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Synergistic Activation of the Pathogenicity-Related Proline Iminopeptidase Gene in *Xanthomonas campestris* pv. *campestris* by HrpX and a LuxR Homolog

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*Xanthomonas campestris* pv. *campestris* strain 8004 contains an orphan quorum-sensing (QS) locus, *xccR-pip*Xcc, in which the proline iminopeptidase (*pip*) gene (where "Xcc" indicates that the *pip* gene is from *X. campestris* pv. *campestris*) is positively regulated by the LuxR homologue XccR by binding to the luxXcc promoter of the *pip*Xcc promoter. The disruption of *pip*Xcc significantly attenuated the virulence of *X. campestris* pv. *campestris*. An imperfect plant-inducible promoter (PIP) box is located in the upstream region of the *pip*Xcc promoter, which is the putative binding site of the transcriptional activator HrpX. To explore whether the expression of the *pip*Xcc gene is regulated by HrpX, the expression level of a *pip*Xcc promoter-*gusA* fusion gene was assayed in an *hrpX* disruption mutant. The results showed that the lack of HrpX dramatically decreased the β-glucuronidase (GUS) activity. Further analyses using an electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP)-PCR indicated that the imperfect PIP box in *X. campestris* pv. *campestris* is specifically bound to HrpX. These data demonstrated that the *pip*Xcc gene belongs to the HrpX regulon and that the imperfect PIP box of the *pip*Xcc promoter could be a cis element for the HrpX protein. We further showed in a pulldown assay that XccR can bind HrpX, suggesting that these two regulatory proteins coactivate the virulence factor by binding to the different cis elements of the *pip*Xcc gene and adapt to the host environment during *X. campestris* pv. *campestris* infection.

Diseases caused by members of the genus *Xanthomonas* contribute to devastating losses of cultivated crops worldwide (27). Many phytopathogenic bacteria elicit a hypersensitive response (HR) in nonhost plants or pathogenicity in host plants, depending on *hrp* (hypersensitive reaction and pathogenicity) and *hrc* (*hrp*-conserved) genes (2). The *hrp* gene cluster in phytopathogens is regulated by two types of regulators (2, 13). Group I *hrp* genes in *Erwinia amylovora* and *Pseudomonas syringae* are activated by an alternative sigma factor (30), whereas the group II *hrp* genes of *Ralstonia solanacearum* and *Xanthomonas campestris* are activated by an AraC family regulator (HrpX for *Xanthomonas* and HrpB for *Ralstonia*). In many *Xanthomonas* species, HrpX regulates the expression of a genome-wide regulon, including type II and type III secretion systems (14), which also exist in many bacterial pathogens of humans and animals to secrete effector proteins and degradation enzymes (6, 17). The promoters controlled by HrpX often carry a conserved motif called plant-inducible promoter (PIP) box and a −10 box (11, 12). HrpX regulates the PIP box-containing promoters by directly binding to the conserved cis element (*TTGCN*-115-*TTGGC*) in xanthomonads (11, 33). A similar sequence (*TTGCN*-16-*TTGGC*), called *hrp* box, discovered in *R. solanacearum*, was regulated by HrpB (7). The central cytidine of each half-site is essential for the function of the cis element, while the other nucleotides are more flexible (7). Notably, genes with an imperfect PIP box or without a PIP box have also been shown to be expressed in an HrpX-dependent manner (7, 20, 26). Thus, HrpX is believed to be a global regulator, and there are more genes belonging to the HrpX regulon than previously expected.

*X. campestris* pv. *campestris* is the causal agent of black rot on most cultivated crucifer plants (27). Our previous study showed that the *xccR-pip*Xcc locus (where "Xcc" indicates that the *pip* gene is from *X. campestris* pv. *campestris*) is related to pathogenicity, and disruption of either of the two genes results in significantly attenuated virulence of *X. campestris* pv. *campestris* (34). The proline iminopeptidase (*pip*) gene was regulated by quorum-sensing (QS) LuxR homolog XccR, and the *pip* promoter-*gusA* fusion gene was significantly induced when the bacteria grew in planta (34). QS enables bacterial cell-cell communication via signal molecules, and it monitors the density of bacterial populations (10). In Gram-negative bacteria, the classic QS regulation is mediated by N-acylhomoserine lactone (AHL) signal molecules, and LuxR and LuxL are responsible for producing and sensing signals, respectively (10). Only a few isolates of *Xanthomonas* produced detectable AHLs (5). A genome survey showed that *X. campestris* pv. *campestris* strain 8004 has no cognate LuxL synthase for AHLs (28), and in consequence, no AHL activity was detected. Instead, XccR activates the expression of *pip* which encodes a hydrolase rather than a LuxI synthase, by binding to the *luxXcc* box, highly similar to the *lux* box in the promoter region of the LuxI genes.

