutant strain $\Delta hpaP_{Xoo}$ and the wild-type strain PXO99[A] induced similar levels of electrolyte leakage at all the test time-points (Figure 4aii). However, the Xoc *hpaP* mutant strain $\Delta hpaP_{Xoc}$ induced much lower levels a 24 and 36 h, compared to the wild type, whereas the complemented strain C$\Delta hpaP_{Xoc}$ generated a very similar wild-type level (Figure 4bii). These data indicate that HpaP affects the ability of HR elicitation in Xoc, but not in Xoo.

## 2.4 | HpaP plays a role in the expression of numerous virulence genes of Xoo and Xoc in XVM2 minimal medium

The above data reveal that loss of HpaP affects a series of phenotypes including the virulence of Xoo and Xoc as well as HR induction by Xoc. To gain more understanding of the regulatory scope of the HpaP, a set of global gene expression profiles was generated using RNA-sequencing (RNA-Seq). Here Xoo strains (the wild-type strain PXO99[A] and the mutant $\Delta hpaP_{Xoo}$) or Xoc strains (the wild-type strain GX01 and the mutant $\Delta hpaP_{Xoc}$) were grown in XVM2, a minimal medium inducing the expression of a series of virulence-related genes of xanthomonads (Astua-Monge et al., 2005).

The Xoo transcriptomic data indicated that 720 genes were differentially expressed by two-fold or more in the mutant $\Delta hpaP_{Xoo}$ relative to the wild-type strain. Among these differentially expressed genes (DEGs), 564 and 156 were up-regulated and down-regulated in $\Delta hpaP_{Xoo}$, respectively (Figure 5ai and Table S3). In the Xoc transcriptome, the expression of a total of 271 genes was significantly changed, of which 108 and 163 were up-regulated and down-regulated in the mutant $\Delta hpaP_{Xoc}$, respectively (Figure 5aii and Table S4). To confirm the transcriptome changes, reverse transcription-quantitative real-time PCR (RT-qPCR) was used to estimate the relative expression levels of several selected genes. The results showed that the expression of these selected genes was consistent with the data from the transcriptome analyses (Figure 5b).

According to the genomic annotations of the Xoo strain PXO99[A] (accession CP000967.2) and the Xoc strain GX01 (accession CP043403.1), the DEGs were assigned to functional categories on gene ontology (GO) terms (Gene Ontology, 2019) and KEGG pathways (Kyoto Encyclopedia of Genes and Genomes,
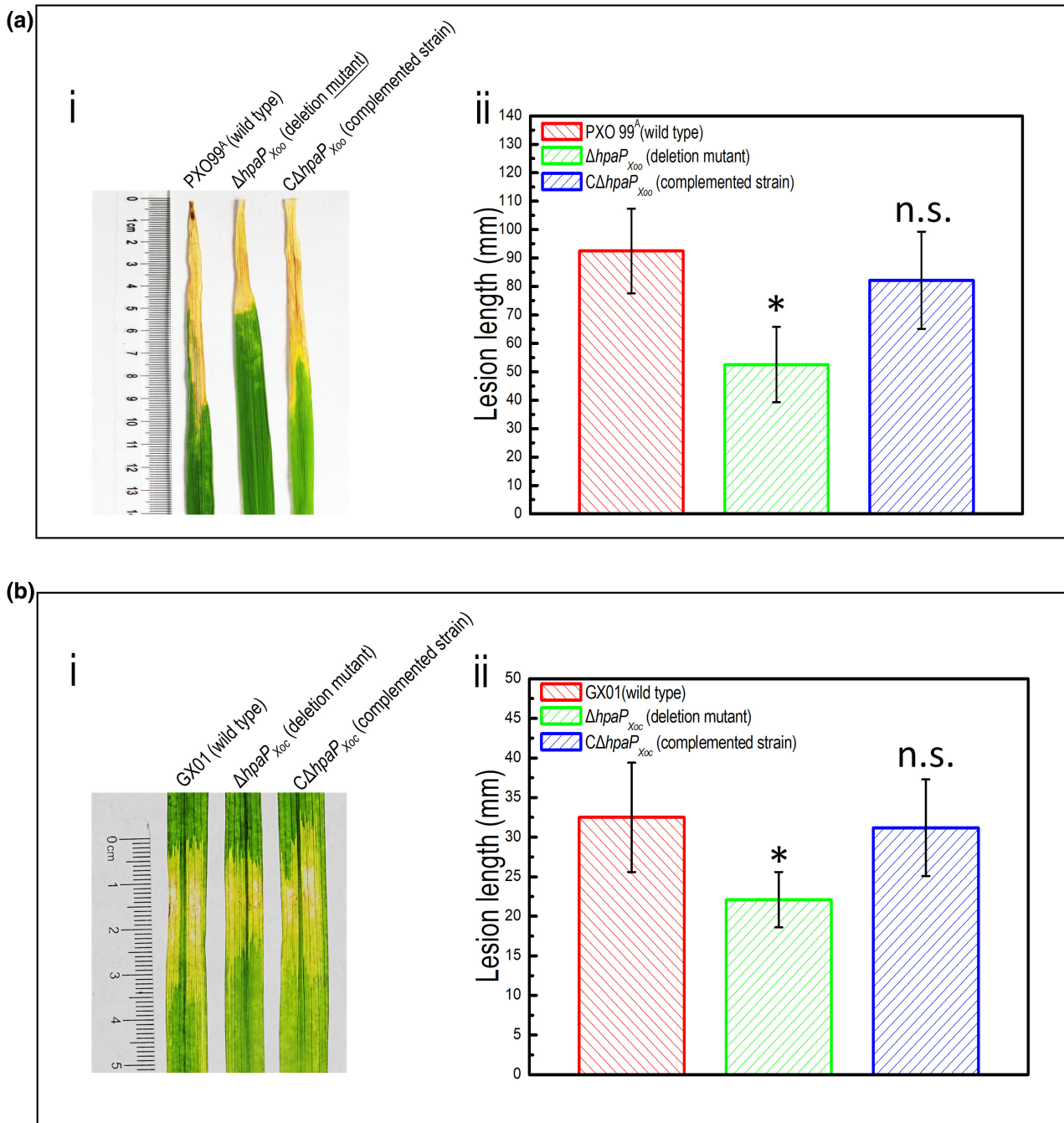
**FIGURE 2** HpaP homologues in *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc) are required for full virulence. (a) Virulence test of Xoo strains. The Xoo wild-type strain PXO99[A], and its derivatives the *hpaP* deletion mutant Δ*hpaP*$_{Xoo}$ and the complemented strain CΔ*hpaP*$_{Xoo}$ were cultured in nutrient broth for 72 h and then adjusted to a concentration of $OD_{600} = 0.3$ in sterile ultrapure water. Susceptible 6-week-old rice plants *Oryza sativa* subsp. *japonica* 'Nipponbare' were inoculated with bacterial suspensions of the Xoo strains by the leaf-clipping method. (i) Representative rice leaves infected with Xoo strains showing bacterial blight symptoms as lesions 14 days postinoculation (dpi). (ii) Lesion lengths were scored 14 dpi. Values given are the mean and *SD* from 25 inoculated leaves in one experiment. Significance was determined by analysis of variance (ANOVA) and Dunnett's post hoc test for comparison to the wild type. *$p < 0.05$; n.s., not significant. The experiment was repeated three times with similar results. (b) Virulence test of Xoc strains. Bacterial suspensions ($OD_{600} = 0.3$) of the Xoc wild-type strain GX01 and its derivatives were tested on susceptible Nipponbare rice plant using leaf infiltration method. (i) Representative rice leaves infected with Xoc strains showing bacterial leaf streak symptoms as lesions 14 dpi. (ii) Lesion lengths were scored at 14 dpi. Values given are the mean and *SD* from 25 inoculated leaves in one experiment. Significance was determined by ANOVA and Dunnett's post hoc test for comparison to the wild type. *$p < 0.05$; n.s., not significant. The experiment was repeated three times with similar results.
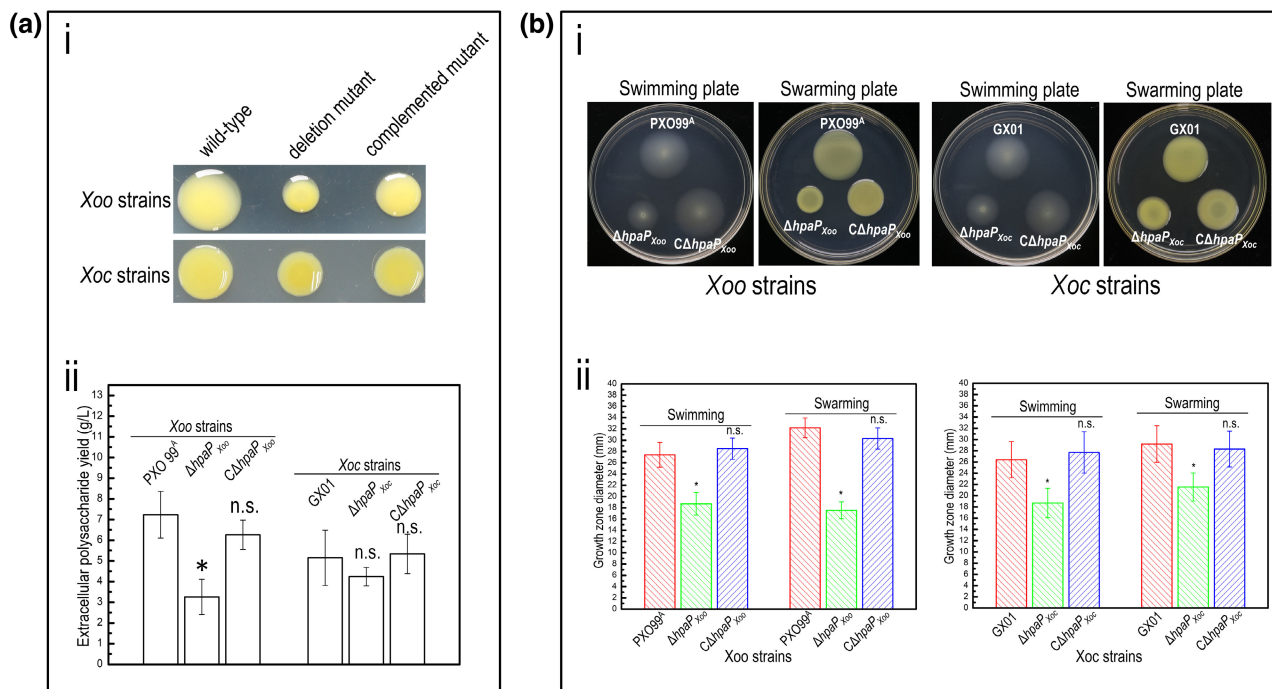
**FIGURE 3** Mutation in *hpaP* reduces extracellular polysaccharide (EPS) production and cell motilies in *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc). (a) The effect of *hpaP* mutation on EPS production. (i) Xoo and Xoc strains grown on nutrient agar plates supplemented with 2% glucose for 5 days. The Xoo *hpaP* deletion mutant strain Δ*hpaP*$_{Xoo}$ displayed obvious small colonies compared to the wild-type strain, while the Xoc *hpaP* deletion mutant strain Δ*hpaP*$_{Xoc}$ formed colonies of moderately small size compared to that of the wild type. (ii) Xoo and Xoc strains cultured in nutrient broth supplemented with 2% (wt/vol) glucose for 3 days, and EPS was precipitated from the culture supernatant. Values given are the means ± *SD* of triplicate measurements from a representative experiment, significance was determined by analysis of variance (ANOVA) and Dunnett's post hoc test for comparison to the wild type. *$p < 0.05$; n.s., not significant. Similar results were obtained in two other independent experiments. (b) The effects of *hpaP* mutation on cell motility. Two microlitres of Xoo or Xoc culture suspension ($10^9$ cfu/ml) was stabbed into "swimming" plate or inoculated onto "swarming" plate and incubated for 4 days at 28°C. (i) Example photographs of bacterial strains. (ii) Mean measurements of colony diameters of each strain on the different media. Data shown are mean ± *SD* from 10 measurements in a representative experiment. Significance was determined by ANOVA and Dunnett's post hoc test for comparison to the wild type. *$p < 0.05$; n.s., not significant. Similar results were obtained in two other independent experiments.

