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e-Xtra*

A Novel Regulatory Role of HrpD6 in Regulating *hrp-hrc-hpa* Genes in *Xanthomonas oryzae* pv. *oryzicola*

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***Xanthomonas oryzae* pv. *oryzicola*, the causal agent of bacterial leaf streak in the model plant rice, possesses a hypersensitive response and pathogenicity (*hrp*), *hrp*-conserved (*hrc*), *hrp*-associated (*hpa*) cluster (*hrp-hrc-hpa*) that encodes a type III secretion system (T3SS) through which T3SS effectors are injected into host cells to cause disease or trigger plant defenses. Mutations in this cluster usually abolish the bacterial ability to cause hypersensitive response in nonhost tobacco and pathogenicity in host rice. In *Xanthomonas* spp., these genes are generally assumed to be regulated by the key master regulators HrpG and HrpX. However, we present evidence that, apart from HrpG and HrpX, HrpD6 is also involved in regulating the expression of *hrp* genes. Interestingly, the expression of *hpa2*, *hpa1*, *hpaB*, *hrcC*, and *hrcT* is positively controlled by HrpD6. Transcriptional expression assays demonstrated that the expression of the *hrcC*, *hrpD5*, *hrpE*, and *hpa3* genes was not completely abolished by *hrpG* and *hrpX* mutations. As observed in analysis of their corresponding mutants, HrpG and HrpX exhibit contrasting gene regulation, particularly for *hpa2* and *hrcT*. Other two-component system regulators (Zur, LrpX, ColR/S, and Trh) did not completely inhibit the expression of *hrcC*, *hrpD5*, *hrpE*, and *hpa3*. Immunoblotting assays showed that the secretion of HrpF, which is an HpaB-independent translocator, is not affected by the mutation in *hrpD6*. However, the mutation in *hrpD6* affects the secretion of an HpaB-dependent TAL effector, AvrXa27. These novel findings suggest that, apart from HrpG and HrpX, HrpD6 plays important roles not only in the regulation of *hrp* genes but also in the secretion of TAL effectors.**

Encoded by the hypersensitive response and pathogenicity (*hrp*) genes, the type III secretion system (T3SS) delivers numerous T3SS effectors from plant-pathogenic bacteria into plant cells to cause diseases in susceptible host plants and to trigger a hypersensitive response (HR) in nonhost plants or resistant host plants (Alfano and Collmer 1997; Bonas 1994; Cunnac et al. 2009; Gürlebeck et al. 2006; He 1998; Hueck 1998). In xanthomonads, the *hrp-hrc-hpa* genes are highly conserved and clustered within the genome (Alfano and Collmer 1997; Bonas et al. 1991; Kim et al. 2003; Niño-Liu and Bogdanove 2006; Zou et al. 2006). The elucidation of the *hrp-hrc-hpa* cluster in *Xanthomonas oryzae* pv. *oryzicola* RS105 (Zou et al. 2006) and sequencing the complete genome of *X. oryzae* pv. *oryzicola* BLS256 (Niño-Liu and Bogdanove 2006) revealed that the core *hrp* cluster is composed of 10 *hrp*, 9 *hrp*-conserved (*hrc*), and 8 *hrp*-associated (*hpa*) genes, which is similar in organization in different *X. oryzae* pv. *oryzae* strains (Lee et al. 2005; Ochiai et al. 2005; Salzberg et al. 2008). However, in spite of the common synteny of *hrp* genes in two closely related *Xanthomonas* pathovars (*X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*), the latter has *hrpE3*, which is specific to *X. oryzae* pv. *oryzicola* and is homologous to *hpaE* in *X. campestris* pv. *vesicatoria* (Büttner et al. 2007).

Expression of the *hrp-hrc-hpa* genes is highly regulated and is generally suppressed in rich media but induced in planta and in certain synthetic media which mimic conditions inside the host plant (Brito et al. 1999; Schulte and Bonas 1992; Wei et al. 2000; Xiao et al. 1992; Xiao et al. 2007). The detection of expression of *hrp* genes is traditionally assessed by polar transposon insertions using the β-glucuronidase *gusA* gene as a reporter (Wengelnik and Bonas 1996; Wengelnik et al. 1996a and b) but it is believed that polar insertional mutagenesis may interfere in the crosstalk involving intergenic regions and genes in the *hrp* operon or regulon (Ortiz-Martin et al. 2010a and b).

Typically, the expression of each *hrp-hrc-hpa* gene is controlled mainly by two key master regulators, HrpG and HrpX, whose genetic loci are distant from the *hrp-hrc-hpa* cluster on the chromosome in *Xanthomonas* spp. (Büttner and Bonas 2006; Kim et al. 2003; Tang et al. 2006; Weber et al. 2007; Zou et al. 2006). HrpG is predicted to be an OmpR family response regulator of two-component signal transduction systems and, presumably, perceives an environmental signal via an as-yet-unknown sensor kinase (Wengelnik et al. 1996a and b, 1999). HrpX is an AraC-type transcriptional activator (Wengelnik et al. 1996a and b) which forms a homodimer, a helix-turn-helix motif that interacts with each TTCGC of the

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plant-inducible promoter (PIP)-box (TTCGC-N_{15}-TTCGC) in *hrp* transcripts to activate transcription of *hrp* genes (Furutani et al. 2006). In *X. campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease in pepper and tomato, HrpG and HrpX form a regulatory cascade in which HrpG regulates the expression of the *hrpA* operon and *hrpX*. HrpX subsequently activates the expression of other PIP-box containing *hrp* operons (*hrpB* to *hrpF*) (Wengelnik and Bonas 1996; Wengelnik et al. 1996a and b). Further investigation revealed that *hrcC* expression is regulated both by HrpG and HrpX (Huang et al. 2009). Although the *hrp* clusters among *Xanthomonas* spp. are highly conserved (Büttner et al. 2003; Gürlebeck et al. 2006), very little information is available about the regulatory cascade controlling expression of the *hrp* operons in different species or pathovars of *Xanthomonas*.

It has been shown that other regulatory genes may also be involved in the transcriptional regulation to the expression of *hrpG* gene along with other *hrp* genes. The *hrpG* gene expression in *X. oryzae* pv. *oryzae* is repressed by multiple regulatory pathways, including the two-component system PhoP/Q (Lee et al. 2008), the H-NS protein XrvA (Feng et al. 2009), and Trh, which is a member of the GntR family of regulators (Tsuge et al. 2006). In *X. campestris* pv. *campestris*, expression of the *hrpC* and *hrpE* operons located in the *hrp* cluster is controlled by the two-component regulatory system ColR/S, suggesting that various signal transduction pathways are involved in the regulation of *hrp* gene expression and that individual *hrp* operons might be regulated by different two-component signaling transduction pathways (Zhang et al. 2008). Consistent with this finding is that several *hrp* genes from *X. axonopodis* pv. *citri* are induced in the minimal medium XVM2, whereas *hrpB1* is repressed (Astua-Monge et al. 2005). This indicates that there might also be pathovar-specific differences in *hrp* gene expression in *Xanthomonas* spp. (Seo et al. 2008). The *Xanthomonas* bacterial regulatory network controlling *hrp* expression has been reviewed recently by Büttner and Bonas (2010).

Recent studies have shown that other two-component regulatory systems influence the expression of *hrp* genes which, in turn, are fine tuned by several environmental factors. In *X. oryzae* pv. *oryzae*, PhoP, a member of the well-characterized PhoP/Q two-component bacterial signal transduction system, is involved in regulation of *hrpG* expression in *X. oryzae* pv. *oryzae* in response to low Ca^{2+} concentration (Lee et al. 2008). LrpX, a putative leucine-rich protein, regulates the expression of *hrp* genes independently of the regulatory pathway for HrpG-HrpX in *X. oryzae* pv. *oryzae* (Islam et al. 2009). A recent report revealed that Zur, a zinc uptake regulator belonging to the Fur family of transcription factors, from *X. campestris* pv. *campestris* 8004, positively regulates expression of *hrp* genes, presumably via the transcriptional activator HrpX (Huang et al. 2009).

Although the coordinated expression of *Xanthomonas hrp-hrc-hpa* expression is orchestrated by multiple two-component systems and transcriptional regulators such as Trh, Clp, Zur, LrpX, and ColR/S, there is always a basal expression level of *hrp* genes that is not influenced by these regulators. There is no evidence that an internal Hrp protein encoded by the *hrp-hrc-hpa* cluster has the ability to control *hrp-hrc-hpa* expression in *Xanthomonas* spp. In this study, we used the *hrp-hrc-hpa* cluster of a model plant-pathogenic bacterium *X. oryzae* pv. *oryzicola*, the causal agent of bacterial leaf streak disease in rice, to demonstrate that all of the *hrp-hrc-hpa* genes except *hpa2*, *hrcC*, *hrcT, hrpD5*, *hrpE*, and *hpa3* are positively regulated in a coordinated fashion by HrpG and HrpX. The expression of *hpa2* was positively regulated by HrpG but not by HrpX, and the expression of *hrcT* was positively regulated by HrpX but negatively regulated by HrpG. We also provide experimental evidence that the expression of *hpa2*, *hpa1*, *hpaB, hrcC*, and *hrcT* is positively controlled by HrpD6. The *hrpD6* mutants are deficient in secretion of Hpa2, Hpa1, and HpaB-dependent T3SS effectors, such as the TAL effector AvrXa27. This is the first report that the expression of *hrcC*, *hrpD5*, *hrpE*, and *hpa3* is HrpG- and HrpX-independent and the internal *hrp* gene product HrpD6 has the novel regulatory role in the expression of Hrp proteins and also is involved in the secretion of Hpa proteins and TAL effectors in *Xanthomonas* spp.

RESULTS

Nonpolar mutations in the *hrp* system of *X. oryzae* pv. *oryzicola*.

To precisely evaluate the expression profiles of the individual genes, 27 *hrp-hrc-hpa* genes, 2 key *hrp* regulatory genes (*hrpG* and *hrpX*) (Table 1; Fig. 1A), and 5 two-component system regulator genes (*trh*, *zur*, *lrpX*, *colR*, and *colS*) (Table 1; Supplementary Fig. S2) in *X. oryzae* pv. *oryzicola* RS105 were deleted individually by using vector pKMS1 containing the *sacB* gene (Jiang et al. 2009). Two flanking fragments located at various distances upstream and downstream of the target genes were fused in pKMS1 (Table 1), and the deletions were obtained by SacB/sucrose counterselection (details below). Each mutation was confirmed by polymerase chain reaction (PCR) and Southern hybridization (data not shown). Based on pathogenicity assays in rice cv. IR24, which is susceptible to *X. oryzae* pv. *oryzicola*, and the ability to elicit HR response in nonhost tobacco cultivar Xanthi, the phenotypes (Fig. 1A) exhibited by the 26 *hrp-hrc-hpa* mutants (excluding *hrpE3*) and 2 key *hrp* regulatory gene mutants, *hrpG* and *hrpX*, were consistently similar to those caused by 26 *hrp-hrc-hpa* gene mutants and *hrpG* and *hrpX* mutants in *X. oryzae* pv. *oryzae* (Cho et al. 2008), the causal agent of bacterial blight in rice. A nonpolar mutation in the *hrpE3* gene of *X. oryzae* pv. *oryzicola*, homologous to *hpaE* in *X. campestris* pv. *vesicatoria* (Büttner et al. 2007), led to reduced virulence in host rice but retained the ability to elicit HR in nonhost tobacco (Fig. 1A). These results indicate that *hrp-hrc* genes (except *hrpE3*) in the *hrp* cluster and *hrpG* and *hrpX* genes are essential for both the pathogenicity of *X. oryzae* pv. *oryzicola* in host rice and HR induction in nonhost tobacco. Disruption of *hpa* genes (except *hpaB*) had little effect on pathogenicity in rice and HR induction in tobacco but was deficient in virulence in comparison to the wild-type strain (Fig. 1A), suggesting that these *hpa* genes are required for full virulence of *X. oryzae* pv. *oryzicola* in rice. On the other hand, mutations in either the promoter of the *hrpB* operon (*phrcT1*) or in the promoter of the *hrpD* operon (*phrpD51*) (Table 1), which contains a PIP-box, resulted in loss of pathogenicity of *X. oryzae* pv. *oryzicola* in rice and HR induction in tobacco (data not shown).

Among the mutants with deletions in the *zur*, *lrpX*, *colR*, *colS*, and *trh* genes (Table 1), only the *colR* mutant lost pathogenicity in rice. The remainder exhibited HR in tobacco but showed reduced virulence in rice. Homologs of *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* have been reported to cause delayed pathogenicity in host plants and HR in nonhost tobacco (Huang et al. 2009; Islam et al. 2009; Tang et al. 2005; Tsuge et al. 2006; Yang et al. 2007; Zhang et al. 2008).

HrpG and HrpX exhibit differential regulatory roles in the expression of *hrp-hrc-hpa* genes in *X. oryzae* pv. *oryzicola.*

Expression of the *hrp-hrc-hpa* genes is induced upon interaction of pathogenic *Xanthomonas* strains with host plants (Schulte and Bonas 1992; Zou et al. 2006). Though it has been considered that HrpG and HrpX regulate the expression of the

hrp cluster, it is unclear whether these two regulators control the expression of all the *hpa-hrp-hrc* genes in *Xanthomonas* spp. Our previous report demonstrated that the *hrp*-inducing medium XOM3 (XOM2-modified medium) could induce *hrp* gene expression similar to that observed when the bacterium was co-cultured in rice suspension cells (Xiao et al. 2007). To mimic the growth conditions of *X. oryzae* pv. *oryzicola* in rice tissue, we used rice suspension cells to investigate whether the expression of all the *hrp-hrc-hpa* genes is regulated by HrpG and HrpX by reverse-transcription (RT)-PCR. After 16 h of interaction with rice suspension cells, 27 *hpa-hrp-hrc* genes and 2 regulatory genes (*hrpG* and *hrpX*) were induced in the wild-type strain *X. oryzae* pv. *oryzicola* RS105 (Fig. 1B). However, in the *hrpG* mutant RΔ*hrpG*, expression of *hrcC, hrcT, hrpD5, hrpE*, and *hpa3* genes is still observed (Fig. 1B). The expression of *hpa2, hrcC, hrpD5, hrpE*, and *hpa3* was not completely attenuated in the *hrpX* mutant RΔ*hrpX* (Fig. 1B). This result indicates that the expression of *hrcT* was positively regulated by HrpX but negatively regulated by HrpG. In contrast, *hpa2* expression was positively controlled by HrpG but not by HrpX. Comparison of *hrp-hrc-hpa* gene expression profiles in *hrpG* and *hrpX* mutants suggests that the expression of *hrcC, hrpD5, hrpE*, and *hpa3* is partially HrpG and HrpX independent in *X. oryzae* pv. *oryzicola*.

