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HrcT Is a Key Component of the Type III Secretion System in *Xanthomonas* spp. and Also Regulates the Expression of the Key *hrp* Transcriptional Activator HrpX

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The type III secretion system (T3SS), encoded by *hrp* (hypersensitive response and pathogenicity) genes in Gram-negative phytopathogenic bacteria, delivers repertoires of T3SS effectors (T3SEs) into plant cells to trigger the hypersensitive response (HR) in nonhost or resistant-host plants and promote pathogenicity in susceptible plants. The expression of *hrp* genes in *Xanthomonas* is regulated by two key regulatory proteins, HrpG and HrpX. However, the interactions between *hrp* gene products in directing T3SE secretion are largely unknown. Here we demonstrated that HrcT of *X. oryzae* pv. oryzicola functions as a T3SS component and positively regulates the expression of *hrpX*. Transcription of *hrcT* occurs via two distinct promoters; one (T1) is with the *hrpB* operon and the second (T3) within *hrpB7* Via either promoter T1 or T3, the defect in Hrp phenotype by *hrcT* deletion was corrected in the presence of *hrcT* only from *Xanthomonas* species but not from other phytopathogenic bacteria. An N-terminally truncated HrcT was able to bind the *hrpX* promoter and activate the expression of *hrpX*, supporting that HrcT is a positive regulator of *hrpX*. A revised model showing the regulatory interactions between HrcT, HrpX, and HrpG is proposed.

The successful infection of crop plants by Gram-negative plant-pathogenic bacteria is largely dependent on the type III secretion system (T3SS), which delivers repertoires of T3SS effectors (T3SEs) into plant cells to promote disease development (1). It has been well documented that the *hrp* genes encode the T3SS and control the ability of phytopathogenic bacteria to trigger the hypersensitive response (HR) in resistant-host or nonhost plants and pathogenicity in susceptible hosts (2, 3). Based on their genetic organization and transcriptional regulation, *hrp* gene clusters have been divided into two main groups (4, 5). The *hrp* genes of *Erwinia amylovora*, *Pseudomonas syringae*, and *Dickeya* spp. are typical representatives of group I. The expression of *hrp* genes in group I is modulated by the alternative sigma factor HrpL, which binds to a conserved *hrp* box to activate transcription (6–8). The *hrp* genes in group II, which includes *Ralstonia solanacearum* and *Xanthomonas* spp., are regulated by HrpG and HrpX (the latter protein is designated HrpB in *Ralstonia*) (9–12). HrpG is an OmpR family protein that belongs to response regulators of the two-component signal transduction system and putatively receives phosphorylation from HpaS (12, 13). HrpX is an AraC-type transcriptional activator that forms a homodimer and contains a helix-turn-helix (HTH) motif (11). The HTH motif of HrpX interacts with the plant-inducible promoter (PIP) box (TTCGC-N_{15}-TTCGC) in *hrp* transcripts by binding to the TTCGC sequence (14, 15). However, evidence demonstrating that HrpX is directly regulated by HrpG is lacking from the literature.

In xanthomonads, the *hrp-hrc-hpa* genes are highly conserved and clustered within several sequenced genomes (16–20). Our previous studies revealed that the *hrp* cluster in *X. oryzae* pv. oryzicola, which causes bacterial leaf streak (BLS) in rice, is composed of 10 *hrp*, nine *hrc* (*hrp*-conserved), and eight *hpa* (*hrp*-associated) genes (3, 21). Comparative genomic analysis has revealed that at least nine *hrc* genes (e.g., *hrcC*, *hrcT*, *hrcN*, *hrcJ*, *hrcU*, *hrcV*, *hrcQ*, *hrcR*, and *hrcS*) are conserved among plant- and animal-pathogenic bacteria (22). It has been proposed that the T3SS consists of ring structures spanning the inner membrane (IM) and outer membrane (OM); these structures comprise a transport channel with an inner diameter of 2 to 3 nm (23). In *Xanthomonas* spp., the IM rings and export apparatus are comprised of HrcR, HrcS, HrcT, HrcU, HrcQ, and HrcV. These six proteins assemble into a structure that is connected to a predicted cytoplasmic C ring (HrcC) and an ATPase complex (HrcN) via an inner membrane protein, HrcJ (22). The core secretion apparatus is presumably associated with an extracellular Hrp pilus that serves as a transport channel for secreted T3SEs into the host cell cytosol (24–26).

hrcT is the eighth gene in the *hrpB* operon, which consists of *hrpB1*, *hrpB2*, *hrcJ*, *hrpB4*, *hrpB5*, *hrcN*, *hrcB7*, and *hrcT* (21, 27). Previous studies indicated that the expression of the *hrpB* operon is HrpX dependent because a PIP box was present in the *hrpB* operon promoter (11). Since the expression of HrpX is presumably controlled by HrpG, *hrcT* should not be transcribed in a *hrpG* mutant. However, our previous results showed that *hrcT* is expressed in the *hrpG* mutant of *X. oryzae* pv. oryzicola (2), implying a more complex regulatory paradigm for HrcT in bacterial pathogenesis.

In *X. campestris* pv. vesicatoria, topology analysis indicates that 41 amino acid residues at the N terminus of HrcT span the bacterial IM (1). We previously demonstrated that the *hrcT* mutant of *X. oryzae* pv. oryzicola was unable to induce HR in tobacco and failed to cause BLS in susceptible rice cultivars (2). In the present

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study, we conduct a more detailed analysis of *hrcT* expression and its contribution to pathogenicity. We also explore whether other *hrp* genes are regulated by HrcT and evaluate the impact of an *hrcT* deletion on the secretion of T3SEs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured in LB (Luria-Bertani) medium at 37°C (28). All strains of *X. oryzae* pv. oryzicola and *X. axonopodis* pv. citri strain 306 were grown in nutrient agar (NA), nutrient broth (NB), NA without sucrose (NAN), NA with 10% sucrose (NAS), or XOM3 at 28°C (2, 29). *R. solanacearum* GMI1000 was grown in BG medium at 30°C (30), and *P. syringae* pv. tomato DC3000 was grown in King's B (KB) medium at 28°C (31). Antibiotics were used at the following final concentrations (μg/ml) when required: rifampin (Rif), 50; kanamycin (Km), 25; ampicillin (Ap), 100; spectinomycin (Sp), 50; and gentamicin (Gm), 20.

DNA manipulations. DNA isolation and cloning and PCR were performed using standard procedures (32). The mobilization of plasmids into *X. oryzae* pv. oryzicola was performed as described previously (2). Restriction enzymes and DNA ligases were used as recommended by the manufacturer (Promega, Shanghai). Primers (see Table S1 in the supplemental material) were synthesized by Invitrogen. Ex-Taq (TaKaRa Bio Inc.) was used in PCR assays as recommended by the manufacturer.

Mutation construction in the *hrpB* operon promoter. To inactivate the *hrpB* operon promoter, an 876-bp open reading frame (ORF) encoding gentamicin resistance (primers Gm-F/Gm-R) was inserted into the *hrpB* operon promoter region in the opposite direction of transcription. Two primer pairs, Gm-IF/Gm-IR and Gm-IIF/Gm-IIR (see Table S1 in the supplemental material), were used to amplify the left and right fragments flanking the *hrpB* operon promoter using RS105 genomic DNA as the template. PCR products were digested based on the restriction sites incorporated into primers and cloned into a vector pKMS1, resulting in the construct pKMSΔGm (Table 1), according to our previous method (33). The deletion of the *hrpB* operon promoter and insertion of the Gm^r ORF was achieved using the *sacB* mutagenesis procedure described previously (33). The mutant containing a Gm^r insertion in the *hrpB* operon promoter was verified by PCR with the primers Gm-IF/Gm-IIR (see Table S1 in the supplemental material) and named RΔ*BP* (Table 1).

Complementation of mutants. An 831-bp DNA fragment containing the intact *hrcT* gene was amplified by PCR using genomic DNA of *X. oryzae* pv. oryzicola RS105 as the template and primer pairs hrcT-F/hrcT-R (see Table S1 in the supplemental material). Similarly, a 350-bp fragment containing the *hrpB* operon promoter region (designated pT1) was amplified using primers phrcT1F/phrcT1R (see Table S1). A 500-bp fragment located upstream of the *hrcT* start codon (pT3) was amplified with primers phrcT3F/phrcT3R (see Table S1). After confirmation by sequence analysis, the T1 and T3 promoters and the promoterless *hrcT* were cloned into pBBR1MCS-5 vector at KpnI and HindIII sites to create recombinant plasmids pChrcT1 and pChrcT3, respectively (Table 1). The promoters of pT1 and pT3 were also fused with promoterless *hrcT* homologs from *X. axonopodis* pv. citri strain 306, *R. solanacearum* GMI1000, and *P. syringae* pv. tomato DC3000 to respectively create $pC1HrcT_{Xac}$, $pC1HrcT_{Rs}$, and $pC1HrcT_{Pst}$ (with pT1) and $pC2HrcT_{Xac}$, $pC2HrcT_{Rs}$, and $pC2HrcT_{Pst}$ (with PT3). The recombined plasmids were then transferred into the mutant RΔ*hrcT* by electroporation, and transformants were selected on NA containing Gm. Representative transformants containing the different promoter constructs and *hrcT* homologs were verified by colony PCR and named C1RΔ*hrcT*, C2RΔ*hrcT*, $C1R\Delta hrcT_{Xac}$, $C1R\Delta hrcT_{Rs}$, $C1R\Delta hrcT_{Pst}$, $C2R\Delta hrcT_{Xac}$, $C2R\Delta hrcT_{Rs}$, and $C2R\Delta hrcT_{Pst}$ (Table 1).