https://www.kegg.jp/kegg/pathway.html). Beside a total of 62 genes in Xoo and 75 genes in Xoc that were predicted to encode hypothetical proteins or have not been given a functional category to date (Figure 5a, Tables S3 and S4), the most dominant genes were assigned to several categories, such as "type III secretion system (*hrp/hrc* genes) and its effectors", "other secretion systems", "extracellular enzymes", "surface polysaccharides, lipopolysaccharides and antigens", "toxin and detoxification", "adhesion and biofilm", "quorum sensing and regulation of virulence factors", "adaptation to atypical conditions" and "bacterial motility", which have been identified as influencing host specificity and bacterial pathogenicity in several *Xanthomonas* spp. A total of 206 genes in Xoo and 56 genes in Xoc were identified as belonging to these categories (Table S5).

The EPS xanthan, a polymer of repeating pentasaccharide units, contributes to virulence in xanthomonads. The products of the *gum* gene cluster, which is composed of 12 genes (*gumB* to *gumM*) and has a major promoter upstream of the first gene, *gumB*, has been demonstrated to be responsible for the assembly of the pentasaccharide repeating unit, polymerization, and the export of the EPS in Xcc (Katzen et al., 1996, 1998). Similarly, the Xoo *gum* cluster contains 14 open reading frames (ORFs) that are controlled mainly by

a promoter located upstream of *gumB*, but the cluster also has internal promoters upstream of *gumG* (Yoon & Cho, 2007). The transcription profile data revealed that nine genes were differentially expressed in the mutant Δ*hpaP*$_{Xoo}$ compared with the wild type (Table S5); these DEGs were all up-regulated. The expression level of other *gum* genes was also altered in Δ*hpaP*$_{Xoo}$, but the change was less than 2-fold compared to the wild type (data not shown). Interestingly, our transcription profiles showed that loss of the HpaP in Xoc had no impact on the expression of the *gum* operon. These observations are inconsistent with the findings that deletion of HpaP reduced EPS production in Xoo and Xoc. This inconsistency is probably due to the employment of bacterial cells from different growth conditions such as cultured media and growth phases in the experiments.

Most of the xanthomonads produce an array of extracellular enzymes, including proteases, endoglucanases, amylase and pectate lyases, that collectively contribute to the degradation of host plant cells. All *Xanthomonas* spp. possess a T2SS that plays a role in the secretion of these plant cell wall-degrading enzymes. In Xoo *hpaP* mutant, four genes involved in the T2SS and seven genes encoding plant cell wall-degrading enzymes (Table S5) were up-regulated.
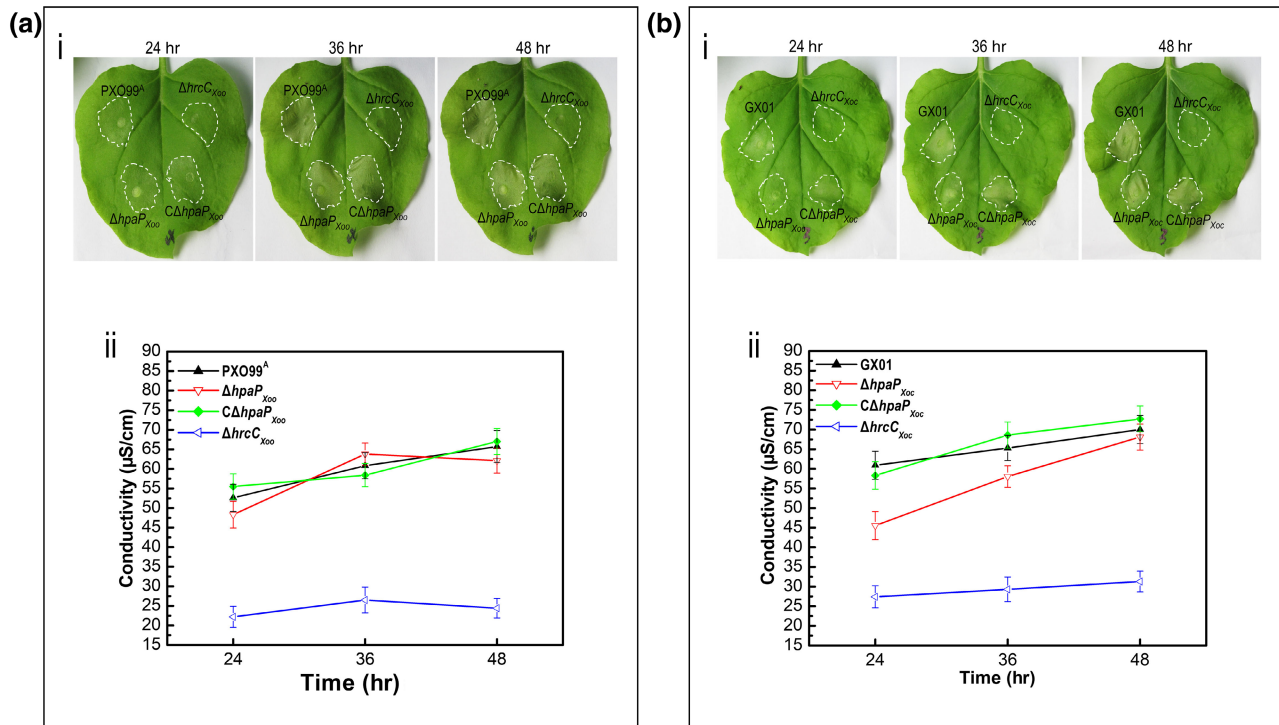
**FIGURE 4** HpaP in *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc) has different effect on the bacterial ability of hypersensitive response (HR) induction. Bacterial cells were cultured in nutrient broth and resuspended in sodium phosphate buffer to a concentration of $OD_{600}$ of 0.5. The bacterial resuspensions were infiltrated into the mesophyll tissue of *Nicotiana benthamiana* leaves. The *hrcC* deletion mutant strains $\Delta hrcC_{Xoo}$ (derivative of Xoo) and $\Delta hrcC_{Xoc}$ (derivative of Xoc) were used as negative controls. (a) Xoo strains; (b)Xoc strains. (i) HR symptoms observed at 24, 36 and 48 h postinoculation (hpi). Three replicates were done in each experiment and the experiment was repeated three times. The results presented are from a representative experiment and similar results were obtained in all other independent experiments. (ii) Electrolyte leakage from *N. benthamiana* leaves inoculated with Xoo or Xoc strains. For each sample, four $0.4\,cm^2$ leaf disks were collected from the bacteria-inoculated area and incubated in 5 ml of ultrapure water. Conductivity was measured at 24, 36 and 48 hpi. Three samples were taken for each measurement in each experiment. The results presented are the mean $\pm SD$ of three replicates from a representative experiment and similar results were obtained in two other independent experiments.