Genes with PIP-box promoters are induced under *hrp*-inducing conditions.

In *Xanthomonas* spp., the expression of *hrp-hrc-hpa* genes is induced by the regulators *hrpG* and *hrpX* and it has been seen to be induced in nutrient-deficient media but repressed in nutrient-rich media (Tsuge et al. 2001; Wengelnik et al. 1996a and b; Xiao et al. 2007). The expression profiles of the *hrcC*, *hrpD5*, *hrpE*, and *hpa3* genes which are partially HrpG and HrpX independent prompted us to investigate whether their promoters are possibly regulated by HrpX by searching for the presence of a PIP-box sequence using online software. The

Table 1. Strains and plasmids mainly used in this study

Strains	Relevant characteristics[a]	Source
Escherichia coli		
DH5α	F⁻ Φ80d*lacZ* ΔM15Δ (*lacZYA-argF*)U169 *endA1 deoR recA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Clontech, Palo Alto, CA, U.S.A.
Xanthomonas oryzae pv. *oryzicola*		
RS105	Wild type, Chinese race 2; Rif^r	Lab collection
RΔ*hrpG*	*hrpG* knock-out mutant of strain RS105, 792 bp, 1 to 792 (–); Rif^r	Jiang et al. 2009
RΔ*hrpX*	*hrpX* knock-out mutant of strain RS105, 1,431 bp, 1 to 1431(+); Rif^r	Jiang et al. 2009
RΔ*hrcV*	*hrcV* knock-out mutant of strain RS105,1,938 bp, 372 to 1920 (+); Rif^r	Wang et al. 2009
RΔ*hrpD6*	*hrpD6* knock-out mutant of strain RS105, 243 bp, 1 to 243 (+); Rif^r	This work
RΔ*hpaB*	*hpaB* knock-out mutant of strain RS105, 471 bp, 1 to 471 (+); Rif^r	This work
RΔ*zur*	*zur* knock-out mutant of strain RS105, 507 bp, 60 to 465(+); Rif^r	This work
RΔ*lrpX*	*lrpX* knock-out mutant of strain RS105, 1,959 bp, 120 to 1811(+); Rif^r	This work
RΔ*colR*	*colR* knock-out mutant of strain RS105, 678 bp, 73 to 622(–); Rif^r	This work
RΔ*colS*	*colS* knock-out mutant of strain RS105, 1,335 bp, 85 to 1172(–); Rif^r	This work
RΔ*trh*	*trh* knock-out mutant of strain RS105, 759 bp, 56 to 105(+); Rif^r	This work
RΔ*hrpD51*	*phrpD51* knock-out mutant of strain RS105; Rif^r	This work
RΔ*hrpD52*	*phrpD52* knock-out mutant of strain RS105; Rif^r	This work
CRΔ*hrpD6*	RΔ*hrpD6* containing pC*hrpD6*; Sp^r	This work
RS106(*hrpD6*)	RS105 containing pC*hrpD6*; Sp^r	This work
RΔ*hrpX*(pD6hpa1GUS)	RΔ*hrpX* containing pD6hpa1GUS; Kan^r	This work
RΔ*hrpX*(phpa1GUS)	RΔ*hrpX* containing phpa1GUS; Kan^r	This work
Plasmids		
pKMS1	6.4 kb, derivative from pK18mobGII, *sacB*$^+$; Kan^r	Lab collection
pHM1	Broad-host range cos parA IncW derivative of pRI40; Sp^r, Sm^r	Lab collection
pKMSΔ*hpaB*	A 1,109-bp fusion ligated in pKMS1 with a 471-bp deletion in *hpaB*; Kan^r	This work
pKMSΔ*zur*	A 934-bp fusion ligated in pKMS1 with a 406-bp deletion in *zur*; Kan^r	This work
pKMSΔ*lrpX*	A 1,026-bp fusion ligated in pKMS1 with a 1,692-bp deletion in *lrpX*; Kan^r	This work
pKMSΔ*colR*	A 865-bp fusion ligated in pKMS1 with a 550-bp deletion in *colR*; Kan^r	This work
pKMSΔ*colS*	A 732-bp fusion ligated in pKMS1 with a 1,088-bp deletion in *cols*; Kan^r	This work
pKMSΔ*trh*	A 837-bp fusion ligated in pKMS1 with a 450-bp deletion in *trh*; Kan^r	This work
pKMSΔ*phrpD51*	A 809-bp fusion ligated in pKMS1 with deletion in *phrpD51*; Kan^r	This work
pKMSΔ*phrpD52*	A 696-bp fusion ligated in pKMS1 with deletion in *phrpD52*; Kan^r	This work
pAvrXa27-FLAG	pHM1 expressing AvrXa27 under the control of *lacZ* promoter with a FLAG tag, Sp^r	Gu et al. 2005
pHrpF-c-Myc	pHM1 expressing HrpF under the control of its own promoter with a c-Myc tag; Sp^r	This work
pHpa1-c-Myc	pHM1 expressing Hpa1 under the control of its own promoter with a c-Myc tag; Sp^r	This work
pHpa2-c-Myc	pHM1 expressing Hpa2 under the control of its own promoter with a c-Myc tag; Sp^r	This work
pC*hrpD6*	pHM1 expressing *hrpD6* under the control of the *hrpD* operon promoter; Sp^r	This work
pD6hpa1GUS	pUFR034 expressing GUS under the promoter of *hpa1* with the *hrpD6* constitutively expressed; Kan^r	This work
phrpGGUS	pUFR034 expressing GUS under the promoter of *hrpG*; Kan^r	This work
phrpXGUS	pUFR034 expressing GUS under the promoter of *hrpX*; Kan^r	This work
phpa3GUS	pUFR034 expressing GUS under the promoter of *hpa3*; Kan^r	This work
phrcCGUS	pUFR034 expressing GUS under the promoter of *hrcC*; Kan^r	This work
phrpD51GUS	pUFR034 expressing GUS under the promoter of *hrpD* operon; Kan^r	This work
phrpD52GUS	pUFR034 expressing GUS under the predicted promoter region upstream of *hrpD5*; Kan^r	This work
phrcT1GUS	pUFR034 expressing GUS under the promoter of *hrpB* operon; Kan^r	This work
phrcT2GUS	pUFR034 expressing GUS under the predicted promoter region upstream of *hrcT*; Kan^r	This work
phpa2GUS	pUFR034 expressing GUS under the promoter of *hpa2*; Kan^r	This lab
phpa1GUS	pUFR034 expressing GUS under the promoter of *hpa1*; Kan^r	This work

[a] Length of the genes measure in base pairs (bp), and range indicates deleted position from site (bp) to site (bp) of the target open reading frames; + = positive chain and – = negative chain. Rif^r, Sp^r, Kan^r, and Sm^r indicate resistant to rifampin, kanamycin, spectinomycin, and streptomycin, respectively; GUS = β-glucuronidase.

deduced promoters—*phrcC* for the *hrcC* gene in *hrpA* operon from –285 to –1 bp (no PIP-box sequence) upstream of the *hrcC* open reading frame (ORF), *phrcT1* for the *hrpB* operon from –177 to 155 bp (with PIP-box sequence) overlap and upstream of the *hrpB1* gene, *phrcT2* possible for the *hrcT* gene from 1,200 to 1,427 bp (no PIP-box sequence) within the *hrcN* gene, *phrpD51* for the *hrpD* operon from –606 to –237 bp (with PIP-box sequence) upstream of the *hrcQ* gene, *phrpD52* possible for the *hrpD5* gene from 613 to 820 bp (no PIP-box sequence) within the *hpaA* gene and upstream of the *hrpD5* gene, and *phpa3* for the *hpa3* operon from –129 to 83 bp overlap and upstream of the *hpa3* gene (Fig. 2A; Table 2)—were PCR amplified with the corresponding primers (Table 2) and cloned upstream from a promoterless *gusA* gene preceded by a termination codon in a reporter plasmid (Table 1). The plasmids were introduced into *X. oryzae* pv. *oryzicola* wild-type strain RS105, and each transformants were incubated in rice suspension cells, an *hrp*-inducing medium XOM3, and nutrient agar without agar (NB) nutrient-rich medium for 16 h, respectively. Then, the β-glucuronidase (GUS) activity was

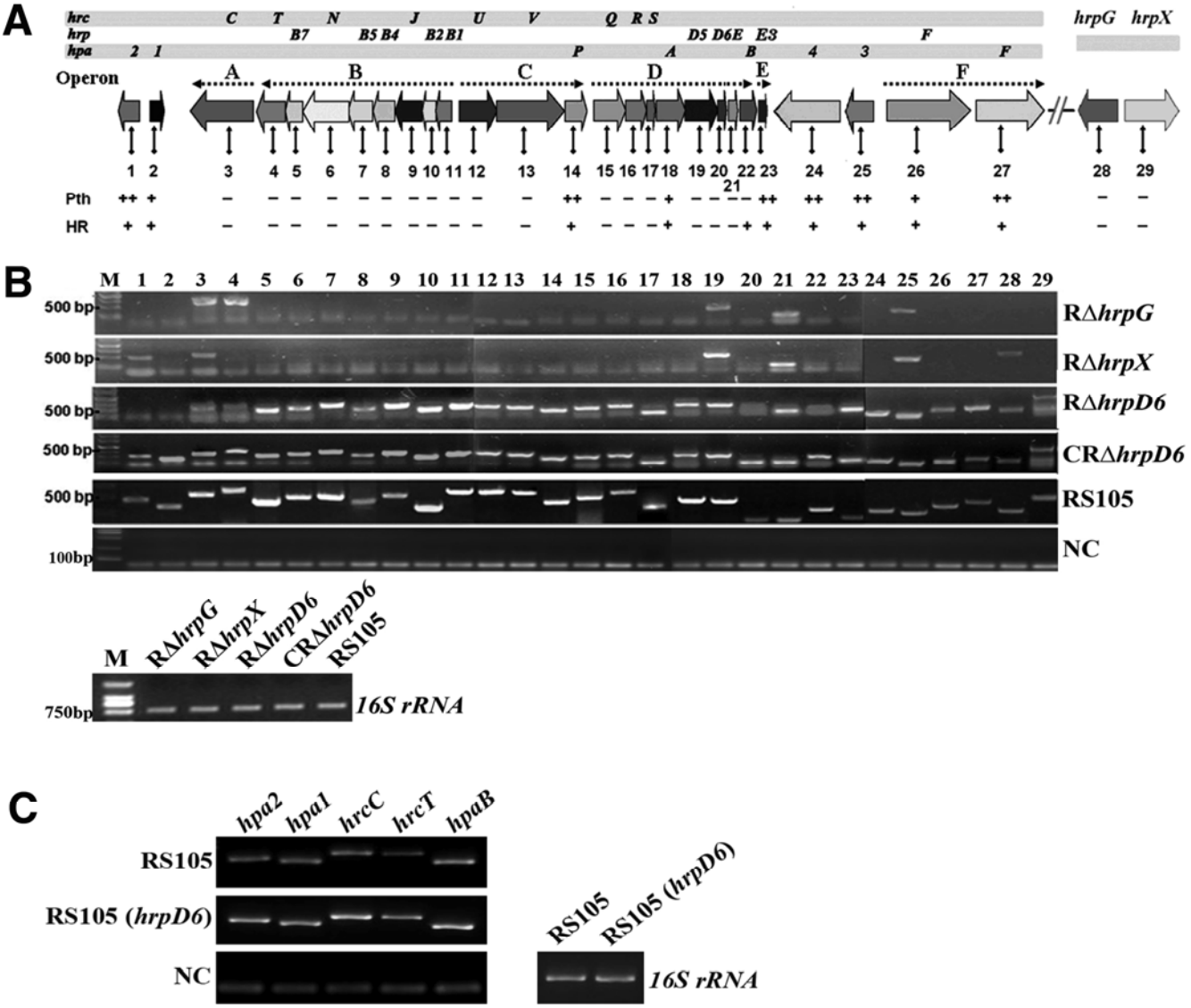

Fig. 1. Expression profiles of the hypersensitive response and pathogenicity (*hrp*), *hrp*-conserved (*hrc*), *hrp*-associated (*hpa*) gene cluster (*hrp-hrc-hpa*) of *Xanthomonas oryzae* pv. *oryzicola* in the *hrpG*, *hrpX*, and *hrpD6* mutant backgrounds. **A,** Genetic organization of 27 *hrp-hrc-hpa* genes and two key regulatory genes (*hrpG* and *HrpX*) and their contribution to pathogenicity in susceptible host rice cv. IR24, and hypersensitive response (HR) induction in the non-host tobacco cultivar Xanthi. Transcript units of these *hrp-hrc-hpa* genes are modified based on the reference (Zou et al. 2006) and the data in this report. Above the *hrp* clusters, each gene is indicated correspondingly by *hrp*, *hrc*, and *hpa*. Below each *hrp-hrc-hpa* gene, the vertical arrows display the deletion mutants achieved and numbered in this report. Under the numbers, the pathogenicity (Pth) in rice and HR in tobacco by each mutant are demonstrated by "+", "++", and "–"; + indicates that the mutants still triggered pathogenicity in rice and HR in tobacco similar to the wild-type strain RS105 and – means that the mutants lost the ability to trigger pathogenicity and HR in plants. The pathogenicity of each *hrp* mutant is represented as follows (mm refers to lesion length): –, no lesion formed; +, less than 5 mm; ++, 5 to 10 mm. As the control, the wild-type strain RS105 formed a 15-mm lesion length. **B,** Expression profiles of the *hrp-hrc-hpa* genes revealed at transcriptional level by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). **C,** HrpD6 overexpression increased the expression of *hpa2*, *hpa1*, *hrcC*, *hrcT*, and *hpaB*. RT-PCR was performed to assay the transcription levels of the tested genes in the wild-type RS105, the *hrpG* mutant RΔ*hrpG*, the *hrpX* mutant RΔ*hrpX*, the *hrpD6* mutant RΔ*hrpD6*, the complementation strain CRΔ*hrpD6* of RΔ*hrpD6* with the *hrpD6* gene driven by the *hrpD* operon promoter, and the *hrpD6* overexpression strain RS105 (*hrpD6*), grown in rice suspension cells for 16 h, by using the primer pairs of the individual *hrp-hrc-hpa* genes of *X. oryzae* pv. *oryzicola*. In addition, the extracted RNAs were used for PCR to ensure that samples were free of DNA contamination as the negative control (NC) where only primer dimers formed. The PCR products were analyzed in 1.2% agarose gels. The *16S rRNA* gene of the pathogen was used as the internal control to verify the absence of significant variation at the cDNA level in samples. Lanes numbered on the top of the gel match the numbers indicating each of the *hrp* genes. The experiment was repeated at least three times and similar results were obtained.

