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The *xrvA* gene of *Xanthomonas oryzae* pv. *oryzae*, encoding an H-NS-like protein, regulates virulence in rice

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Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes bacterial blight disease in rice, one of the most serious rice diseases. The *xrvA* gene from *Xoo* strain 13751 encodes a protein containing a histone-like nucleoid-structuring protein (H-NS) domain. The expression of *xrvA* in strain 13751 was enhanced in XOM2 minimal medium. Mutation of the *xrvA* gene of strain 13751 led to a significant reduction in virulence in the host plant rice, a delayed hypersensitive response in the nonhost castor-oil plant, a decrease in extracellular polysaccharide and diffusible signal factor production, and an increase in intracellular glycogen accumulation. Northern hybridization analyses revealed that the virulence-associated genes *hrpG*, *hrpX*, *rpfC*, *rpfF*, *rpfG* and *gumB* were downregulated in the *xrvA* mutant compared to the wild-type and complemented strains. Interestingly, increase of copy number of *xrvA* in the wild-type strain 13751 resulted in a strain showing similar phenotypes as the *xrvA* mutant and a reduction of the expression of *gumB*, *hrpX*, *rpfC*, *rpfF* and *rpfG*. These findings indicate that the *xrvA* gene, which is highly conserved in the sequenced strains of *Xanthomonas*, encodes an important regulatory factor for the virulence of *Xoo*.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (hereafter *Xoo*), a member of the γ-subdivision of the Gram-negative *Proteobacteria*, is the causative agent of bacterial blight in rice (*Oryza sativa* L.), the most serious bacterial disease of rice in many rice-growing areas worldwide. The pathogen invades rice leaves through hydathodes or wounds (Ou, 1985). After sufficient multiplication in the intercellular spaces of the underlying epitheme, the bacteria enter the xylem and spread in the vascular system (Mew *et al.*, 1993). Bacterial cells and extracellular polysaccharide (EPS) fill the xylem vessels within a few days (Nino-Liu *et al.*, 2006).

A considerable effort is being made to identify genes involved in the pathogenesis of *Xoo* to plants and to understand the roles of the gene products in the disease process. This work will undoubtedly be facilitated by the recent determination of the full genome sequence of *Xoo* strains KACC10331, MAFF311018 and PXO99A (Lee *et al.*, 2005; Ochiai, *et al.*, 2005; Salzberg *et al.*, 2008). It has already been established that the outcome of interactions of *Xoo* with plants is determined by hypersensitive response and pathogenicity (*hrp*) genes, which are required for pathogenicity in susceptible host plants and for the hypersensitive response (HR) in resistant and nonhost plants (Alfano & Collmer, 1997; Nino-Liu *et al.*, 2006), and avirulence (*avr*) genes that determine host specificity via gene-for-gene interactions (Bai *et al.*, 2000; Lee *et al.*, 2005; Nino-Liu *et al.*, 2006). Other factors that contribute to the virulence of *Xoo* include EPS, the type II general secretion system and its secreted proteins, and regulation involving genes within the *rpf* cluster (Dharmapuri & Sonti, 1999; Dharmapuri *et al.*, 2001; Nino-Liu *et al.*, 2006; Tang *et al.*, 1996; Chatterjee & Sonti, 2002; Jeong *et al.*, 2008).

In xanthomonads, the *hrp* gene cluster comprises six operons (*hrpA* to *hrpF*) and is positively regulated by HrpG and HrpX (Bonas *et al.*, 1991; Wengelnik *et al.*, 1996; Wengelnik & Bonas, 1996), repressed in nutrient-rich

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Abbreviations: DSF, diffusible signal factor; EPS, extracellular polysaccharide; H-NS, histone-like nucleoid-structuring protein; GUS, β-glucuronidase; HR, hypersensitive response; *Xcc*, *Xanthomonas campestris* pv. *campestris*; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*.

A supplementary table of primers is available with the online version of this paper.

media, but induced in nutrient-limited media and inside the host (Arlat *et al.*, 1991; Schulte & Bonas, 1992a, b; Tsuge *et al.*, 2002). HrpG regulates the expression of *hrpX*, and HrpX then activates the expression of other *hrp* genes (Wengelnik *et al.*, 1996; Wengelnik & Bonas, 1996). The highly conserved *hrp* genes named *hrc* encode the proteins of the apparatus of the type III secretion system and are critical for pathogenicity and the initiation of disease (Bogdanove *et al.*, 1996; Alfano & Collmer, 1997; Lahaye & Bonas, 2001).