In this study, an imperfect PIP box could be found in the *xccR-pip*Xcc intergenic region upstream of the *luxXcc* box by sequence analysis. We provide evidence for direct binding of HrpX to the imperfect PIP box in vivo and in vitro. We further show the *in vitro* binding of HrpX and XccR in pulldown assays, suggesting that the
two proteins are coactivators of pipXcc. This conclusion is consistent with the results that deletion of either hrpX or xccR abolished the pipXcc activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacteria and plasmids used in this work are listed in Table 1. Escherichia coli strains were grown in Luria-Bertani (24) medium at 37°C. E. coli hrpX, the X. campestris pv. campestris strains were cultured at 28°C either in NYG medium (5 g/liter tryptone, 3 g/liter yeast extract, 20 g/liter glycerol, pH 7.2) as a nutrient-rich condition or in MMX [4 g/liter K_2PO_4, 6 g/liter KH_2PO_4, 2 g/liter (NH_4)_2SO_4, 1 g/liter tricine -Na_2, 0.2 g/liter MgSO_4 · 7 H_2O, 5 g/liter glucose, pH 7.0] as a minimal medium. Bacterial cell density was monitored by measuring the optical absorbance at 600 nm. Antibiotics were used at the following final concentrations: 50 μg/ml rifampin, 20 μg/ml kanamycin, 100 μg/ml ampicillin, 80 μg/ml spectinomycin, and 3 μg/ml tetracycline for liquid medium and 10 μg/ml for solid medium.

Plasmid construction. To determine pipXcc promoter activity, plasmid pFR421 was generated, which contains a 438-bp EcoRI-BspHI fragment PCR amplified with primers pip-PF and pip-PR (Table 1) from the mid pFR421 was generated, which contains a 438-bp EcoRI-BspHI fragment with the results that deletion of either hrpX or xccR abolished the pipXcc activity.

To construct plasmid that expressed the chimeric HrpX protein in E. coli, the hrpX gene was amplified by PCR with primers jxhrpXFwd and jxhrpXRev (Table 1). The fragment was digested with NdeI and XhoI restriction enzymes and ligated into pHM1 vector. The 6×His tag was fused to the C terminus of HrpX. The hrpX HindIII/EcoRI fragment was blunt ended by DNA polymerase I Klenow fragment (NEB, Hitchin, Hertfordshire, United Kingdom) and ligated into the flush-ended PstI site of pMAL-p2x to generate phrpX-MBP (maltose binding protein).

To make the plasmid that expressed the chimeric HrpX protein in E. coli, the hrpX gene was amplified by PCR with primers jxhrpXFwd and jxhrpXRev (Table 1). The fragment was digested with NdeI and XhoI restriction enzymes and ligated into PET30a to generate PET30a-hrpX.

Protein expression, purification, and antibody preparation. Prokaryotic expression plasmids PET30a-hrpX and phrpX-MBP were transformed into E. coli BL21 or TB1. Fusion proteins were expressed in E. coli cells after induction of an early log culture overnight by isopropyl-D-thiogalactopyranoside (IPTG) (0.1 mM) at 16°C. The MBP-tagged protein was purified by affinity chromatography with amylase resin (NEB) and eluted with maltose. The His-tagged protein was purified by Ni-nitrilotriacetic acid (NTA) resin (Novagen) under denaturing conditions. Amicon YM10 (Millipore) was used for protein concentration or for changing the protein suspension buffer. For production of antibody against HrpX, the His-tagged protein expressed from PET30a-hrpX was purified and pooled to immunize and boost rabbits, and serum was taken after the fourth booster injection.

Construction of hrpX mutant strain. The hrpX deletion mutant was generated using a selection/counterselection suicide vector, pK18mobsacB (21). To construct the strain with disrupted hrpX, two fragments corresponding to the hrpX coding regions 1 to 431 and 1008 to 1431 were PCR amplified from the strain 8004 genomic DNA by two pairs of primers,
HrpX and XccR Promote X. campestris pv. campestris pip

The xcr-Rpip pip region and structure of the intergenic sequence upstream of the pip gene. The imperfect PIP box and luxC box sequences are indicated.

FIG 1  Genomic organization of the xcr-Rpip pip region and structure of the intergenic sequence upstream of the pip gene. The imperfect PIP box and luxC box sequences are indicated.

HrpX del F1/hrpX del R1 and HrpX del F2/hrpX del R2, respectively (Table 1). The resulting EcoRV-Sall and Sall-HindIII fragments were fused into the Smal/HindIII-cleaved pK18mobsacB vector in one ligation reaction. The hrpX gene in the strain 8004 genome was truncated by homologous recombination. To detect positive clones with the truncated hrpX gene, a PCR primer pair, hrpX outup and hrpX outdown, flanking the recombination. To detect positive clones with the truncated hrpX gene, a PCR primer pair, hrpX outup and hrpX outdown, flanking the hrpX coding region (Table 1), was designed.