However, no plant cell wall-degrading enzyme gene was found to be differentially expressed in the Xoc transcriptome.

Bacterial survival depends on the ability to respond and adapt to changing environmental conditions. Many bacteria use chemotaxis to migrate towards environments that are better for growth. In this process, chemoreceptors detect changes in attractant (or repellant) levels and signals are transduced through a two-component signalling pathway to the flagellar motor, which then drives the rotation of the flagellum (Porter et al., 2011). The transcriptional profiles revealed that a total of 22 genes involved in the flagellar structure and 17 genes involved in chemotaxis were differentially expressed in the mutant strain $\Delta hpaP_{Xoo}$ compared with the wild-type PXO99[A] (Table S5); all of these genes were up-regulated. Similarly, in the Xoc transcriptome, three flagellar structure genes and eight chemotaxis genes were up-regulated in the mutant strain $\Delta hpaP_{Xoc}$ (Table S5). Although swimming motility decreased in the *hpaP* deletion mutants, the expression levels of flagellar-related genes were up-regulated. It is possible that these contradictory phenomena are due to the different test conditions. A second possibility, but not mutually exclusive, is that HpaP could control the flagellar rotation with unknown mechanisms.

The type IV pili are strong, long and flexible filaments that extend outward from the surface of gram-negative bacteria. These structures contribute to bacterial virulence, swarming/twitching motility and biofilm formation (Köhler et al., 2000; Mhedbi-Hajri et al., 2011). The RNA-Seq results revealed that 24 genes involved in pilus biogenesis/fimbrial assembly were up-regulated in the mutant $\Delta hpaP_{Xoo}$ compared with the wild type (Table S5). Similarly, 10 genes involved in pilus biogenesis/fimbrial assembly were up-regulated in the mutant $\Delta hpaP_{Xoc}$ (Table S5).

In addition, four genes involved in "adhesion and biofilm" were up-regulated in the Xoo mutant, while no genes in Xoc mutant were differentially expressed (Table S5).

TonB-dependent receptors (TBDRs) of gram-negative bacteria are located in the outer membrane and are mainly known to transport iron-siderophore complexes, vitamin $B_{12}$, nickel complexes, and carbohydrates into the periplasm (Noinaj et al., 2010). In *Xanthomonas* spp., TonB-dependent receptors are related to the nutrition uptake for bacterial growth under in planta infection conditions (Blanvillain et al., 2007). Here, 14 TonB-dependent receptors were differentially expressed in the Xoo *hpaP* mutant (Table S5). All these DEGs were up-regulated when the bacterial cells were cultured in XVM2 medium. In
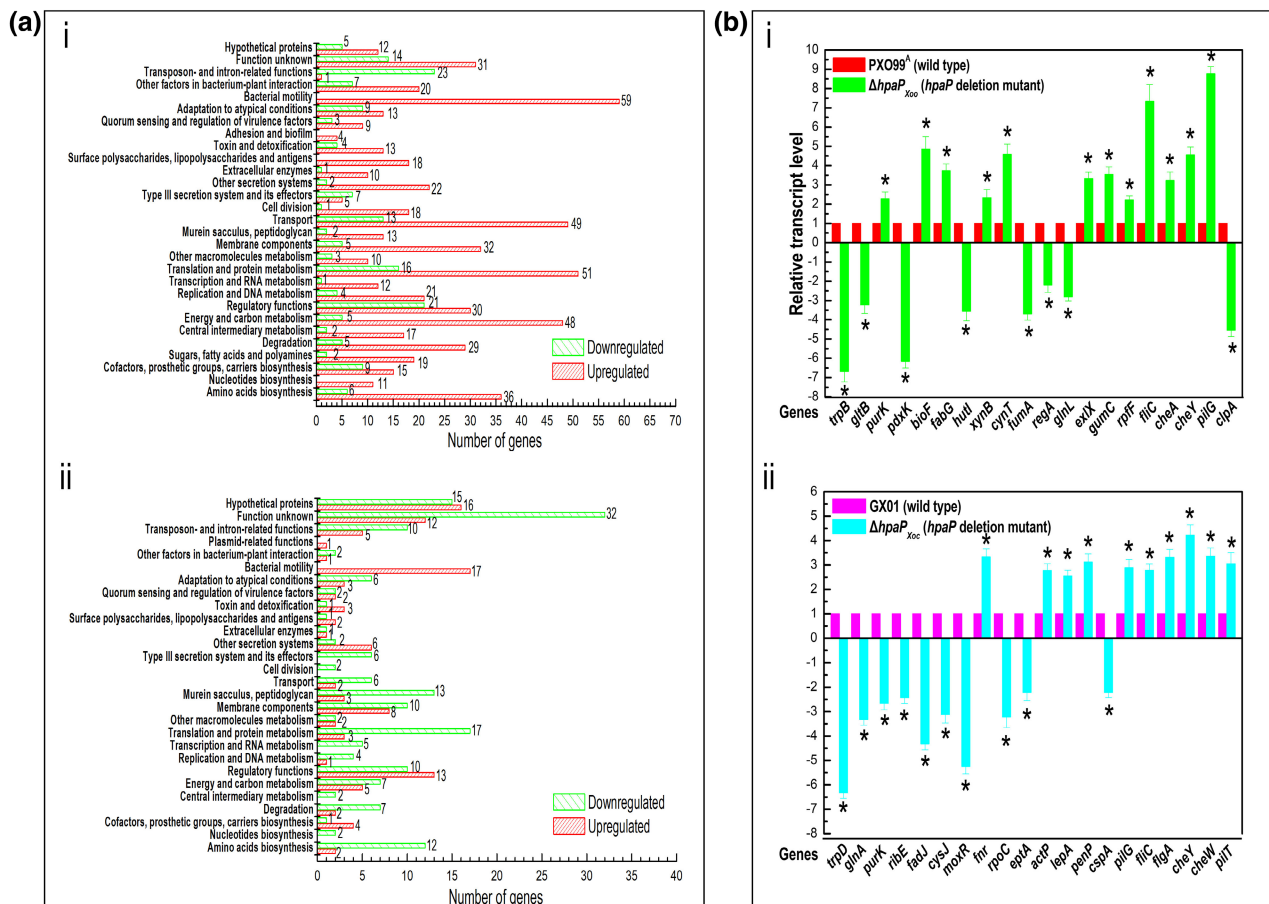
**FIGURE 5** HpaP acts as a negative regulatory factor that affects a number of genes involved in pathogenicity and virulence in *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc) cultured in a minimal medium. (a) Functional categories of differentially expressed genes (DEGs) (|log$_2$(fold change)| ≥ 1) in the *hpaP* deletion mutant of Xoo (i) and Xoc (ii). The transcriptomes of Xoo and Xoc strains cultured in XVM2 medium were investigated by RNA-Seq. In the 720 DEGs of Xoo, 564 and 156 were up-regulated and down-regulated in the mutant Δ*hpaP*$_{Xoo}$, respectively. Of the 271 Xoc DEGs, 108 and 163 were up-regulated and down-regulated in the mutant Δ*hpaP*$_{Xoc}$, respectively (Tables S3 and S4). These DEGs were broadly categorized according to the gene ontology (GO) annotations and KEGG pathways. Most of the genes associated with pathogenicity and virulence determinants, such as extracellular polysaccharide (EPS), extracellular enzymes, cell motility, bacterial adhesion, and biofilm, TonB-dependent receptors and the type VI secretion system, were up-regulated in the *hpaP* mutants of Xoo and Xoc (Table S5). (b) Reverse transcription-quantitative PCR assays of the expression level of several virulence-related genes regulated by HpaP in Xoo or Xoc strains. RNA was isolated from the cultures of Xoo or Xoc strains grown in XVM2 medium to a concentration of OD$_{600}$ of 0.6. Relative gene expression with respect to the corresponding transcript levels in the wild-type strain (PXO99$^A$ for Xoo and GX01 for Xoc) was calculated. Values given are the means ± SD of triplicate measurements from a representative experiment. Genes were considered to be differentially expressed if |log$_2$ (fold change)| ≥ 1 compared to the wild type (indicated by asterisks). Similar results were obtained in two other independent experiments.

the Xoc mutant, three TonB-dependent receptors were differentially expressed, two up-regulated and one down-regulated (Table S5).