The *rpf* gene cluster (for regulation of pathogenicity factors) is involved in diverse regulatory actions contributing to virulence in different xanthomonads. Several of these *rpf* genes encode elements of a cell–cell communication mechanism which has been best studied in the crucifer pathogen *Xanthomonas campestris* pv. *campestris* (hereafter *Xcc*). The virulence of *Xcc* depends upon cell–cell signalling mediated by a diffusible signal factor (DSF) that has been characterized as *cis*-11-methyl-2-dodecenoic acid (Barber *et al.*, 1997; Wang *et al.*, 2004). The synthesis of the DSF in *Xcc* is fully dependent on RpfF and partially dependent on RpfB (Barber *et al.*, 1997; Slater *et al.*, 2000). The RpfC/RpfG two-component regulatory system is implicated in DSF perception and signal transduction. RpfG is an HD-GYP domain-containing protein that functions in cyclic di-GMP turnover in *Xcc* (Slater *et al.*, 2000; Ryan *et al.*, 2006). The *rpfB*, *rpfC*, *rpfF* and *rpfG* genes of *Xoo* have been shown to affect virulence in rice, EPS production, xylanase production and motility (Tang *et al.*, 1996; Chatterjee & Sonti, 2002; Jeong *et al.*, 2008).

EPS synthesis in *Xcc* is directed by genes within the *gum* cluster, which contains 12 genes and has a major promoter upstream of the first gene, *gumB* (Katzen *et al.*, 1996). Similarly, the *Xoo gum* cluster is composed of 14 ORFs that constitute an operon expressed from a promoter located upstream of *gumB*, but which also has internal promoters upstream of *gumG*, *gumH* and *gumM*, respectively (Lee *et al.*, 2005; Yoon & Cho, 2007; Lee *et al.*, 2008a).

The work described in this paper concerns the *xrvA* gene of *Xoo*, which encodes a protein containing an H-NS domain. H-NS and H-NS-like proteins are modular proteins associated with the bacterial nucleoid. These proteins have been identified in a number of bacteria, including *Escherichia coli*, *Erwinia amylovora*, *Erwinia chrysanthemi*, *Proteus mirabilis*, *Salmonella typhimurium*, *Shigella flexneri* and *Vibrio cholerae*, where they are known to act mainly as transcriptional repressors of a wide range of genes (reviewed by Tendeng & Bertin, 2003; Dorman, 2004, 2007; Fang & Rimsky, 2008). The phenotypes of *hns* mutants are highly pleiotropic. For example, *hns* mutants of *E. coli* are able to use β-glucoside as a carbon source, are strongly susceptible to serine, mucoid, non-motile, and show increased resistance to low pH and to high osmolarity (reviewed by Tendeng & Bertin, 2003). The H-NS proteins of *E. coli*, *Er. amylovora*, *Er. chrysanthemi*, *Sal. typhimurium* and *Actinobacillus pleuropneumoniae* have been shown to be involved in virulence (Müller *et al.*, 2006; Hildebrand *et al.*, 2006; Nasser *et al.*, 2001; Harrison *et al.*, 1994; Dalai *et al.*, 2009). Here, we present genetic evidence to demonstrate that the *xrvA* gene of *Xoo* plays an important role in virulence and regulates the expression of a number of virulence genes.

METHODS

Bacterial strains, plasmids and growth conditions. Table 1 lists the strains and plasmids used throughout this work. *E. coli* strains were grown in LB medium (Miller, 1972) at 37 °C. *Xoo* strains were grown in OB medium (Tang *et al.*, 1996), containing (g l^{-1}) polypeptone, 2; tryptone, 5; sucrose, 10; sodium glutamate, 1; L-methionine, 0.1; K_2HPO_4, 0.72; KH_2PO_4, 0.28; NH_4Cl, 1; $MgCl_2$, 1; Fe^{2+}-EDTA, 1 p.p.m., or XOM2 medium (Tsuge *et al.*, 2002) at 28 °C. *Xcc* strains were grown in NYGB medium (Daniels *et al.*, 1984) at 28 °C. Antibiotics were used at the following final concentrations as required: streptomycin at 250 μg ml^{-1}, kanamycin at 25 μg ml^{-1}, ampicillin at 100 μg ml^{-1}, rifampicin at 50 μg ml^{-1}, and tetracycline at 15 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *Xoo*.