GUS assay. The β-glucuronidase (GUS) activity of different X. campestris pv. campestris strains grown in medium and in planta was measured by the fluorometric method using the substrate 4-methylumbelliferyl β-d-glucuronide (MUG) (34). GUS activity was normalized to bacterial cell numbers. One unit of enzyme activity is defined as the amount of enzyme that releases 1 pmol of 4-methylumbelliferone (MU) min⁻¹ at pH 7.0 at 37°C. The experiments were repeated at least three times for each of the conditions, each time in triplicate.

ChIP-PCR. Strain 8004 harboring the pHM1-hrpX-his plasmid was grown in 10 ml NYG medium to an optical density at 600 nm of 1.5. The proteins and chromatin DNA were cross-linked by adding formaldehyde to a final concentration of 1% for 10 min. The cross-linking reaction was stopped by the addition of glycine. The assay was performed using a chromatin immunoprecipitation (ChIP) assay kit (Millipore, Billerica, MA), following the manufacturer’s instructions. The resulting purified DNA was used for PCR analysis. A small aliquot of untreated sonicated chromatin was reverse cross-linked and used as the total input DNA control. The experiments were repeated at least three times.

EMSA. MBP-HrpX fusion protein was purified through an amylose resin chromatography column (NEB) according to the manufacturer’s instructions. The 45-bp DNA duplex containing the PIP box sequence was generated by annealing the synthetic oligonucleotides box45F and box45R. The product was then end labeled with [α-32P]dATP. For the electrophoretic mobility shift assay (EMSA), 0.65 μg of the labeled probe and the MBP-HrpX protein were incubated in a binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol (DTT), 2.5% glycerol, and 50 ng ml⁻¹ poly(dI · dC)] for 20 min at room temperature. For competition, a certain amount of the unlabeled probe was coincubated with the labeled probe and the MBP-HrpX protein. Samples were size fractionated by using a 4% non-denaturing polyacrylamide gel in 0.5× TBE buffer (45 mM Tris-borate and 1 mM EDTA) at 4°C. The gel was dried, and the shifted bands were detected by autoradiography.

His pulldown assay. Purified XcrR-MBP, HrpX-His, and MBP were subjected to His tag pulldown analysis. HrpX–his was loaded onto a Ni-NTA column using binding buffer (300 mM NaCl, 50 mM Na-phosphate, pH 8.0, 10 mM imidazole). XcrR-MBP was then loaded onto the same column. The column was washed with four volumes of wash buffer (300 mM NaCl, 50 mM Na-phosphate, pH 8.0, 20 mM imidazole), and then the protein was eluted in elution buffer (300 mM NaCl, 50 mM Na-phosphate, pH 8.0, 300 mM imidazole). The eluted proteins were analyzed by SDS-PAGE and verified by Western blotting.

RESULTS

Sequence analysis of the pip promoter region. The 438-bp intergenic sequence between xcrR and pip in X. campestris pv. campestris 8004 was analyzed, and an imperfect PIP box (TTCGC-N4-TTCGC-N2-TTGGCC) was found at positions −307 to −287 relative to the pip translational start site (TSS) (Fig. 1). While the consensus sequence of a perfect PIP box (TTCGC-N15-TTGGCC) has two conserved half-sites (TTCGC), the imperfect PIP box comprises not only the two conserved half-sites but also one nonconserved half-site (TTGGCC). The three sites were separated in the imperfect PIP box by 4 bp and 2 bp. Including the spacing base pairs, the imperfect PIP box may be presented as TTCGC-N11-TTGGCC for HrpX dimer binding.

HrpX is essential for pip expression. The Pip box is known to be the cis element for HrpX binding in the promoter region that regulates gene expression. The existence of the imperfect PIP box in the pip promoter may indicate that the expression of pip is HrpX dependent. To verify the role of HrpX in pip expression, an hrpX mutant was generated by double crossover steps using the pK18mobsacB vector (21). The GUS reporter plasmid pFR421 that carried a 438-bp pip promoter-gus fusion (34) was introduced into the wild-type 8004 and hrpX mutant strains. The GUS activity assay showed that disruption of hrpX resulted in decreased GUS activities in NYG medium, MMX medium, and in planta (Fig. 2). Compared with that in strain 8004/pFR421, the GUS activity in ΔhrpX/pFR421 decreased by 6.7-, 12.8-, and 8.2-fold, respectively, under the three growth conditions. These results suggested that HrpX is indispensable for pip expression.