The type VI secretion system (T6SS) is defined as a cell envelope-spanning machine that bacteria use to translocate toxic effector proteins into eukaryotic and prokaryotic cells (Costa et al., 2015). Recent works have demonstrated that the T6SS is involved in virulence or bacterial competition in several *Xanthomonas* pathogens (Choi et al., 2020; Liyanapathiranage et al., 2022; Montenegro Benavides et al., 2021; Zhu et al., 2020). Here, the transcription profile showed that the HpaP in Xoo and Xoc affects the T6SS. Mutation in the HpaP in Xoo and Xoc enhanced the expression of one gene and five genes involved in the T6SS of Xoc and Xoo (Table S5), respectively, indicating that HpaP controls the T6SS in Xoo and Xoc. Differences

in the expression of genes encoding the T3SS and related effectors are detailed below.

## 2.5 | HpaP plays a role in the expression of numerous virulence genes of Xoo and Xoc in planta

The above transcriptomic data revealed that HpaP in Xoo and Xoc repressed the expression of a series of pathogenicity factors when cultured in XVM2 medium, which is believed to mimic the plant environment. To better understand the role of HpaP during the pathogenesis of Xoo and Xoc, we analysed the global transcriptome profiles of the Xoo and Xoc wild-type and *hpaP* deletion mutant
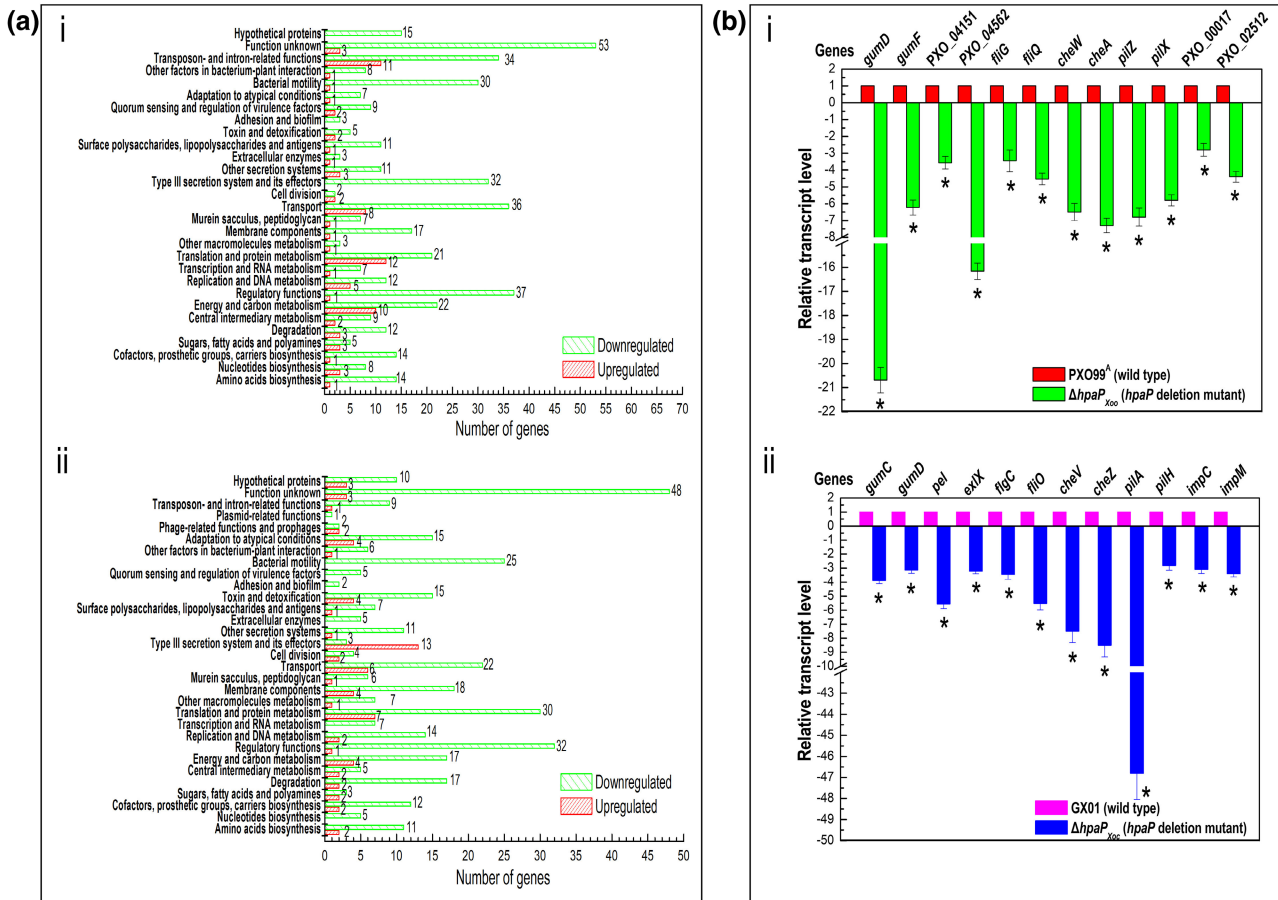
**FIGURE 6** HpaP mainly acts as a positive regulatory factor in the transcriptomes of *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc) grown in host plants. (a) Functional categories of differentially expressed genes (DEGs) (|log$_2$(fold change)| ≥ 1) in the *hpaP* deletion mutant of Xoo (i) and Xoc (ii). The transcriptomes of Xoo and Xoc strains infiltrated into the host plant rice for 24 h were investigated by RNA-seq. In total, 527 Xoo DEGs (81 up-regulated and 446 down-regulated) and 413 Xoo DEGs (67 up-regulated and 346 down-regulated) were identified (Tables S6 and S7). Most of the genes associated with virulence determinants, such as extracellular polysaccharide (EPS), extracellular enzymes, cell motility, bacterial adhesion and biofilm, TonB-dependent receptors and the type VI secretion system, were down-regulated in the *hpaP* mutants of Xoo and Xoc (Table S8). (b) Reverse transcription-quantitative PCR assays of the expression level of several virulence-related genes regulated by HpaP in Xoo strains (i) or Xoc strains (ii). RNA was isolated from plant samples inoculated Xoo strains or Xoc strains. Relative gene expression with respect to the corresponding transcript levels in the wild-type strain (PXO99$^A$ for Xoo and GX01 for Xoc) was calculated. Values given are the means ± SD of triplicate measurements from a representative experiment. Genes were considered to be differentially expressed if |log$_2$ (fold change)| ≥ 1 compared to the wild type (indicated by asterisks). Similar results were obtained in two other independent experiments.

strains during plant infection. To do this, Xoo and Xoc strains were inoculated into the rice plant leaves by infiltration and plant samples were taken at 24 h after inoculation for RNA extraction. RNA-Seq and data analysis were carried out according to the method previously described (Liao et al., 2019).

Comparison of Xoo gene expression profiles in the wild-type strain PXO99$^A$ and the *hpaP* mutant strain Δ*hpaP*$_{Xoo}$ under in planta infection conditions revealed a total of 527 DEGs, of which 81 and 446 were up-regulated and down-regulated, respectively (Figure 6ai Table S6), indicating that HpaP in Xoo mainly acts as an activating factor in planta. Similarly, 413 genes were differentially expressed in the Xoc *hpaP* mutant Δ*hpaP*$_{Xoc}$ compared with the wild-type strain GX01. Among these DEGs, 67 and 346 were up-regulated and down-regulated, respectively (Figure 6aii and Table S7). To verify the

transcriptomic data, several pathogenicity-associated genes were selected randomly to estimate the relative expression levels using RT-qPCR. The expression of these selected genes was consistent with the data from the transcriptome analyses (Figure 6b, Tables S6 and S7).

Three *gum* genes (*gumC*, *gumD*, *gumF*) were down-regulated in the Xoo *hpaP* mutant. Interestingly, the *PXO_01224* gene (*udgH*), which codes for a UDP-glucose dehydrogenase catalysing the conversion of UDP-glucose to UDP-glucuronic acid, was previously shown to be required for the biosynthesis of EPS in *Xanthomonas* spp. (Lin et al., 1995). The expression level of *PXO_01224* was much lower (fold change = 1056.9) in the *hpaP* mutant strain Δ*hpaP*$_{Xoo}$ compared with that in the Xoo wild-type strain PXO99$^A$. Similarity, two *gum* genes (*gumD*, *gumF*) were repressed in the Xoc *hpaP* mutant

(Table S8). Based on these data we inferred that the HpaP in Xoo and Xoc positively regulates the EPS production during pathogen colonization in plants.

As described above, several plant cell wall-degrading enzymes were differentially expressed in the Xoo *hpaP* mutant, but not the Xoc mutant when cultured in medium (Table S5). Nevertheless, three and five plant cell wall-degrading enzyme genes in the Xoo *hpaP* mutant and the Xoc *hpaP* mutant were found down-regulated within the host plant, respectively (Table S8). These data suggested that HpaP positively controls the synthesis of several plant cell wall-degrading enzymes in both Xoo and Xoc during growth in plants.

In the Xoo *hpaP* mutant, 18 flagellar genes, 13 chemotaxis genes and seven type IV pili genes were found to be down-regulated (Table S8), while in the Xoc mutant $\Delta hpaP_{Xoc}$, nine flagellar, 11 chemotaxis, and five type IV pili genes were down-regulated. Overall, these expression profiles indicate that HpaP positively controls Xoo and Xoc motility and chemotaxis within the host plant.