DNA manipulations and primer extension. DNA manipulations were carried out as described by Sambrook & Russell (2001). The transcriptional start site of the *xrvA* gene was determined by primer extension analysis using the AMV reverse transcriptase Primer Extension System (Promega). The end-labelled primer (5′-ACCGGCGAACTGTTCGAG-3′) was annealed to total RNA isolated from the wild-type strain 13751 carrying pGXN3400 and extended.

Construction of a nonpolar mutant of *xrvA* in *Xoo* strain 13751. The nonpolar mutant of *xrvA* in *Xoo* strain 13751 (Tang *et al.*, 1996) was constructed by homologous suicide plasmid integration as described by Windgassen *et al.* (2000), using pK18MobGII as the vector (Katzen *et al.*, 1999). The DNA sequence containing *xrvA* nucleotides 4–255 was amplified using the total DNA of strain 13751 as the template and the primer pair *xrvA*NF/*xrvA*NR, which was designed based on the *xrvA* sequence (GenBank accession no. X97866) (primer sequences are listed in Supplementary Table S1, available with the online version of this paper). The amplified DNA fragment was ligated to the plasmid vector pGEM-3Zf(+) (Table 1) and confirmed by sequencing. The correct insert DNA was cloned into the suicide plasmid pK18MobGII to create the recombinant plasmid pK18MobGII*xrvA* (Table 1). To ensure the creation of a nonpolar mutant, the 252 bp fragment was inserted such that the transcription orientation of the fragment was the same as that of the *lac* promoter in the vector. The plasmid pK18MobGII*xrvA* was transferred from *E. coli* strain DH5α (Hanahan, 1983) into *Xoo* strain 13751 by triparental conjugation using pRK2073 (Leong *et al.*, 1982) as the helper plasmid, as described by Turner *et al.* (1985). Transconjugants were screened on OA [OB with 1.5 % (w/v) agar] plates supplemented with streptomycin and kanamycin. The transconjugants obtained with a mutation in the *xrvA* gene were confirmed by PCR using the total DNAs of the transconjugants as the templates and the primer pair pK18F/Mu577R (Table S1) (pK18F is located in pK18MobGII, and Mu577R is located downstream of the 252 bp internal fragment of *xrvA*). The expected 843 bp PCR products were further confirmed by sequencing. One of the confirmed mutants, named GXN1280 (Table 1), was used for further study.

Complementation of the *xrvA* mutant GXN1280. In order to complement the *xrvA* mutant GXN1280, a 901 bp DNA fragment containing the entire *xrvA* gene (from 264 bp upstream of the start codon to 235 bp downstream of the stop codon) was amplified by PCR using the total DNA of the wild-type strain 13751 as the

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics*	Reference or source
E. coli		
DH5α	*recA* ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 deoR hdsR17* ($r_K^- m_K^+$) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Hanahan (1983)
Xoo		
13751	Laboratory wild-type, Chinese isolate, Sm^r	Tang *et al.* (1996)
GXN1280	As 13751, but *xrvA*::pK18MobGII; Sm^r Kan^r	This work
GXC2088	GXN1280 harbouring pLAFRJ*xrvA*; Sm^r Kan^r Tc^r	This work
GXO3098	13751 harbouring pLAFRJ*xrvA*; Sm^r Kan^r Tc^r	This work
13751hrcV	As 13751, but *hrcV*::Tn*5*; Sm^r Kan^r	Feng *et al.* (2001)
13751*xrvA*GUS	13751 harbouring pGUS*xrvA*; Sm^r Tc^r	This work
Xcc		
8523	As wild-type strain 8004, but *rpfF*::Tn*5lac*; Rif^r Kan^r	Tang *et al.* (1991)
Plasmids		
pGEM-3Zf(+)	Has multiple cloning site (MCS); Amp^r	Promega
pRK2073	Helper plasmid; Tra^+ Mob^+ ColE1; Spc^r	Leong *et al.* (1982)
pLAFRJ	Broad-host-range cloning vector; as pLAFR3 but *Hin*dIII–*Eco*RI MCS replaced by that of pUC19; Tc^r	Laboratory collection
pLAFR1	Broad-host-range cloning vector; Tc^r	Huynh *et al.* (1989)
pGXN3400	pLAFR1 containing a 35 kb fragment from the chromosomal DNA of *Xoo* strain 13751; Tc^r	This work
pK18MobGII	Mob^+ ColE1 *gusA*; Kan^r	Katzen *et al.* (1999)
pK18MobGII*xrvA*	pK18MobGII containing a 252 bp internal fragment of *xrvA* gene; Kan^r	This work
pLAFRJ*xrvA*	pLAFRJ containing a 901 bp fragment including the *xrvA* gene; Tc^r	This work
pGUS*xrvA*	pLAFR6 containing an *xrvA* promoter-*gusA* fusion fragment	This work