HrpX binding to the imperfect PIP box. A previous report showed that even an imperfect PIP box could be recognized by cognate regulatory factors (12). As a result, more genes with imperfect cis elements in the promoters could be assigned to the
HrpX regulon, such as the \( \text{pipXcc} \) gene of strain 8004, in which a potential PIP box-like element is located \( -307 \) to \( -287 \) upstream from the TSS (Fig. 1). In order to detect the interaction between HrpX and this potential PIP box, ChIP-PCR and EMSA were used to assess the presence of the HrpX protein bound to the cis element. ChIP-PCR of strain 8004/pHM1-hrpX-his bacterial extract was performed. The DNA fragments (spanning \( -438 \) to \( -1 \) and \( -438 \) to \( -287 \) relative to TSS of \( \text{pipXcc} \)) immunoprecipitated with the His monoclonal antibody were amplified by PCR. The amplification of a no-antibody sample was the negative control. The results revealed that the HrpX protein is bound to the potential PIP box (Fig. 3). The result of \( \text{in vitro} \) EMSA was consistent with the ChIP-PCR result. The migrated bands of 45-bp duplex DNA and HrpX-MBP complexes were observed in nondenaturing polyacrylamide gel. Various amounts of unlabeled probe (Fig. 4, right) and various amounts of purified HrpX-MBP (Fig. 4, left) were used as competitors. MBP, as the negative control, did not bind the DNA probe.

**HrpX/XccR interplay is responsible for \( \text{pipXcc} \) induction.** As previously reported, an \( \text{xccR} \) mutant decreased the induction of the \( \text{pipXcc} \) promoter in host plants (34). This indicated that the disruption of either \( \text{xccR} \) or \( \text{hrpX} \) can result in a failed induction of \( \text{pipXcc} \). To investigate the relationship between XccR and HrpX in regulating \( \text{pipXcc} \) expression, a pulldown assay was carried out to test the binding of the two proteins. The MBP-tagged XccR was expressed in \( \text{E. coli} \) and \( \text{X. campestris pv. campestris} \) harboring the plasmid-expressed HrpX-His. The pulldown result showed that the XccR protein interacted with the HrpX protein (Fig. 5). The MBP protein used as a negative control did not bind any of the tested proteins. This result indicates that the complex formed by XccR and HrpX may contribute to the activation of \( \text{pipXcc} \).

### DISCUSSION

In \( \text{Xanthomonas} \) species, the essential role of HrpX in virulence-related gene expression, through binding by a plant-inducible element, the PIP box, has been well defined. According to bioinformatics analysis results, an imperfect PIP box (TTCGC-N4-TTCGC) was found in the \( \text{pipXcc} \) promoter region in strain 8004 (Fig. 1). The \( \text{X. campestris pv. campestris} \) genome has 12 candidate promoters with perfect PIP boxes (TTCGC-N15-TTCGC) (13), and an earlier study showed that a substitution in...
and genes without PIP boxes (26). As a member of the HrpX regulon, the disruption of pip_{Xcc} impaired the virulence of X. campestris pv. campestris in a host plant cabbage. It is worth exploring whether PIP_{Xcc} is a secretory virulence protein or a modification enzyme to arrest the other virulence factors.

Our previous observations showed that the pip_{Xcc} gene could be induced in host plants and that the increased GUS activity of strain 8004/pFR421 depends on the activation of XccR by binding to the luxXc box in planta (34). Disruption of xccR on the 8004 chromosome (strain 8515/pFR421) resulted in failure of the in planta induction of gusA (34). In this study, the pip_{Xcc} promoter activity also decreased drastically in the hrpX mutant. The results of the pulldown assay indicated that XccR can bind HrpX directly. It is likely that HrpX and XccR form a complex as coactivators to regulate pip_{Xcc} expression and that HrpX/XccR interplay is responsible for pip_{Xcc} induction.

Quorum-sensing systems exist widely in bacteria, and QS-dependent functions include virulence, sporulation, plasmid transfer, biosynthesis of antibiotics, and plant nodulation (3, 4, 25). In the xccR-pip_{Xcc} locus, XccR is an unpaired LuxR homolog of QS because the cognate LuxI synthase gene is lacking. The LuxR orphan, such as ExpR of Sinorhizobium, BisR of Rhizobium, QscR of Pseudomonas, and SdiA in Salmonella, Escherichia, and Klebsiella, respond to AHL signals (1, 9, 22, 23). However, XccR could not respond to AHLS. Instead, XccR activates the expression of pip_{Xcc}, which encodes a hydrolase, by binding the luxXc box (34). In this study, XccR recruited HrpX to coregulate pip_{Xcc} expression, which indicated that at least two transcriptional factors mediate the function of the xccR-pip_{Xcc} locus.

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