In addition, there were three genes in Xoo and two genes in Xoc involved in adhesion and biofilm formation that were down-regulated (Table S8), implying that HpaP positively regulates bacterial adhesion and biofilm formation in Xoo and Xoc during infection.

Compared to the wild type, although a total of 14 TonB-dependent receptors were differentially expressed and up-regulated in the Xoo *hpaP* mutant cultured in medium, seven TonB-dependent receptors were found differentially expressed in the Xoo *hpaP* mutant in planta. However, all these DEGs were down-regulated (Table S8). Similarly, three TonB-dependent receptors in the Xoc mutant were down-regulated. These data imply that HpaP positively regulates the nutrition uptake for Xoo and Xoc growth under in planta infection conditions.

In the Xoo mutant, only one gene encoding a type VI protein was down-regulated, while in the Xoc mutant, seven genes were differentially expressed and all of these genes were down-regulated compared with the wild-type strain GX01 (Table S8). The expression levels of these DEGs changed from a range of −2.47- to −230.42-fold, implying that HpaP in Xoc might control the ability of bacteria to compete with other rivals during infection.

## 2.6 | HpaP divergently regulates the expression of the T3SS genes in Xoo and Xoc

The above transcriptomic data revealed that a subset of pathogenicity-related genes such as those involved in cell motility, extracellular enzymes, and the T6SS were similarly regulated by HpaP orthologues in Xoo and Xoc (Figures 5a and 6a; Tables S5 and S8). Notably, analysis of the DEGs in the bacteria cultured in XVM2 medium (Tables S3 and S4) revealed that the expression of four T3SS genes, *hrcN*, *hrcJ*, *hrpB2*, *hrpB1*, and one effector gene, *tal7b*, was increased in the Xoo *hpaP* mutant $\Delta hpaP_{Xoo}$ compared to the wild type. However, two T3SS genes, *hpa2* and *hpa1*, and five effector genes, *tal3b*, *tal6a*, *xopL*, *xopAD* and *tal8b*, were down-regulated in

the same mutant ($\Delta hpaP_{Xoo}$). Conversely, the expression of six T3SS genes, *hrpR*, *hrpV*, *hrpU*, *hrpL*, *hrpN* and *hrpB7*, was decreased in the Xoc *hpaP* mutant ($\Delta hpaP_{Xoc}$) compared to the wild type. These data imply that the mechanisms by which HpaP regulates the T3SS in Xoo and Xoc are different.

To confirm the different impacts of HpaP on the expression of the T3SS between Xoo and Xoc, we used RT-qPCR to compare the transcript levels of a set of T3SS-related genes (not limited to the DEGs) between the wild-type strain (PXO99$^A$ or GX01) and the *hpaP* deletion mutant ($\Delta hpaP_{Xoo}$ mutant or $\Delta hpaP_{Xoc}$ mutant) cultured in XVM2 medium. The results demonstrated that, except for the effector gene *xopL*, the expression of most of the selected genes was up-regulated in Xoo but down-regulated in Xoc (Figure 7a). This expression pattern confirmed the results from the transcriptome analyses, indicating that in the bacteria cultured in XVM2 medium, HpaP negatively controls the T3SS in Xoo but positively controls the T3SS in Xoc.

Intriguingly, the bacterial transcriptome profiles in planta showed that 15 T3SS genes (*hrpG*, *hrpT*, *hrpB7*, *hrpL*, *hrpU*, *hrpV*, *hrpQ*, *hrpR*, *hrpD*, *hrpE*, *hrpF*, *hrpJ*, *hpa2*, *hpa1* and *hpaA*), and 17 effector genes (*tal2a*, *tal4*, *tal3b*, *tal3a*, *tal6a*, *tal5a*, *tal5b*, *tal7b*, *xopL*, *xopK*, *xopP1*, *xopC*, *xopAE*, *xopR*, *xopAD*, *tal1* and *tal7a*) were differentially expressed (Table S6); all these genes were down-regulated in the mutant strain $\Delta hpaP_{Xoo}$ compared with the wild-type strain PXO99$^A$. In the mutant $\Delta hpaP_{Xoc}$, 15 genes belonging to the category "T3SS and its effectors" were differentially expressed compared with the wild type. Except for two effector genes (*tal3* and *tal8b*), which were down-regulated, seven T3SS genes (*hrpF*, *hrpU*, *hrpB1*, *hrpL*, *hrpN*, *hrpC* and *hpaA*) and six effector genes (*xopR*, *tal2a*, *xopA*, *tal8a*, *tal9a* and *avrBs2*) were up-regulated (Table S7).

To verify the expression pattern of the T3SS in plants after infection, the expression level of several T3SS-related genes was estimated using RT-qPCR, as previously described (Liao et al., 2019). The expression trends of the selected genes in each sample were largely consistent (Figure 7b), although the variations in their expression levels were not exactly the same between the results from RT-qPCR and RNA-Seq. This might due to the differences in the test conditions and the sensitivity and/or specificity between the two approaches. Overall, the combined data revealed a divergent regulation in the expression of T3SS genes by HpaP in Xoo and Xoc. These effects were seen in minimal medium and in planta, although the scope of regulation was much broader in planta.

## 3 | DISCUSSION

Previous work revealed that HpaP in Xcc influences cell motilities (swimming and swarming), but has no impact on the production of EPS and extracellular enzymes (Cui et al., 2018). This study revealed that the HpaP orthologues in Xoo and Xoc influence EPS production and cell motilities, but not the activities of extracellular enzymes, indicating that the physiological role of HpaP orthologues
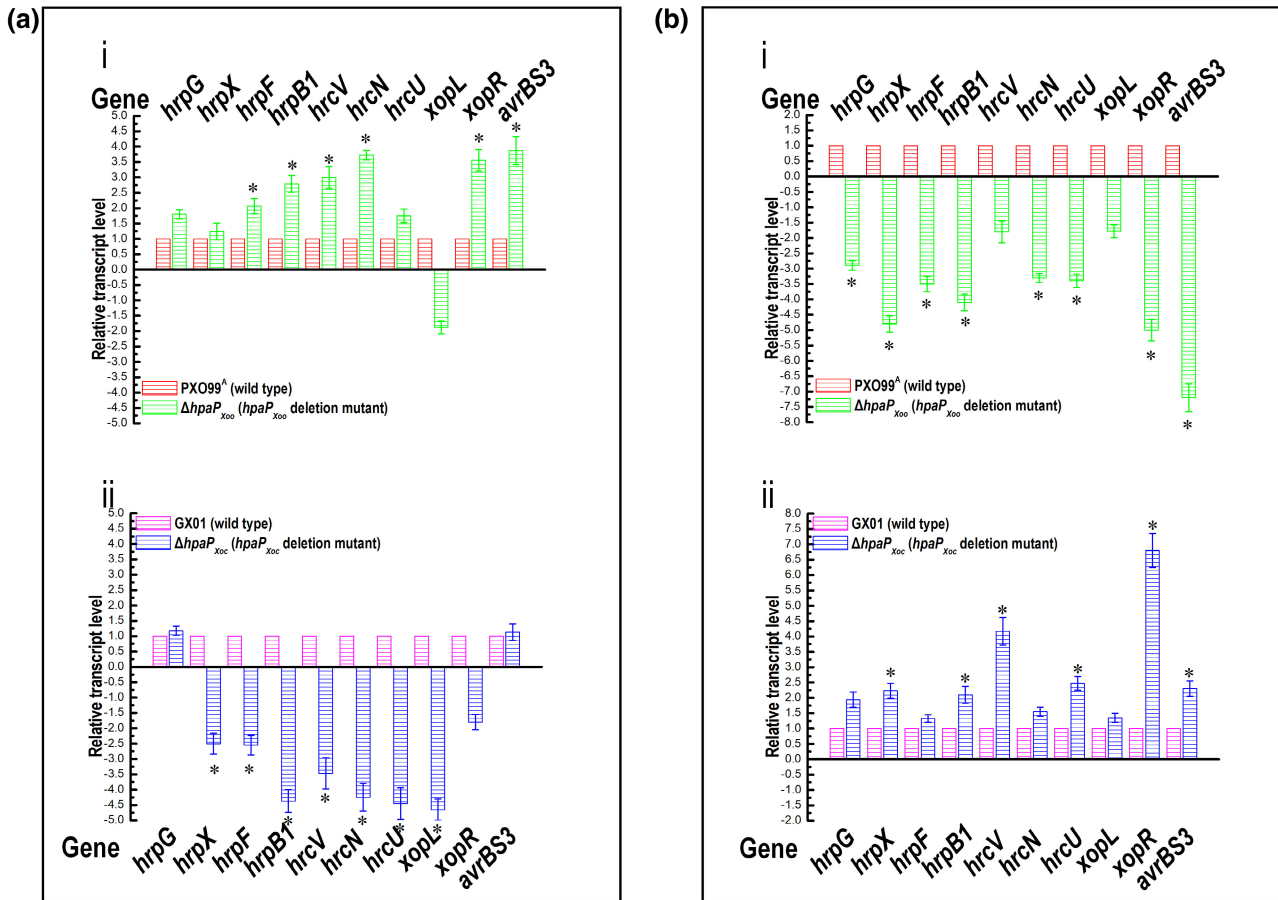
**FIGURE 7** HpaP divergently regulates the expression of type III secretion system (T3SS) in *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc). Several *hrp/hrc* genes encoding the components of T3SS apparatus (*hrpF*, *hrpB1*, *hrcV*, *hrcN* and *hrcU*) and effector genes (*xopL*, *xopR* and *avrBs3*), as well as the T3SS key regulator genes (*hrpG* and *hrpX*) were selected to estimate the expression level in Xoo and Xoc by reverse transcription-quantitative PCR. (a) The expression levels of the tested genes in Xoo wild-type strain PXO99$^A$ and the *hpaP* deletion mutant Δ*hpaP*$_{Xoo}$ (i), and Xoc wild-type strain GX01 and the *hpaP* deletion mutant Δ*hpaP*$_{Xoc}$ (ii) cultured in XVM2 medium. Xoo or Xoc strains grown in XVM2 to an OD$_{600}$ of 0.6, and total RNA was isolated. Relative gene expression with respect to the corresponding wild-type levels were calculated. Values given are the means ± *SD* of triplicate measurements from a representative experiment. Genes were considered to be differentially expressed if |log$_2$ (fold change)| ≥ 1 compared to the wild type (asterisks indicate). Similar results were obtained in two other independent experiments. (b) The expression levels of the tested genes in Xoo wild-type strain PXO99$^A$ and the *hpaP* deletion mutant Δ*hpaP*$_{Xoo}$ (i), and Xoc wild-type strain GX01 and the *hpaP* deletion mutant Δ*hpaP*$_{Xoc}$ (ii) grown in host plants. Xoo and Xoc strains were inoculated into 2-week-old rice seedlings by infiltrating with a syringe. The infiltrated leaf part was collected 24 h after inoculation and total RNA was extracted. Values given are the means ± *SD* of triplicate measurements from a representative experiment; genes were considered to be differentially expressed if |log$_2$ (fold change)| ≥ 1 compared to the wild type (asterisks indicate). Similar results were obtained in two other independent experiments.