Determination of the *hrcT* promoter. 5′ rapid amplification of cDNA ends (5′-RACE) was used to determine the transcriptional start site of *hrcT*. *X. oryzae* pv. oryzicola strain RΔ*BP* was incubated in XOM3 medium at 28°C for 16 h, and total RNA was extracted from the mutant RΔ*BP* using an RNAiso Plus kit (TaKaRa, Dalian, China). Isolated RNA was treated with RNase-free DNase I at 37°C for 2.5 h, followed by a second purification using an RNase-free column. cDNA fragments were obtained using the 5′-Full RACE kit (TaKaRa, Dalian, China), and an anchor sequence was added to the 5′ end of the cDNA using terminal deoxynucleotide transferase. The tailed cDNA was then amplified using nested gene-specific primers hrcTO-R and hrcTI-R and RACE outer primer O-F and inner primer I-F (see Table S1 in the supplemental material). 5′-RACE products were cloned into pMD18-T and sequenced.

Pathogenicity and HR assays. HR and pathogenicity assays were performed as described previously (3). Briefly, *X. oryzae* pv. oryzicola strains were grown in NB, adjusted to 3×10^8 CFU/ml (optical density at 600 nm [OD_{600}], 0.3), and inoculated into leaves of rice seedlings (*Oryza sativa* cv. IR24, 2 weeks old) with needleless syringes to assess the formation of water-soaked lesions. Adult rice plants (cv. IR24, 2 months old) were inoculated by leaf needling for lesion length measurement. The T3SS mutant RΔ*hrcV* was used as a negative control, and bacterial growth was monitored during the experiment as described previously (3). Strains (OD_{600} = 0.01) were also tested for the ability to elicit HR on *Nicotiana benthamiana* (3). All plants were maintained in a greenhouse at 25°C with a 12-h photoperiod and 75 to 80% relative humidity. Experiments were repeated three times.

qRT-PCR. The cultivation of rice suspension cells and real-time quantitative RT-PCR (qRT-PCR) were performed as described previously (34) using the primers listed in Table S1 in the supplemental material. Total RNA was extracted from each treatment using TRIzol, and cDNA synthesis and PCR were conducted as described previously (34); *gyrB* was used as an internal standard. qRT-PCR was performed using the Applied Biosystems 7500 real-time PCR system and SYBR Premix Ex Taq (TaKaRa, China). The comparative threshold method was used to calculate the relative mRNA levels. All qRT-PCR experiments were performed three or more times.

GUS activity assays. To construct transcriptional fusions to glucuronidase, the promoter regions of target genes were fused to a promoterless *gusA* with its ribosome binding site (35). Promoters T1 (350 bp upstream of *hrpB1* translational start codon), T3 (500 bp upstream of *hrcT* translational start codon), and pG and pX (located 300 bp upstream of the *hrpG* or *hrpX* translational start codon) were amplified by PCR using total genomic DNA of the wild-type RS105 as the template with primer pairs phrcT1gF/phrcT1gR, phrcT3gF/phrcT3gR, phrpGgF/phrpGgR, and phrpXgF/phrpXgR (see Table S1 in the supplemental material), respectively, fused to the promoterless *gusA* gene, and then cloned into the EcoRI and BamHI sites of pUFR034 (36), resulting in pT1GUS, pT3GUS, pGGUS, and pXGUS (Table 1). Bacterial strains containing GUS transcriptional fusions were incubated in either NB or XOM3 at 28°C for 12 h and examined for GUS activity as described previously (2).

***In vivo* binding of HrcT to *hrpX* promoter.** To examine whether or not HrcT binds the *hrpX* promoter *in vivo*, a 300-bp promoter of *hrpX* (300 bp upstream of the *hrpX* translational start codon) was fused to a promoterless *gusA* in a vector, pBI121, that was transferred into tobacco leaves (*N. benthamiana*) mediated by *Agrobacterium* (37). The *hrcT* ORF was then fused under the CaMV 35S promoter in a vector, pCAMBIA1300, that was also used for transient expression of a tested gene in tobacco mediated by *Agrobacterium* (37). The primers for the above-described constructs were pXF/pXR and TF/TR (see Table S1 in the supplemental material), respectively. The PCR-amplified fragments were digested with HindIII and XbaI and cloned into HindIII/XbaI sites in pBI121 or pCAMBIA1300, respectively, generating phrpX and HrcT (Table 1). The *Agrobacterium*-mediated transient expression assays were performed as described previously (37, 38). For the control, the plasmids PthXo1 containing *pthXo1* gene in pCAMBIA1300 (37) and pOs8N3 harboring the *Os8N3* promoter (targeted by PthXo1 and fused with the promoterless *gusA*) in pBI121 (37) (Table 1) were used. The GUS activity was determined 2 days postinfection (dpi) by stained leaf disks (0.8 cm in diameter) with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide)

TABLE 1 Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristics	Source
X. oryzae pv. oryzicola		
RS105	Wild type, causal agent of bacterial leaf streak in rice, Rif^r	This lab
RΔ*hrpG*	*hrpG* deletion mutant of RS105, Rif^r	2
RΔ*hrpX*	*hrpX* deletion mutant of RS105, Rif^r	2
RΔ*hrpV*	*hrcV* deletion mutant of RS105, Rif^r	2
RΔ*hrcT*	*hrcT* deletion mutant of RS105, Rif^r	2
RΔBP	RS105 mutant containing deletion of *hrpB1* promoter and insertion of Gm^r, Rif^r	This study
C1RΔ*hrcT*	Complemented strain of *R*Δ*hrcT* with plasmid pChrcT1, Rif^r Gm^r	This study
C2RΔ*hrcT*	Complemented strain of RΔ*hrcT* with plasmid pChrcT3, Rif^r Gm^r	This study
C1RΔ$hrcT_{Xac}$	Complemented strain of RΔ*hrcT* with plasmid pC1HrcT_{Xac}, Rif^r Gm^r	This study
C1RΔ$hrcT_{Rs}$	Complemented strain of RΔ*hrcT* with plasmid pC1HrcT_{Rs}, Rif^r Gm^r	This study
C1RΔ$hrcT_{Pst}$	Complemented strain of RΔ*hrcT* with plasmid pC1HrcT_{Pst}, Rif^r Gm^r	This study
C2RΔ$hrcT_{Xac}$	Complemented strain of RΔ*hrcT* with plasmid pC2HrcT_{Xac}, Rif^r Gm^r	This study
C2RΔ$hrcT_{Rs}$	Complemented strain of RΔ*hrcT* with plasmid pC2HrcT_{Rs}, Rif^r Gm^r	This study
C2RΔ$hrcT_{Pst}$	Complemented strain of RΔ*hrcT* with plasmid pC2HrcT_{Pst}, Rif^r Gm^r	This study
X. axonopodis pv. citri	Strain 306	Collected by this lab
R. solanacearum	Strain GMI1000	Collected by this lab
P. syringae pv. tomato	Strain DC3000	Collected by this lab
E. coli		
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44*λ^- *thi-1 gyrA96 relA1*	Clontech
BL21(DE3)	F^- *ompT hsdS20 gal*	Novagen
Plasmids		
pMD18-T	pUC *ori*, cloning vector, Ap^r	TaKaRa
pUFR034	*incW mob(p) mob*$^+$ *lacZA*$^+$, PK2 replicon, Km^r	36
pBBR1MCS-5	Broad-host-range cloning vector, Gm^r	49
pKMS1	Suicide vector derived from pK18mobGII, *sacB*$^+$, Km^r	This lab
pT1GUS	pUFR034 expressing *gusA* under 350-bp promoter region of *hrpB* operon, Km^r	2
pT3GUS	pUFR034 expressing *gusA* under 500-bp promoter region of *hrcT*, Km^r	This study
pXGUS	pUFR034 expressing *gusA* under 300-bp promoter region of *hrpX*, Km^r	This study
pGGUS	pUFR034 expressing *gusA* under 490-bp promoter region of *hrpG*, Km^r	This lab
pKMSΔGm	1,977-bp fragment containing the left and right border fragments of the *hrpB* operon promoter, flanks the Gm ORF in pKMS1, Km^r	This study
pChrcT1	pBBR1MCS-5 expressing *X. oryzae* pv. oryzicola *hrcT* from a 350-bp promoter region derived from *hrpB* operon, Gm^r	This study
pChrcT3	pBBR1MCS-5 expressing *hrcT* from a 500-bp promoter region of *hrcT* Gm^r	This study
pC1HrcT_{Xac}	pBBR1MCS-5 expressing *hrcT* from *X. axonopodis* pv. citri 306; promoter was derived from 350-bp promoter region derived from *hrpB* operon, Gm^r	This study
pC1HrcT_{Rs}	pBBR1MCS-5 expressing *hrcT* from *R. solanacearum* GMI1000; promoter was derived from 350-bp promoter region derived from *hrpB* operon, Gm^r	This study
pC1HrcT_{Ps}	pBBR1MCS-5 expressing *hrcT* from *P. syringae* pv. tomato DC3000; promoter was derived from 350-bp promoter region derived from *hrpB* operon, Gm^r	This study
pC2HrcT_{Xac}	pBBR1MCS-5 expressing *hrcT* from *X. axonopodis* pv. citri 306; promoter was derived from 500-bp region of *X. oryzae* pv. oryzicola *hrcT*, Gm^r	This study
pC2HrcT_{Rs}	pBBR1MCS-5 expressing *hrcT* from *R. solanacearum* GMI1000; promoter was derived from 500-bp region of *X. oryzae* pv. oryzicola *hrcT*, Gm^r	This study
pC2HrcT_{Pst}	pBBR1MCS-5 expressing *hrcT* from *P. syringae* pv. tomato DC3000; promoter was derived from 500-bp region of *hrcT*, Gm^r	This study
pHZWavrXa27	AvrXa27 with Flag tag under the control of *lacZ* promoter, Sp^r, Ap^r	Yang Bing's lab
pET30a(+)	pBR322 origin, *lacI*, His tag/S tag; Km^r	Novagen
pETHrcTΔ41N	*hrcT* from RS105 cloned in pET30a(+) as a 711-bp fragment, His tag at C terminus, Km^r	This study
phrpX	The 300-bp promoter of *hrpX* fused to a promoterless *gusA* in pBI121, Km^r	This study
HrcT	*hrcT* from RS105 fused under CaMV 35S promoter in pCAMBIA1300, Km^r	This study
pOs8N3	Os8N3 promoter fused to a promoterless *gusA* in pBI121, Km^r	37
PthXo1	*pthXo1* fused under CaMV 35S promoter in pCAMBIA1300, Km^r	37