measured by using ρ-nitrophenyl β-D-glucuronide (PNPG) as the substrate (Fig. 2B). The transformants harboring the predicted promoters, *phrcC*, *phrcT1*, *phrpD51*, and *phpa3*, exhibited significantly higher activity in both rice suspension cells and the *hrp*-inducing XOM3 compared with expression in NB medium (*t* test, $P = 0.01$) (Fig. 2B), indicating that the transcription of the *hrcC*, *hrcT*, *hrpD5*, and *hpa3* genes in *X. oryzae* pv. *oryzicola* is induced in *hrp*-inducing conditions and repressed in nutrient-rich medium. On the other hand, *gusA* expression driven by the *phrcT2* or the *phrpD52* promoter was similar among cells induced in rice suspension cells, XOM3, or NB media (Fig. 2B).

Global regulators Trh, Zur, LrpX, and ColR/S have regulatory effects on the expression of *hrcC*, *hrpE*, *hrpG*, and *hrpX* but not *hrcT*, *hrpD5*, and *hpa3*.

Previous reports showed that Trh transcriptionally activates *hrpG* expression (Tsuge et al. 2006), and LrpX negatively regulates the transcripts of *hrpX* and most *hrp* operons in *X. oryzae* pv. *oryzae* (Islam et al. 2009). It is also known that, in *X. campestris* pv. *campestris*, Zur regulates the expression of the *hrp-hrc-hpa* cluster via *hrpX* but not *hrpG* (Huang et al. 2009), and ColR/S positively regulates expression of the *hrpC* and *hrpE* operons (Zhang et al. 2008). To see whether these regulators are involved in regulation of *hrpG*, *hrpX*, *hrcC*, *hrcT*, *hrpD5*, *hrpE*, and *hpa3*, we first generated *trh*, *lrpX*, *zur*, *colR*, and *colS* deletions in *X. oryzae* pv. *oryzicola* using the suicide vector pKMS1 (Jiang et al. 2009), resulting in RΔ*trh*, RΔ*lrpX*, RΔ*zur* RΔ*colR*, and RΔ*colS* mutants (Table 1), respectively. Subsequently, the reporter plasmids mentioned above were introduced into the mutant strains RΔ*hrpX*, RΔ*hrpG*, RΔ*zur*, RΔ*lrpX*, RΔ*colR*, RΔ*colS*, and RΔ*trh*. The GUS activities of the resulting reporter strains were measured after 16 h of growth in the *hrp*-inducing XOM3. The results indicated that *hrcC* expression was downregulated in RΔ*hrpG*, RΔ*zur*, RΔ*colR*, and RΔ*trh* compared with the wild-type strain RS105 and strains RΔ*hrpX*, RΔ*lrpX*, and RΔ*colS* (Fig. 3).

In the case of the *hrpB* operon, the GUS activity of *phrcT1* and *phrcT2* was not significantly affected in these five global regulator mutant backgrounds and in the *hrpG* mutant, RΔ*hrpG*, whereas the GUS activity of *phrcT1* was considerably (*t* test, $P = 0.01$) reduced in the RΔ*hrpX* background (Fig. 3). This suggests that expression of the *hrpB* operon, including the *hrcT* gene, is positively regulated by HrpX, and the mutations in *hrpG*, *zur*, *lrpX*, *colR*, *colS*, and *trh* have no effect on transcription of the *hrpB* operon in *X. oryzae* pv. *oryzicola*. Additionally, no change in GUS activity was observed in any of the strains with mutations in these regulatory genes harboring phrpD52GUS whereas the GUS activity of *phrpD51* was significantly decreased in the RΔ*hrpX* background (Fig. 3), which indicates that the *hrpD* operon is possibly regulated by HrpX. In addition, *hpa3* expression was not significantly affected in any of the mutants with deletions in the above regulatory genes (Fig. 3), suggesting that *hpa3* expression is not controlled by HrpG, HrpX, Zur, LrpX, CorR, ColS, or Trh. On the other hand, the mutation in *trh* led to significantly lower GUS activity with the *hrpG* promoter, compared with that in other regulatory gene mutants and the wild type (Fig. 3C). This is consistent with the observation that Trh positively regulates the expression of *hrpG* in *X. oryzae* pv. *oryzae* (Tsuge et

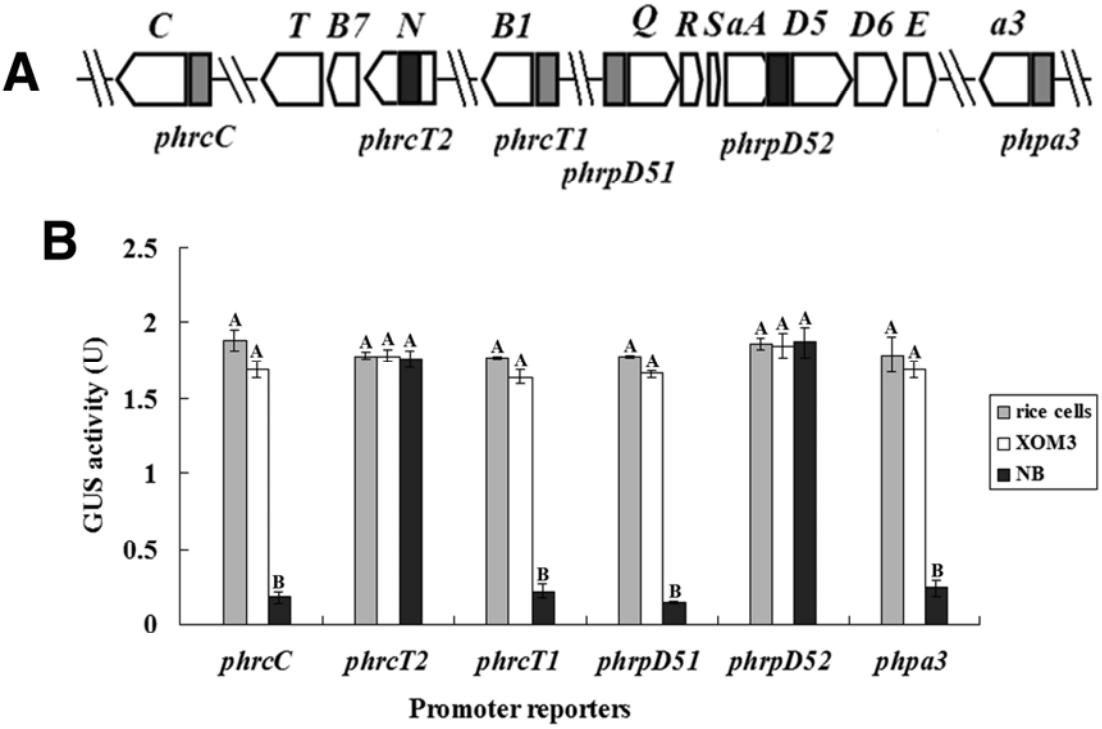

Fig. 2. Transcriptional organization and promoter location analysis of the hypersensitive response and pathogenicity (*hrp*)*A* operon for the *hrp*-conserved (*hrc*)*C* gene; the *hrpB* operon from the *hrpB1* to *hrcT* genes; the *hrpD* operon for the *hrcQ*, *hrcR*, *hrcS*, *hrp*-associated (*hpa*)*A*, *hrpD5*, *hrpD6*, *hrpE*, and *hpaB* genes; and an uncertain operon for *hpa3* gene in *Xanthomonas oryzae* pv. *oryzicola*. The locations of promoter regions in the *hrp-hrc-hpa* cluster from *hrpA* to the uncertain *hpa3* operon (containing *hpa3* gene). Gray boxes, designated *phrcC*, *phrcT1*, *phrpD51*, and *phpa3*, indicate the promoters containing perfect or imperfect plant-inducible promoter-boxes (TTCGC-N_{15}-TTCGC) (Zou et al. 2006). Black boxes represent the predicted promoters, *phrcT2* within the *hrcN* gene, and *phrpD52* in the *hpaA* gene, analyzed by online software. β-Glucuronidase (GUS) activity of the *hrp* promoter-*gusA* reporters in the wild-type RS105 of *X. oryzae* pv. *oryzicola* grown in rice suspension cells, *hrp*-inducing medium XOM3, and the nutrient-rich medium NB for 16 h were determined by measurement of optical density at 415 nm using ρ-nitrophenyl-β-D-glucuronide as a substrate. Data are the mean ± standard deviations of triplicate measurements. The experiment was repeated three times, and similar results were obtained. Different letters in each horizontal data column indicate significant differences at $P = 0.01$ by *t* test.

Table 2. Primer pairs used for gene mutagenesis and expression detection in *Xanthomonas oryzae* pv. *oryzicola*