*Sm^r, Kan^r, Tc^r, Spc^r and Rif^r indicate resistance to streptomycin, kanamycin, tetracycline, spectinomycin and rifampicin, respectively.

template and the primer pair *xrvA*CF/*xrvA*CR (Table S1). After being confirmed by sequencing, the amplified DNA fragment was cloned into pLAFRJ (Table 1) to obtain the recombinant plasmid pLAFRJ*xrvA*. Plasmid pLAFRJ*xrvA* was transferred into the mutant GXN1280 by triparental conjugation. The transconjugants carrying pLAFRJ*xrvA* were screened on OA plates containing streptomycin, kanamycin and tetracycline. A representative transconjugant, named GXC2088 (Table 1), was chosen for further study.

Construction of strain GXO3098, carrying an additional copy of *xrvA*. Plasmid pLAFRJ*xrvA* was introduced into the wild-type 13751 by triparental conjugation. The transconjugants carrying pLAFRJ*xrvA* were screened on OA plates containing streptomycin and tetracycline. After confirmation by restriction analyses of the extracted plasmid, a transconjugant was designated GXO3098 (Table 1) and chosen for further study.

Construction of the *xrvA* reporter plasmid pGUS*xrvA*. The *xrvA* reporter plasmid pGUS*xrvA* was constructed by cloning the promoter region of the *xrvA* gene into the broad-host-range cloning vector pLAFRJ (Table 1), which harbours the promoterless β-glucuronidase (*gusA*) gene in its MCS (multiple cloning site). The 246 bp region upstream of the *xrvA* GTG start codon (not including GTG) was amplified by PCR using the total DNA of the wild-type strain 13751 as the template and the primer pair *xrvA*PF/*xrvA*PR (Table S1). The amplified DNA fragment, confirmed by sequencing, was inserted 9 bp upstream of the promoterless *gusA* ATG start codon in the vector pLAFRJ to create the recombinant plasmid pGUS*xrvA* (Table 1). The recombinant plasmid obtained was further confirmed by restriction analysis and PCR. Confirmation PCR was performed using the recombinant plasmid pGUS*xrvA* as the template and the primer pair *xrvA*PF/*xrvA*PR (Table S1).

Northern hybridization. *Xoo* strains were incubated in OB medium to an OD_{600} of 1.0 before being harvested for RNA extraction. For the test in minimal medium XOM2, *Xoo* strains were first incubated in OB to OD_{600} 1.0 and then the collected cells were washed twice with XOM2, resuspended in XOM2 to OD_{600} 0.1, and cultured in XOM2 to OD_{600} 0.5 before being harvested for RNA extraction. The total RNAs were extracted from the *Xoo* strains with TRIzol Reagent (Invitrogen). Partial sequences of candidate gene probes were amplified by PCR using the total DNA of the wild-type strain 13751 as the template and the respective primer pairs (Table S1), and then cloned into pGEM-T easy vector (Promega) and confirmed by sequencing. The cloned DNA was recovered and used as the probe after restriction digestion and agarose gel electrophoresis. Probes were labelled with [α-^{32}P]dCTP according to the protocol of the Prime-a-Gene Labelling System (Promega). Unincorporated nucleotides were removed by size-exclusion chromatography using Sephadex G-100 columns. Northern hybridization was carried out in a Hybaid Shake 'n' Stack Hybridization Oven (Thermo) according to the procedures described by Alwine *et al.* (1977).