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Rif^r, rifampin resistance; Sp^r, spectinomycin resistance; Gm^r, gentamicin resistance.

as previously described (38). The experiment was repeated three times at least.

HrcTΔ41N protein production and purification. The partial coding region of *hrcT* (nucleotides 124 to 831) was amplified from genomic DNA of *X. oryzae* pv. oryzicola RS105 by PCR using primers T-F and T-hisR (see Table S1 in the supplemental material); the latter fragment includes a hexahistidine tag code. The amplified fragment was ligated into pMD18-T and then subcloned into the EcoRI/XhoI sites of pET30a (Novagen) to generate a construct pETHrcTΔ41N (Table 1), which expresses HrcT with a C-terminal His tag. This construct was transformed into *E. coli* strain BL21(DE3) (Invitrogen) to generate strain BLHrcTΔ41N (Table 1). The overexpression and purification of HrcTΔ41N were performed as previously described (39).

EMSA. DNA fragments of different sizes upstream of *hrpX* with respect to the translational start codon were used as probes in electrophoretic mobility shift assays (EMSA). The 3′-end of the probes was biotinylated using the biotin 3′ end DNA labeling kit (Thermo Scientific). The biotinylated DNA fragments and protein HrcTΔ41N were incubated as recommended by the LightShift chemiluminescent EMSA kit (Thermo Sci). The reaction mixture was separated by electrophoresis in a 5% polyacrylamide gel (acrylamide/bisacrylamide, 29:1 [wt/wt]) in 0.5× Tris-borate-EDTA (TBE) buffer (44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA, pH 8.0). Samples were electrophoresed at 100 V after prerunning the gel for 30 min, and the gel was then transferred to nylon membranes (GE Healthcare). After UV cross-linking (15 min), the biotinylated probes were detected using the chemiluminescent nucleic acid detection kit (Thermo Scientific) as described by the manual.

Type III secretion assays. Plasmid pHZWavrXa27 containing *avrXa27* with a FLAG tag code (Table 1) was introduced into *X. oryzae* pv. oryzicola RS105, the mutant RΔ*hrcT*, the complemented strains C1RΔ*hrcT* and C2RΔ*hrcT*, and RΔ*hrcV*, separately. Strains (OD_{600} = 0.3) were inoculated into rice cv. 87-15 (containing *Xa27*) (40), and symptoms were assessed 48 hpi. To examine secretion of AvrXa27 via the T3SS *in vitro*, transformants were incubated in XOM3 at 28°C for 12 h, and total cell extracts (TEs) and culture supernatants (SNs) were analyzed by immunoblotting using a Flag antibody as described previously (21).

RESULTS

***hrcT* is transcribed by a promoter located within the *hrpB* operon.** In *Xanthomonas*, the *hrpB* operon consists of eight *hrp* genes, *hrpB1*, *hrpB2*, *hrcJ*, *hrpB4*, *hrpB5*, *hrcN*, *hrcB7*, and *hrcT*, which are transcribed from a promoter region located upstream of *hrpB1* (Fig. 1A). This promoter region contains a PIP box, and the expression of these eight genes is positively regulated by the transcriptional activator HrpX (2). Since the expression of HrpX is controlled by HrpG (12), a mutation in *hrpG* should result in impaired expression of these eight genes. However, the expression of *hrcT* in *X. oryzae* pv. oryzicola was still detectable in the *hrpG* mutant, and a putative promoter (T2) within *hrpB5* was not responsible for *hrcT* expression (2), implying that *hrcT* is differentially regulated via an unknown promoter. To determine whether the *hrpB* operon contains an internal promoter, we generated a mutant, RΔ*BP* (Table 1), which contains a deletion in the *hrpB* promoter and an insertion of the *Gm* gene cassette in opposition to the *hrpB* operon. After incubation in the *hrp*-inducing medium XOM3 (29) at 28°C for 12 h, mRNAs of the wild-type and mutant RΔ*BP* strains were extracted and used for RT-PCR with primer sets designed to amplify intergenic regions within the *hrpB* operon (Fig. 1A; see Table S1 in the supplemental material). RT-PCR results indicated that transcription of *hrpB1*, *hrpB2*, *hrcJ*, *hrpB4*, *hrpB5*, and *hrcN* was dependent on the *hrpB* operon promoter (T1), since the transcription of these genes was not detected in the promoter T1 mutant (Fig. 1B). Interestingly, RT-PCR products of

