Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2013) 14(7), 678–692 DOI: 10.1111/mpp.12039

HrpE3 is a type III effector protein required for full virulence of *Xanthomonas oryzae* pv. *oryzicola* in rice

YIPING CUI[1], LIFANG ZOU[2], HUASONG ZOU[2], YURONG LI[2], MUHAMMAD ZAKRIA[2] AND GONGYOU CHEN[1,2,*]

[1]*Department of Plant Pathology, Nanjing Agricultural University/Key Laboratory of Monitoring and Management for Plant Diseases and Insects, Ministry of Agriculture of China, Nanjing 210095, China*

[2]*School of Agriculture and Biology, Shanghai Jiao Tong University/Key Laboratory of Urban (South) by Ministry of Agriculture of China, Shanghai 200240, China*

SUMMARY

Xanthomonas oryzae pv. *oryzicola* (*Xoc*) is the causal agent of bacterial leaf streak, a devastating disease in rice. *Xoc* uses a type III secretion (T3S) system, which is encoded by the *hrp–hrc–hpa* (hypersensitive response and pathogenicity, *hrp*-conserved and *hrp*-associated) genes, to inject repertoires of T3S effectors (T3Es) into plant cells. Many of the *hrp–hrc–hpa* genes have roles in pathogenesis, but the role of *hrpE3*, which shows homology to *hpaE* in *X. campestris* pv. *vesicatoria* (*Xcv*), is poorly understood. In this study, *hrpE3* was shown to be transcribed independent of the *hrpD* operon, and its expression was dependent on a promoter within *hpaB*. The expression of *hrpE3* was positively regulated by HrpG and HrpX, a finding probably caused by an imperfect plant-inducible promoter (PIP) box (TTCGT-N_{16}-TTCGA) in the *hrpE3* promoter. The secretion of HrpE3 was dependent on T3S, and subcellular localization of HrpE3 was cytoplasmic and nuclear in plant cells. A mutation in *hrpE3* reduced the virulence of *Xoc* by decreasing disease lesion length and bacterial growth *in planta*. Full virulence was restored to the mutant when *Xoc hrpE3*, but not *Xcv hpaE*, was expressed *in trans*. The differences in transcription, secretion via the T3S system and bacterial virulence in plants were attributed to N-terminal amino acid differences between *Xoc* HrpE3 and *Xcv* HpaE. Collectively, the results demonstrate that *hrpE3* encodes a T3E protein which is delivered into the plant cell through the T3S system, localizes to the cytoplasm and nucleus, and is required for full virulence in rice.

INTRODUCTION

In Gram-negative phytopathogenic bacteria, the *hypersensitive response and pathogenicity* (*hrp*) genes are normally required for the activation of defence, e.g. the hypersensitive response (HR), in nonhost plants, and for parasitic growth in susceptible host plants. The *hrp* genes encode a type III secretion (T3S) system, which enables a bacterium to deliver T3S effector proteins (T3Es) into plant cells (Alfano and Collmer, 1997; Cunnac *et al*., 2009; Gürlebeck *et al*., 2006; He, 1998; Li *et al*., 2011b). The *hrp* genes in *Xanthomonas* species have been classified as *hrp* group II, which differs from *hrp* group I of *Erwinia amylovora* and *Pseudomonas syringae* (Alfano and Collmer, 1997; Tang *et al*., 2006). The elucidation of the *hrp–hrc–hpa* cluster in *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strain RS105 (Li *et al*., 2011a; Zou *et al*., 2006) and the completed genome sequence of *Xoc* BLS256 (Bogdanove *et al*., 2011) have revealed that the core *hrp* cluster contains 10 *hrp*, nine *hrc* (*hrp-conserved*) and eight *hpa* (*hrp-associated*) genes. The organization of the *Xoc hrp* cluster is similar to that of *X. oryzae* pv. *oryzae* (*Xoo*) (Cho *et al*., 2008; Lee *et al*., 2005; Ochiai *et al*., 2005; Salzberg *et al*., 2008), *X. campestris* pv. *vesicatoria* (*Xcv*) (Bonas *et al*., 2000), *X. campestris* pv. *campestris* (*Xcc*) and *X. axonopodis* pv. *citri* (*Xac*) (da Silva *et al*., 2002). Although there is shared synteny in *hrp* genes of closely related *Xanthomonas* spp., only *Xoc* contains *hrpE3*, which is homologous to *hpaE* in *Xcv* (Büttner *et al*., 2007; Weber *et al*., 2007). In *Xcv*, *hpaE* is not shown to be secreted via the T3S system, but its expression is dependent on HrpG and HrpX (Büttner *et al*., 2007). In *Xoc*, *hrpE3* is flanked by *hpaB* and *hpa4* in the *hrp–hrc–hpa* cluster (Li *et al*., 2011a; Zou *et al*., 2006). It is unclear whether *hrpE3* is independently transcribed or co-transcribed as part of an operon.

The expression of the *hrp–hrc–hpa* genes is generally suppressed in rich media, but highly induced *in planta* and in *hrp*-inducing media (Brito *et al*., 1999; Schulte and Bonas, 1992; Wei *et al*., 2000; Xiao *et al*., 1992, 2007). Typically, the expression of *hrp–hrc–hpa* genes is controlled by two key regulatory genes: *hrpG* and *hrpX* (Büttner and Bonas, 2006; Kim *et al*., 2003; Tang *et al*., 2006; Zou *et al*., 2006). HrpG is related to OmpR, a response regulatory protein in the highly conserved, two-component signal transduction paradigm. Presumably, HrpG perceives an environmental signal via a histidine protein kinase (HPK), but the cognate HPK for HrpG has not been identified (Wengelnik *et al*., 1996, 1999). HrpX is an AraC-type transcriptional activator (Wengelnik and Bonas, 1996), which forms a homodimer and contains a helix–turn–helix (HTH) motif. The HTH motif interacts with the plant-inducible promoter (PIP) box (TTCGC-N_{15}-TTCGC) in *hrp* transcripts by binding and activating transcription (Furutani *et al*.,

*Correspondence: Email: gyouchen@sjtu.edu.cn

2006; Koebnik *et al*., 2006). The PIP box was later described as TTCGB-N_{15}-TTCGB, where 'B' refers to any base except adenine (Cunnac *et al*., 2004; Mole *et al*., 2007; Tsuge *et al*., 2005).

In *Xanthomonas*, 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 PIP box-containing *hrp* transcripts (*hrpB* to *hrpF*) (Wengelnik and Bonas, 1996; Wengelnik *et al*., 1996, 1999) and other HrpX-regulated genes (Furutani *et al*., 2009; Jiang *et al*., 2009b; Li *et al*., 2012). Another *cis* element resembling the −10 binding sequence (YANNRT: Y, C/T; N, A/T/G/C; R, A/G) of RNA polymerase σ^{70} factor is present 30–32 bp downstream of the PIP box in *Xoo* (Furutani *et al*., 2006). However, the intergenic region between *hpaB* and *hrpE3* in *Xoc* is only 38 nucleotides and does not contain the PIP or −10 box (Li *et al*., 2011a; Zou *et al*., 2006). It is unclear whether a PIP box and/or σ^{70}-binding sequence is present further upstream of *hrpE3*.

In addition to the PIP box, some T3Es regulated by HrpX have the following characteristics: (i) their expression is co-regulated with *hrp* genes (Tang *et al*., 2006); and (ii) the first 50 N-terminal amino acids of the translated genes contain T3S signals [containing 20% serine (Ser) or proline (Pro) residues] (Furutani *et al*., 2009; Schechter *et al*., 2004). T3Es in *Xoo*, *Ralstonia solanacearum* and *P. syringae* are enriched in Ser and Pro residues in the first 50 N-terminal amino acids (Alfano and Collmer, 2004; Cunnac *et al*., 2004; Furutani *et al*., 2009; Schechter *et al*., 2006; Tampakaki *et al*., 2004). The transcription and translocation of T3Es have been examined using several reporter systems, including the calmodulin-dependent adenylate cyclase (Cya) from *Bordetella pertussis* (Casper-Lindley *et al*., 2002; Cunnac *et al*., 2004; Schechter *et al*., 2006), the glucuronidase protein encoded by *gusA* (Mukaihara *et al*., 2010) and avirulence proteins lacking T3S signal sequences, e.g. AvrBs1 (Jiang *et al*., 2009b; Roden *et al*., 2004) and AvrXa10 (Li *et al*., 2011b). It remains unclear whether HrpE3 is secreted via the T3S system in a manner consistent with other T3Es.

Although *hpa* gene products contribute to virulence, *hpa* mutations generally have a mild effect on disease severity compared with other *hrp–hrc* mutations (Cho *et al*., 2008; Huguet *et al*., 1998; Kim *et al*., 2003; Li *et al*., 2011a; Zou *et al*., 2006). However, some Hpa proteins are indispensable for pathogenicity, including HpaB and HpaC, which work as exit control proteins for the secretion of some T3Es into plant cells (Büttner *et al*., 2004; Gürlebeck *et al*., 2006; Li *et al*., 2011b). Generally, T3Es in *Xanthomonas* spp. are classified into two types: TALEs (transcriptional activator-like effectors) and NTALEs (non-TAL effectors) (Jiang *et al*., 2009b; Song and Yang, 2010; Yang and White, 2004). *tale* genes, such as *avrXa10*, trigger HR in an HpaB-dependent manner in rice cultivars containing the corresponding resistance (*R*) genes (Cunnac *et al*., 2009; Gürlebeck *et al*., 2006; Sugio *et al*., 2005). This avirulence (*avr*)–*R* gene-mediated response has been used previously to explore the secretion of NTALEs by fusing the first 50 amino acids of NTALES with an N-terminally-truncated AvrXa10 (Li *et al*., 2011b).

In this study, we investigated the transcription, regulation and secretion of HrpE3. The contribution of *hrpE3* to virulence was investigated using a genetic approach, and the gene was shown to be required for a full level of pathogenicity in the *Xoc*–rice interaction.

RESULTS

hrpE3 is required for full virulence in rice and cannot be replaced by *hpaE* from *Xcv*

Sequence analysis of the *hpaB–hpa4* intergenic region in *Xoc* RS105 led to the identification of *hrpE3* (Zou *et al*., 2006), which is homologous to *hpaE*, a virulence factor in *Xcv* (Büttner *et al*., 2007). To determine whether *hrpE3* is required for virulence in *Xoc*, we constructed the *hrpE3* deletion mutant RΔ*hrpE3* (Table 1; Fig. S1, see Supporting Information). The wild-type strain RS105 and mutant RΔ*hrpE3* were inoculated to seedlings and adult plants of the susceptible rice line IR24. Strain RΔ*hrpE3* displayed significantly (*t*-test, $P = 0.01$) smaller lesions (Fig. 1A,B) and reduced bacterial growth (Fig. 1C) relative to the wild-type. When *hrpE3* (under the control of a 397-bp promoter region, see construct pP1HrpE3-c-Myc in Table 1) was introduced into the deletion mutant, virulence was restored (see strain designated CRΔ*hrpE3*, Fig. 1).