Virulence assay and determination of bacterial load *in planta* and media. Virulence was tested on the hybrid rice cultivar Teyou 63 grown in a greenhouse with a 12 h day–night cycle of illumination at temperatures of 28 °C during the day and 25 °C at night. Bacteria were grown in OB medium at 28 °C with shaking at 200 r.p.m. for 20 h with the specific purpose of reaching the exponential phase of growth. The concentrations of bacterial inocula were adjusted to an OD_{600} of 0.001 (10^6 c.f.u. ml^{-1}) or 0.1 (10^8 c.f.u. ml^{-1}). After maintenance at 100 % humidity for 24 h, the inoculated plants were maintained in the growth conditions described above. Lesion length was measured 14 days after inoculation. Thirty leaves were inoculated for each *Xoo* strain in each treatment by the leaf-clipping method

(Kauffman *et al.*, 1973). Each treatment was carried out in triplicate per experiment. The same experiment was repeated three times.

The growth of bacteria in rice leaf tissues was measured by homogenizing a group of leaves (five leaves for each sampling) in 9 ml sterile water. Diluted homogenates were plated on OA plates supplemented with streptomycin (for the wild-type), streptomycin plus kanamycin (for the mutant), streptomycin plus kanamycin and tetracycline (for the complemented strain) and streptomycin plus tetracycline (for the overexpression strain GXO3098). The number of bacterial colonies on these plates was counted after incubation at 28 °C for 3 days.

HR test. HR was tested in the nonhost castor-oil plant (*Ricinus communis*). The plants were inoculated by infiltrating approximately 20 μl of bacterial suspension (5×10^7 c.f.u. ml^{-1} or 5×10^8 c.f.u. ml^{-1}) in 10 mM sodium phosphate buffer (5.8 mM Na_2HPO_4 and 4.2 mM NaH_2PO_4, pH 7.0) into the leaves by using a blunt-end plastic syringe. The inoculated plants were maintained in a greenhouse with a 12 h day–night cycle of illumination with a fluorescent lamp and a constant temperature of 28 °C. The HR symptoms were observed and photographed at 10, 20, 30 and 40 h after inoculation. At least ten leaves were inoculated for each treatment per experiment. The same experiment was repeated four times.

Test of extracellular enzyme activity and EPS production of *Xoo* strains. To measure the extracellular activity of endoglucanase, xylanase and amylase, 100 μl culture supernatant was added to 400 μl buffer containing 1 % (w/v) carboxymethylcellulose (for endoglucanase), 1 % (w/v) oat spelt xylan (for xylanase) or 1 % (w/v) soluble starch (for amylase). Reactions were carried out for 30 min at 28 °C. The released reducing sugars were measured as D-glucose equivalent as described by Miller (1959). One unit (U) of the endoglucanase/xylanase/amylase activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar per minute. The activity of protease was quantitatively assayed as described by Swift *et al.* (1999).

To measure EPS production, strains were cultured in 100 ml OB medium containing 2 % (w/v) glucose in place of sucrose at 28 °C in a conical flask with shaking at 200 r.p.m. for 4 days. EPS was precipitated from the culture with ethanol, dried, and weighed as described by Tang *et al.* (1991).

Measurement of glycogen accumulation by *Xoo* strains. Intracellular glycogen accumulation was preliminarily determined by the iodine vapour staining method as described by Chao *et al.* (2008). Intracellular glycogen accumulation was quantified as follows. Cells of *Xoo* strains were collected by centrifugation, resuspended in fresh OB medium and lysed by ultrasonication. Trichloroacetic acid was added to the solution to a final concentration of 10 % and the mixture was shaken. After centrifugation, the glycogen was precipitated from the collected supernatant by adding an equal volume of 95 % ethanol and dried.

DSF extraction and activity bioassay. DSF was extracted into ethyl acetate from culture supernatants of *Xoo* strains grown in OB medium (Barber *et al.*, 1997). The ethyl acetate extracts were evaporated to dryness and samples were resuspended in methanol. The *Xcc rpfF* mutant strain 8523 (Table 1), which cannot make DSF and is deficient in endoglucanase production, was inoculated into NYGB medium with DSF extracts and grown overnight. The restored extracellular endoglucanase activity produced by strain 8523 was quantitatively measured as described for the measurement of endoglucanase activity above.