in *Xanthomonas* is diverse from species to species. Our data also revealed that even within the same *X. oryzae* species, HpaP orthologues in different pathovars have different impacts on the T3SS. Moreover, HpaP differentially regulated the expression of the T3SS in either Xoo or Xoc under the conditions of in vitro *hrp*-inducing medium and in planta. To our knowledge, this is the first report that shows an upstream conserved regulatory element has divergent effects on the expression of the T3SS in two closely related plant pathogens.

Phylogenetically, Xoo and Xoc are very closely related pathovars (Ochiai & Kaku, 1999; Vauterin et al., 1995), but have significant differences at their genome sequences. The two pathovars infect their host rice in distinct ways and elicit different symptoms. Both

Xoo and Xoc produce a range of virulence factors, including EPS, extracellular enzymes and T3Es, that are essential for virulence. However, little is known about the determinants of tissue-specificity and symptom development between Xoo and Xoc. Comparative genome analysis suggested that the difference in pathogenic lifestyle between Xoo and Xoc might be due to subtle changes in nucleotide sequences and/or expression of certain virulence-related genes such as the *gum* (responsible for EPS production), *hrp* (encoding the T3SS), *xps/xcs* (encoding the T2SS), or *rpf* (DSF-dependent cell–cell signal system) genes (Lu et al., 2008). Here, our data have demonstrated that although HpaP is conserved in *Xanthomonas* spp. and required for full virulence in both Xoo and Xoc, this protein has different effects on the expression of certain pathogenicity and

virulence-related genes, in particular the T3SS genes. Moreover, to further ascertain that these observations are really caused by the HpaP homologues, we performed a cross-complementary test and found that the virulence and HR induction of the Xoc HpaP-deficient mutant Δ*hpaP*~Xoc~ could be restored to wild-type level by the innate Xoo *hpaP* gene (Figure S4). These findings suggest differences in the regulatory mechanisms of pathogenicity gene expression between these two pathovars.

The reduced virulence of the Xoo *hpaP* mutant is consistent with a reduced expression of both *hrp* genes and other virulence factors such as the production of EPS and extracellular enzymes and cell motility. In contrast, the reduced virulence of the Xoc *hpaP* mutant is associated with enhanced expression level of *hrp* genes in planta. It has been reported that an increase in transcription levels of the T3SS and T3E coding genes results in enhanced virulence in Xoo (Zhang et al., 2013). We speculate that the reduced virulence of the Xoc *hpaP* mutant strain is due to the reduced expression of other virulence-related genes such as those involved in the production of EPS and extracellular enzymes, flagellar chemotaxis, and membrane proteins. However, we cannot exclude that overexpression of particular *hrp* genes or T3Es may negatively impact virulence. Along similar lines, the different effects of *hpaP* mutation on the expression of T3SS genes in Xoo and Xoc seems at variance with the outcomes of the HR tests in *N. benthamiana*, where the *hpaP* mutant of Xoo had an unaltered response whereas the *hpaP* mutant of Xoc had reduced capacity to induce an HR. Although the T3SS is certainly required for triggering an HR in these nonhost interactions, it is not known which other factors or effectors are involved.

In the present study, the expression level of *hrp* genes in the Xoo *hpaP* mutant is enhanced and reduced under the in vitro *hrp*-inducing medium and in planta, respectively. In contrast, in the Xoc *hpaP* mutant, expression of *hrp* genes is decreased in the *hrp*-inducing medium but increased in planta. This data revealed that the expression patterns of the *hrp* genes in either the Xoo *hpaP* mutant or the Xoc *hpaP* mutant were different when they were grown in XVM2 medium and in planta, implying that the mechanism of *hrp* genes induction under in vitro *hrp*-inducing conditions is different from that in planta. XOM2, a highly efficient *hrp*-inducing medium for Xoo (Tsuge et al., 2002), was also used to test the transcription level of *hrp* genes in Xoo or Xoc strains, and similar findings were obtained in Xoc strains. The expression pattern of the T3SS-related genes in the Δ*hpaP*~Xoc~ mutant grown in XOM2 was different from that grown in planta (Figure S5). Previously, to get insights into bacterial infection and adaption mechanisms, comparative transcriptome analysis was performed on a number of *Xanthomonas* mutant strains with differentially reduced virulence, cultured in artificial media. The minimal medium XVM2, which is suspected to mimic the environment of the plant intercellular spaces, was used as an alternative system for gene expression studies under controlled conditions. In this respect, transcription profiling in vitro was used to explore the role of HpaP in the pathogenicity and virulence of Xoo and Xoc. However, our study revealed a number of differences between the transcriptional responses of the Xoo or

Xoc *hpaP* mutant to artificial plant-mimicking media and the transcriptional responses within the plant host. Similarly, our previous work showed that the GntR-family transcriptional regulator HpaR1 in Xcc negatively regulates the T3SS coding genes in artificial plant-mimicking media while positively regulating their expression in planta (An et al., 2011). Therefore, to compare the interactions with host plants between different microbial pathogen strains, transcription profiling under in planta conditions, rather than under in vitro conditions, will be preferable to provide a better in-depth understanding of the plant–microbe interactions.

# 4 | EXPERIMENTAL PROCEDURES

## 4.1 | Bacterial strains, plasmids, culture media and growth conditions

The strains and plasmids used in this study are listed in Table S2. Xoc strains were grown at 28°C in nutrient broth (NB; per litre: 1 g yeast extract, 3 g beef extract, 5 g polypeptone, 10 g sucrose), nutrient agar (NA; NB with 15 g agar per litre) or XVM2 medium (Astua-Monge et al., 2005). *Escherichia coli* strains were grown in LB (Luria-Bertani) medium (per litre: 5 g yeast extract, 10 g NaCl, 10 g tryptone, 15 g agar) or on LB agar plates (LB with 15 g agar per litre) at 37°C. Antibiotics were used at the following concentrations as required: kanamycin (Kan) at 25 μg/ml, rifampicin (Rif) at 50 μg/ml, ampicillin (Amp) at 100 μg/ml, spectinomycin (Spc) at 50 μg/ml, streptomycin (Sm) at 50 μg/ml, and tetracycline (Tet) at 5 μg/ml for *Xanthomonas* strains or 15 μg/ml for *E. coli* strains.

## 4.2 | Nucleic acid manipulations

The nucleic acid manipulations followed the procedures described by Sambrook et al. (1989). Conjugation between the Xoo/Xoc and *E. coli* strains was performed as described by Turner et al. (1985). The restriction endonucleases, T4 DNA ligase, and *Pfu* DNA polymerase were provided by Promega. Total RNA was extracted from cultures of Xoo or Xoc strains with a total RNA extraction kit (Invitrogen) and cDNA was generated using a cDNA synthesis kit (Invitrogen). For qPCR, the obtained cDNA was diluted and used as a template with selected primers for target genes (Table S1).