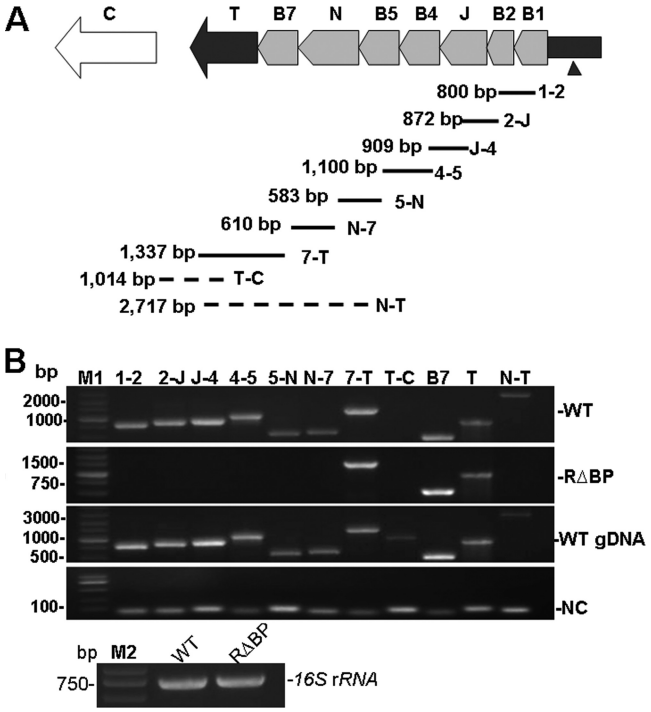

FIG 1 Transcription of genes in the *hrpB* operon. (A) Graphic representation of the *hrpB* operon and RT-PCR products amplified using primers designed to span intergenic junctions. Arrows shaded in gray represent *hrpB1* (B1), *hrpB2* (B2), *hrcJ* (J), *hrpB4* (B4), *hrpB5* (B5), *hrcN* (N), and *hrpB7* (B7). The black arrow represents *hrcT* (T), and the open white arrow depicts *hrcC* (C). The black rectangle adjacent to *hrpB1* represents the *hrpB* operon promoter, and the black triangle indicates the insertion of the Gm cassette in opposition to the *hrpB* operon promoter (mutant RΔ*BP*). The black lines indicate the sizes of RT-PCR products amplified using the intergenic primers (see Table S1 in the supplemental material). The dashed lines indicate that no RT-PCR products were generated in the mutant RΔ*BP*. (B) RT-PCR products detected by agarose gel electrophoresis. Abbreviations: WT, cDNA from wild-type RS105; RΔ*BP*, cDNA from the mutant in the *hrpB* operon promoter; WT gDNA, genomic DNA from wild-type RS105; NC, the extracted RNAs were used for PCR to ensure no residual genomic DNA in samples; M1, DL5000 DNA ladder (TaKaRa); M2, DL2000 DNA ladder (TaKaRa). 16S rRNA was used as an internal control to verify cDNA levels.

hrpB7 and *hrcT* were detected in both the wild-type and the mutant (RΔ*BP*) strains, whereas the RT-PCR products from *hrcN* to *hrcT* were generated only in the wild type (Fig. 1B). These results indicate that the transcription of *hrcT* gene might be controlled by two promoters, the *hrpB* operon promoter (T1) and the other, unknown promoter upstream of *hrcT* (named T3) (Fig. 2A).

To identify the second promoter (T3) driving the expression of *hrcT*, 5′-RACE-PCR was explored using nested primers (see Table S1 in the supplemental material) and total RNA from the mutant RΔ*BP* was cultured in XOM3 medium. 5′-RACE indicated that *hrcT* was indeed transcribed from the T3 promoter as a 750-bp PCR product (Fig. 2B). Sequence analysis of the *hrcT* upstream region revealed typical promoter elements, including a −10 box, a potential transcriptional start site (TSS), and a putative Shine-Dalgarno sequence (41) prior to the translation start codon (ATG) (Fig. 2C). It is noteworthy that the region upstream of *hrcT* lacked a PIP box.

Expression of *hrcT* is HrpG independent. To investigate T3 activity in various backgrounds, this promoter was fused with a

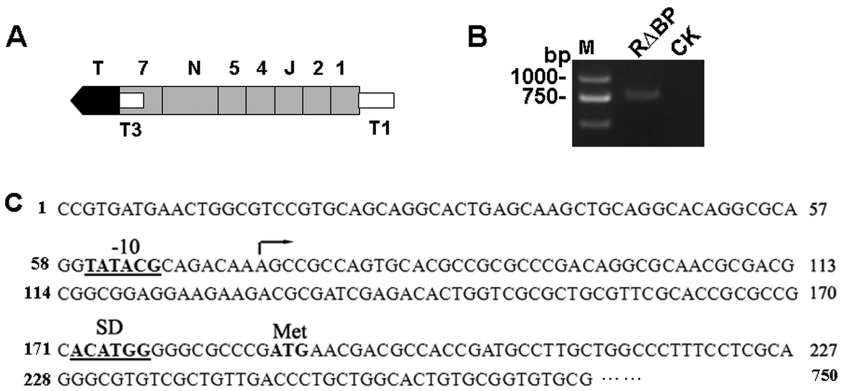

FIG 2 Identification of the second promoter (T3) of *hrcT* gene by 5′-RACE-PCR. (A) Graphical representation of the *hrpB* operon. Gray or black regions represent eight genes in the *hrpB* operon as in Fig. 1A. The open rectangle T1 shows the location of the *hrpB* operon promoter upstream of *hrpB1* (350 bp), and T3 shows the location of a promoter region (186 bp) upstream of *hrcT*. (B) Detection of the 750-bp 5′-RACE PCR product of *hrcT* in the T1 promoter mutant RΔ*BP* by agarose gel electrophoresis. CK, RΔ*BP* RNA used as a negative control. (C) Nucleotide sequence of T3 upstream of *hrcT*. Elements in the 5′ region include a −10 box (underlined), a potential transcriptional start site (arrow), and a putative ribosome binding site (Shine-Dalgarno [SD]). The boldface ATG represents the transcription start code of *hrcT*.

promoterless β-glucuronidase (*gusA*) gene, and the fusion (pT3GUS) (Table 1) was introduced into the wild-type RS105 and the mutants RΔ*hrpG* and RΔ*hrpX*. The T1 promoter fused to *gusA* (pT1GUS) (Table 1) was used as a control. The GUS activity of transformants containing pT1GUS or pT3GUS was measured. The results showed that GUS activity of the T1::GUS fusion was significantly lower in RΔ*hrpG* and RΔ*hrpX* than in the wild-type RS105 ($P = 0.05$, *t* test) (Fig. 3A), indicating that HrpG and HrpX positively regulate the expression of the *hrpB* operon genes via the T1 promoter. However, transcriptional activity of the T3::GUS fusion in RΔ*hrpG* and RΔ*hrpX* was significantly higher than that observed in the wild-type RS105 (Fig. 3A), suggesting that HrpG or HrpX may negatively regulate *hrcT* via the T3 promoter. To investigate this further, the posttranscript of *hrcT* was evaluated by qRT-PCR in the wild type, RΔ*hrpG*, and RΔ*hrpX*. The results showed that *hrcT* expression was significantly ($P = 0.05$, Student's *t* test) lower in the *hrpX* mutant than in both the *hrpG* mutant and the wild type, while the expression of *hrcT* in the *hrpG* mutant was almost the same as in the wild type (Fig. 3B). As predicted, the expression of *hrpF*, an *hrpX*-regulated gene, was significantly lower in RΔ*hrpG* and RΔ*hrpX* than in the wild-type RS105 (Fig. 3B). All these results together suggest that the expression of *hrcT* is HrpG independent.

HrcT positively regulates the expression of *hrpX* but not *hrpG*. Our previous qRT-PCR data demonstrated that the deletion in *hrcT* dramatically affected the expression of some *hrp* genes in *X. oryzae* pv. oryzicola (unpublished data). We speculated that the possibility was that the mutation in *hrcT* may impact the expression of *hrpG* or *hrpX*. To test this hypothesis, the promoter activities of *hrpG* and *hrpX* were measured in the wild type and the *hrcT* mutant RΔ*hrcT* using *gusA* as a reporter. Similar expression levels of *hrpG* were observed in the wild type and RΔ*hrcT* in the *hrp*-inducing medium XOM3 (see Fig. S1 in the supplemental material). In addition, analysis by qRT-PCR showed no difference in the *hrpG* mRNA between the wild type and RΔ*hrcT* ($P \leq 0.05$, *t* test) (see Fig. S1 in the supplemental material). This result suggests that the expression of *hrpG* is not influenced by the deletion of *hrcT*. However, the promoter activity of *hrpX* was significantly lower ($P = 0.01$, *t* test) in RΔ*hrcT* than those in the wild type

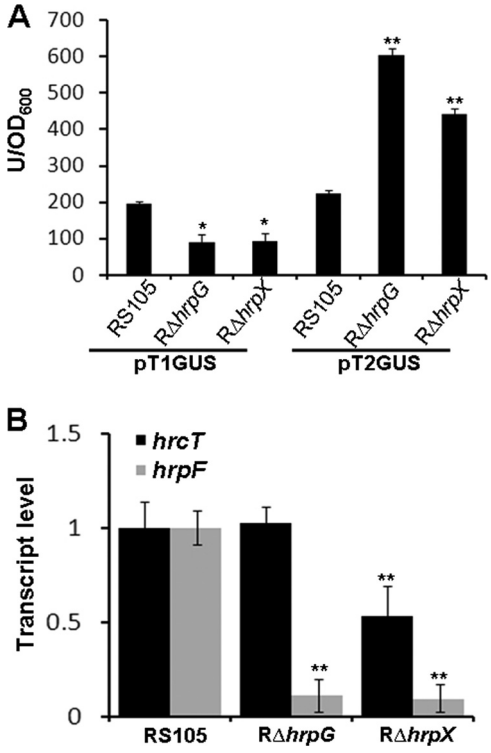

FIG 3 The expression of *hrcT* is HrpG independent. (A) β-Glucuronidase activities of transcriptional fusions designated pT1GUS and pT3GUS in the wild-type RS105, RΔ*hrpG*, and RΔ*hrpX*. All strains were cultured in XOM3 medium at 28°C for 12 h, and GUS activities were determined by measuring the optical density at 415 nm using 4-MUG (4-methylumbelliferyl-β-glucuronide) as a substrate. (B) Expression analysis of *hrcT* and *hrpF* by real-time quantitative RT-PCR. RNAs were isolated from cultures of *X. oryzae* pv. oryzicola RS105, RΔ*hrpG*, and RΔ*hrpX*, which were incubated in rice suspension cells at 25°C for 16 h. Data represent the means ± standard deviations of triplicate measurements. Asterisks above bars indicate significance relative to the wild type using a paired, two-tailed Student's *t* test. **, $P = 0.01$; *, $P = 0.05$. The experiment was repeated three times with similar results.