Purpose, prime pair	Sequence (5′ to 3′)[a]	Description
Mutagenesis		
*hpaB*IF/	ATACCCGGGGCACATGACATCCCT/	
*hpaB*IR	TATGGATCCCGTTGGTGAGACATGCA	A 705-bp left fragment targeting *hpaB*
*hpaB*IIF/	ATAGGATCCTACAGGCCTGTTGGCAT/	
*hpaB*IIR	TATGTCGACAACCCTGACGCGCAA	A 404-bp right fragment targeting *hpaB*
*zur*IF/	AATGGATCCGTGCCGAAGTGCCGCTC/	
*zur*IR	ATATCTAGACGACGTGGTGATGCGGTG	A 328-bp left fragment targeting *zur*
*zur*IIF/	TTATCTAGAGAAGTGCATGGGTTATGC/	
*zur*IIR	TATGTCGACCCTGGAACGGTGCGTCGAT	A 606-bp right fragment targeting *zur*
*lrpX*IF/	ATAGGATCCCGGCCTGCATTGGCAGC/	
*lrpX*IR	TATTCTAGACGCTCAGGCGCGGGCTG	A 621-bp left fragment targeting *lrpX*
*lhrpX*IIF/	AATTCTAGAAGTCCAGATGTGGAAGACC/	
*lrpX*IIR	TAAGTCGACTGCGACAGGCCGGTGACC	A 405-bp right fragment targeting *lrpX*
*colR*IF/	AATGGATCCCCAATACCGACAAGATCAG/	
*colR*IR	ATATCTAGAAAGCCGTTCGATGTGCCG	A 566-bp left fragment targeting *colR*
*colR*IIF/	AATTCTAGATAATCGCCAAGATTTGC/	
*colR*IIR	ATAGTCGACGTGGCCAGATATCCAC	A 305-bp right fragment targeting *colR*
*colS*IF/	AATGGATCCCGTACGGGCGAGTTG/	
*colS*IR	ATACTGCAGTGTGCGATCTATA	A 220-bp left fragment targeting *colS*
*colS*IIF/	AATCTGCAGACTGGTTTTCCACG/	
*colS*IIR	ATTTCATGCGAAGTGGAAGTGC	A 512-bp right fragment targeting *colS*
*trh*IF/	AATGGATCCTGTACGCGTGGCTGTTCTAT/	
*trh*IR	ATATCTAGACTGCAATCTGTGCGGAGATC	A 630-bp left fragment targeting *trh*
*trh*IIF/	AATTCTAGATCGGCCTGTTTCTCAGC/	
*trh*IIR	ATAGTCGACTCGGTGCCTCTGTCC	A 207-bp right fragment targeting *trh*
*phrpD51*IF/	TTAGGATCCTTATCTTGCGCATACCGCC/	
*phrpD51*IR	ATATCTAGAAGGTTCCGGTCGATCCTCG	A 534-bp left fragment targeting *phrpD51*
*phrpD51*IIF/	AATTCTAGATTCAATACCAGTTCGCCAGAC/	
*phrpD51*IIR	ATAGTCGACGCGTGCGGCGCGTGGATCG	A 275 right fragment targeting *phrpD51*
*phrpD52*IF/	AATGGATCCGTCGCTGGAAGGCCAGGA/	
*phrpD52*IR	ATATCTAGACCGCCGCGGTGGCCCTGTC	A 428-bp left fragment targeting *phrpD52*
*phrpD52*IIF/	AATTCTAGACGCCCATGACCATGCAGCTT/	
*phrpD52*IIR	TATGTCGACCAATCGGCACCAGCATCACC	A 268-bp right fragment targeting *phrpD51*
Western blot constructs		
Hpa1F/	TATGAGCACGCGAAAAAAACTTTTCTCAAC/	
Hpa1-c-MycR	ATAAAGCTTCTACAGATCTTCTTCAGAAATAAGTTTTTGTTCC TGCATCGATCCGCTGTC	A 676-bp fragment of *hpa1* with its own promoter fused with a c-Myc tag
Hpa2F/	TATGAGCACCAGAGGGGGAAGTGGAAAAT/	
Hpa2-c-MycR	AAAAGCTTCTACAGATCTTCTTCAGAAATAAGTTTTTGTTCTT CTCCAATCACACCA	A 810-bp fragment of *hpa2* with its own promoter fused with a c-Myc tag
HrpFF/	GAGCACGTTTAAGTTACCCAACCAAAACCTG/	
HrpF-c-MycR	TATAAGCTTTTACAGATCTTCTTCAGAAATAAGTTTTTGTTCT CTGCGACGTATCCTGAC	A 3,106-bp fragment of *hrpF* with its own promoter fused with a c-Myc tag
Reporter constructs		
*phrcC*F/	ATAGAATTCTCGGCCTGGTGGCGCGAG/	
*phrcC*R	ATTAAGCTTTGACGTTCCCTCTGCTAG	A 285-bp promoter of *hrpA operon*
*phpa3*F/	ATAGAATTCAACGCTGACGCTGATGAA/	
*phpa3*R	ATTAAGCTTGAGCGGGCCGCATATTG	A 291-bp promoter of *hpa3*
*phrpG*F/	ATAGAATTCGCCGGTCTCTCTCTTGGG/	
*phrpG*R	AATAAGCTTCAGGTGGGCGTCCCGTGG	A 760-bp promoter of *hrpG*
*phrpX*F/	ATAAAGCTTGCCGGTCTCTCTCTTGGG/	
*phrpX*R	AATGAATTCCAGGTGGGCGTCCCGTGG	A 760-bp promoter of *hrpX*
*phrpD51*F/	TATGAATTCACCACAGTGCGACGACGAG/	
*phrpD51*R	ATTAAGCTTACTGGTATTGAAGCGAAGCG	A 370-bp *hrpD* operon promoter
*phrpD52*F/	ATAGAATTCGAAAACCTGCAGGCCTTG/	
*phrpD52*R	ATTAAGCTTGAACCTCCTGAGCCGCCTG	A 209-bp promoter-like region upstream of *hrpD5*
*phrcT1*F/	TATGAATTCTCATACCATTCCCAGG/	
*phrcT1*R	TATAAGCTTAGCTGATGCAGCAACGACC	A 261-bp promoter of *hrpB* operon
*phrcT2*F/	TTAGAATTCACATTGCCTTCTCCTTC/	
*phrcT2*R	ATAAAGCTTTTGAATCTTCTCCACACTG	A 233-bp promoter-like region upstream of *hrcT*
*phpa1*F/	TATGAATTCGCGAAAAAAACTTTTCTCAA/	
*phpa1*R	ATTAAGCTTCGTGGCGATTCCTCTCTGATT	A 232-bp promoter of *hpa1*
*phpa2*F/	TATGAATTCAGAGGGGGAAGTGGAAAAT/	
*phpa2*R	TATAAGCTTGTTTCGTTACCTCGATCTC	A 216-bp promoter of *hpa2*
*pecpA*F/	ATAGAATTCAGGCGCAGTCGGCAAT/	
*pecpA*R	TAAGGATCCCAGTGACTCTCCGTGT	A 466-bp promoter of *Xoryp_01688*
*hrpD6*F1/	ATAGGATCCATGTTCGATGCAATGAC/	
*hrpD6*R1	ATTGGTACCTTACCGCATGCTGGC	A 243-bp ORF of *hrpD6*
phpa1GUSF/	TATGGTACCGCGAAAAAAACTTTTCTCAAC/	
phpa1GUSR	TAAGAATTCTCATTGTTTGCCTCCCTGCTG	A 2,044-bp *hpa1* promoter-β-glucuronidase (GUS) fusion
*gus*F/	ATAAAGCTTTTACGTCCTGTAGAAACCC/	
*gus*R	TAAGAATTCTCATTGTTTGCCTCCC	A 1,830-bp *gusA* gene

[a] Added restriction sites are underlined.

al. 2006). Interestingly, the GUS activity driven by the *hrpX* promoter in the *lrpX* mutant was significantly higher than that in *zur* and *colR* mutants, which was significantly lower than that in *hrpX*, *colS*, and *trh* mutant backgrounds and in the wild-type RS105, respectively (Fig. 3C). The GUS activity of the *hrpX* promoter was lowest in the *hrpG* mutant compared with the other mutants (Fig. 3C). The above data suggest that not only does Trh positively regulate the expression of HrpG which, in turn, plays roles in regulation of *hrpX* and other *hrp-hrc-hpa* gene expression but Zur, ColR, ColS, and Trh also have impacts on the expression of HrpX, which subsequently controls other *hrp-hrc-hpa* gene expression. However, the decrease in *hrpX-gusA* expression in the *colR* mutant is contradictory to the results observed in *X. campestris* pv. *campestris*, in which a mutation in *colR* has no effect on *hrpX* expression (Zhang et al. 2008).

To assess whether the expression of *hrcC*, *hrpD5*, *hrcT*, *hrpE*, and *hpa3* was influenced by mutations in *hrpG*, *hrpX*, *zur*, *lrpX*, *colR*/*S*, and *trh* genes, we used Northern blot analysis to detect transcript levels of the target genes with RNA isolated from the mutant strains and the wild-type RS105 grown in XOM3 for 16 h. The results showed that *hrpE* was still expressed in the *hrpX* mutant (RΔ*hrpX*), albeit at lower levels than in the wild-type strain (Fig. 4), but *hrpE* was strongly induced in the *lrpX* mutant (RΔ*lrpX*). Compared with the wild-type strain, mutations in *hrpG* (RΔ*hrpG*), *trh* (RΔ*trh*), *zur* (RΔ*zur*), and *colR* (RΔ*colR*) had little effect on *hrpE* expression (Fig. 4). The Northern blot results also revealed that the mutation in either *hrpG* or *hrpX* did not completely abolish transcription of *hrpE*, implying that other factors may be involved in regulation of *hrpE* expression. Furthermore, no hybridization signals were detected for *hrcC*, *hrcT*, *hrpD5*, and *hpa3* transcripts and, therefore, we were unable to determine the effects of the mutations on these targets. This could be due to expression levels of *hrcC*, *hrcT*, *hrpD5*, and *hpa3* that may be suboptimal for detection by Northern blot.

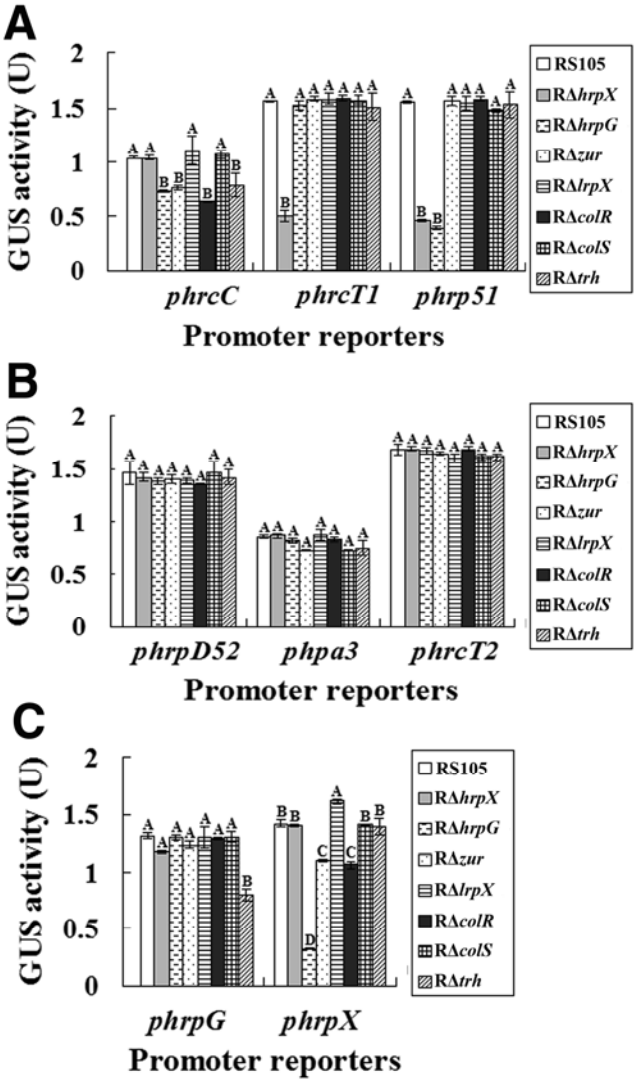

Fig. 3. Regulatory roles of other global regulators (Zur, LrpX, ColR, ColS, and Trh) in the expression of hypersensitive response and pathogenicity (*hrp*)*A*, *hrpB*, *hrpD*, and *hrp*-associated (*hpa*)*3* operons and *hrpG* and *hrpX* genes of *Xanthomonas oryzae* pv. *oryzicola*. **A** and **B,** β-Glucuronidase (GUS) activities of *hrp* promoter-*gusA* reporters, phrcCGUS, phrcT1GUS, phrp51GUS, phrpD52GUS, phpa3GUS, and phrcT2GUS in different genetic backgrounds of the wild-type RS105 and mutants of *hrpG* (RΔ*hrpG*), *hrpX* (RΔ*hrpX*), *zur* (RΔ*zur*), *lrpX* (RΔ*lrpX*), *colR* (RΔ*colR*), *colS* (RΔ*colS*), and *trh* (RΔ*trh*). **C,** Effects of Zur, LrpX, ColR, ColS, and Trh on the expression of *hrpG* and *hrpX* genes revealed by GUS activity of *hrpG* promoter- and *hrpX* promoter-*gusA* reporters, respectively, in the deletion mutant backgrounds of RΔ*zur*, RΔ*lrpX*, RΔ*colR*, RΔ*colS*, and RΔ*trh*. *X. oryzae* pv. *oryzicola* strains were cultured in XOM3 medium for 16 h and GUS activities were determined by measurement of optical density at 415 nm using ρ-nitrophenyl-β-D-glucuronide as a substrate. Data are the mean ± standard deviations of triplicate measurements. The experiment was repeated twice, and similar results were obtained.

The *hrpD* operon contains eight genes from *hrcQ* to *hrpE* in *X. oryzae* pv. *oryzicola*.

We examined the sequence between the *hrpA* and *hrpD5* genes of *X. oryzae* pv. *oryzicola*. Within the ribosomal binding site (RBS) for *hrpE,* the RBS sequence has been changed from GGA in *X. campestris* pv. *vesicatoria* (Weber et al. 2007) to GCC in *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* (data not shown). In *X. oryzae* pv. *oryzicola*, the putative PIP-box promoter sequence overlaps with the start codon of the *hrpD5* gene, as is the case in *X. campestris* pv. *vesicatoria* (Weber et al. 2007). This indicates that the *hrpE* gene belongs to the *hrpD* operon in *X. oryzae* pv. *oryzicola*, as seen in *X. oryzae* pv. *oryzae* (Cho et al. 2008).

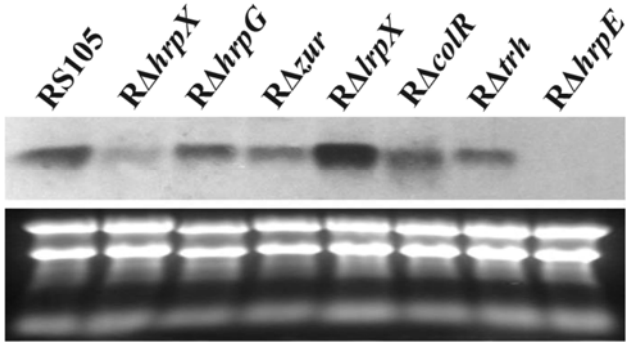

Fig. 4. Expression profiles of the hypersensitive response and pathogenicity (*hrp*)*E* gene in the mutant strains of other global regulatory factors Zur, LrpX, ColR, and Trh compared with the mutants of the key regulators HrpG and HrpX of *Xanthomonas oryzae* pv. *oryzicola*. RS105 (wild-type), RΔ*hrpX* (*hrpX* mutant), RΔ*hrpG* (*hrpG* mutant), RΔ*zur* (*zur* mutant), RΔ*lrpX* (*lrpX* mutant), RΔ*colR* (*colR* mutant), RΔ*colS* (*colS* mutant), RΔ*trh* (*trh* mutant), and RΔ*hrpE* (*hrpE* mutant, used for negative control) were incubated in *hrp*-inducing medium XOM3 for 16 h and then the total RNAs were extracted after incubation. Northern hybridization was performed to analyze transcript levels of *hrpE* in *X. oryzae* pv. *oryzicola* strains. Approximately 30 μg of the total RNAs of each sample were loaded onto each lane and the blot was probed with Biotin-labeled *hrpE*, which was polymerase chain reaction-amplified. Total RNAs were electrophoretically separated on 1.2% agarose gel and stained with ethidium bromide to detect the quality of the extracted RNAs (bottom). The experiment was repeated twice with similar results.