RT-qPCR was carried out as previously described (Li et al., 2014) with minor modification. Briefly, PCR was performed in qPCR thermal cycler (Analytik jena qTOWER2.0). Reactions included ChamQ universal SYBR qPCR master mix (Vazyme), corresponding primers (Table S1) and cDNA templates. Reactions with no template were used as negative controls. The melting curve analysis was performed at temperature ranges from 60 to 95°C by raising 0.5°C at each second. The relative mRNA level was calculated with respect to the level of the corresponding transcript in the wild-type strain (equalling 1). The expression level of the 16S rRNA gene was used as an internal standard. The RT-qPCR tests were performed in triplicate.

## 4.3 | Deletion mutant construction and complementation

The construction of an in-frame deletion mutant of *hpaP* gene in Xoo or Xoc was carried out using the previously described method (Cui et al., 2018). Fragments of 851-bp upstream and 805-bp downstream flanking *hpaP* in Xoo were PCR-amplified with the corresponding primer sets (Table S1), respectively. The two fragments were cloned together into the vector pK18*mobsacB* (Schäfer et al., 1994), and the resulting plasmid (pKΔ*hpaP*$_{Xoo}$) was introduced into the Xoo strain PXO99$^A$. The candidate mutants were screened on selective agar plates. For Xoc *hpaP* deletion mutant construction, 833-bp upstream and 698-bp downstream fragments amplified by PCR with the corresponding primer sets (Table S1) were cloned into pK18*mobsacB*. The obtained plasmid (pKΔ*hpaP*$_{Xoc}$) was introduced into the Xoc strain GX01.

For mutant complementation, an 870-bp DNA fragment of the *hapP* coding sequence was PCR-amplified from the Xoo wild-type strain PXO99$^A$ or the Xoc wild-type strain GX01 with the selected primer set (Table S1). The obtained DNA fragments were cloned into the plasmid pLAFR3 (Staskawicz et al., 1987) to generate the recombinant plasmids pLC*hpaP*$_{Xoo}$ or pLC*hpaP*$_{Xoc}$ (Table S2). The recombinant plasmids were transferred into the *hpaP* mutants by triparental conjugation, generating the complemented strains CΔ*hpaP*$_{Xoo}$ and CΔ*hpaP*$_{Xoc}$, or cross-complemented strains Δ*hpaP*/pLC*hpaP*$_{Xoo}$, Δ*hpaP*/pLC*hpaP*$_{Xoc}$ and Δ*hpaP*$_{Xoc}$/pLC*hpaP*$_{Xoo}$ (Table S2).

## 4.4 | Plant assays

The virulence of Xcc to its host cabbage plant (*Brassica oleracea* 'Jingfeng No. 1') was tested by the leaf-clipping method as previously described (Dow et al., 2003). Concentrations of Xcc strains were adjusted to $10^6$ cfu/ml. Leaves were cut with scissors dipped in the bacterial suspensions. The lesion and symptoms were measured 10 days postinoculation. HR assays were performed as previously described (Li et al., 2014). Bacterial suspensions ($10^7$ cfu/ml) were infiltrated into the pepper leaves (*Capsicum annuum* 'ECW-10R'). The inoculated plants were kept in the greenhouse to observe the HR symptoms and measure conductivity at several time points. For conductivity measurements, samples (leaf discs of 0.4 cm$^2$) were collected and soaked in ultrapure water and its conductivity was measured.

Xoo and Xoc virulence assays were performed as previously described (Li et al., 2017; Liao et al., 2019) with minor modifications. Xoo or Xoc wild-type strains and their derivatives were grown at 28°C in NB medium with appropriate antibiotics, and the cells were collected by centrifugation and resuspended in sterile ultrapure water to a concentration of OD$_{600}$ = 0.3 (approximately $10^8$ cfu/ ml) prior to inoculation. The bacterial suspensions were inoculated onto 6-week-old leaves of susceptible rice plant *O. sativa* subsp. *japonica* 'Nipponbare' by the leaf-clipping method (for Xoo) or by infiltrating with needleless syringe (for Xoc) under relevant conditions. Symptoms were recorded by photography and the disease lesion lengths were measured 14 days postinoculation.

The HR test of Xoo and Xoc was conducted as previously described (Li et al., 2017). Briefly, Xoo or Xoc cells from cultures were washed with 10 mM sodium phosphate buffer (5.8 mM Na$_2$HPO$_4$, 4.2 mM NaH$_2$PO$_4$, pH 7.0) and resuspended in the same buffer to an OD$_{600}$ of 0.5 ($5 \times 10^8$ cfu/ml). Bacterial suspensions were infiltrated into the leaves of greenhouse-grown nonhost plant *N. benthamiana*, and the symptoms were observed at 24, 36 and 48 h after infiltration. Simultaneously, the electrolyte leakage test was performed by measuring the conductivity of the infiltrated spot of *N. benthamiana* leaves.

For transcriptome analysis, 2-week-old rice seedlings were inoculated with the bacterial suspension at a concentration of OD$_{600}$ of 0.5 by infiltration. For each plant, the second and third leaves were picked. The infiltrated leaf part was collected 24 h after inoculation, snap frozen in liquid nitrogen, and stored at −80°C immediately.

## 4.5 | Exopolysaccharide and extracellular enzyme assays

EPS and extracellular enzyme assays were performed as previously described (Su et al., 2016; Tang et al., 1991). Briefly, for estimation of EPS production, Xoo or Xoc strains were spotted onto NA with 2% sucrose and grown for 5 days. For quantification of EPS production, Xoo or Xoc strains were cultured in 100 ml NB containing 4% glucose at 28°C with shaking at 200 rpm for 3 days. For estimation of the activity of the extracellular enzymes endoglucanase (cellulase) and protease, strains on NA plates containing carboxymethylcellulose (for endoglucanase) or skim milk (for protease) were incubated at 28°C for 24 h (endoglucanase) or 48 h (protease). For quantification of enzymes, bacterial cells were cultured in NB medium for 24 h and adjusted to the same concentration.

## 4.6 | Motility assay

Cell motility was detected as previously described (Li et al., 2020). Two microlitres of bacterial suspension ($10^9$ cfu/ml) was stabbed into 0.28% agar plates composed of 0.03% Bacto peptone and 0.03% yeast extract (for swimming), or spotted on NA plates containing 2% glucose and 0.6% agar (for swarming). The diameters of the area occupied by the bacterial cells were measured after 4 days of incubation at 28°C, and these values were used to indicate the motility of the Xoo or Xoc strains.

## 4.7 | Transcriptome analysis

Transcriptome analysis of Xoo or Xoc cultured in medium was performed as previously described (Cui et al., 2018). Briefly, Xoo or Xoc

strains were cultured in XVM2 medium to an OD$_{600}$ of 0.6. RNA was prepared and the contaminating genomic DNA was removed. After the quantity determination and quality assessment, total RNA was sent to Novogene for library construction and strand-specific RNA sequencing. Clean reads were mapped to the genome of Xoo strain PXO 99$^A$ (Assembly ID = 357931) or Xoc strain GX01 (Assembly ID = 4726371) and the RPKM (reads per kilobase per million mapped reads) method was used to calculate the gene expression levels. False discovery rate (FDR) ≤0.05 and |log$_2$FC| (log$_2$ of the fold changes) ≥1 were considered for DEGs.

Transcriptome analysis of the Xoo or Xoc during infection of rice leaves was performed using the dual RNA-Seq according to the method previously described (Liao et al., 2019). Briefly, Xoo or Xoc strains were inoculated into 2-week-old rice seedlings by infiltrating with a syringe. The infiltrated leaf part was collected 24 h after inoculation. Total RNA was extracted from infected plant samples using RNAiso Plus Kit (TaKaRa). After RNA integrity assessment and rRNA depletion, a cDNA library was prepared and quality assessed. The library was sequenced on the Illumina Hiseq 2000 platform, and reads were generated. Clean reads were mapped to the genome of *O. sativa japonica* group (Assembly ID = 22512) or the genome of Xoo strain PXO99$^A$ or Xoc strain GX01.

## 4.8 | Phylogenetic analysis

The amino acid sequences of HpaP homologues were analysed by BLAST searches against the NCBI database. The selected amino acid sequences were obtained from the NCBI database and aligned using ClustalW, and trees were generated using PHYLIP and displayed using MEGA 7.

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

### ORCID
*Rui-Fang Li* https://orcid.org/0000-0001-6974-7266
*Guang-Tao Lu* https://orcid.org/0000-0001-5082-9755

### REFERENCES
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.

Andrade, M.O., Farah, C.S. & Wang, N. (2014) The post-transcriptional regulator *rsmA/csrA* activates T3SS by stabilizing the 5′ UTR of *hrpG*, the master regulator of *hrp/hrc* genes, in *Xanthomonas*. *PLoS Pathogens*, 10, e1003945.

Astua-Monge, G., Freitas-Astua, J., Bacocina, G., Roncoletta, J., Carvalho, S.A. & Machado, M.A. (2005) Expression profiling of virulence and pathogenicity genes of *Xanthomonas axonopodis* pv. *citri*. *Journal of Bacteriology*, 187, 1201–1205.

Blanvillain, S., Meyer, D., Boulanger, A., Lautier, M., Guynet, C., Denancé, N. et al. (2007) Plant carbohydrate scavenging through tonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. *PLoS One*, 2, e224.