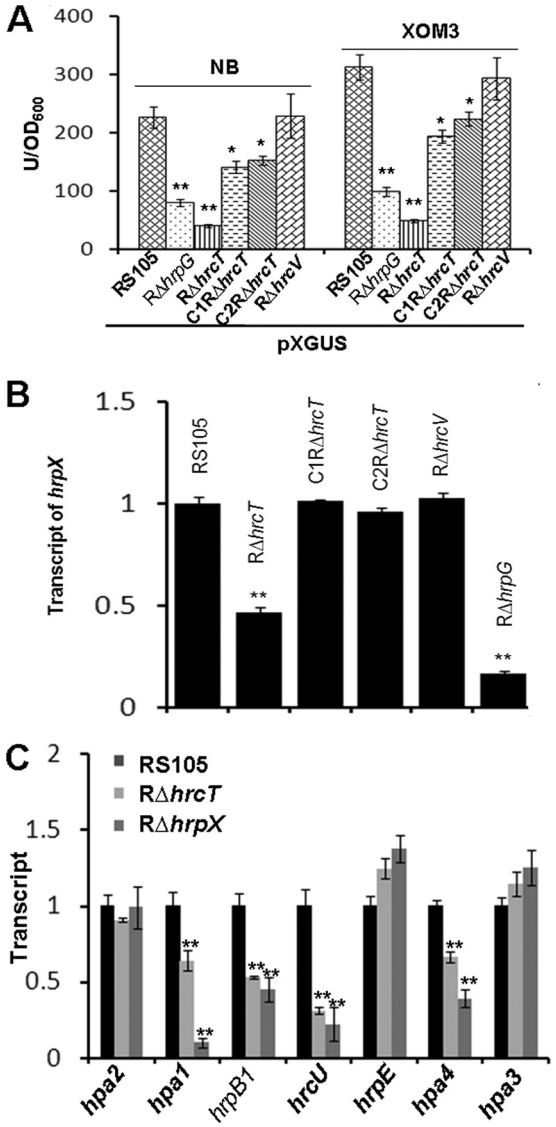

FIG 4 HrcT functions in regulating the expression of *hrpX*. (A) pXGUS-mediated glucuronidase activity in *X. oryzae* pv. oryzicola RS105, RΔ*hrpG*, RΔ*hrcT*, RΔ*hrcV*, and the complemented strains C1RΔ*hrcT* and C2RΔ*hrcT*. Strains were incubated in NB or XOM3 medium for 12 h at 28°C. GUS activity was determined by measuring the OD at 415 nm using 4-MUG as a substrate. (B) Expression analysis of *hrpX* by real-time qRT-PCR. RNAs were isolated from cultures of *X. oryzae* pv. oryzicola RS105, RΔ*hrcT*, RΔ*hrcV*, complemented strains C1RΔ*hrcT* and C2RΔ*hrcT*, and RΔ*hrpG*. All strains were incubated in rice suspension cells at 25°C for 16 h, and relative mRNA levels were calculated with respect to the expression level of the corresponding transcript in the wild-type RS105. (C) Expression analysis of *hrp* genes by qRT-PCR. RNAs were isolated from cultures of the wild-type RS105, RΔ*hrcT*, and RΔ*hrpX*, which were incubated in rice suspension cells at 25°C for 16 h. The relative mRNA levels were calculated with respect to the corresponding transcript in RS105. All the experiments were repeated three times, and similar results were obtained. Data represent the means ± standard deviations of triplicate measurements. Asterisks above columns represent significance based on a paired, two-tailed Student *t* test relative to the wild type. **, $P = 0.01$; *, $P = 0.05$.

grown in both NB and XOM3 (Fig. 4A). Given that HrpG is necessary for the expression of *hrpX* (12), the *hrpG* mutant RΔ*hrpG* was included as a negative control to determine the *hrpX* promoter activity. The expression of *hrpX* in RΔ*hrcT* could be partially restored to the wild-type levels in C1RΔ*hrcT* and C2RΔ*hrcT*, which contain a promoterless *hrcT* gene driven by promoters T1 and T3, respectively (Fig. 4A). Compared to the wild type tested by qRT-PCR, a reduction in the *hrpX* mRNA was detected in RΔ*hrcT* and restored to the wild-type level in complemented strains C1RΔ*hrcT* and C2RΔ*hrcT* (Fig. 4B). To exclude the possibility that the lack of a functional T3SS leads to reduced expression of *hrpX*, we tested the promoter activity and transcript level of *hrpX* in another *hrc* deletion mutant, RΔ*hrcV*. No difference in the promoter activity and the transcript of *hrpX* was observed between wild-type RS105 and RΔ*hrcV* (Fig. 4A and B). These results suggest that the deletion of *hrcT* affecting the expression of *hrpX* is not due to the lack of the functional T3SS.

Since our results indicated that HrcT positively regulates the expression of *hrpX*, we sought to investigate whether the expression of other *hrp-hrc-hpa* genes is positively regulated by HrcT. The wild-type RS105 and RΔ*hrcT* were inoculated into rice, and bacterial mRNAs were extracted and used as the templates in qRT-PCR assays 12 hpi. We chose some *hrp* genes as representation to confirm the conclusion by referring to the study in which the expression of some *hrp* genes was reported to be obviously influenced when *hrpX* is mutated (2). Primers for this experiment were specific for *hpa2*, *hpa1*, *hrpB1*, *hrcU*, *hrpE*, *hpa4*, and *hpa3* (see Table S1 in the supplemental material). qRT-PCR results showed that the expression of *hpa1*, *hrpB1*, *hrcU*, and *hpa4* was significantly ($P \leq 0.01$, *t* test) reduced in RΔ*hrcT* compared to the wild-type RS105, while the expression of *hpa2*, *hrpE*, and *hpa3* was similar in RΔ*hrcT* and RS105 (Fig. 4C). This is consistent with our previous finding that the expression of *hpa1*, *hrpB1*, *hrcU*, and *hpa4* is positively regulated by HrpX while the expression of *hpa2*, *hrpE*, and *hpa3* is not obviously influenced when *hrpX* is mutated (2). Thus, our current results suggest that HrcT positively regulates the expression of *hrpX*, which in turn controls the expression of *hrp-hrc-hpa* genes mentioned above.

HrcT binds the *hrpX* promoter and regulates the expression of *hrpX*. Given that HrcT regulates the expression of *hrpX* as demonstrated above, we speculated that HrcT may bind the *hrpX* promoter for regulation. The promoter of *hrpX* was fused to the promoterless *gusA* gene as a reporter. GUS activity was determined in the presence (+) or absence (−) of HrcT in *N. benthamiana* leaves by using an *Agrobacterium*-mediated transient expression system *in planta* (38). HrcT indeed induced stronger GUS activity when the *hrpX* promoter was present, like the positive control indicating that PthXo1 bound the *Os8N3* promoter to activate the expression of *gusA*, than that when the *hrpX* promoter was absent (Fig. 5A). This indicates that HrcT binds the *hrpX* promoter *in vivo*. In order to find the specific region of the *hrpX* promoter bound by HrcT *in vitro*, electrophoretic mobility shift assays (EMSAs) were performed. Initially, we attempted to overproduce the entire HrcT protein in *E. coli* but were unsuccessful (data not shown). Topology analysis indicated that HrcT contains a 41-amino-acid (aa) region at the N terminus that spans the IM (42), which might lead to our failure in trying to overproduce the intact HrcT in *E. coli*. We then sought to overproduce and purify an N-terminally truncated HrcT (a 41-aa region was deleted, designated HrcTΔ41N) in *E. coli*. The truncated HrcT was tagged with hexahistidine at the C terminus, and the purified HrcTΔ41N-His6 was used in EMSAs. Different lengths of the target *hrpX* promoter were PCR amplified or synthesized, biotinylated, and used as probes. These included probes A, B, and C, which comprised 149-, 78-, and 57-bp fragments upstream of the *hrpX* transcription start codon (ATG), re-