We then investigated whether *hpaE* from *Xcv* could restore the virulence defect in the *Xoc hrpE3* mutant. Two constructs were used for this objective: *hpaE* under the control of the *hrpE3* promoter (201-bp promoter region upstream of the *hrpE3* predicted start codon; construct pP2HpaE-c-Myc, Table 1) and *hpaE* under the control of its native promoter (987-bp region upstream of the *hpaE* predicted start codon; construct p987hpaE, Table 1). Regardless of the promoter used, *hpaE* failed to complement the virulence deficiency in mutant RΔ*hrpE3* (Fig. 1A,B).

The predicted protein sequences of HrpE3 (ABH07404) and HpaE (YP_362146) differ primarily at their N-termini. Thus, we sought to determine whether a chimeric gene containing the N-terminus of HrpE3 fused to the C-terminus of HpaE could restore virulence to RΔ*hrpE3*. Plasmid p2nE3-cE was designed to investigate this possibility; this chimeric construct contains 351 nucleotides from *Xoc* RS105 (spanning 201 bp upstream of *hrpE3* and extending 150 bp into the coding region) fused to a 132-bp region containing the C-terminus of *hpaE* from *Xcv* 23-1. Notably, the impaired virulence of the *hrpE3* mutant was restored to wild-type levels when p2nE3-cE was introduced into RΔ*hrpE3* (Fig. 1A–C). These results indicate that the C-terminal functions of HrpE3 and HpaE are functionally interchangeable in *Xoc*, but that the N-terminal regions are not.

Table 1 Strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Reference or source
Xanthomonas oryzae pv. *oryzae*		
PXO99[A]	Wild-type, Philippine race 6	This laboratory
PΔ*hrcU*	*hrcU* deletion mutant of strain PXO99[A]	Li *et al*. (2011b)
Xanthomonas oryzae pv. *oryzicola*		
RS105	Wild-type, Chinese race 2; Rif^r	Zou *et al*. (2006)
RΔ*hrpE3*	*hrpE3* deletion mutant of strain RS105; Rif^r	This work
CR*hrpE3*	RΔ*hrpE3* containing pP2HrpE3-c-Myc; Rif^r Km^r	This work
CR*hrpE3*(201hpaE)	RΔ*hrpE3* containing pP2HpaE-c-Myc; Rif^r Km^r	This work
RΔ*hrcC*	*hrcC* deletion mutant of strain RS105; Rif^r	Li *et al*. (2011a)
RΔ*hrpG*	*hrpG* deletion mutant of strain RS105, Rif^r	Jiang *et al*. (2009a)
RΔ*hrpX*	*hrpX* deletion mutant of strain RS105; Rif^r	Jiang *et al*. (2009a)
RΔ*hpaB*	*hpaB* deletion mutant of strain RS105, Rif^r	Li *et al*. (2011b)
RΔ*hrcV*	*hrcV* deletion mutant of strain RS105; Rif^r	This laboratory
RΔ*hrpD5*	*hrpD5* deletion mutant of strain RS105, Rif^r	Li *et al*. (2011a)
RΔ*hrpD6*	*hrpD6* deletion mutant of strain RS105, Rif^r	Li *et al*. (2011a)
RΔ*hrpE*	*hrpE* deletion mutant of strain RS105, Rif^r	Li *et al*. (2011a)
Xanthomonas campestris pv. *vesicatoria*		
23-1	Wild-type, Chinese race	Laboratory collection
Escherichia coli		
DH5α	F$^-$ Φ80d*lacZ* ΔM15Δ (*lacZYA-argF*)U169 *endA1 deoR recA1 hsdR17*(r_K^- m_K+) *phoA supE44* λ$^-$ *thi-l gyrA96 relA1*	Clontech
Agrobacterium tumefaciens		
EH105	Rif^r	This laboratory
Plasmids		
pMD18-T	pUC *ori*, cloning vector; Ap^r	TaKaRa
pUFR034	*IncW*, *Mob(p)*, *Mob*$^-$, *LacZa*$^+$, PK2 replicon, cosmid; Km^r	De Feyter *et al*., 1990
pKMS1	6.4-kb derivative from pK18mobGII, *sacB* $^+$; Km^r	Zou *et al*., 2011
pKΔ*hrpE3*	1250-bp fusion ligated in pKMS1 with a 285-bp deletion in *hrpE3*; Km^r	This work
pP1HrpE3-c-Myc	pUFR034 expressing *hrpE3* under the control of its own 397-bp promoter with a c-Myc tag; Km^r	This work
pP2HrpE3-c-Myc	pUFR034 expressing *hrpE3* under the control of its own 201-bp promoter with a c-Myc tag; Km^r	This work
pP3HrpE3-c-Myc	pUFR034 expressing *hrpE3* under the control of its own 101-bp promoter with a c-Myc tag; Km^r	This work
p987HpaE-c-myc	pUFR034 expressing *hpaE* under the control of its own 987-bp promoter with a c-Myc tag; Km^r	This work
pP1HpaE-c-myc	pUFR034 expressing *hpaE* under the control of *hrpE3* 398-bp promoter with a c-Myc tag; Km^r	This work
pP2HpaE-c-myc	pUFR034 expressing *hpaE* under the control of *hrpE3* 201-bp promoter with a c-Myc tag; Km^r	This work
pP2nE3-cE	pUFR034 expressing the fusion encoding the 50 N-terminal amino acids of HrpE3 and the last 44 C-terminal amino acids of HpaE under a 201-bp *hrpE3* promoter; Km^r	This work
pP1GUS	pUFR034 expressing GUS under the 397-bp promoter of *hrpE3*; Km^r	This work
pP2GUS	pUFR034 expressing GUS under the 201-bp promoter of *hrpE3*; Km^r	This work
pP3GUS	pUFR034 expressing GUS under the 101-bp promoter of *hrpE3*; Km^r	This work
p987GUS	pUFR034 expressing GUS under the 987-bp promoter of *hpaE*; Km^r	This work
p397GUS	pUFR034 expressing GUS under the 397-bp promoter of *hpaE*; Km^r	This work
p201GUS	pUFR034 expressing GUS under the 201-bp promoter of *hpaE*; Km^r	This work
p101GUS	pUFR034 expressing GUS under the 101-bp promoter of *hpaE*; Km^r	This work
pavrXa10	pUFR034 expressing AvrX10, Km^r	Li *et al*. (2011b)
pavrXa10Δ	pUFR034 expressing AvrXa10Δ, Km^r	Li *et al*. (2011b)
pP2avrXa10Δ	avrXa10Δ expression from the 205-bp promoter of *hrpE3* in pUFR034; Km^r	This work
pP1E3avrXa10Δ	The first 50 amino acids of HrpE3 fused with AvrXa10Δ under a 397-bp promoter region of *hrpE3* in pUFR034; Km^r	This work
pP2E3avrXa10Δ	The first 50 amino acids of HrpE3 fused with AvrXa10Δ under a 201-bp promoter region of *hrpE3* in pUFR034; Km^r	This work
pP3E3avrXa10Δ	The first 50 amino acids of HrpE3 fused with AvrXa10Δ under a 101-bp promoter region of *hrpE3* in pUFR034; Km^r	This work
pP2EavrXa10Δ	The first 50 amino acids of HpaE fused with AvrXa10Δ under a 201-bp promoter region of *hrpE3* in pUFR034; Km^r	This work
pEGAD	*lacZa*$^+$, contains GFP; Km^r	This laboratory
pHrpE3-GFP	A 285-bp *hrpE3* was fused in frame with *gfp* in vector pEGAD; Km^r	This work
pHpaE-GFP	A 300-bp *hpaE* was fused in frame with *gfp* in vector pEGAD; Km^r	This work
pXopR-GFP	A 1314-bp *XopR* was fused in frame with *gfp* gene in vector pEGAD; Km^r	Guo *et al*. (2012)

Ap^r, ampicillin resistance; GFP, green fluorescent protein; GUS, β-glucuronidase; Km^r, kanamycin resistance; Rif^r, rifampicin resistance.

hrpE3 is transcribed by a promoter located within *hpaB*

In *Xcv*, the *hrpE*, *hpaB* and *hpaE* genes are in the *hrpE* operon, and *hpaB* and *hpaE* are co-transcribed, but they are not in the same operon with *hrpE* (Büttner *et al*., 2007; Rossier *et al*., 2000; Weber *et al*., 2007). However, the transcriptional organization of *hrpE3* and the upstream regions in *Xoc* remain unclear. A 38-bp intergenic region is present between *hpaB* and *hrpE3* in *Xoc* (Fig. S1), which prompted us to determine the location of the