To determine the transcript unit between *hpaP* and *hrpE3*, we first generated deletions in the *phrpD51* and *phrpD52* promoters, RΔ*hrpD51* and RΔ*hrpD52* (Table 1; Fig. 5A), respectively. The wild-type and mutant strains were incubated in XOM3 for 16 h, and then RT-PCR was performed to assess the expression of individual genes from *hrcQ* to *hrpE3* in the RΔ*hrpG*, RΔ*hrpX*, RΔ*hrpD51*, and RΔ*hrpD52* mutants compared with the expression of those in the wild-type RS105. The results demonstrated that the expression of *hrcR*, *hrcS*, *hrcQ*, *hpaA*, and *hrpD6* genes is controlled by the *hrpD* operon promoter *phrpD51*, as was seen in the *hrpG* and *hrpX* mutants. Particularly, *hrpD5* expression was unaffected when the promoters *phrpD51* and *phrpD52* and the *hrpG* and *hrpX* genes were mutated (Fig. 5A), implying that the *hrpD5* expression is *hrpG* and *hrpX* independent and controlled by other unknown regulatory factors. The RT-PCR results also showed that a mutation in the *hrpD52* promoter completely abolished *hpaA* expression (Fig. 5A). More importantly, the *hrpE* and *hpaB* genes were not expressed in the *phrpD51* promoter mutant, RΔ*hrpD51* (Fig. 5A), which suggests that they are members of the *hrpD* operon. Previously, we believed they were part of the *hrpE* operon based on homology analysis of the core *hrp* cluster sequence (Zou et al. 2006).

To confirm the above results, we also used RT-PCR to determine the transcriptional linkage in different regions of the *hrp* gene cluster from *hpaP* through *hrpE3* in RS105. Primer pairs in Supplementary Table 3 were used to detect the intergenic junction between these *hrp* genes, and the results indicated that the *hrpD* operon is transcribed as a single polycistronic mRNA extending from *hrcQ* to *hpaB* (Fig. 5B). PCR products spanning *hrcQ-hrcR*, *hrcR-hrcS*, *hrcS-hpaA*, *hpaA-hrpD5*, *hrpD5-hrpD6*, *hrpD6-hrpE*, *hrpE-hpaB*, and *hrcS-hpaB* were obtained, suggesting that they are transcribed as one transcriptional unit. These results also proved that, although *phrpD52* shows promoter activity in the GUS assay, it is not the promoter of the *hrpD5* gene. Furthermore, this information helps identify the members of the *hrpD* operon in *X. oryzae* pv. *oryzicola*. These results are consistent with observations in *X. oryzae* pv. *oryzae* (Cho et al. 2008) but different from those in *X. campestris* pv. *vesicatoria*, where *hrpD6* is one of the genes in the *hrpE* operon (Weber et al. 2007).

HrpD6 controls the expression of *hpa1*, *hpa2*, *hrcC*, *hrcT*, and *hpaB*.

Our previous work showed that, 48 h after infiltration with a bacterial suspension of 1×10^8 CFU/ml, the *hrpD6* mutant RΔ*hrpD6* is unable to elicit HR in nonhost tobacco (cultivar Xanthi) or cause water-soaking symptoms in susceptible rice (IR24) (Guo et al. 2010). Moreover, HR induction in tobacco and pathogenicity in rice could be restored in the *hrpD6* mutant upon complementation with a construct expressing the *hrpD6* gene driven by the *hrpD* operon promoter (Fig. 2, *phrpD51*) (Guo et al. 2010). To evaluate the role of the *X. oryzae* pv. *oryzicola* HrpD6 in HR induction in tobacco and

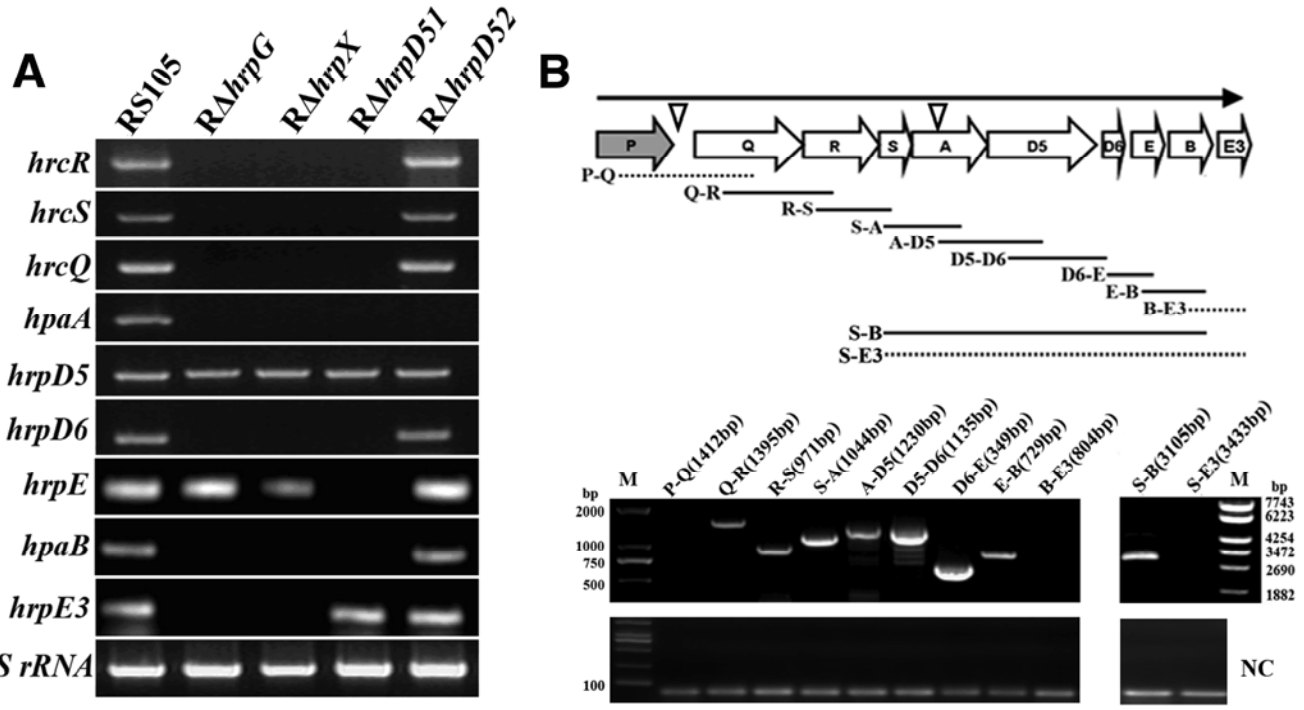

Fig. 5. Reverse-transcription polymerase chain reaction (RT-PCR) analysis of the transcriptional units of hypersensitive response and pathogenicity (*hrp*)-associated (*hpa*)*P* to *hrpE3* genes in *Xanthomonas oryzae* pv. *oryzicola*. **A,** Transcriptional detection of *hrp*-conserved (*hrc*)*Q* to *hrpE3* genes revealed by RT-PCR with specific primers in the deletion mutants of *hrpG* and *hrpX* genes (RΔ*hrpG* and RΔ*hrpX*), *phrpD51* and *phrpD52* promoter deletion mutants (RΔ*hrpD51* and RΔ*hrpD52*), and the wild-type *X. oryzae* pv. *oryzicola* RS105 after incubation in the *hrp*-inducing medium XOM3 for 16 h. The *phrpD51* and *phrpD52* promoter regions are shown to the right of this figure marked as delta. The *16S rRNA* gene was used as the standard control. **B,** Schematic representation of the *hrpD* operon and RT-PCR products amplified using primers designed to span the intergenic junctions. The black arrow indicates the extension and transcription direction of the *hrpD* operon, and the operon arrows represent the open reading frames of *hrp* genes: *hrcQ* (Q), *hrcR* (R), *hrcS* (S), *hpaA* (A), *hrpD5* (D5), *hrpD6* (D6), *hrpE* (E), *hpaB* (B), and *hrpE3* (E3) after the *hpaP* (P) gene of the *hrpC* operon. The thick black lines indicate the 11 PCR products and the expected sizes of the corresponding RT-PCR products after the strain was incubated in XOM3 for 16 h. P-Q denotes PCR reactions with primer pairs 14F-15Q, Q-R is 15F-16R, R-S is 16F-17R, S-A is 17F-18R, A-D5 is 18F-19R, D5-D6 is 19F-20R, D6-E is 20F-21R, E-B is 21F-22R, B-E3 is 22F-23R, S-B is 17F-22R, and S-E3 is 17F-23R. The latter two were used to confirm whether the *hrcS* and *hrpE3* genes are intergenic. The lower panel shows the agarose gel analysis of the RT-PCR products. In addition, the extracted RNAs were used directly as the templates for PCR to ensure samples were free of DNA contamination as the negative control (NC) where only primer dimers formed. M, DL 2000 DAN ladder (left) and λ-*Eco*T14 DNA ladder (right) (TaKaRa). All the experiments were repeated three times and similar data were obtained.

pathogenicity in rice, we investigated the expression of all the *hrp-hrc-hpa* genes in the *hrpD6* mutant RΔ*hrpD6* using RT-PCR, following interaction with rice suspension cells for 16 h. The result showed that, in the *hrpD6* mutant, *hpa2*, *hpa1*, and *hpaB* transcripts were undetectable, and the expression levels of *hrcC* and *hrcT* were reduced (Fig. 1B). Meanwhile, the expression of *hpa2*, *hpa1*, *hpaB*, *hrcC*, and *hrcT* was restored to the wild type in the complementation strain CRΔ*hrpD6*, which harbors a plasmid carrying the *hrpD6* gene controlled by the *hrpD* operon promoter (Fig. 1B). On the other hand, the expression of other *hrp-hrc-hpa* genes, including *hrpG* and *hrpX* in either RΔ*hrpD6* or CRΔ*hrpD6*, was similar to that in the wild-type RS105 (Fig. 1B). Additionally, the expression of *hpa2*, *hpa1*, *hpaB*, *hrcC*, and *hrcT* in the *hrpD6* overexpression strain, RS105(*hrpD6*), was higher than that in the wild-type RS105 (Fig. 1C). These results strongly suggest that HrpD6 upregulates the expression of *hpa2*, *hpa1*, *hrcC*, *hrcT*, and *hpaB* and that *hrpD6* expression is positively regulated by HrpX.

In order to confirm whether HrpD6 is involved in the regulation of *hpa2*, *hpa1*, *hrcC*, *hrcT*, and *hpaB*, constructs were generated in which their promoters were fused with *gusA* to yield phpa1GUS, phpa2GUS, phrcCGUS, phrcT1GUS, and phrpD51GUS (Table 1). These constructs were then introduced into the *hrpD6* mutant (RΔ*hrpD6*) and the wild-type RS105. The GUS activity of the resulting reporter stains was measured after 16 h of growth in the *hrp*-inducing medium XOM3. With the exception of phrpD51GUS, the results showed that the GUS activity of each reporter plasmid was significantly lower than that in the wild type (Fig. 6A), indicating that HrpD6 regulates the expression of *hpa2*, *hpa1*, and *hpaB* and partially controls the transcript levels of *hrcC* and *hrcT*. We also investigated whether HrpD6 has some regulatory effect on *hrpG* and *hrpX* expression by introducing constructs with *hrpG* and *hrpX* promoters fused to *gusA* (phrpGGUS and phrpXGUS) into RΔ*hrpD6* and the wild-type RS105. The GUS activity of these two reporter plasmids in the *hrpD6* mutant background was similar to that in the wild type (Fig. 6A), suggesting that HrpD6 has no obvious influence on *hrpG* or *hrpX* expression in *X. oryzae* pv. *oryzicola*.

The PIP-box in the *hrp* promoter regions is normally the *cis*-element where HrpX binds in *Xanthomonas* spp. (Furutani et al. 2006; Koebnik et al. 2006; Wengelnik and Bonas 1996; Wengelnik et al. 1996). The presence of a perfect PIP-box in the *hpa1* promoter region, analyzed elsewhere in this work, suggests that the expression of *hpa1* is positively regulated by HrpX as detected at the transcriptional level in our previous (Zou et al. 2006) and present (Fig. 1B) reports. However, the *hpa1* transcript was undetectable in the *hrpD6* mutant (Fig. 1B), which led us to assume that *hpa1* expression is not regulated by HrpX but by HrpD6. For this, a constitutive promoter that lacks a PIP-box from *Xoryp_01688* was used here. *Xoryp_01688* encodes an extracellular protease whose transcription is not regulated by HrpX (*unpublished data*). To sup-