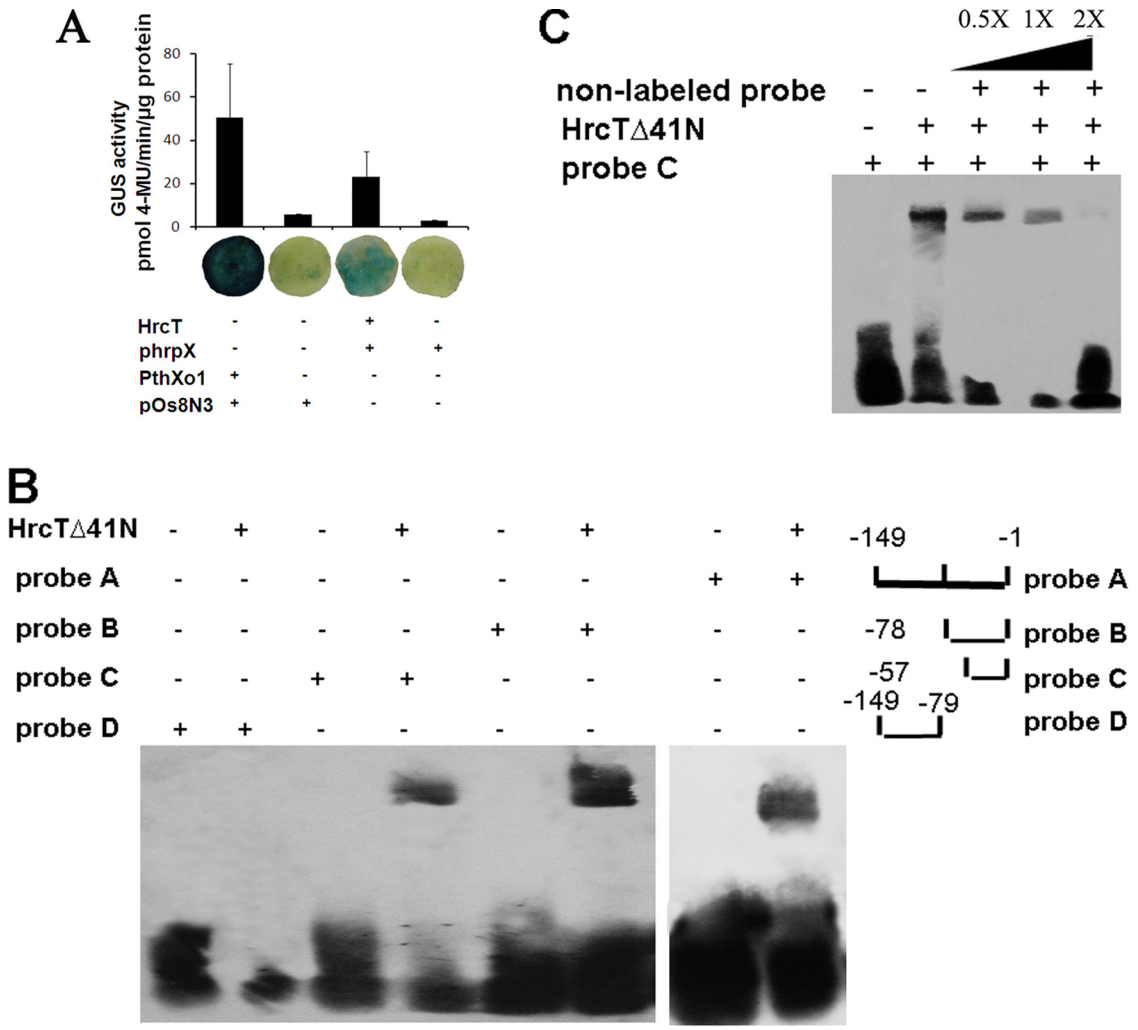

FIG 5 Binding of HrcT to *hrpX* promoter *in vivo* and *in vitro*. (A) *Agrobacterium*-mediated transient expression assay of *hrpX* activated by HrcT. GUS reporter constructs are codelivered via *A. tumefaciens* into *N. benthamiana* with (+) and without (−) the construct HrcT. PthXo1 of *X. oryzae* pv. oryzae and its target *Os8N3* (pOs8N3) were used as a positive control. The GUS activity was determined 2 dpi by stained leaf disks (0.8 cm in diameter) with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide). Blue indicates a positive reaction. Error bars indicate standard deviations ($n = 3$ samples). 4-MU, 4-methyl-umbelliferone. (B) Binding of HrcTΔ41N to the *hrpX* promoter by EMSA. Twenty femtomoles of biotinylated probes was used to react with 1 μg of purified HrcTΔ41N (presence indicated by +, absence by −). Different sizes of the *hrpX* promoter upstream of the *hrpX* transcriptional start codon were used as probes, displayed on the right. Probe A is 149 bp long, probe B 78 bp, probe C 57 bp, all upstream of the *hrcT* transcription start code, and probe D is 70 bp long, upstream of the probe B as displayed on the left. (C) Competition of biotinylated probe C with unlabeled probe C bound to HrcTΔ41N by EMSA. The unlabeled probe C with higher concentration, at 0.5×, 1×, and 2× more than 20 fmol of biotinylated probe C, was used. +, present; −, absent. The above-described experiments were repeated three times, and results from one representative experiment are shown.

spectively, and probe D, a 70-bp fragment upstream of probe B (Fig. 5B). Each probe was incubated with purified HrcTΔ41N-His6 (1 μg) for 20 min at room temperature. After EMSA, we found that HrcTΔ41N-His6 bound to probes A, B, and C but not probe D (Fig. 5B). The observed shift was prevented by nonlabeled probe C when it competed with the labeled probe C (Fig. 5C). These results indicate that HrcT binds the *hrpX* promoter and binding occurs within a 57-bp region upstream of the *hrpX* transcript start codon.

The Hrp phenotype can be partially restored when *hrcT* is expressed under promoters T1 and T3 in the *hrcT* mutant. Our previous work has revealed that the *hrcT* mutant RΔ*hrcT* lost the ability to trigger HR in tobacco and pathogenicity in rice (2). To investigate whether the RΔ*hrcT* complemented strains (C1RΔ*hrcT* and C2RΔ*hrcT*, containing promoters T1 and T3, respectively) have the ability to trigger HR in tobacco and pathogenicity in rice, the tested strains were infiltrated into rice seedlings and inoculated into adult plants. The RΔ*hrcT* complemented strains, C1RΔ*hrcT* and C2RΔ*hrcT*, induced water-soaked symptoms as the wild-type RS105 did 3 dpi (Fig. 6A), but the lesion lengths induced by C1RΔ*hrcT* and C2RΔ*hrcT* were significantly shorter than those induced by the wild-type RS105 (Fig. 6B). In contrast, RΔ*hrcT*, like RΔ*hrcV*, failed to trigger water-soaked lesions (Fig. 6A and B). Bacterial growth in rice tissue was compromised in RΔ*hrcT* and was partially restored to the wild-type level in the complemented strains C1RΔ*hrcT* and C2RΔ*hrcT* (Fig. 6C). Regarding HR induction in tobacco, the *hrcT*-complemented strains C1RΔ*hrcT* and C2RΔ*hrcT* elicited strong HRs 24 hpi when the concentration of bacterial cells was adjusted to an