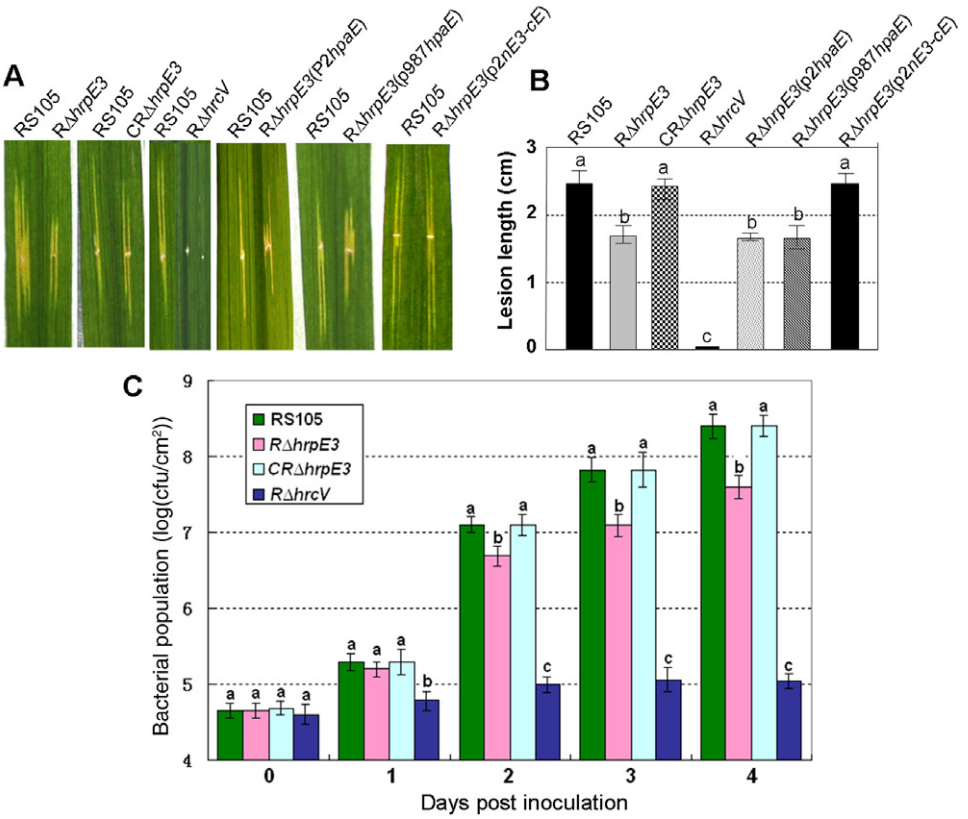

Fig. 1 *hrpE3* is required for the virulence and growth of *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) in rice. (A) Bacterial leaf streak (BLS) symptoms caused by *Xoc* strains RS105 (wild-type), RΔ*hrpE3* (*hrpE3* mutant), CRΔ*hrpE3* (complemented *hrpE3* mutant with pP1hrpE3-c-Myc), RΔ*hrpE3*(pP2hpaE-c-Myc), RΔ*hrpE3*(p987hpaE), RΔ*hrpE3*(p2nE3-cE) and RΔ*hrcV* (*hrcV* mutant; nonpathogenic control). *Xoc* strains [1×10^8 colony-forming units (cfu)/mL] were inoculated to rice cultivar IR24 (2 months old) by leaf needling, and lesions were measured at 14 days post-inoculation (dpi). The left half of each leaf was inoculated with the wild-type RS105, and the right half was inoculated with *Xoc* derivatives. (B) BLS lesion lengths formed in rice (2 months old) inoculated with *Xoc* strains and assessed at 14 dpi. (C) Bacterial population in rice leaves inoculated with *Xoc* and derivatives. Leaf discs (0.5 cm in diameter) were excised from the inoculated areas, homogenized in sterile water, diluted and plated on nutrient agar (NA) plates. Data points represent the means ± standard deviation (SD) from three replicates.

hrpE3 promoter. We first determined the transcriptional linkages between the *hrpD5* and *hrpE3* regions in the wild-type RS105 and deletion mutants RΔ*hrpD5*, RΔ*hrpD6*, RΔ*hrpE*, RΔ*hpaB* and RΔ*hrpE3* (Table 1, Fig. 2). *Xoc* RS105 and mutant strains were incubated in XOM3, an *hrp*-inducing medium, for 16 h, and reverse transcription-polymerase chain reaction (RT-PCR) was performed to assess the expression of the intergenic junctions using primer pairs flanking each open reading frame (ORF) (Table S1, see Supporting Information). If the entire *hrpD5* to *hrpE3* region was co-transcribed, a 3302-bp PCR product would be predicted; however, this was not detected in the wild-type RS105 or the five mutant strains (Fig. 2). However, when primers encompassing *hrpD5* to *hpaB* were used in RT-PCR, a 2295-bp PCR product detected in RS105 was larger than those in the *hrpD6*, *hrpE*, *hpaB* and *hrpE3* mutants (Fig. 2B, see lane designated D5-aB). These results suggest that *hrpD5*, *hrpD6*, *hrpE* and *hpaB* are co-transcribed as polycistronic mRNA. This hypothesis was supported by the PCR products observed when smaller regions were amplified (e.g. 349-bp PCR product when primers extended from *hrpD6* to *hrpE*; 729-bp product using primers spanning *hrpE* to *hpaB*; see products amplified from strain RS105, lanes D6-E and E-aB, Fig. 2B). The predicted 804-bp PCR product spanning *hpaB–hrpE3* (lane designated aB-E3) was not obtained in the wild-type RS105 or mutants, suggesting that *hpaB* and *hrpE3* are independently transcribed (Fig. 2B, lane ab-E3). However, a 285-bp PCR product was amplified from RS105, RΔ*hrpD5*, RΔ*hrpD6* and RΔ*hrpE* when primers specific to *hrpE3* were used in RT-PCR (Fig. 2B, lane E3). The absence of the 285-bp PCR product in the *hpaB* and *hrpE3* mutants suggests that transcription of *hrpE3* begins in the coding region of *hpaB*. Thus, *hrpE3* is transcribed independently from a promoter located within *hpaB*, an organization that is clearly different from *hpaE* in *Xcv*, where *hpaE* and *hpaB* are co-transcribed (Büttner *et al*., 2007).

5′-Rapid amplification of cDNA ends (5′-RACE) was used to further define the promoter region for *hrpE3*. When RACE outer

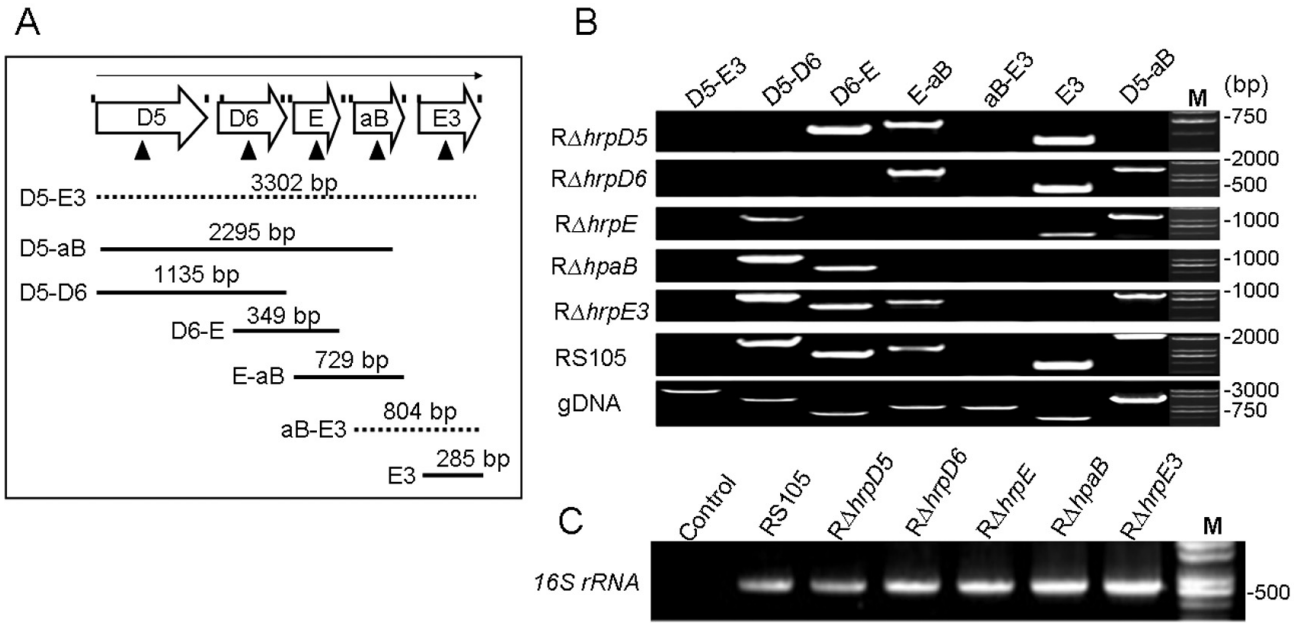

Fig. 2 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of transcriptional units in the *hrpD5–hrpE3* region of *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*). (A) Schematic representation of the *hrpD5–hrpE3* region; RT-PCR primers are indicated by vertical lines above the open arrows and were designed to span the intergenic junctions. Triangular arrows present the open reading frame (ORF)-deleted mutations in the region from *hrpD5* to *hrpE3*. The open arrows represent *hrpD5* (D5), *hrpD6* (D6), *hrpE* (E), *hpaB* (aB) and *hrpE3* (E3). The full lines indicate the physical map location of PCR products amplified in this experiment; the dotted lines represent the deletions in PCR products D5-E3 and aB-E3. D5-E3 denotes PCRs with the primer pair hrpD5F-hrpE3R, D5-aB with hrpD5F-hpaBR, D5-D6 with hrpD5F-hrpD6R, D6-E with hrpD6F-hrpER, E-aB with hrpEF-hpaBR, aB-E3 with hpaBF-hrpE3R and E3 with hrpE3F-hrpE3R. (B) Transcripts detected by RT-PCR using cDNA isolated from RS105, RΔ*hrpD5*, RΔ*hrpD6*, RΔ*hrpE*, RΔ*hpaB* and RΔ*hrpE3*; bacterial strains were incubated in the *hrp* (*hypersensitive response and pathogenicity*)-inducing medium XOM3 for 16 h. (C) The 16S *rRNA* gene was used to verify the absence of significant variation in cDNA levels. Lane M represents a molecular weight marker (TaKaRa). All experiments were repeated three times, and similar results were obtained each time.

and inner primers (O-F and I-F) (Table S1) were used in nested PCR with the anchor-specific *hrpE3* outer and inner primers (*hrpE*3O-R and *hrpE*3I-R, Table S1), a 201-bp fragment was amplified (Fig. 3A). Sequence analysis indicated that this 201-bp region was located in the coding region of *hpaB*. Included within this 201-bp region were an imperfect PIP box (TTCGTT-N_{16}-TTCGA) sequence (as in *Xcv*) and a −10 box (GAGCCT), 32 bp downstream of the PIP box, that is changed to GCGCCT in the corresponding region upstream of *hpaE* (Fig. 3A). In comparing the predicted translational start codons of *hpaE* and *hrpE3*, we note that *hrpE3* contains an additional 14 nucleotides of leader sequence, including a predicted Shine Dalgarno (SD) site, which are not present in *hpaE* (Fig. 3A). The differences above may help to explain the divergent transcriptional organization of the two genes.

The information presented above (see Figs 2 and 3A) suggests that *hrpE3* is transcribed under its own promoter. To test this hypothesis, three transcriptional fusions were constructed between the *hrpE3* promoter region and a promoterless *gusA* gene. The constructs included pP1GUS, pP2GUS and pP3GUS (Table 1). The *gusA* gene was fused to the 397-, 201- and 101-bp regions upstream of the *hrpE3* translational start codon shown in Fig. 3B. These constructs were then introduced into the wild-type *Xoc* strain RS105. β-Glucuronidase (GUS) activity was measured after 16 h of growth in *hrp*-inducing medium (XOM3) and a nutrient-rich medium (NB). GUS activity of *Xoc* containing either pP1GUS or pP2GUS was significantly higher than *Xoc* (pP3GUS), regardless of the growth medium (*t*-test, $P = 0.01$). This result suggests that the 101-bp DNA region in pP3GUS does not contain the promoter region necessary for *hrpE3* transcription. Furthermore, GUS activity was higher when *Xoc* RS105 containing pP1GUS or pP2GUS was cultured in XOM3 relative to NB (Fig. 3C). These results suggest that the *hrpE3* promoter is plant inducible, and that the promoter region is located in the 201-bp region upstream of the *hrpE3* translational start site.