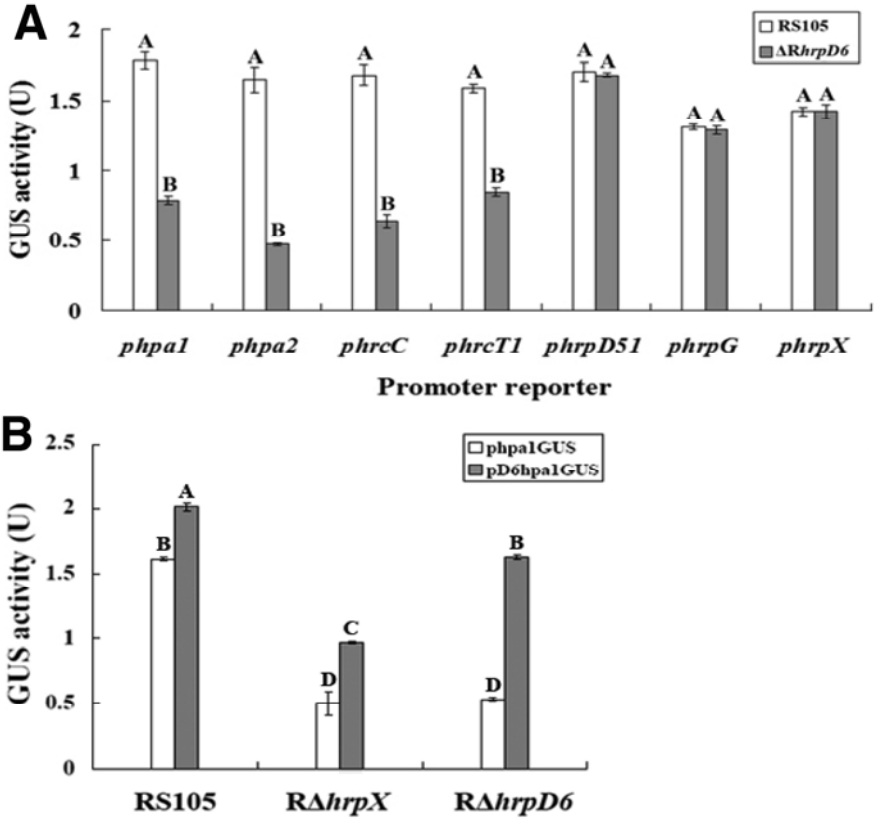

Fig. 6. Regulation of HrpD6 on expression of hypersensitive response and pathogenicity (*hrp*)-associated (*hpa*)*1*, *hpa2*, *hrp*-conserved (*hrc*)*C*, *hrcT*, *hpaB*, *hrpG*, and *hrpX* in *Xanthomonas oryzae* pv. *oryzicola*. **A,** Comparison of β-glucuronidase (GUS) activities in the wild-type RS105 and the *hrpD6* mutant *R*Δ*hrpD6* backgrounds driven by *hpa1*, *hpa2*, *hrcC*, *hrcT*, *hrpE*, *hpaB*, *hrpG*, and *hrpX* promoter-GUS reporters phpa1GUS, phpa2GUS, phrcCGUS, phrcT1GUS, phrpD51GUS, phrpGGUS, and phrpXGUS, respectively. **B,** HrpX-independent expression of *hpa1* regulated by HrpD6. A constitutive promoter of *Xoryp_01688* that lacks a plant-inducible promoter-box was fused with the *hrpD6* open reading frame in the vector phpa1GUS which is the *hpa1* promoter-GUS reporter, giving pD6hpa1GUS. The GUS activities of pD6hpa1GUS and phpa1GUS in the wild-type RS105, the *hrpX* mutant RΔ*hrpX*, and the *hrpD6* mutant were investigated. All the reporter strains above were cultured in XOM3 medium for 16 h and GUS activities were then determined by measurement of optical density at 415 nm using ρ-nitrophenyl-β-D-glucuronide as a substrate. Data are the mean ± standard deviations of triplicate measurements. Different letters in each horizontal data column indicate significant differences at $P = 0.01$ by *t* test. The experiment was repeated twice and yielded similar results.

port the above hypothesis, we used the *Xoryp_01688* promoter to drive the expression of *hrpD6* with *hpa1*::*gusA* as a reporter (pD6hpa1GUS) (Table 1) in the *hrpX* mutant RΔ*hrpX*, the *hrpD6* mutant RΔ*hrpD6*, and the wild-type RS105, respectively. For comparison, the *hpa1::gusA* reporter (phpa1GUS) (Table 1) was used as a control. Indeed, the GUS activity of pD6hpa1GUS was significantly ($P = 0.01$, *t* test) higher than that of phpa1GUS in either the wild-type strain RS105, the *hrpX* mutant RΔ*hrpX*, or the *hrpD6* mutant RΔ*hrpD6* (Fig. 6B), demonstrating that HrpD6 regulates the expression of *hpa1*. The GUS activity of pD6hpa1GUS in the *hrpD6* mutant RΔ*hrpD6* was almost the same as that of phpa1GUS in the wild-type RS105 but was significantly lower than that in either the wild-type RS105 or the *hrpX* mutant RΔ*hrpX* (Fig. 6B), implying that HrpD6 controls *hpa1* expression in an HrpX-independent manner.

HrpD6 controls the secretion of the HpaB-dependent TAL effector AvrXa27 but not HrpF.

The loss of expression of *hpa2* and *hpa1* in *hrpD6* mutant background suggests that this mutation may also effect Hpa1 and Hpa2 secretion. To assess this, we used *X. oryzae* pv. *oryzicola* RS105, an *hrpD6* deletion mutant (RΔ*hrpD6*), an *hrcV* T3SS deletion mutant (RΔ*hrcV*) deficient in secretion of T3SS effectors, and a deletion mutant lacking *hpaB* (RΔ*hpaB*) in which HpaB works as an exit control protein in T3SS-dependent protein secretion in *X. campestris* pv. *vesicatoria* (Büttner et al. 2004). For the in vitro analysis of the T3SS effectors Hpa2 and Hpa1, strains RS105, RΔ*hrpD6*, RΔ*hpaB*, and RΔ*hrcV* were incubated in secretion medium XOM3. Total cell extracts (TE) and culture supernatants (SN) were analyzed by immunoblotting. All proteins tested were secreted by the wild-type strain but were not detectable in the SN of the *hrcV* deletion mutant (Fig. 7), indicating that secretion of these proteins is through the T3SS of *X. oryzae* pv. *oryzicola*. Because the mutation in *hrpD6* attenuated *hpaB* expression (Fig. 1B), we tested the secretion of Hpa2, Hpa1, AvrXa27, and HrpF in both the *hpaB* nonpolar mutant RΔ*hpaB* and the *hrpD6* deletion mutant RΔ*hrpD6*. The result showed that Hpa2 and Hpa1 were not detected in TE and SN of the *hrpD6* mutant (Fig. 7C and D) but Hpa2 and AvrXa27 were undetectable only in the SN of *hpaB* and *hrpD6* deletion mutants (Fig. 7A and D). The data also showed that Hpa1 was only secreted in SN of the *hpaB* deletion mutant (Fig. 7B), and that HrpF secretion was observed in both *hpaB* and *hrpD6* deletion mutants (Fig. 7B). This suggests that the mutation in *hrpD6* inhibits transcription of *hpa2* and *hpa1* (Fig. 1B) and, hence, Hpa2 and Hpa1 proteins are not detected in the SN. However, HrpD6 acts upstream in the HpaB regulatory cascade (Fig. 1B) and, therefore, HpaB-dependent T3SS effectors AvrXa27 and Hpa2 are not secreted in SN of *hrpD6* and *hpaB* mutants, whereas the secretion of the HpaB-independent translocator, HrpF, is not affected by the *hrpD6* mutation.

DISCUSSION

In this study, we investigated the role of *hrpG* and *hrpX* in the regulation of the *hrp-hrc-hpa* gene cluster of *X. oryzae* pv. *oryzicola* and identified a novel regulator, HrpD6, within this *hrp-hrc-hpa* cluster. The expression of most of the *hrp-hrc-hpa* genes, excluding *hpa2*, *hrcC*, *hrcT*, *hrpD5*, *hrpE*, and *hpa3*, is regulated by both HrpG and HrpX. This follows the general principle that HrpG regulates the expression of the *hrpA* operon and *hrpX*; and HrpX, in turn, regulates the expression of the *hrpB* to *hrpF* operons, in which PIP-box promoters are presumably activated by HrpX (Furutani et al. 2006; Koebnik et al. 2006) in diverse *Xanthomonas* spp. (Arlat et al. 1991; Furutani et al. 2006; Kamdar et al. 1993; Koebnik et al. 2006; Oku et al. 1995; Wengelnik and Bonas 1996; Wengelnik et al. 1996a and b; Zou et al. 2006). However, in *X. oryzae* pv. *oryzicola*, transcription of the *hrcC*, *hrpD5*, *hrpE*, and *hpa3* genes is still observed when the *hrpG* and *hrpX* genes were mutated (Figs. 1, 2,

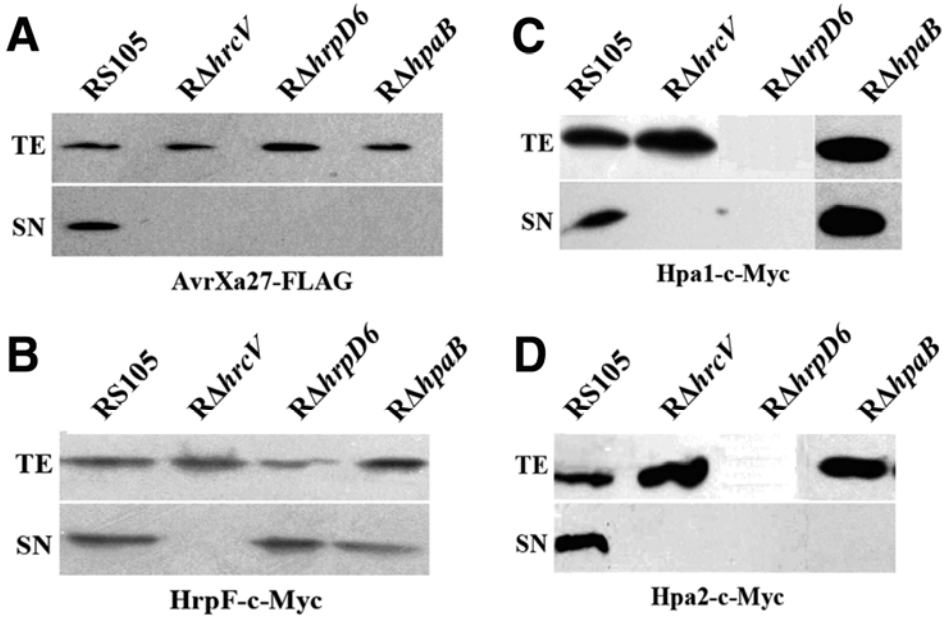

Fig. 7. Influence of hypersensitive response and pathogenicity (Hrp)D6 on secretion of type III secretion system (T3SS) effectors AvrXa27, *hrp*-associated (Hpa)1, and Hpa2, and T3SS translocator HrpF in *Xanthomonas oryzae* pv. *oryzicola*. The *hrpD6* mutant RΔ*hrpD6*, the *hrp*-conserved (*hrc*)*V* mutant RΔ*hrcV*, the *hpaB* mutant RΔ*hpaB*, and the wild-type RS105, harboring pAvrXa27-FLAG, pHrpF-c-Myc, pHpa1-c-Myc, or pHpa2-c-Myc, respectively, were induced in the *hrp*-inducing XOM3 medium for 16 h. Bacterial total cell extracts (TE) and culture supernatants (SN) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. **A,** In vitro secretion of the TAL effector AvrXa27 was analyzed using a monoclonal anti-FLAG antibody. **B,** In vitro secretion of the T3S translocon HrpF protein was detected by using a monoclonal anti-c-Myc antibody. **C,** In vitro secretion of a harpin protein Hpa1 was immunoblotted with a monoclonal anti-c-Myc antibody. **D,** In vitro secretion of a lytic transglycosylase Hpa2 was assayed by using a monoclonal anti-c-Myc antibody. This experiment was repeated three times. Results from one representative experiment are shown.

and 4), indicating that other unknown regulators control their expression. We do not know whether these novel regulatory features exist in other *Xanthomonas* spp. For instance, *hrcC* is the only member in the *hrpA* operon that is highly conserved in many gram-negative animal- and plant-pathogenic bacteria and works as an outer membrane protein of T3SS for secretion of T3SS effectors (Alfano and Collmer 1997; Büttner and Bonas 2002; Büttner et al. 2006; Deng and Huang 1999; Kim et al. 1997; Rossier et al. 2000). In *X. campestris* pv. *vesicatoria,* the *hrpA* operon is activated by HrpG but not by HrpX (Wengelnik and Bonas 1996). In this report, we found that HrpG does not completely abolish the expression of *hrcC* (Fig. 1B), indicating that other unknown factors may work together with HrpG to regulate expression of *hrcC* in *X. oryzae* pv. *oryzicola*. In *X. campestris* pv. *campestris,* the expression of *hrcC* is regulated by both HrpG and HrpX (Huang et al. 2009). The novel expression profiles of *hrp-hrc-hpa* genes regulated by HrpG and HrpX support the hypothesis that there may be pathovar-specific differences in *hrp* gene expression in *Xanthomonas* spp.

In *X. campestris* pv. *vesicatoria*, *hrpD5* (*hrcD*), *hrpD6*, and *hrpE* belong to the *hrpE* operon. The *hrpE* promoter lies within the *hpaA* coding sequence and harbors a thus-far-not-considered PIP-box that is conserved in all *Xanthomonas* strains (Weber et al. 2007). However, the transcriptional unit of the *hrcQ* to *hpaB* region in *X. oryzae* pv. *oryzicola* is not consistent with that of *X. campestris* pv. *vesicatoria*, because there was no expression of the *hrcQ* to *hrpD6* genes, except *hrpD5*, when the *hrpD* operon promoter was deleted. Furthermore, expression of the genes from *hrcQ* to *hrpD6* was not impaired by the deletion of the PIP-box promoter within *hpaA* (Fig. 5), suggesting that the eight genes from *hrcQ* to *hpaB* are transcribed as one unit, with the exception of *hrpD5*. This is consistent with that in *X. oryzae* pv. *oryzae* (Cho et al. 2008). However, at this time, we do not know why transcription of *hrpD5* and *hrpE* genes is still observed when *hrpG*, *hrpX*, and the PIP-box promoter of the *hrpD* operon are mutated. In *X. oryzae* pv. *oryzicola* as in *X. campestris* pv. *vesicatoria*, the sequences between *hpaA* and *hrpD5* share 4 bp for the start codon of *hrpD5* (ATG) and the stop codon of *hpaA* (TGA). Furthermore, the sequences spanning *hpaA-hrpD5* has an exact match of 46 bp (5′-AACAGGCGGCTCAGGAGGTTCG CCCATGACCATGCAGCTTCGCGTA-3′), including the PIP box (underlined sequences) newly reported in *X. campestris* pv. *vesicatoria* (Weber et al. 2007). Interestingly, the intergenic primers between the contiguous genes among *hpaA*, *hrpD5*, *hrpD6*, *hrpE*, *hpaB*, *and hrpE3* could detect the expression of *hpaA* to *hpaB* by RT-PCR amplification (Fig. 5), indicating that these junctions are expressed as mRNAs. The discrepancy between *X. oryzae* pv. *oryzicola* and *X. campestris* pv. *vesicatoria* with regard to *hpaA* to *hpaB* transcription might be a simple difference as species but the mechanisms of the expression of *hrpD5* and *hrpE* in *hrpG-* and *hrpX*-independent profiles remains a mystery.