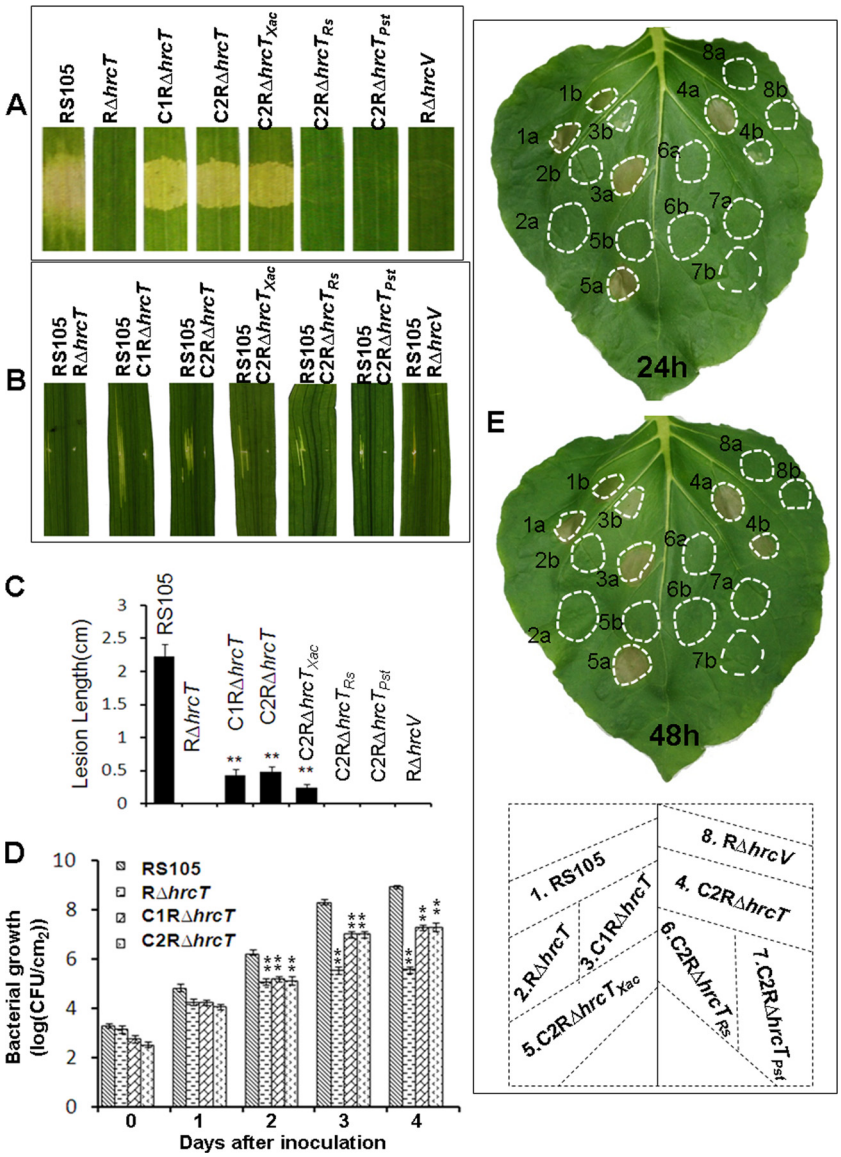

FIG 6 Detection of the HR in tobacco and pathogenicity in rice. (A and B) Symptoms induced by infiltration of *X. oryzae* pv. oryzicola strains (suspended in water, OD_{600} = 0.3) into leaves of rice cv. IR24 (susceptible to *X. oryzae* pv. oryzicola) with a needleless syringe. Photographs were taken 3 dpi. (C) BLS lesion lengths in adult rice (2 months old) inoculated by the leaf-needling method was measured 14 dpi. (D) Bacterial growth in inoculated rice leaves. Bacteria were recovered from inoculated leaves daily for a period of 4 days. (E) Assay for the HR in tobacco (*N. benthamiana*) inoculated with bacterial strains adjusted to the following concentrations: OD_{600} = 0.3 (3×10^8 CFU/ml) and OD_{600} = 0.01 (1×10^5 CFU/ml). Strains are identified by numbers as follows: 1, *X. oryzae* pv. oryzicola RS105; 2, mutant RΔ*hrcT*; 3, *hrcT*-complemented strain C1RΔ*hrcT*; 4, C2RΔ*hrcT*; 5, C2RΔ$hrcT_{Xac}$, RΔ*hrcT* containing *hrcT* from *X. axonopodis* pv. citri in *trans* under the T3 promoter; 6, C2RΔ$hrcT_{Rs}$, RΔ*hrcT* containing *hrcT* from *R. solanacearum* GMI1000 in *trans* under the T3 promoter; 7, CRΔ$hrcT_{Pst}$, RΔ*hrcT* containing *hrcT* from *P. syringae* pv. tomato DC3000 in *trans* under the T3 promoter; 8, RΔ*hrcV* (*hrcV* mutant, negative control). Tobacco leaves were inoculated with the above-listed strains by a needleless syringe, and HRs were scored 24 and 48 hpi, respectively. The experiments were repeated three times. Data represent the means ± standard deviations from three replicates. Asterisks above columns represent significance based on a paired, two-tailed Student *t* test relative to the wild type. **, $P = 0.01$; *, $P = 0.05$.

OD_{600} of 0.3. However, the HR was delayed (e.g., did not appear until 48 hpi) when the OD_{600} was 0.01. As predicted, the T3SS mutant RΔ*hrcV* did not elicit HR in tobacco (Fig. 6D). Collectively, these data indicate that expression of *hrcT* via the T1 or T3 promoter partially restores the HR in tobacco and bacterial virulence in rice.

HrcT is functionally interchangeable between *Xanthomonas* spp. HrcT, a key component of the T3SS, is conserved in both animal- and plant-pathogenic bacteria (26). Phylogenetic analysis of HrcT orthologs from various bacterial pathogens showed that HrcT proteins could be classified into four groups (see Fig. S2 in the supplemental material). Group I contained HrcT proteins from closely related *Xanthomonas* spp.: group II from *Acidovorax citrulli*, *R. solanacearum*, and *Burkholderia* sp.; group III from *P. syringae* pv. tomato DC3000, *E. amylovora*, and *Dickeya dadantii* 3937; and group IV from animal pathogenic *Shigella boydii*, *Salmonella enterica*, and *Yersinia pseudotuberculosis* (see Fig. S2 in the supplemental material). This prompted us to investigate whether HrcT proteins from other bacteria can functionally complement

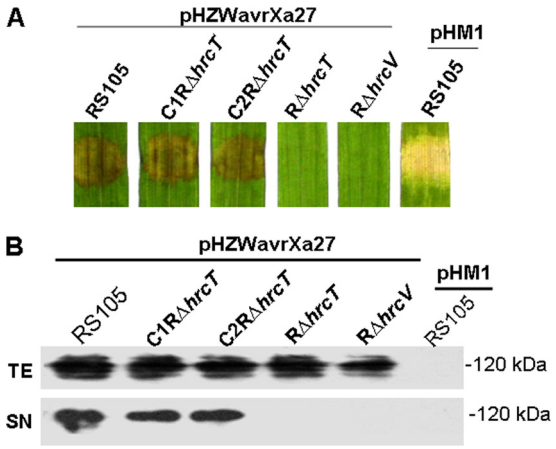

FIG 7 Expression of *hrp* genes and secretion of T3SEs in the *hrcT*-deleted mutant. (A) Symptoms induced in seedlings of rice cv. 87-15 (containing *Xa27*) inoculated with *X. oryzae* pv. oryzicola derivatives. Strains containing pHZWavrXa27 (suspended in water, $OD_{600} = 0.3$) were inoculated into leaves as described in Materials and Methods. (B) Western blot assays to examine the secretion of effector AvrXa27 using a monoclonal anti-FLAG antibody. Strains included *X. oryzae* pv. oryzicola RS105, RΔ*hrcT* (*hrcT* deletion mutant), C1RΔ*hrcT* and C2RΔ*hrcT* (complemented strains of RΔ*hrcT* with *hrcT* under the T1 or T3 promoter), and RΔ*hrcV*, a T3SS mutant (negative control). With the exception of the empty vector control (pHM1), all strains contained pHZWavrXa27 (*avrXa27* is fused to a Flag tag code).

the *X. oryzae* pv. oryzicola *hrcT* mutant for HR and pathogenicity in plants. The promoterless *hrcT* genes from *X. axonopodis* pv. *citri* 306, *R. solanacearum* GMI1000, and *P. syringae* pv. tomato DC3000 were used in this experiment, and these genes were cloned as transcriptional fusions whereby the expression was driven by T1 or T3 promoter from *X. oryzae* pv. oryzicola *hrcT* (Table 1). The transcriptional fusions were introduced into RΔ*hrcT*, and the constructs were designated C1RΔ$hrcT_{Xac}$, C1RΔ$hrcT_{Rs}$, C1RΔ$hrcT_{Pst}$, C2RΔ$hrcT_{Xac}$, C2RΔ$hrcT_{Rs}$, and C2RΔ$hrcT_{Pst}$ (Table 1), respectively. The constructs containing *hrcT* from *X. axonopodis* pv. citri triggered HR in tobacco and BLS symptoms in rice (only the case driven by promoter T3 is shown) (Fig. 6A, B, and D), implying that *hrcT* is interchangeable among *Xanthomonas* spp. HrcT orthologs from *R. solanacearum* and *P. syringae* pv. tomato failed to functionally complement the *hrcT* mutation for HR or BLS symptoms in rice.

The deletion in *hrcT* impairs the secretion of AvrXa27. Considering that HrcT is an inner membrane protein for the T3SS (27, 42), we speculated that an *hrcT* deletion might impair the secretion of T3SEs. To test this hypothesis, we used AvrXa27 to monitor secretion because AvrXa27 also induces HR in rice cv. 87-15 containing the resistance gene *Xa27* (40). Plasmid pHZWavrXa27 containing *avrXa27* (Table 1) was introduced into *X. oryzae* pv. oryzicola RS105, mutant RΔ*hrcT*, complemented strains C1RΔ*hrcT* and C2RΔ*hrcT*, and RΔ*hrcV*, respectively. Three days after infiltration ($OD_{600} = 0.3$) into rice cv. 87-15, tissues inoculated with RS105, C1RΔ*hrcT*, and C2RΔ*hrcT* (each containing pHZWavrXa27) exhibited dark, HR-like symptoms (Fig. 7A). In contrast, RΔ*hrcT* and RΔ*hrcV* containing pHZWavrXa27 did not induce visible symptoms in rice. *X. oryzae* pv. oryzicola RS105 harboring the empty vector pHM1 elicited typical BLS symptoms (Fig. 7A). These results suggest that HrcT functions as a key component of the T3SS and is essential for the secretion of T3SEs into plant cells.