For comparative purposes, four transcriptional fusions were constructed between putative *hpaE* promoter regions and the promoterless *gusA*. These included *gusA* fused to a 987-bp fragment (containing a 263-bp region upstream of *hrpE* and the intact *hrpE* and *hpaB* genes) and the 397-, 201- and 101-bp regions upstream of the *hpaE* predicted start codon; these plasmids were designated p987GUS, p397GUS, p201GUS and p101GUS, respectively (Table 1). These constructs were transferred into the wild-type RS105, and GUS activity was measured in XOM3 medium. GUS activity of *Xoc* containing p987GUS was significantly higher

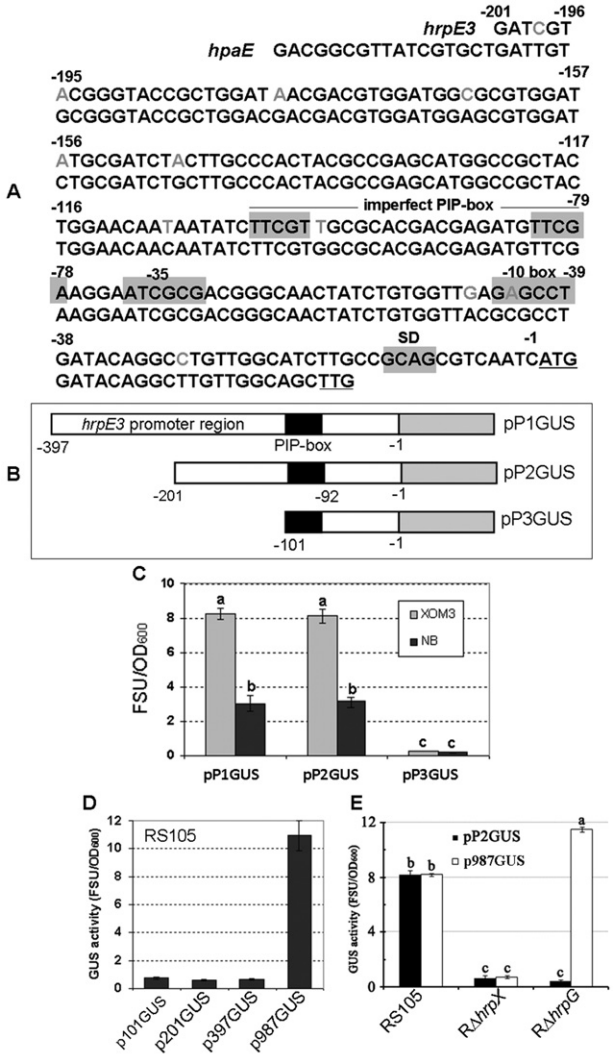

Fig. 3 Analysis of the *hrpE3* promoter region. (A) The comparison of the nucleotide sequences 201 bp upstream of the *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) *hrpE3* start codon (ATG) and the *X. campestris* pv. *vesicatoria* (*Xcv*) *hpaE* start codon (TTG). The letters underlined indicate the predicted start codon of *hrpE3* or *hpaE*. A predicted Shine Dalgarno (SD) sequence is shaded in grey upstream of the *hrpE3* start codon and is absent in *hpaE*. A putative imperfect plant-inducible promoter (PIP) box is shaded in grey and a −10 box 32 bp downstream of the PIP box is marked in grey, but some important nuclear acids are altered correspondingly in the upstream of *hpaE*. The grey letters in the *hrpE3* promoter region display the different nucleotides from those in the upstream of *hpaE*. (B) Schematic map of the *hrpE3* promoter region containing the PIP box (in black) fused to a promoterless *gusA* (GUS). Three different sized fragments upstream of *hrpE3* in *Xoc* were subcloned as three promoter regions containing 397 (pP1), 201 (pP2) or 101 (pP3) nucleotides, to form pP1GUS, pP2GUS and pP3GUS, respectively. (C) β-Glucuronidase (GUS) activity of the pP1GUS, pP2GUS and pP3GUS constructs in the wild-type *Xoc* RS105. GUS activity was measured after 16 h of growth in the *hrp* (*hypersensitive response and pathogenicity*)-inducing medium (XOM3) and the nutrient-rich medium (NB). (D) Detection of GUS activity in *Xoc* RS105 strain containing transcriptional fusions to fragments upstream of the *hpaE* start codon of *Xcv*. Upstream fragments included 987 (p987), 397 (p987), 201 (p201) and 101 (p101) nucleotides fused to a promoterless *gusA* to generate p987GUS, p397GUS, p201GUS and p101GUS, respectively. (E) The comparison of GUS activity in *Xoc* RS105, RΔ*hrpX* and RΔ*hrpG* containing either pP2GUS or p987GUS. Bacterial strains were cultured in XOM3 medium for 16 h, and GUS activity was assayed as described in Experimental procedures. All experiments were repeated three times with similar results. Statistical differences in (C) and (D) are indicated by different letters.

(*t*-test, $P = 0.01$) than *Xoc* harbouring p397GUS, p201GUS or p101GUS, with no significant differences among the last three constructs (Fig. 3D). The data established that *hpaE* is transcribed as part of an *hrpE–hpaB–hpaE* operon, an organization that is clearly different from *hrpE3* in *Xoc*.

hrpE3 expression is positively regulated by HrpG and HrpX

In *Xcv*, the expression of *hpaE*, which is homologous to *hrpE3* in *Xoc*, is regulated by HrpG and HrpX (Büttner *et al*., 2007). To investigate whether *hrpE3* is regulated similarly in *Xoc*, we transferred pP2GUS, which contains the 201-bp region upstream of *hrpE3* fused to a promoterless *gusA*, into the *hrpG* and *hrpX* mutants, RΔ*hrpG* and RΔ*hrpX*, respectively. The bacterial strains were cultured in XOM3 medium for 16 h, and GUS activity was assayed. GUS expression in RΔ*hrpG* and RΔ*hrpX* containing pP2GUS was approximately sevenfold lower ($P = 0.01$, *t*-test) than in RS105 (pP2GUS) (Fig. 3E). For comparative purposes, we transferred the transcriptional fusion p987GUS into *Xoc* RS105, RΔ*hrpG* and RΔ*hrpX* to investigate promoter activity in the 987-bp region upstream of *hpaE*. In XOM3 medium, GUS expression in RΔ*hrpX* (pP987GUS) was significantly lower ($P = 0.01$, *t*-test) than in RS105 and RΔ*hrpG* containing pP987GUS (Fig. 3E). In summary, these data indicate that the *hrpE3* promoter is positively regulated by both HrpG and HrpX, whereas the transcript containing *hpaE* is positively regulated by HrpX, but not HrpG.

The expression of *hrpE3* is induced *in planta*

Rice seedlings of cultivar IR24 were infiltrated with *Xoc* RS105, and the expression of *hrpE3* was monitored by RT-PCR using the *hrpE3*-specific primers *hrpE3*-F/*hrpE3*-R (Table S1) at 0, 3, 6, 12 and 24 h post-inoculation (hpi). The expression of *hrpE3* was elevated at 0, 3, 6 and 12 hpi, with maximal expression detected at 12 hpi (Fig. 4A), indicating that expression of *hrpE3* is induced *in planta*. We also investigated the *in planta* expression of *hrpE3* in mutants RΔ*hrcC*, RΔ*hrpX*, RΔ*hrpG* and RΔ*hpaB*, which are defective in *hrcC*, *hrpX*, *hrpG* and *hpaB*, respectively. Although *hrpE3* was highly expressed in RS105 and mutant RΔ*hrcC*, there was no obvious expression of *hrpE3* detected in RΔ*hrpG*, RΔ*hrpX* and RΔ*hpaB* strains (Fig. 4B).

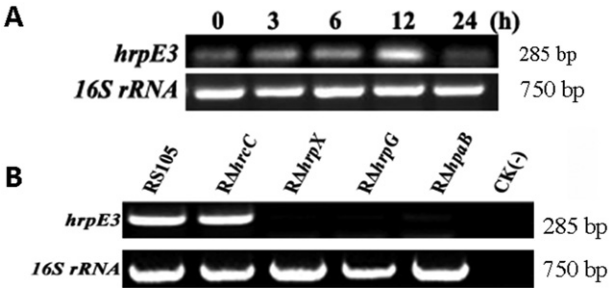

Fig. 4 Detection of *hrpE3* expression *in planta* by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). (A) Time point detection of *hrpE3* expression in rice. Seedlings of rice cultivar IR24 (2 weeks old) were inoculated with *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) using a needleless syringe and assayed by semi-quantitative RT-PCR at 0, 3, 6, 12 and 24 h post-inoculation (hpi). PCR products were electrophoretically separated on a 1.2% agarose gel. 16S *rRNA* was amplified as a constitutive control. (B) Detection of *hrpE3* expression in *Xoc* RS105 and *hrcC*, *hrpG*, *hrpX* and *hpaB* mutants. Strains were infiltrated into rice seedlings (as described above), incubated for 12 h and total RNA was extracted for RT-PCR. Water [CK(-)] was used as a negative control. PCR products were electrophoretically separated as described above, and 16S *rRNA* was amplified as a control. The experiments were repeated three times with similar results, and one experiment is displayed.