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

Choi, Y., Kim, N., Mannaa, M., Kim, H., Park, J., Jung, H. et al. (2020) Characterization of type VI secretion system in *Xanthomonas oryzae* pv. *oryzae* and its role in virulence to rice. *Plant Pathology Journal*, 36, 289–296.

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.

Cui, P., Li, R.F., Zhang, D.P., Tang, J.L. & Lu, G.T. (2018) HpaP, a novel regulatory protein with ATPase and phosphatase activity, contributes to full virulence in *Xanthomonas campestris* pv. *campestris*. *Environmental Microbiology*, 20, 1389–1404.

Dow, J.M., Crossman, L., Findlay, K., He, Y.Q., Feng, J.X. & Tang, J.L. (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 10995–11000.

Feng, J.X., Song, Z.Z., Duan, C.J., Zhao, S., Wu, Y.Q., Wang, C. et al. (2009) The *xrvA* gene of *Xanthomonas oryzae* pv. *oryzae*, encoding an H-NS-like protein, regulates virulence in rice. *Microbiology*, 155, 3033–3044.

Ikawa, Y., Ohnishi, S., Shoji, A., Furutani, A. & Tsuge, S. (2018) Concomitant regulation by a LacI-type transcriptional repressor XylR on genes involved in xylan and xylose metabolism and the type III secretion system in rice pathogen *Xanthomonas oryzae* pv. *oryzae*. *Molecular Plant-Microbe Interactions*, 31, 605–613.

Kametani-Ikawa, Y., Tsuge, S., Furutani, A. & Ochiai, H. (2011) An H-NS-like protein involved in the negative regulation of *hrp* genes in *Xanthomonas oryzae* pv. *oryzae*. *FEMS Microbiology Letters*, 319, 58–64.

Katzen, F., Becker, A., Zorreguieta, A., Pühler, A. & Ielpi, L. (1996) Promoter analysis of the *Xanthomonas campestris* pv. *campestris gum* operon directing biosynthesis of the xanthan polysaccharide. *Journal of Bacteriology*, 178, 4313–4318.

Katzen, F., Ferreiro, D.U., Oddo, C.G., Ielmini, M.V., Becker, A., Pühler, A. et al. (1998) *Xanthomonas campestris* pv. *campestris gum* mutants: effects on xanthan biosynthesis and plant virulence. *Journal of Bacteriology*, 180, 1607–1617.

Köhler, T., Curty, L.K., Barja, F., van Delden, C. & Pechère, J.C. (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *Journal of Bacteriology*, 182, 5990–5996.

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, L., Li, R.F., Ming, Z.H., Lu, G.T. & Tang, J.L. (2017) Identification of a novel type III secretion-associated outer membrane-bound protein from *Xanthomonas campestris* pv. *campestris*. *Scientific Reports*, 7, 42724.

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 *hrp* 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 HpaS co-opts the orphan response regulator VemR to form a branched two-component system that regulates motility. *Molecular Plant Pathology*, 21, 360–375.

Liao, Z.X., Ni, Z., Wei, X.L., Chen, L., Li, J.Y., Yu, Y.H. et al. (2019) Dual RNA-seq of *Xanthomonas oryzae* pv. *oryzicola* infecting rice reveals novel insights into bacterial-plant interaction. *PLoS One*, 14, e0215039.

Lin, C.S., Lin, N.T., Yang, B.Y., Weng, S.F. & Tseng, Y.H. (1995) Nucleotide sequence and expression of UDP-glucose dehydrogenase gene required for the synthesis of xanthan gum in *Xanthomonas campestris*. *Biochemical and Biophysical Research Communications*, 207, 223–230.

Lindgren, P.B. (1997) The role of *hrp* genes during plant-bacterial interactions. *Annual Review of Phytopathology*, 35, 129–152.

Liu, Y., Long, J., Shen, D. & Song, C. (2016) *Xanthomonas oryzae* pv. *oryzae* requires H-NS-family protein XrvC to regulate virulence during rice infection. *FEMS Microbiology Letters*, 363, fnw067.

Liyanapathiranage, P., Jones, J.B. & Potnis, N. (2022) Mutation of a single core gene, *tssM*, of type VI secretion system of *Xanthomonas perforans* influences virulence, epiphytic survival and transmission during pathogenesis on tomato. *Phytopathology*, 112, 752–764.

Lu, H., Patil, P., Van Sluys, M.A., White, F.F., Ryan, R.P., Dow, J.M. et al. (2008) Acquisition and evolution of plant pathogenesis-associated gene clusters and candidate determinants of tissue-specificity in *Xanthomonas*. *PLoS One*, 3, e3828.

Lu, Y., Rashidul, I.M., Hirata, H. & Tsuyumu, S. (2011) KdgR, an IclR family transcriptional regulator, inhibits virulence mainly by repression of *hrp* genes in *Xanthomonas oryzae* pv. *oryzae*. *Journal of Bacteriology*, 193, 6674–6682.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P. et al. (2012) Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*, 13, 614–629.

Mhedbi-Hajri, N., Jacques, M.A. & Koebnik, R. (2011) Adhesion mechanisms of plant-pathogenic *Xanthomonadaceae*. *Advances in Experimental Medicine and Biology*, 715, 71–89.

Montenegro Benavides, N.A., Alvarez B,A., Arrieta-Ortiz, M.L., Rodriguez-R, L.M., Botero, D., Tabima, J.F., Castiblanco, L., Trujillo, C., Restrepo, S., & Bernal, A. (2021) The type VI secretion system of *Xanthomonas phaseoli* pv. *manihotis* is involved in virulence and in vitro motility. *BMC Microbiology*, 21, 14.

Niño-Liu, D.O., Ronald, P.C. & Bogdanove, A.J. (2006) *Xanthomonas oryzae* pathovars: model pathogens of a model crop. *Molecular Plant Pathology*, 7, 303–324.

Noinaj, N., Guillier, M., Barnard, T.J. & Buchanan, S.K. (2010) TonB-dependent transporters: regulation, structure, and function. *Annual Review of Microbiology*, 64, 43–60.

Ochiai, H. & Kaku, H. (1999) Genetic relationships among *Xanthomonas* species and pathovars based on RFLP analyses of PCR-amplified 16S, 23S rDNA and rDNA internal transcribed spacer. *Annals of the Phytopathological Society of Japan*, 65, 437–446.

Ou, S.H. (1985) *Rice diseases*. Kew, Surrey: Commonwealth Agricultural Bureau.

Porter, S.L., Wadhams, G.H. & Armitage, J.P. (2011) Signal processing in complex chemotaxis pathways. *Nature Reviews Microbiology*, 9, 153–165.

Rashid, M.M., Ikawa, Y. & Tsuge, S. (2016) GamR, the LysR-Type galactose metabolism regulator, regulates *hrp* gene expression via transcriptional activation of two key *hrp* regulators, HrpG and HrpX, in *Xanthomonas oryzae* pv. *oryzae*. *Applied and Environmental Microbiology*, 82, 3947–3958.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edition. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

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

Staskawicz, B., Dahlbeck, D., Keen, N. & Napoli, C. (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *Journal of Bacteriology*, 169, 5789–5794.

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.

Tang, J.L., Liu, Y.N., Barber, C.E., Dow, J.M., Wootton, J.C. & Daniels, M.J. (1991) Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*. *Molecular & General Genetics*, 226, 409–417.

Timilsina, S., Potnis, N., Newberry, E.A., Liyanapathiranage, 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 hrp* genes in XOM2, a novel synthetic medium. *Journal of General Plant Pahtology*, 68, 363–371.

Tsuge, S., Nakayama, T., Terashima, S., Ochiai, H., Furutani, A., Oku, T. et al. (2006) Gene involved in transcriptional activation of the *hrp* regulatory gene *hrpG* in *Xanthomonas oryzae* pv. *oryzae*. *Journal of Bacteriology*, 188, 4158–4162.

Turner, P., Barber, C.E. & Daniels, M.J. (1985) Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. *Molecular and General Genetics*, 199, 338–343.

Vauterin, L., Hoste, B., Kersters, K. & Swings, J. (1995) Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology*, 45, 472–489.

Wengelnik, K. & Bonas, U. (1996) HrpXv, an AraC-type regulator, activates expression of five of the six loci in the *hrp* cluster of *Xanthomonas campestris* pv. *vesicatoria*. *Journal of Bacteriology*, 178, 3462–3469.

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.

White, F. (2016) *Xanthomonas* and the TAL effectors: nature's molecular biologist. *Methods in Molecular Biology*, 1338, 1–8.

Yoon, K.H. & Cho, J.Y. (2007) Transcriptional analysis of the *gum* gene cluster from *Xanthomonas oryzae* pathovar *oryzae*. *Biotechnology Letters*, 29, 95–103.

Zhang, F., Du, Z., Huang, L., Vera Cruz, C., Zhou, Y. & Li, Z. (2013) Comparative transcriptome profiling reveals different expression patterns in *Xanthomonas oryzae* pv. *oryzae* strains with putative virulence-relevant genes. *PLoS One*, 8, e64267.

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.

## SUPPORTING INFORMATION

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