In *X. campestris* pv. *vesicatoria*, HrpD5 is named as HrcD, which is encoded by the first ORF in the *hrpE* operon (Weber et al. 2007). HrcD is a biotic membrane protein that exhibits weak homology to members of the YscD family, to which HrpW from *Ralstonia solanacearum* and PrgH from *Salmonella typhimurium* belong (Pallen et al. 2005). PrgH is an essential component of T3SS needle complex associated with T3SS and has been co-purified with the needle subunit PrgI (Kubori et al. 1998). In comparison, in the genus *Xanthomonas*, HrpE is the major subunit of the Hrp pilus which possesses a two-domain structure with a surface-exposed, positively selected N terminus and a C-terminal polymerization domain (Weber and Koebnik 2005; Weber et al. 2005). In *X. oryzae* pv. *oryzicola*, a mutation in the *hrpE* gene leads to lack of Hrp pili formation in rice parenchyma and the inability to attach to rice callus during host cell interactions (Wang et al. 2009). As for Hpa3, little is known about its role in the T3SS. There are two homologs of *hpa3* in *X. campestri*s pv. *vesicatoria* (*hpaI* and *hpaD*) that are regulated by HrpG and HrpX (Büttner et al. 2007). A mutation in *hpa3* has no apparent effect on pathogenicity of *X. oryzae* pv. *oryzae* (Cho et al. 2008) or of *X. oryzae* pv. *oryzicola* but transcription of *hpa3* was HrpG and HrpX independent, as are *hrcC*, *hrpD*5, and *hrpE* (Fig. 1B). It has been suggested that Hpa3 may be a chaperone for secretion of Hpa4 by the T3SS, because it is a small acidic and leucine-rich protein (Sugio et al. 2005). Intriguingly, *hpa3* expression was almost completely inhibited in the mutants of *hrpB1*, *hrpB2*, *hrpB4*, and *hrpB5* in *X. oryzae* pv. *oryzicola* (*unpublished data*), indicating that Hpa3 may play important roles in formation of the T3SS or the Hrp pilus. Considering the results above, we postulate that the HrcC outer-membrane protein, the extracellular Hrp pilus composed of HrpD5 and HrpE, and the Hpa3 chaperone may be essential for pathogen survival and are expressed independently of HrpG and HrpX in either nutrient-rich medium (NB), *hrp*-inducing medium XOM3, or in planta.

Another interesting finding is that *hpa2* expression was positively regulated by HrpG and HrpD6 but not by HrpX. In addition, *hrcT* transcription was positively controlled by HrpX but negatively controlled by HrpG and partially regulated by HrpD6 (Fig. 1B). Our experimental evidence also showed that *hpa1* and *hpaB* expression seemed to be positively regulated by both HrpG and HrpX (Fig.1B) but, actually, this is not the case. Expression of *hpa2*, *hpa1*, and *hpaB* genes was undetectable, and expression levels of the *hrcC* and *hrcT* genes were reduced when *hrpD6* was mutated in *X. oryzae* pv. *oryzicola* RS105 (Figs. 1B, 5, and 6). These data strongly suggest that HrpD6, whose transcription is positively controlled by HrpG and HrpX, positively regulates the expression of *hpa2*, *hpa1*, and *hpaB* and upregulates the transcription of *hrcC* and *hrcT*. Our hypothesis is confirmed by the following aspects. i) HrpD6 has no homologs among genes encoding the T3SS of other gram-negative plant and animal pathogenic bacteria except in *Xanthomonas* spp. (Weber et al. 2007), implying that HrpD6 is not involved in T3SS formation. ii) HrpD6 controls the *hpa1* expression in an HrpX-independent manner. This was confirmed by using the constitutive promoter of the extracellular protease gene *Xoryp_01688* to drive *hrpD6* expression which, in turn, regulates the perfect PIP-box promoter of *hpa1*, all of which was monitored by measuring *gusA* expression in the wild-type RS105, the *hrpX* deletion mutant *R*Δ*hrpX*, and the *hrpD6* deletion mutant RΔ*hrpD6* (Fig. 6B). This is consistent with previous reports which found that, just because the HrpX-regulon candidates have promoters containing a perfect or imperfect PIP-box does not necessarily mean they are true HrpX regulons (Furutani et al. 2006, 2009). iii) The HpaB-dependent T3SS effectors, like AvrXa27 (Gu et al. 2005) and AvrBs3 (Büttner et al. 2004, 2006), are not detectable in the *hrpD6* and *hpaB* mutants (Fig. 7). These results are consistent with previous studies which showed that *hpaB* or *hrpD6* mutations result in lack of TAL effector secretion. iv) The HpaB-independent T3SS translocator HrpF (Büttner et al. 2004) is detectable in both *hpaB* and *hrpD6* mutants (Fig. 7B), demonstrating that HrpD6 has no effect on the secretion of HpaB-independent T3SS effectors. v) Inhibition of *hpa2* and *hpa1* expression and downregulation of *hrcC* and *hrcT* in the *hrpD6* mutant (Figs. 1B and 6) led to a deficiency in Hpa2 and Hpa1 secretion (Fig. 7) and, possibly, a complete lack of T3SS secretion (*not assessed in this report*). This indicates that the expression of *hpa2*, *hpa1*, and *hpaB* is HrpG- and HrpX-independently regulated by HrpD6 and the expression of *hrcC* and *hrcT* genes is co-regulated by HrpG, HrpX, and HrpD6.

Integrating accumulated knowledge and current expression profiles of the *hrp-hrc-hpa* genes in *Xanthomonas* spp., we propose a new working model for regulation of the *X. oryzae* pv. *oryzicola hrp* system (Fig. 8). Trh, a member of the GntR regulator family (Tsuge et al. 2006), represses the expression of HrpG (Fig. 2B), indirectly resulting in lower expression of HrpX which, in turn, directly regulates the expression of most of the *hrp-hrc-hpa* genes, including *hrpD6* but excluding the *hrcC*, *hrcT*, *hrpD5*, *hrpE*, and *hpa3* genes. The leucine-rich protein LrpX (Islam et al. 2009) may negatively regulate certain *hrp* genes (e.g.,, in *hrpA* and *hrpE* operons) indirectly by regulating an as-yet-unidentified repressor for the expression of HrpG and HrpX, which subsequently impacts the expression of *hrp-hrc-hpa* genes; for example the *hrpE* gene (Fig. 8). The expression of *hrpX* but not *hrpG* is repressed by Zur (Fig. 4), a zinc uptake regulator (Huang et al. 2009), which results in downregulation of the *hrp-hrc-hpa* genes. The ColR/S two-component signal transduction system (Zhang et al. 2008) downregulates the expression of the *hrpX*, *hrpA*, and *hpa3* operons, whereas Zur positively regulates the expression of the *hrpC* and *hrpE* operons in *X. campestris* pv. *campestris* (Zhang et al. 2008). Moreover, the expression of *hrcC*, *hrpD5*, *hrpE*, and *hpa3* may be positively regulated by other unidentified factors, outside of the differential regulation by HrpG, HrpX, Trh, Zur, ColR/S, or LrpX, leading us to assume that some positive regulatory proteins have become negative regulators depending on where they bind in unknown promoter regions for *hrcC*, *hrpD5*, *hrpE*, and *hpa3* gene expression. This possibility is worthy of being scientifically investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains used in this study are listed in Table 1 and Supplementary Table S1. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C (Miller 1972). All *X. oryzae* pv. *oryzicola* strains were grown in nutrient agar (NA) (1% polypeptone, 0.5% yeast extract, 1% sucrose, and 1.5% agar), NB, NAN (NA without sucrose), or NAS (NA with 10% sucrose) medium at 28°C when required. The *hrp*-inducing medium for *X. oryzae* pv. *oryzicola* strains is XOM3 (D-xylose,1.8 g/liter; D,L-methionine, 670 μM; sodium L-glutamate, 10 mM; $NaFe^{2+}$-EDTA, 240 μM; $MgCl_2$, 5 mM; KH_2PO_4, 14.7 mM; and $MnSO_4$, 40 μM; pH6.0) (Xiao et al. 2007). Antibiotics were used at the following final concentrations as required: rifampin, 50 μg/ml; kanamycin, 25 μg/ml; ampicillin, 100 μg/ml; and spectinomycin, 50 μg/ml.

Recombinant DNA techniques.

DNA manipulations and PCR were performed according to standard procedures (Sambrook et al. 1989). Mobilization of plasmids into *X. oryzae* pv. *oryzicola* was performed as de-

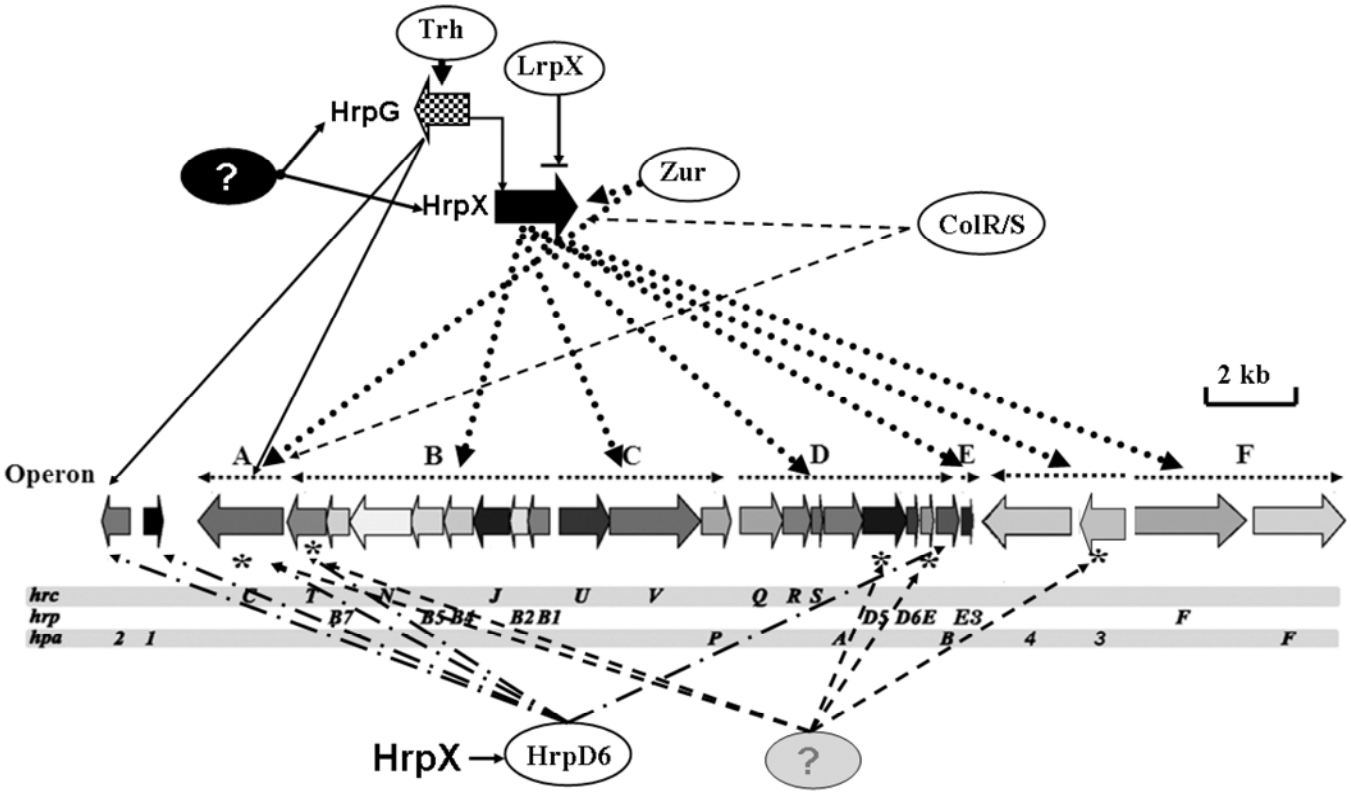

Fig. 8. Working model of hypersensitive response and pathogenicity (*hrp*), *hrp*-conserved (*hrc*), *hrp*-associated (*hpa*) gene cluster (*hrp-hrc-hpa*) regulation in *Xanthomonas oryzae* pv. *oryzicola*. Horizontal dashed-line arrows indicate the transcriptional units, from the *hrpA* to *hrpF* operons, of the *hrp-hrc-hpa* cluster. Single letters or letters plus numbers or numbers stand for individual *hrp*, *hrc*, and *hpa* genes beneath the cluster. The *hrpD* operon consists of eight genes from *hrcQ* to *hpaB* and the *hrpE* operon only contains the *hrpE3* gene. Trh positively regulates (black arrow) the expression of *hrpG*. LrpX negatively regulates (black arrow with a stop line) the expression of *hrpX*, which then may lead to higher expression of *hrpE*. Zur significantly represses (dotted line arrows) the expression of *hrpX,* and ColR/S is required (dashed-line arrow) for the expression of the *hrpX* and *hrpA* operons. The unknown regulatory factors question-marked with a black oval may regulate (short black arrow) the expression of *hrpG* and *hrpX*. HrpG positively regulates (long black arrow) the expression of *hrcT* and *hrpX*. HrpX then activates (dotted-line arrow) the *hrp* cluster of genes (*hrpB* to *hrpF* operons) except *hrcC*, *hrcT*, *hrpD5*, *hrpE*, and *hpa3*. HrpG also partially controls (long black arrow) the expression of the *hpa2* and *hrpA* operons. Simultaneously, HrpD6, is positively regulated by HrpX, which is activated by HrpG, and can positively control (dashed line arrow) the expression of *hpa1*, *hpa2*, and *hpaB* and partially downregulate the transcription of *hrcC* and *hrcT*. Genes *hrcC*, *hrcT*, *hrpD5*, *hrpE*, and *hpa3* are marked with stars to indicate that their expression is HrpG or HrpX independent and may be regulated by one or more other unidentified factors. Ovals with a question mark represent one or more as-yet-uncharacterized regulatory proteins.

scribed by Turner (2004). Restriction digestion and DNA ligation were performed in accordance with the manufacturer's instructions (Promega, Shanghai, China). Oligonucleotide primers (Table 2) for sequencing or PCR reaction kits were purchased from Jinsite Biotechnology. PCR was performed with *Ex*-Taq (Takara Bio Inc., Dalian, China). DNA sequences were analyzed with the VECTOR NTI software package (Infomax; Invitrogen, Shanghai, China).