HrpE3 is secreted through the T3S system, but HpaE is not

In many Gram-negative phytopathogenic bacteria, T3Es contain a secretion signal within the N-terminal 50 amino acids that is highly enriched in Ser and Pro residues (Alfano and Collmer, 2004; Cunnac *et al*., 2004; Furutani *et al*., 2009; Schechter *et al*., 2006; Tampakaki *et al*., 2004). For example, Furutani *et al*. (2009) showed that *Xanthomonas* effectors contained up to 20% Ser/Pro residues in their N-terminal sequences. One strategy for the investigation of secretion via the T3S system is to fuse the N-terminus of a candidate effector with an Avr protein that lacks a secretion signal, and investigate whether the fused effector can be translocated into plant cells (Jiang *et al*., 2009b; Li *et al*., 2011a). Sequence analysis of the predicted translational product of HrpE3 indicated the presence of three Ser and six Pro residues (18% Ser/Pro) in the first 50 amino acids (Fig. 5A). In comparison, HpaE from *Xcv* contains six Ser and seven Pro residues in the N-terminal 50 amino acids (Fig. 5A), suggesting that HpaE may also be a T3E. However, it is important to note that the N-terminal 50 amino acids of HrpE3 and HpaE are very different from each other (Fig. 5A). To investigate whether these proteins are secreted by the T3S system, the N-terminal 50 amino acids of HrpE3 and HpaE were fused with a truncated AvrXa10 that lacked 28 amino acid residues at the N-terminus (*avrXa10*Δ). The chimeric protein E3avrXa10Δ was expressed from the three putative *hrpE3* promoters described in Fig. 3B, resulting in pP1E3avrXa10Δ, pP2E3avrXa10Δ and pP3E3avrXa10Δ (Fig. 5B). The fusion protein EavrXa10Δ, consisting of the first 50 amino acids of HpaE fused to AvrXa10Δ, was expressed under the control of the 201-bp promoter of *hrpE3* in plasmid pP2EavrXa10Δ (Table 1). It is important to mention that the wild-type RS105 harbouring *avrXa10* does not trigger an HR in rice line IRBB10, which carries the cognate *R* gene *Xa10* (Li *et al*., 2011b). Therefore, we used *Xoo* strain PXO99^A to investigate whether the fused protein is translocated into IRBB10. The T3S-deficient mutant PΔ*hrcU* was used as a negative control.

PXO99^A containing pP1E3avrXa10Δ or pP2E3avrXa10Δ produced an HR in IRBB10, as did PXO99^A (pavrXa10) (Fig. 5C). However, an HR was not elicited by PXO99^A containing pP3E3avrXa10Δ, pP2EavrXa10Δ or pP2avrXa10Δ (Fig. 5C); the latter construct contains the 201-bp *hrpE3* promoter fused to *avrXa10* with the N-terminal deletion. As predicted, the T3S-deficient strain PΔ*hrcU* did not elicit bacterial blight symptoms in rice cultivar IRBB10. *Xoo* strain PXO99^A containing the constructs mentioned above induced water-soaked lesions in rice cultivar IR24, which does not contain the *Xa10* gene (Fig. 5C).

We then used semi-quantitative RT-PCR to investigate whether or not the translational fusions present in pP2E3avrXa10Δ and pP2EavrXa10Δ were expressed in the infiltrated leaves. The *E3::avrXa10Δ* fusion was amplified in rice leaves inoculated with PXO99^A containing either pP1E3avrXa10Δ or pP2E3avrXa10Δ, and the *E::avrXa10* fusion was detected in rice leaves inoculated with PXO99^A (pP2EavrXa10Δ) (Fig. 5D). To summarize, the *hrpE3* promoter and the first 50 N-terminal amino acids of HrpE3, but not HpaE, enable the N-terminal truncated AvrXa10 to be secreted through the T3S system and translocated into rice cells for HR induction.

To confirm secretion via the T3S system, HrpE3 was expressed as a C-terminal Myc epitope-tagged derivative in plasmid pP2HrpE3-c-Myc (Table 1), which was introduced into *Xoc* strains RS105, RΔ*hrcC*, RΔ*hrpG*, RΔ*hrpX* and RΔ*hpaB*. Bacterial cells were incubated in XOM3 medium, and total protein extracts (TE) and culture supernatants (SN) were analysed for the presence of HrpE3-c-Myc using an epitope-specific antibody. The HrpE3 protein was detected in the TE and SN of the wild-type RS105 (Fig. 6A). However, HrpE3 was only detected in the TE of *hrp* deletion mutants (RΔ*hrcC*, RΔ*hrpG*, RΔ*hrpX* and RΔ*hpaB*) (Fig. 6A); this indicates that the secretion of HrpE3 did not occur in the mutants and was *hrp* dependent.

Using a similar strategy, the secretion of HpaE was evaluated in *Xoc* RS105 and the four *hrp* deletion mutants. The HpaE-c-Myc protein was detected in the TE of the *hrpG* mutant when expressed under the control of the 987-bp *hpaE* promoter region (p987HpaE-c-Myc) (Fig. 6B); however, it was not detectable in TE or SN from other strains. When expressed from the 201-bp *hrpE3* promoter, the HpaE-c-Myc fusion was detected in TE of all strains, but was undetectable in SN (see pP2HpaE-c-Myc) (Fig. 6C). The undetectable HpaE in the SN fractions of the wild-type and mutant strains implies that HpaE is not secreted via the T3S system.

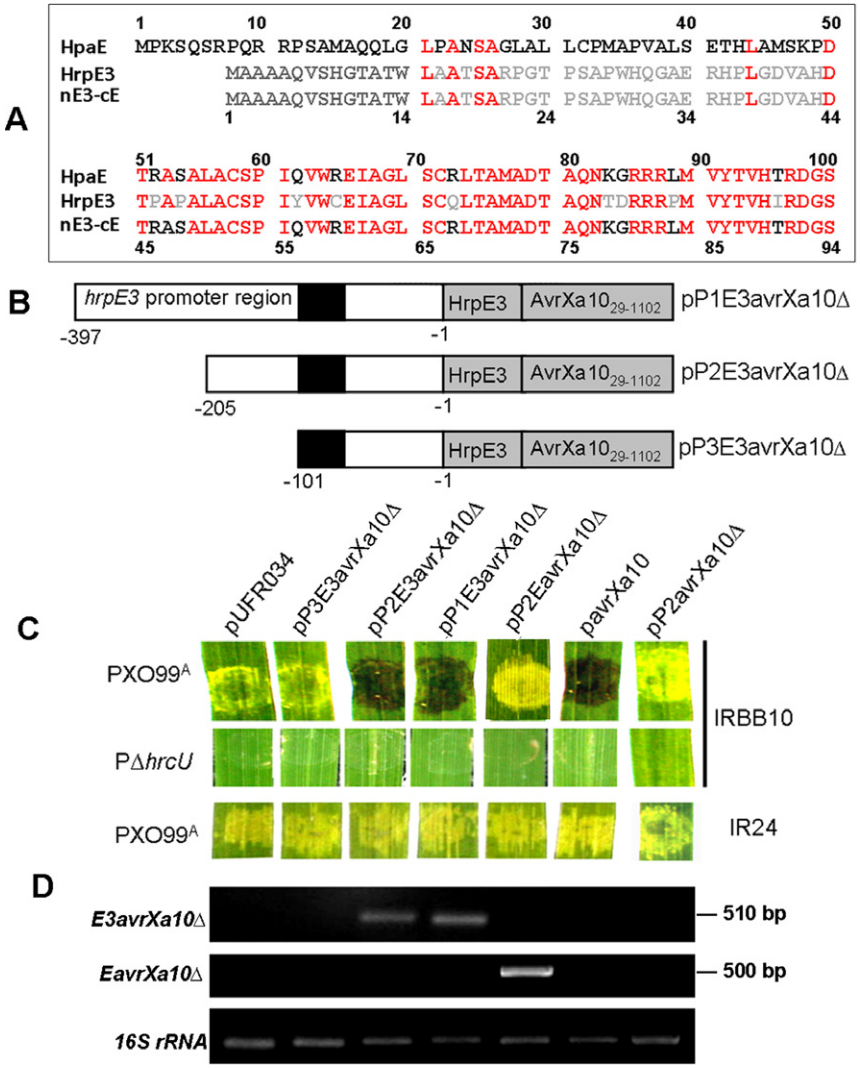

Fig. 5 Detection of HrpE3 secretion via the type III secretion (T3S) system. (A) Amino acid sequence alignment of *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) HrpE (ABH07404), *X. campestris* pv. *vesicatoria* (*Xcv*) HpaE (YP_362146) and the fused chimeric protein nE3-cE; the different amino acids among HrpE3, HpaE and nE3-cE are shown in grey and black letters, and the same in red letters. (B) Schematic maps showing HrpE3 constructs fused to AvrXa10. The 50 amino acids at the N-terminus of HrpE3 contain a higher percentage of serine (S) and proline (P) residues, and may comprise a T3S signal. The 50 N-terminal amino acids of HrpE3 were fused to a truncated version of AvrXa10Δ in which the first 28 amino acids were deleted, generating a fusion designated E3avrXa10Δ. The transcription of this fusion was driven by three fragments containing 397, 201 and 101 bp of *hrpE3* upstream DNA; these were designated pP1E3avrXa10Δ, pP2E3avrXa10Δ and pP3E3avrXa10Δ, respectively. The black rectangle indicates the location of the putative plant-inducible promoter (PIP) box in the promoter region of *hrpE3*. (C) The *X. oryzae* pv. *oryzae* (*Xoo*)–rice pathosystem was used to evaluate secretion and translocation of the HrpE3-AvrXa10 chimeric proteins. For comparative purposes, an HpaE-AvrXa10 chimeric protein was included. This construct was designated pP2EavrXa10Δ and contained the 50 N-terminal amino acids from HpaE fused to AvrXa10Δ; the promoter driving transcription was the 201-bp region upstream of *hrpE3*. *Xoo* strains containing the fusions were inoculated into rice cultivar IRBB10 or IR24 (see Experimental procedures). The native *avrXa10* was used as a positive control (construct pavrXa10) and the N-terminal truncated *avrXa10Δ* under control of the 201-bp *hrpE3* promoter region (pP2*avrXa10Δ*) served as a negative control. PXO99^{A} and P*ΔhrcU* containing the empty vector pURF034 were also included. Phenotypes were photographed 3 days after inoculation in three independent experiments. (D) Detection of the fused genes E3avrXa10Δ and EavrXa10Δ in rice IRBB10 leaves infiltrated with *Xoo* strains and analysed by reverse transcription-polymerase chain reaction (RT-PCR) at 12 h post-inoculation (hpi) (detailed in Experimental procedures). The 16S *rRNA* gene of *Xoo* was used as an internal control.