To examine secretion of AvrXa27 via the T3SS *in vitro*, the strains mentioned above were incubated in XOM3 at 28°C for 12 h, and total cell extracts (TEs) and culture supernatants (SNs) were analyzed by immunoblotting using Flag antibodies. Analysis of the SN fractions revealed that AvrXa27 was secreted into the medium by *X. oryzae* pv. oryzicola RS105, C1RΔ*hrcT*, and C2RΔ*hrcT*; however, the protein was not detectable in the SNs of RΔ*hrcT* or RΔ*hrcV* (Fig. 7B). These results indicate that expression of *hrcT* via promoter T1 or T3 in *trans* can complement the secretion defect in the *hrcT* mutant.

DISCUSSION

Previous studies have indicated that the regulation of *hrp-hrc-hpa* genes in *Xanthomonas* requires multiple factors. For example, HrpG regulates the expression of *hrpX* and the *hrpA* transcript, and HrpX activates the expression of the *hrpB* to *hrpF* operons (11, 12). In this study, we investigate potential regulatory roles of HrcT protein, which is a structural component of the T3SS. HrcT functioned as a positive regulator of HrpX, which adds another layer of complexity to *hrp* gene regulation. In our revised model (Fig. 8) (referring to our previous one, described in reference 2), an unknown regulatory factor, possibly regulated by HrpG, may switch on the expression of HrcT via promoter T3; HrcT binds to the *hrpX* promoter to activate *hrpX* transcription; HrpX then binds the PIP box promoters of multiple HrpX regulons to regulate the expression of *hrp-hrc-hpa* genes, including our newly identified *hrp* regulator gene *hrpD6* (2); once the *hrpB* operon is activated by HrpX via promoter T1, the binding of HrcT to HrpX or the mRNA level of *hrcT* may possibly be degraded by another unknown factor (Fig. 8).

Precisely how HrcT modulates HrpX remains unclear. HrcT is a highly conserved component of the T3SS in *Xanthomonas* spp. (27). The N-terminal portion of HrcT presumably spans the IM of bacterial cells (42). Considering our results, we hypothesize that HrcT was possibly synthesized first in cytoplast, where it can bind the *hrpX* promoter to activate the expression of *hrpX*. Meanwhile, it is integrated into the cell membrane for the T3SS. We also show that the expression of *hpa1*, *hrpB1*, *hrcU*, and *hpa4* is reduced in both the *hrcT* and *hrpX* mutants (Fig. 4), which is consistent with coordinated regulation via HrcT/HrpX.

Based on the fact that the expression of promoter T1 is attenuated in the *hrpX* mutant but that of promoter T3 is not (Fig. 3A), we propose that the expression of *hrcT* may be controlled by both promoters T1 and T3. The expression of *hrcT* under promoter T1 (containing the PIP box) was positively regulated by HrpX (2), while *hrcT* under promoter T3 was positively regulated by an unknown factor that may possibly be activated by HrpG (Fig. 8). Activation by an unknown regulatory protein would help explain why *hrcT* can be transcribed independently of the *hrpB* operon promoter. We speculate that HrpX binds to the PIP box in promoter T1 to produce the polycistronic *hrpB* operon, which would include *hrcT* (Fig. 1B). These speculations are consistent with the fact that the *hrcT* mutant can be complemented by expression via either promoter T1 or T3 as shown by partial restoration of HR and pathogenicity *in planta* (Fig. 6). These hypotheses remain highly speculative until the unknown regulator is identified.

Intensive studies have been undertaken to elucidate components of the Hrp regulon in *Xanthomonas* spp. (1, 2, 13). In addition to the regulators HrpG, HrpX, and HrpD6 (2, 11, 12), additional two-component regulatory system (TCS) proteins have

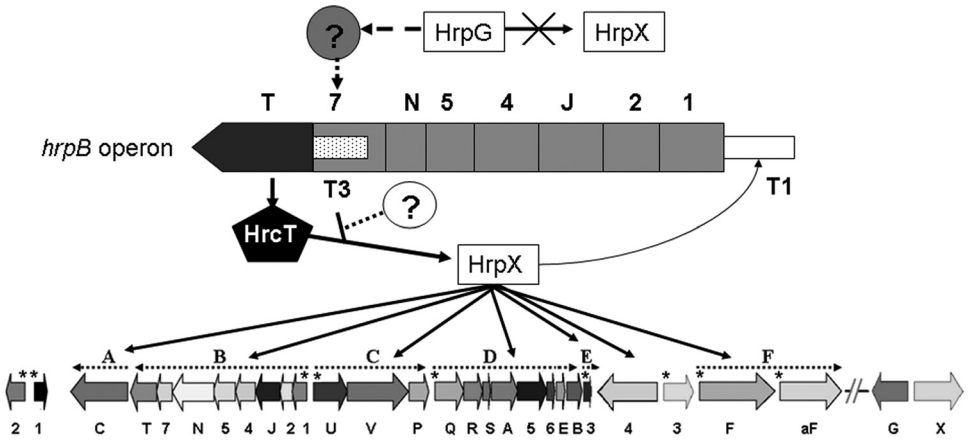

FIG 8 Working model showing the proposed regulatory role of HrcT. Our results, together with our previous report (2), show that HrcT expression at the T3 promoter is HrpG independent. Thus, we propose that an unidentified regulator (question mark in a gray circle), possibly regulated by HrpG, which does not directly regulate the expression of *hrpX* (cross on a black arrow), promotes *hrcT* transcription at the T3 promoter. HrcT then positively regulates the expression of *hrpX* by binding the *hrpX* promoter. The transcriptional activator HrpX regulates multiple *hrp* operons (black-lined arrows) by binding to the PIP box promoter as indicated by asterisks above the *hrp-hrc-hpa* cluster. Another unknown protease-like protein (question mark in a white circle), possibly regulated by HrpX, may degrade the mRNA of *hrcT* or HrcT to repress the expression of *hrpX*. The dashed horizontal arrows represent *hrp* operons (A to F) consisting of individual *hrp-hrc-hpa* genes, which were assigned by choosing the last letter or number of a gene name under differentially shaded arrows, referring to references 2 and 3.

been shown to modulate expression of the T3SS in *Xanthomonas* spp. For example, Li and coworkers (13) recently identified HpaS, the histidine protein kinase that interacts with HrpG via phosphorylation. Other regulators of the TCS include Trh and HpaR1, which were shown to positively regulate the expression of *hrpG* (43, 44). ColS/ColR constitute a TCS that is implicated in virulence and HR *in planta*; these proteins were shown to repress the expression of *hrpG* and the *hrpC* and *hrpE* operons but not other *hrp* genes (45, 46). Zur, a key regulator for zinc homeostasis, positively influenced the expression of *hrp* operons via *hrpX* but not *hrpG* (47). HpaR1 is a GntR family transcriptional activator that regulates the expression of all five operons in the *hrp* cluster via HrpG (48). It seems unlikely that the unknown regulator for HrcT is one of the preceding TCSs, particularly because the expression of *hrpG* is repressed by Trh, HpaR1, ColS/ColR, Zur, and HpaR, and the expression of *hrcT* is occasionally HrpG independent and HrpX dependent (Fig. 3B). In addition to the numerous regulatory loci that map elsewhere in the *Xanthomonas* genomes (16–20), our findings highlight the existence of key regulatory loci that map within the *hrp-hrc-hpa* genes in *X. oryzae* pv. oryzicola. An excellent example is the HrpD6 protein recently identified in our lab. We previously showed that the expression of *hrcT* is reduced in the *hrpD6* mutant; furthermore, *hrpD6* is positively controlled by HrpX (2). It is tempting to speculate that HrpD6 may serve as a negative regulator for the unknown regulatory factor shown in Fig. 8, and experiments to test this hypothesis are under way in our laboratory.

The expression of *hrcT* may be stimulated by an environmental or plant signal that is sensed by membrane-associated proteins. For example, the OM portion of HpaS presumably senses plant stimuli during the early stages of infection and transphosphorylates HrpG (13). As noted in our model, the activated form of HrpG may function via an unknown factor to activate HrcT expression via promoter T3. More detailed studies of genes that are expressed independently of HrpG and/or HrpX (e.g., HrcC, HrcT, HrpD5, HrpE, and Hpa3) will help further elucidate *hrp* regulatory networks in *Xanthomonas*.

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