Rice suspension cell cultures.

Oryza sativa subsp. *indica* cv. Shanyou 63, susceptible to *X. oryzae* pv. *oryzicola* RS105, was used for callus induction. Seed were dehulled and sterilized in 70% ethanol for 10 min, 50% commercial bleach with a few drops of Tween 20 for 30 min, and then 1% $HgCl_2$ for 15 min. The sterilized seed were washed five times with sterile distilled water and placed on N6 medium (Chu 1978) with 2,4-D (5 mg/liter) for induction of rice callus in the dark at 28°C. The actively growing calli were selected and transferred to liquid N6 medium supplemented with 2,4-D at 5 mg/liter and kinetin at 1 mg/liter. The cells were maintained in the dark on a 7-day subculture schedule at a dilution of 1:5 (inoculum/fresh medium). Generally, large amounts of rice suspension cells can be obtained after 4 to 5 weeks of subculture and then dispersed, or single round rice cells could be observed under the microscope.

Construction of nonpolar mutants of *X. oryzae* pv. *oryzicola* RS105.

According to gene loci referenced in the *hrp* clusters (AY875714 and AY272885) of *X. oryzae* pv. *oryzicola* RS105 (Zou et al. 2006) and in the genome sequence of *X. oryzae* pv. *oryzicola* BLS256, two pairs of primers, ϕIF/ϕIR and ϕIIF/ϕIIR (ϕ represents a target gene) (Table 2), were used to amplify the upstream and downstream fragments flanking the target gene for a two-step integration mutagenesis by using the wild-type RS105 genomic DNA as the template (Table 2). PCR was performed using *Ex*-Taq polymerase (Takara Bio Inc.), and the following PCR parameters: step 1, 94°C for 5 min; step 2, 94°C for 50 s; step 3, 55°C for 45 s; step 4, 72°C for 1 min/kb; 32 cycles repeating from steps 2 to 4; and step 5, 72°C for 7 min. After purification, all PCR products were digested based on restriction sites incorporated into the primer design and cloned into the suicide vector pKMS1 (Jiang et al. 2009) to construct the recombinant deletion vector pKMSΔϕ for each gene (Table 1). The expected PCR products were further confirmed by sequencing.

The above deletion vectors (50 ng/μl) were electroporated into competent cells of the RS105 recipient strain and plated for single-colony selection on NAN plates containing kanamycin at 25 μg/ml. Colonies from the initial homologous crossover event which grew on NAN/kanamycin were transferred to NBN broth, grown for 12 h at 28°C, and plated on NAS medium. Sucrose-resistant colonies were replica streaked onto NA and NA plus kanamycin plates. Recombinants resulting from double crossover events were selected by *sacB* and sucrose-positive selection. Mutations were confirmed by PCR amplification with pair primers (ϕIF/ϕIIR). Subsequent Southern hybridization (digoxigenin; Roche, Mannheim, Germany) was conducted to verify the deletion of the target genes. One of the confirmed deletion colonies (Table 1) was used for further study.

Complementation of mutants.

To sort out the text, some of the complementation constructs were listed in the main text (Table 1) and others in the supplementary material. Complementations were confirmed by restoration of pathogenicity or virulence in rice and HR in tobacco to wild-type levels (data not shown). Mutants were complemented with the corresponding 27 *hrp-hrc-hpa* genes driven by their respective operon promoters of the core *hrp* cluster (Table 1) and the seven regulatory genes *hrpG*, *hrpX*, *trh*, *lrpX*, *zur*, and *colR/S*, driven by their native promoters. For complementation of the *hrpD6* mutant RΔ*hrpD6*, a 243-bp DNA fragment containing the entire *hrpD6* coding region was amplified by PCR with primers *hrpD6*F and *hrpD6*R using the genomic DNA of strain RS105 as a template. Correspondingly, a 370-bp *hrpD* operon promoter was amplified by PCR using primers *phrpD51*F and *phrpD51*R (Table 2). After confirmation by sequencing, the amplified DNA fragments were cloned into pUFR034 to create the recombinant plasmid pC*hrpD6* (Table 1). The recombinant plasmid was transferred into RΔ*hrpD6* by electroporation. Transconjugants carrying pC*hrpD6* were selected on NA plates containing kanamycin, and one representative complementation strain (CRΔ*hrpD6*) was verified by colony PCR and chosen for further study (Table 1).

Pathogenicity and HR assays.

Hrp assays were performed as described (Zou et al. 2006). *X. oryzae* pv. *oryzicola* strains were assessed for their ability to cause disease symptoms and multiply in rice IR24 plants by inoculation of rice seedling leaves (2 weeks old) by infiltration with needleless syringes and adult rice (2 months old) by the leaf needling method, with bacterial suspensions adjusted to 1×10^8 CFU/ml. The strains were also tested for the ability to elicit an HR on *Nicotiana benthamiana* by infiltration of plant tissue with strains adjusted to 1×10^8 CFU/ml with a needleless syringe. Plant responses were scored for HR in tobacco 24 h postinoculation, for water soaking in rice seedlings 3 days postinoculation (dpi), and after 14 dpi for lesion lengths. All plants were grown in growth chambers at 25°C with a 12-h photoperiod. Experiments were repeated at least three times.

Semiquantitative RT-PCR analysis.

Single colonies of *X. oryzae* pv. *oryzicola* RS105 and deletion mutants were inoculated into 20 ml of NB broth and grown at 28°C for 24 to 36 h until the optical density at 600 nm (OD_{600}) value reached 0.8. This culture (2 ml) was subcultured into 100 ml of fresh NB and incubated for another 12 to 16 h until the OD_{600} reached 0.6. After centrifugation at 6,000 rpm for 10 min at 4°C, the pellet was washed in sterilized water twice and each wash followed by centrifugation for 10 min. The pellet was eventually resuspended in sterilized water to $OD_{600} = 2.0$. Then, 40 μl of this bacterial suspension was inoculated into 1 ml of rice suspension cells. After incubation for 16 h at 26°C, bacteria were collected by centrifugation at 12,000 rpm for 5 min and total RNA was extracted using the Trizol method (Invitrogen). The extracted RNA was treated with DNase I (Takara Bio Inc.) and purified. The RNA was then used as the template for PCR amplification of 27 *hrp-hrc-hpa* genes with primers to ensure that RNA samples contained no contaminating genomic DNA. Total RNA (2 μg) was used to synthesize cDNA using the RNA PCR kit (AMV) with random primers (order number D3801) provided by the manufacturer (Takara Bio Inc.). The reaction was performed at 30°C for 10 min, 42°C for 1 h, and inactivated at 75°C for 5 min. The cDNA product (1 μl) and gene-specific primers (Table 3) were used in the RT-PCR reaction with the following PCR program: step 1, 95°C for 5 min; step 2, 95°C for 50 s; step 3, 53°C for 30 s; step 4, 72°C for 40 s; 35 cycles repeating from steps 2 to 4; and step 5, 72°C for 7 min. The RT-PCR products were sequenced to confirm specificity of the primers for the *hrp-hrc-hpa* genes.

Northern blot assay.

After induction in rice suspension cells, total RNA was extracted from *X. oryzae* pv. *oryzicola* strains as described above

at the indicated time point. For each sample, 30 μg of total RNA was subjected to electrophoresis in 1% agarose gels. The Biotin-labeled DNA probe was prepared by the BrightStar Psoralen-Biotin Labeling kit (Ambion, Austin, TX, U.S.A.) according to the manufacturer's instructions. The RNA was transferred to Hybond N^+ positively charged nylon membrane (Amersham Pharmacia, Uppsala, Sweden) and hybridized with specific probes at 42°C using Northern Max (Ambion), and signals were detected using BrightStar BioDetect (Ambion) according to the manufacturer's instructions.

GUS activity assay.

To construct *hrp* promoter-GUS fusion constructs, the promoter regions of target genes in this study were fused to the promoterless *gusA* gene (Mitsuhara et al. 1996) with its ribosome binding site. Regions upstream of the start codon of these genes were amplified by PCR using the total DNA of the wild-type strain RS105 as the template, with primer pairs *phrcC*F/*phrcC*R, *phrpD51*F/*phrpD51*R, *phrpD52*F/*phrpD52*R, *phpa3*F/*phpa3*R, *phrcT1*F/*phrcT1*R, *phpa1*F/*phpa1*R, *phrpG*F/*phrpG*R, and *phrpX*F/*phrpX*R (Table 2) and fused to *gusA*, which was amplified with primer pair *gus*F/*gus*R (Table 2). Promoter-*gusA* fusions were cloned into pUFR034 (De Feyter et al. 1990) with *Eco*RI, giving phrcCGUS, phrpD51GUS, phrpD52GUS, phpa3GUS, phrcT1GUS, phrcT2GUS, phpa1-GUS, phrpGGUS, and phrpXGUS (Table 1).

To investigate whether HrpD6 regulates *hpa1* expression, HrpD6 was expressed in the *hrpX* mutant RΔ*hrpX* by using the constitutive promoter of a *X. oryzae* pv. *oryzicola* RS105 extracellular protease gene (*Xoryp_01688*) to drive *hrpD6* expression, while the *hpa1::gusA* reporter was used as an indicator. *Xoryp_01688* is 100% identical to *Xoryp_01688* of *X. oryzae* pv. *oryzicola* BLS256, which we proved to have extracellular protease activity (*unpublished data*). A 466-bp promoter upstream of *Xoryp_01688* and a 243-bp ORF of *hrpD6* ending with a stop codon were amplified by PCR using the genomic DNA of the wild-type RS105 as the template, with primer pairs *pecpA*F/*pecpA*R and *hrpD6*F1/*hrpD6*R1 (Table 2), respectively. The *Xoryp_01688* promoter region and *hrpD6* ORF were fused together at a *Bam*HI site and subsequently fused at a *Kpn*I site to a 2,044-bp *hpa1* promoter-GUS fusion, which was amplified from phpa1GUS with primer pair phpa1GUSF/phpa1GUSR (Table 2). The entire 2,753-bp fusion was later cloned into pUFR034 at an *Eco*RI site, giving plasmid pD6hpa1GUS (Table 1).

For GUS activity assays, *X. oryzae* pv. *oryzicola* strains were cultured in XOM3 to OD_{600} = 0.5. Bacteria cells were diluted and disrupted in sonic buffer (20 mM Tris-HCl, pH 7.0; 10 mM 2-mercaptoethanol; 5 mM EDTA; and 1% Triton X-100). GUS activities were determined at intervals of 15 min until 4 h by measurement of the OD_{415} using PNPG as the substrate (Jefferson et al. 1987). One unit (U) was defined as 1 nmol of 4-methyl-umbelliferone produced per minute per bacterium.

Type III secretion assays.

To generate Hpa2-c-Myc, Hpa1-c-Myc, and HrpF-c-Myc expression constructs, *hpa2* with the promoter region (–216 to –1 bp upstream), *hpa1* with the promoter region (–231 to –1 bp upstream), and *hrpF* with the promoter region (–667 to –1 bp upstream), respectively, were amplified from the genomic DNA of *X. oryzae* pv. *oryzicola* RS105 strain by PCR with the primers listed in Table 2. The PCR products were cloned into corresponding restriction sites of pHM1 in-frame with a c-Myc epitope-encoding sequence, giving pHpa2-c-Myc, pHpa1-c-Myc, and pHrpF-c-Myc (Table 2), respectively. The AvrXa27-Flag in the pHM1 vector has previously been used in *X. oryzae* pv. *oryzae* for detection of proteins secreted through the T3S apparatus (Gu et al. 2005). These constructs were then transformed individually into the wild-type strain RS105, the *hrcV* mutant RΔ*hrcV*, the *hrpD6* mutant RΔ*hrpD6*, and the *hpaB* mutant RΔ*hpaB* (Table 1), respectively, for protein secretion detection.

For Western blot analysis, *X. oryzae* pv. *oryzicola* strains were preincubated in NB medium, resuspended at OD_{600} = 2.0 in sterilized water, and washed twice. Then, 40 μl of the bacterial suspension was inoculated into 1 ml of the *hrp*-inducing medium XOM3 (pH 6.5) at 28°C for 16 h. Cell and supernatant fractions were separated by centrifugation, and proteins in the supernatant fraction were precipitated with 12.5% trichloroacetic acid (Laemmli 1970). Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to membranes for immunoblotting using primary antibody anti-FLAG or anti-c-Myc (Genescript, Nanjing, China). Primary antibodies were recognized by anti-rabbit secondary antibodies (Genescript) and visualized on autoradiographs with the Western-Light chemiluminescence system (Transgene, Nanjing, China).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Berkeley Drosophila Genome Project website: www.fruitfly.org/seq_tools/promoter.html
NCBI Nucleotide Alphabet of Life website: www.ncbi.nlm.nih.gov/nuccore/NZ_AAQN01000001
Jinsite Biotechnology website: www.croasia.net/company/jinsite_biotechnology_co.html
Takara Bio Inc. website: www.takara-bio.com
Life Technologies Invitrogen website: www.invitrogen.com