HrpE3 localizes to the cytoplasm of host cells

The localization of HrpE3 in plant cells was studied by fusing the *hrpE3* coding region as a translational fusion to *gfp* (encoding green fluorescent protein), whereas HpaE was used for comparison. For comparative purposes, a known T3E, XopR (Akimoto-Tomiyama *et al*., 2012), was also fused with GFP and used as a positive control. To confirm whether the fused proteins

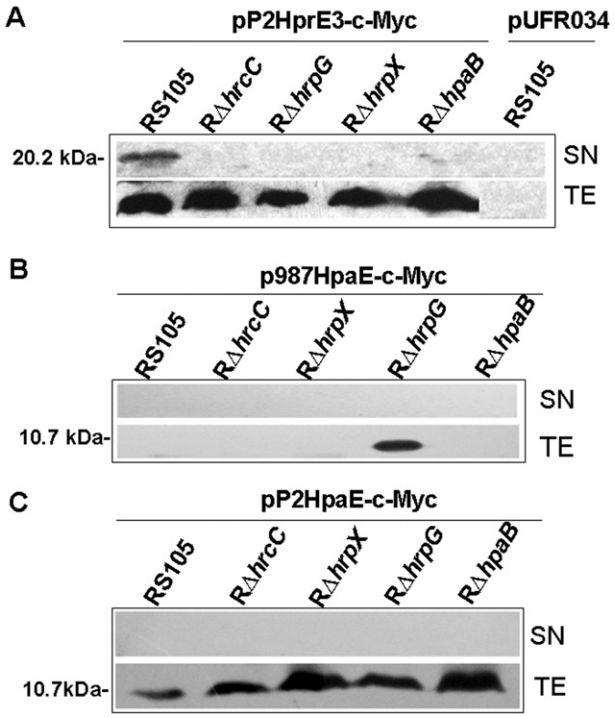

Fig. 6 Comparison of HrpE3 and HpaE secretion by immunoblotting. (A) Detection of HrpE3 secretion in different genetic background strains RS105, R*ΔhrcC*, R*ΔhrpG*, R*ΔhrpX* and R*ΔhpaB* harbouring pP2HrpE3-c-Myc (*hrpE3* under the control of the 201-bp *hrpE3* promoter). (B) Immunodetection of HpaE in *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strains RS105, R*ΔhrcC*, R*ΔhrpG*, R*ΔhrpX* and R*ΔhpaB* harbouring p987HpaE-c-Myc [*hpaE* under the control of the *X. campestris* pv. *vesicatoria* (*Xcv*)-derived 987-bp upstream promoter]. (C) Immunodetection of HpaE in *Xoc* strains RS105, R*ΔhrcC*, R*ΔhrpG*, R*ΔhrpX* and R*ΔhpaB* harbouring pP2HpaE-c-Myc (*hpaE* under the control of the 201-bp *hrpE3* promoter). After incubation in XOM3 medium for 16 h, the total protein extracts (TE) and culture supernatants (SN) of each tested strain were analysed by immunoblotting using anti-c-Myc antibodies (Genescript). The experiments were repeated three times, and representative results are shown.

were synthesized, we first evaluated whether they could be detected in tobacco leaves inoculated with *Agrobacterium tumefaciens* EH105 containing pEGAD, pHrpE3-GFP, pHpaE-GFP or pXopR-GFP (Table 1). After transient expression for 36 h, tobacco leaves were collected, and total proteins were extracted, precipitated and analysed for expression of GFP by immunoblotting (see Experimental procedures). As predicted, a 35.9-kDa HrpE3-GFP, a 36.9-kDa HpaE-GFP and a 75.01-kDa XopR-GFP were expressed as GFP fusions and could be detected using an anti-GFP antibody (Fig. 7A).

Fluorescence microscopy was used to examine the localization of the GFP fusions described above. Although HrpE3-GFP was localized to the cytoplasm and nucleus, HpaE-GFP was only observed in the cytoplasm (Fig. 7B). The localization of XopR-GFP was observed at the plasma membrane and in the cytoplasm, which is consistent with previous results (Akimoto-Tomiyama *et al*., 2012). In general, our results suggest a cytoplasmic location for HrpE3-GFP, although nuclear localization cannot be completely excluded.

DISCUSSION

Previously, we have reported that the *hrpD* operon in *Xoc* contains eight co-transcribed genes (*hrcQ* to *hpaB*; Li *et al*., 2011a), which is similar to the organization of the *hrpD* operon in the closely related pathogen *Xoo* (Cho *et al*., 2008). In the tomato/pepper pathogen *Xcv*, this region is subdivided into several transcriptional units. For example, the *hrpD* operon contains four co-transcribed genes, the *hrpE* operon contains *hrpD5–hrpD6–hrpE* and a third transcript contains *hpaB–hpaE* (Büttner *et al*., 2007; Weber *et al*., 2007). The results presented in this study show that *hrpE3*, the *hpaE* homologue in *Xcv*, is transcribed independently under a promoter located within *hpaB* of the *hrpD* operon. Thus, the transcriptional organization of *hrpE3* is clearly different from *hpaE* in *Xcv*, where *hpaE* and *hpaB* are co-transcribed (Büttner *et al*., 2007). It is important to note that these genes are part of the nonconserved region of the *hrp* pathogenicity island (Büttner *et al*., 2007), and divergent transcription in this region may provide clues regarding host specificity: *Xoc* HrpE3 plays a role in virulence in rice, whereas *Xcv* HpaE plays a role in pepper.

The expression of *hrpE3* was evaluated in various genetic backgrounds to further explore its regulation in *Xoc*. *hrpE3* was expressed in the *hrcC* deletion mutant (R*ΔhrcC*), which was not surprising as *hrcC* encodes a membrane component of the T3S system in *Xanthomonas* spp. (Büttner and Bonas, 2002a, b) and was not expected to have an impact on the regulation of *hrpE3* (Fig. 6A). The absence of *hrpE3* transcription in the R*ΔhrpX* and R*ΔhrpG* mutants is consistent with a role for *hrpX* and *hrpG* at the apex of the Hrp regulon (Guo *et al*., 2012; Li *et al*., 2011a; Wengelnik *et al*., 1996, 1999). The absence of *hrpE3* expression in the *hpaB* deletion mutant (Figs 2B and 4B) is probably a result of the deletion of the *hrpE3* promoter region, which mapped within the *hpaB* coding region (Fig. 3A, Fig. S1).

In this study, we have demonstrated that *hrpE3* is required for full virulence of *Xoc* in rice (Fig. 1). Büttner *et al*. (2007) have shown previously that the *hrpE3* homologue, *hpaE*, contributes to the virulence of *Xcv* in pepper and is required for HR induction in resistant host plants. HrpE3 and HpaE differ in their N-terminal 50 amino acids, but share a high degree of identity in their C-terminal residues (Fig. 5A). Despite their similarities, we found that *hpaE* could not restore full virulence to the *hrpE3* mutant (Fig. 1), and did not interfere with *Xoc* virulence in rice when the wild-type RS105 was overexpressed with *hpaE in trans* (data not shown), regardless of the promoter used to drive expression. To explore whether the C-terminal regions of *hrpE3* and *hpaE* were functionally interchangeable, we designed plasmid p2nE3-cE. This chimeric

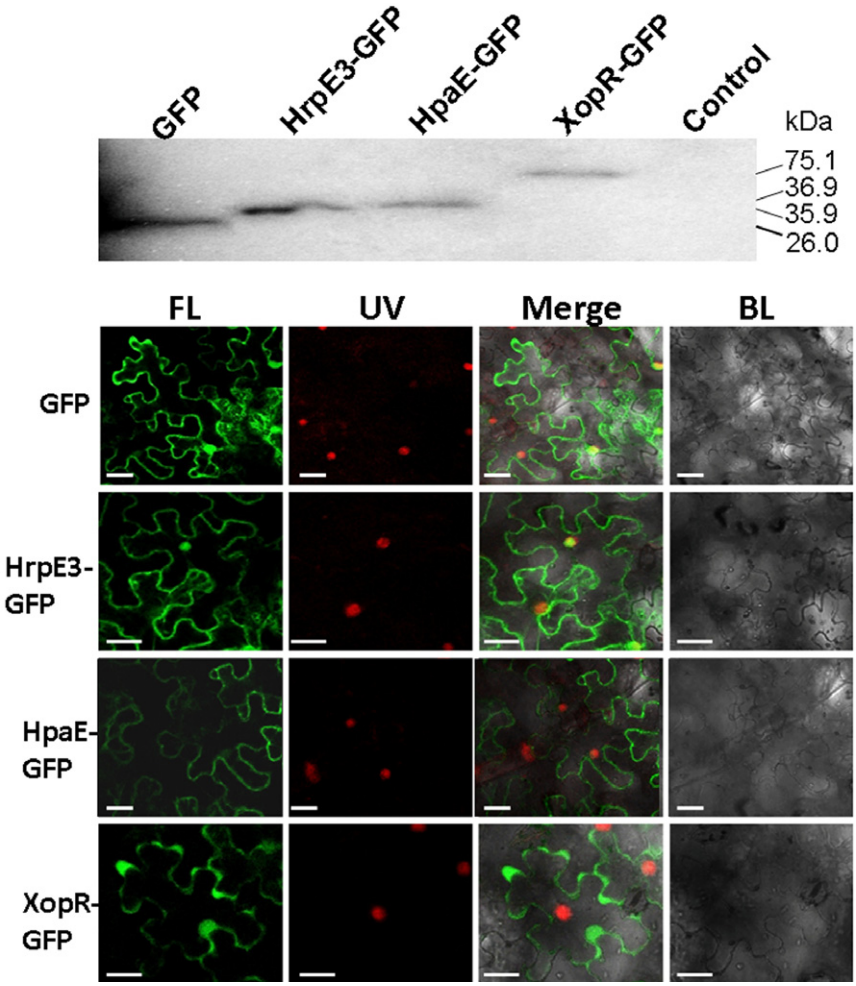

Fig. 7 Subcellular localization of HrpE3 in plant cells. (A) Detection of the fusion protein HrpE3-GFP in tobacco (*Nicotiana benthamiana*). Total proteins were extracted from tobacco leaves infiltrated with *Agrobacterium tumefaciens* containing pEGAD, pHrpE3-GFP, pHpaE-GFP and pXopR-GFP, and analysed by immunoblotting with anti-GFP (Genescript). Tobacco leaves infiltrated with *A. tumefaciens* EH105 containing empty vector were used as a control. The numbers represent the size of proteins in kilodaltons (kDa). (B) Detection of green fluorescent protein (GFP) fused proteins in tobacco cells. Tobacco epidermal cells expressing HrpE3-GFP, HpaE-GFP and XopR-GFP fusions or GFP alone are shown. The XopR-GFP fusion was used as a positive control. Expression was driven by the 35S cauliflower mosaic virus (CaMV) promoter. For confocal laser scanning microscopy, tobacco samples were taken 36 h after *Agrobacterium*-mediated delivery of GFP, HrpE3-GFP, HpaE-GFP and XopR-GFP. Images were acquired by fluorescence (FL) at 503 nm, exposure to ultraviolet light (UV) at 395 nm and using black light (BL). White bars represent 10 nm, and experiments were repeated three times with similar results.

construct contains the *hrpE3* promoter, 150 bp of the 5′ coding region of *hrpE3*, and 132 bp from *hpaE*; the latter segment encodes the 45 amino acid residues at the C-terminus of *hpaE*. Notably, we found that p2nE3-cE complemented the *hrpE3* mutant for virulence in rice (Fig. 1), suggesting that functions at the C-terminal regions of *hrpE3* and *hpaE* are conserved.

In vitro and *in vivo* secretion assays demonstrated that HrpE3 is secreted by the T3S system of *Xoc* (Figs 5 and 6) and translocated into the plant cytoplasm (Fig. 7). This is in sharp contrast with HpaE, which is not secreted by the T3S system (Büttner *et al*., 2007), which is consistent with our findings. As our results suggest that the C-terminal regions of HrpE3 and HpaE are functionally interchangeable, it is probable that the differences in secretion via the T3S system and the delivery into plant cells between HrpE3 and HpaE are attributed to their N-termini. As HpaE is not secreted via the T3S system, it probably functions inside the bacterial cell to influence effector secretion, possibly as a helper for *Xcv* T3E translocation (Büttner *et al*., 2007). The subcellular localization of HrpE3 is cytoplasmic–nuclear in plant cells (Fig. 7B), suggesting that HrpE3 functions as a genuine T3E. Thus, the major functional polymorphisms of HrpE3 and HpaE in bacterial virulence in plants, T3S-dependent secretion and subcellular localizations in plant cells are possibly determined by the polymorphisms in the N-terminal differences between HrpE3 and HpaE. However, the host cell function targeted by HrpE3 remains unknown. Our results do not exclude the possibility that HrpE3 may also function to facilitate the translocation of T3Es in *Xoc*. These possibilities are not mutually exclusive, because individual T3Es often exhibit multiple functions in host–pathogen interactions (Cunnac *et al*., 2009). Thus, whether or not the mutation in *hrpE3* diminishes or compromises the translocation of other T3Es is a question that warrants further investigation.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Xanthomonas* strains and other derivatives were grown in nutrient agar (NA; 0.1% yeast extract, 0.3% beef extract, 0.5% peptone, 1% sucrose

and 1.5% agar) or NB (NA without agar) (Li *et al*., 2011a) at 28 °C. The *hrp*-inducing medium for *X. oryzae* strains was XOM3 (Li *et al*., 2011a; Xiao *et al*., 2007). To obtain the *hrpE3* deletion mutant, NA without sucrose (NAN) was used. *Agrobacterium tumefaciens* strain EH105 and related constructs were grown in Luria–Bertani medium (LB; Miller, 1972) at 28 °C. *Escherichia coli* was grown in LB at 37 °C. Antibiotics were used at the following concentrations (μg/mL) when required: rifampicin (Rif), 50; kanamycin (Km), 25; ampicillin (Ap), 50.

DNA manipulation and plasmid construction

DNA isolation, restriction enzyme digestion, cloning, electroporation, PCR, Southern blots and immunoblotting were performed according to standard procedures (Sambrook *et al*., 1989). The primers used for PCR are listed in Table S1. PCR products were first cloned into pMD18-T (TaKaRa, Dalian, China), verified by sequencing, and analysed using VECTOR NTI software (http://www.invitrogen.com). The promoter analysis of *hrpE3* was conducted using tools available at http://www.fruitfly.org/seq_tools/promoter.html.

Construction of an *hrpE3* deletion mutant

In *Xoc* strain RS105, *hrpE3* (nucleotides 18 351–18 635) is located 38 bp downstream of *hpaB* and 454 bp upstream of *hpa4* (accession no. AY875714) (Zou *et al*., 2006) (Fig. S1). To generate a nonpolar mutation in *hrpE3*, fragments located 500 bp upstream and 750 bp downstream of *hrpE3* (Fig. S1) were amplified from RS105 genomic DNA using the primers E3UP-F/E3UP-R and E3II-F/E3II-R (Table S1), respectively. PCR products were cloned into pMD18-T and verified by DNA sequencing. The constructs were then digested with *Sma*I/*Bam*HI and *Bam*HI/*Hin*dIII, respectively, to release the cloned fragments, which were then subcloned into the suicide vector pKMS1 (Zou *et al*., 2011) at *Sma*I and *Hin*dIII sites, resulting in pK*Δhrp*E3 (Table 1). This construct was then introduced into *Xoc* RS105. A mutant lacking *hrpE3* was initially obtained on NAN medium and then on NA following the procedure described by Jiang *et al*. (2009a). The mutant was verified by PCR amplification with the primers E3UP-F/E3II-R (Table S1) and by Southern blot analysis using the *hrpE3* gene as a probe (Fig. S1).

Complementation of the *hrpE3* mutant

For the complementation of the *hrpE3* mutant (R*Δhrp*E3), a 682-bp DNA fragment containing a 397-bp upstream region and a 285-bp coding region of *hrpE3* was amplified from RS105 genomic DNA using the primers E3P1-F/E3Pm-R (Table S1). After confirmation by sequencing, the amplified DNA fragment was cloned into pUFR034 at *Eco*RI and *Bam*HI sites, resulting in plasmid pP1HrpE3-c-Myc (Table 1).

For comparative studies, the entire ORF of *hpaE* with a c-Myc tag was amplified from genomic DNA of *Xcv* 23-1 (Table 1) using the primers hpaE-F1/aEPm-R (Table S1). This amplified DNA was fused to a 201-bp promoter region of *hrpE3*, which was amplified from the genome of *Xoc* RS105 with the primers E3P2-F/E3P-R (Table S1). This chimeric construct was then cloned into pUFR034 at *Eco*RI and *Bam*HI sites, generating pP2HpaE-c-Myc (Table 1).

To determine whether *hpaE* from *Xcv* could complement the *Xoc hrpE3* mutant for virulence, a 1290-bp fragment, containing *hpaE* (303 bp) and 987 bp of upstream DNA (Büttner *et al*., 2007), was amplified from genomic DNA of *Xcv* 23-1 with the primers aEP987-F and aEPm-R (Table S1). After sequencing, this DNA fragment was cloned into pUFR034 at *Eco*RI sites, creating p987hpaE (Table 1).

A construct was also designed to determine whether a chimeric fusion between the N-terminal and C-terminal regions of HrpE3 and HpaE could restore virulence to the *hrpE3* mutant. For these experiments, a genetic fusion named p2nE3-cE was constructed; this contains the first 50 amino acids of HrpE3 and the C-terminal region of HpaE (44 amino acids). To generate this fusion, primer pair E3P2-F/nE3-R (Table S1) was used to amplify 351 nucleotides from *Xoc* RS105 spanning 201 bp upstream of *hrpE3* and extending 150 bp into the coding region. Next, a 132-bp region containing the C-terminus of HpaE was amplified from *Xcv* 23-1 genomic DNA using the primers cE-F/aEPm-R (Table S1). After *Bam*HI digestion, the two fragments were ligated and used as a template to amplify a 483-bp fusion with the primers E3P2-F/aEPm-R. After confirmation by sequence analysis, this fusion was cloned in pUFR034 at the *Eco*RI site, creating pP2nE3-cE (Table 1).

The constructs described above, pP1HrpE3-c-Myc, pP2hpaE, p987hpaE and pP2nE3-cE, were then transferred into the *hrpE3* mutant, R*Δhrp*E3. One representative transformant was used in subsequent studies after verification by PCR.

Pathogenicity and HR assays

Oryza sativa ssp. *indica* rice cultivars IR24 and IRBB10 (susceptible to *Xoc* infection) were used to assay pathogenicity. IR24 and IRBB10 plants were inoculated with a needleless syringe as seedlings (2 weeks old) and by leaf needling as adults (2 months old). The bacterial suspensions used in pathogenicity assays were adjusted to 1×10^8 colony-forming units (cfu)/mL. Rice cultivar IRBB10, which contains the *Xa10* gene, is resistant to *Xoo* strains with *avrXa10*, and this interaction results in an HR (Li *et al*., 2011b; Yang and White, 2004). The ability of *Xoc* strains to induce an HR was evaluated on tobacco (*Nicotiana benthamiana*) by infiltration of a bacterial suspension at 1×10^8 cfu/mL into fully expanded leaves with a needleless syringe. Plants were observed at 24 hpi for HR in tobacco, at 3 days post-inoculation (dpi) for water soaking or HR induction in rice seedlings, and at 14 dpi for lesion length in adult rice plants. Plants were maintained in a glasshouse, and all experiments were repeated at least three times.

Measurement of bacterial growth in rice

Suspensions of *Xoc* RS105 and derivatives were adjusted to 1×10^8 cfu/mL and infiltrated into newly expanded leaves of rice IR24 (2 weeks old) with a needleless syringe at three locations per leaf. Three leaf discs (0.8 cm in diameter) were excised with a cork borer from each infiltrated area. After sterilization in 70% ethanol and 30% hypochlorite, the discs were macerated using a sterile mortar and pestle in 1 mL of distilled water, diluted and plated to determine cfu/cm^2. Serial dilutions were spotted in triplicate on NA plates with appropriate antibiotics. The plates were incubated at 28 °C for 3–4 days until single colonies could be counted. The bacterial population (cfu/cm^2 of leaf area) was then estimated, and the standard deviation

was calculated using colony counts from three triplicate spots from each of three samples obtained at each time point. Experiments were repeated at least three times.

Semi-quantitative RT-PCR

To evaluate the expression of selected genes in the pathogen, single colonies of *Xoc* RS105 and deletion mutants (Table 1) were cultured, and total bacterial RNAs were extracted as described previously (Li *et al*., 2011a) using the Trizol method (Invitrogen, Shanghai, China). The extracted RNAs were treated with DNase I (TaKaRa) to remove any contaminating genomic DNA, purified and then used as templates for PCR amplification of *hrpD5*, *hrpD6*, *hrpE*, *hpaB* and *hrpE3* with the primers listed in Table S1. cDNA synthesis and semi-quantitative RT-PCR were performed as described by Li *et al*. (2011a). The RT-PCR products were sequenced to confirm the specificity of the primers for the five *hrp–hrc–hpa* genes. The resulting amplified products were analysed on 1.2% agarose gels. The *Xoc* 16S *rRNA* gene was used as an internal control to verify the absence of significant variation at the cDNA level.

To investigate whether *hrpE3* and *hrpE3* fused genes were expressed *in planta*, total RNAs were extracted from plant leaves inoculated with *Xoc* strains (1×10^8 cfu/mL). The procedure outlined above was followed for RNA extraction and cDNA synthesis. Semi-quantitative RT-PCR was performed with primer pairs specific for each gene (Table S1), and the rice 16S *rRNA* gene was used as an internal control. Similar results were obtained from three independent experiments.

Promoter analysis of *hrpE3* by 5′-RACE and GUS assays

5′-RACE was used to determine the transcriptional start site of *hrpE3*. *Xoc* RS105 (10 mL) was incubated in XOM3 medium at 28 °C for 16 h, and total RNA was extracted using an RNeasy Midi kit (TaKaRa). The isolated RNA was treated with RNase-free DNase I (TaKaRa) at 37 °C for 2.5 h, followed by a second purification using an RNeasy column. cDNA fragments were obtained using a 5′-RACE kit (TaKaRa), and an anchor sequence was added to the 5′ end of the cDNA using terminal deoxynucleotide transferase. The tailed cDNA was then amplified using the nested gene-specific primers *hrpE3O*-R and *hrpE3I*-R (Table S1) and RACE outer primer O-F and inner primer I-F (Table S1), as recommended in the 5′-Full RACE Kit (TakaRa). PCR products were then cloned into pMD18-T and sequenced.

To construct transcriptional fusions between the *hrpE3* promoter and *gusA*, the putative promoter-containing regions of *hrpE3* were fused to a promoterless *gusA* (Mitsuhara *et al*., 1996) with its ribosome binding site. Regions upstream of the start codon of *hrpE3* were PCR amplified using total DNA of *Xoc* RS105 as the template. The primer pairs E3P1-F/E3P-R, E3P2-F/E3P-R and E3P3-F/E3P-R (Table S1) were used to amplify the *hrpE3* upstream regions, and these were fused to *gusA*, which was amplified with the primer pair *gus*F/*gus*R (Table S1). Transcriptional fusions were cloned into pUFR034, resulting in pP1GUS, pP2GUS and pP3GUS (Table 1). For comparative analysis of *hpaE* and *hrpE3* promoter activity, 987-, 397-, 201- and 101-bp fragments upstream of the *hpaE* coding region were amplified with the primer pairs aEP987-F/aEP-R, aEP397-F/aEP-R, aEP201-F/aEP-R and aEP101-F/aEP-R (Table S1), respectively, using genomic DNA of *Xcv* 23-1 as a template (Table 1). After sequence verification, these fragments were fused with *gusA* at the *Hin*dIII site and cloned into pUFR034 at *Eco*RI and *Xho*I sites, resulting in p987GUS, p397GUS, p201GUS and p101GUS (Table 1). Constructs were then transferred into RS105, RΔ*hrpX* and RΔ*hrpG* by electroporation and verified by colony-PCR.

For GUS assays, *Xoc* strains were cultured in XOM3 or NB to an optical density at 600 nm (OD_{600}) of 0.5. Bacterial cells were diluted and disrupted in sonication buffer [20 mM Tris-HCl, pH 7.0; 10 mM 2-mercaptoethanol, 5 mM ethylenediaminetetraacetic acid (EDTA) and 1% Triton X-100]. GUS activities were determined at 15-min intervals up to 4 h by evaluating OD_{415} using 4-methylumbelliferone-D-glucuronide as the substrate (Jefferson *et al*., 1987). One unit (U) was defined as 1 nmol of 4-methylumbelliferone produced per min per OD_{600} value of the bacterium.

T3S assays for HrpE3 and HpaE

Two strategies were used to detect the secretion of HrpE3 and HpaE through the T3S system. In one approach, a c-Myc epitope-encoding sequence was fused to the C-terminus of HrpE3 or HpaE prior to the stop codon. To generate HrpE3-c-Myc expression constructs, *hrpE3* with three promoter regions (–397 to –1, –201 to –1 and –101 to –1 bp upstream of the *hrpE3* translational start site) were amplified from genomic DNA of *Xoc* strain RS105 with the primers listed in Table S1. The PCR products were cloned in frame into pURF034 with a c-Myc epitope-encoding sequence, resulting in pP1HrpE3-c-Myc, pP2HrpE3-c-Myc and pP3HrpE3-c-Myc (Table 1). These constructs were then transformed into *Xoc* RS105, RΔ*hrcC*, RΔ*hpaB*, RΔ*hrpG* and RΔ*hrpX* (Table 1) for protein secretion investigation. For comparative purposes, constructs p987HpaE-c-Myc and pP2HpaE-c-Myc (Table 1) were transferred into RS105, RΔ*hrcC*, RΔ*hrpX*, RΔ*hrpG* and RΔ*hpaB* strains for detection of HpaE-mediated secretion.

In the second strategy, we fused a variant of AvrXa10 lacking 28 amino acids at the N-terminus to the first 50 amino acid residues located at the N-terminus of HrpE3 or HpaE. The truncated form of AvrXa10 had been cloned previously into pUFR034 and was designated as pΔ28AvrX10 (Li *et al*., 2011b). To generate HrpE3-AvrXa10 expression constructs, the N-terminal coding sequence of *hrpE3* (1 to 150 bp) plus three putative promoter regions (–397 to –1, –201 to –1 and –101 to –1 bp upstream of the *hrpE3* translational start site) were amplified from genomic DNA of *Xoc* RS105 using the primers listed in Table S1. The PCR products were cloned into the *Eco*RI and *Xho*I sites of pURF034 in frame with the truncated AvrXal10 fragment in pΔ28AvrX10; this resulted in pP1E3avrXa10Δ, pP2E3avrXa10Δ and pP3E3avrXa10Δ (Table 1). For comparative purposes, nucleotides encoding the N-terminal 50 amino acids of HpaE were amplified from genomic DNA of *Xcv* strain 23-1 with the primers hpaE-F1/aEP-R1 (Table S1). This amplified DNA was fused with the 201-bp promoter (P2) of *hrpE3*, which was amplified from *Xoc* RS105 with the primers E3P2-F/E3P-R1 (Table S1). The fused fragment was cloned in frame with pavrXa10Δ at the *Eco*RI/*Xho*I sites, resulting in pP2EavrXa10Δ (Table 1). These constructs were transformed into the wild-type *Xoo* strain PXO99^A and the *Xoo hrcU* mutant PΔ*hrcU* (Table 1). Transformed strains were assayed for HR induction in rice IRBB10.

For Western blot analysis, *Xoc* strains were pre-incubated in NB medium, resuspended in sterile distilled water at $OD_{600} = 2.0$ and washed twice. The bacterial suspension (40 μL) was transferred into 1 mL of the *hrp*-inducing medium XOM3 (pH 6.5) and incubated at 28 °C for 16 h. Bacterial cells and supernatants 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 dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to membranes for immunoblotting using anti-c-Myc (Genescript, Nanjing, China) as the primary antibody. Primary antibodies were recognized using a secondary anti-rabbit antibody (Genescript) and visualized on autoradiographs by chemiluminescence (Transgene, Shanghai, China).

Subcellular localization of HrpE3 *in planta*

GFP was used as a reporter to investigate the subcellular localization of HrpE3 *in planta*. The coding region of *hrpE3* was amplified from genomic DNA of *Xoc* RS105 with the primers hrpE3-F1/hrpE3-R (Table S1). After sequence verification, the intact *hrpE3* ORF without a stop codon was cloned in frame with *gfp* at *Eco*RI and *Hin*dIII sites in vector pEGAD (Guo *et al*., 2012), generating pHrpE3-GFP (Table 1). Using the same strategy, pHpaE-GFP was also constructed (Table 1). Expression of the *hrpE3-gfp* fusion was driven by duplicate cauliflower mosaic virus (CaMV) 35S promoters. As a positive control, *xopR* (PXO_03819), encoding a 239-amino-acid membrane-binding protein (Akimoto-Tomiyama *et al*., 2012), was amplified from genomic DNA of *Xoo* PXO99^{A} using the primers *xopR*-F/*xopR*-R (Table S1); the product was subcloned into pEGAD at *Eco*RI and *Hin*dIII sites, creating pXopR-GFP (Table 1). The constructs described above were individually introduced into *A. tumefaciens* strain EH105 and infiltrated into *N. benthamiana* leaves for transient expression in epidermal cells. At 36 hpi, tobacco leaves were collected and total proteins were extracted as described previously (Akimoto-Tomiyama *et al*., 2012). Proteins in the supernatant fraction were precipitated with 12.5% trichloroacetic acid (Laemmli, 1970), separated on 10% SDS-PAGE gels, and transferred to membranes for immunoblotting using the primary antibody anti-GFP (Genescript). Primary antibodies were recognized using a secondary anti-rabbit antibody (Genescript) and visualized on autoradiographs by chemiluminescence (Transgene).

For observation by fluorescence microscopy, epidermal cells were used to investigate the localization of fusion proteins at 36 hpi with *A. tumefaciens*. Three independently infiltrated leaves expressing GFP under duplicate CaMV 35S promoters were placed in an enzyme solution (2% cellulase, 1% pectinase, 0.2 mol/L $CaCl_2$, 50 mmol/L 2-(*N*-morpholino)ethanesulphonic acid (MES) and 0.6 mol/L mannitol) containing 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI). After incubation for 3 h, the treated leaves were observed using confocal laser scanning microscopy (Leica Microsystems, Solms, Germany). Fluorescence images were acquired after exposure to fluorescent (FL, 509 nm), ultraviolet (UV, 395 nm) and black light (BL) irradiation. The assays were repeated three times with similar results, and the findings of one representative experiment are presented here.

ACKNOWLEDGEMENTS

We are grateful to Dr Carol Bender (Oklahoma State University, Stillwater, OK, USA) for reviewing the manuscript prior to submission. This work was supported by the Natural Science Foundation of China (31230059 and 31071656), the Special Fund for Agro-Scientific Research in the Public Interest of China (201303015) and the Key Basic Research Project of the Shanghai Committee of Science and Technology (11JC1406300).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 (A) Physical and functional map of *hrpE3* and flanking genes (*hpaB* and *hpa4*) in the *hrp* (*hypersensitive response and pathogenicity*) cluster (AY875714) of *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strain RS105. Open arrows indicate length, location and orientation of *hpaB*, *hrpE3* and *hpa4*. (B) Strategy used for the deletion of *hrpE3* by removal of a 285-bp coding region (white) of *hrpE3* using the E3UP (amplifies approximately 500 bp, grey) and E3II (amplifies 750 bp, black) primers. The fused PCR fragments were digested at *Sma*I and *Hind*III, and ligated in pKMS1. (C) Confirmation of deletion in mutant RΔ*hrpE3* by polymerase chain reaction (PCR) amplification with the primer pair (upF and downR) and by Southern hybridization using the left fragment as the probe, confirming that the PCR product and hybridized band in RΔ*hrpE3* is 285 bp shorter than that in the wild-type RS105. Genomic DNAs from *Xoc* RS105 and RΔ*hrpE3* were digested with *Msc*I.

Table S1 List of oligonucleotide primers used in this study.