GUS activity assay. The *Xoo* strains were cultured in OB and XOM2 media. β-Glucuronidase (GUS) activities were assayed with *p*-nitrophenyl β-D-glucuronide as the substrate, measuring the A_{415} after the reaction, as described by Jefferson *et al.* (1986). One unit of GUS activity was defined as the amount of enzyme releasing 1 μmole of *p*-nitrophenol from *p*-nitrophenyl β-D-glucuronide per minute. Histochemical GUS staining was performed using 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) (Promega) as substrate, essentially as described by Jefferson *et al.* (1987).

RESULTS

Discovery and analysis of the *xrvA* gene of *Xoo*

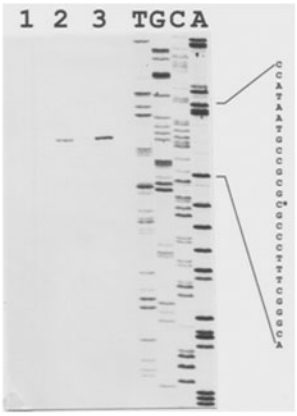
The discovery of *xrvA* as a regulator of virulence factor synthesis came from work aimed at identification of genes involved in EPS production of *Xoo*. The approach was to screen a genomic library of *Xoo* strain 13751 constructed in the broad-host-range vector pLAFR1 (Huynh *et al.*, 1989; Table 1) for clones that could alter the EPS production of the bacterium. The recombinant plasmids from the genomic library were transferred into the *Xoo* wild-type strain 13751 by triparental conjugation with selections for Sm^r (13751) and Tc^r (pLAFR1 derivatives). One of the 480 transconjugants obtained, which harboured the recombinant plasmid designated pGXN3400, displayed colonies that had less EPS than the wild-type on OB medium agar plates (data not shown). The transconjugant strain also showed significant reduction in virulence in rice (data not shown). These findings suggested that the recombinant plasmid pGXN3400 harbours a gene(s) whose increase in copy number could negatively influence the virulence and EPS production of *Xoo*. The gene(s) responsible for these effects was located on a 1.3 kb DNA region within the insert DNA of pGXN3400 as determined by subcloning and Tn*5* mutagenesis (data not shown). Nucleotide sequence and primer extension analyses of the 1.3 kb DNA region revealed the presence of a transcriptionally active gene that we named *xrvA* (*Xanthomonas* regulator of virulence) (GenBank accession no. X97866) (Fig. 1).

The *xrvA* ORF (designated XOO2744, XOO_2588 and PXO_00422 for *Xoo* strains KACC1033, MAFF311018 and PXO99A, respectively) is 402 bp in length and is predicted to encode a protein with 133 amino acids. The XrvA protein is highly homologous with XAC2416 of *Xanthomonas axonopodis* pv. *citri* strain 306 (97 % identity), XCV2614 of *Xanthomonas campestris* pv. *vesicatoria* strain 85-10 (97 % identity), XorYP_010100010090 of *Xanthomonas oryzae* pv. *oryzicola* strain (96 % identity), and XCC2309, XC_1806 and XCCB100_1869 of *Xcc* strains ATCC33913, 8004 and B100 (91 % identity). Protein domain analyses using SMART and the bioinformatic work of Bertin *et al.* (2001) showed that XrvA possesses an H-NS domain at its C terminus, spanning the 78th to 121st amino acids.

XrvA is required for the virulence and HR elicitation of *Xoo*

To facilitate the functional studies of XrvA, a nonpolar *xrvA* mutant, named GXN1280 (Table 1), was constructed by homologous suicide plasmid integration (see Methods

(a)



(b)

complementation plasmid construction
GCGGTCCGGC**GC**GCCGACGTTGCACGTGTCGTCACTCATTGACTGCTCCT
reporter construction

GTATCTTCATGGGCGGGTTTGCGACATCGAGCCCTTCGGCTGGACAGCGC
CCGTAGCGACAGTGCGAAAGCGTTCGAACAGCCGTCGTGAAATGCCTGTT
CACGCCATGAGCAAGCCCCTGTTCC**ATAACG**GCCGCCCCCGGGCCACATG
-35
SD
C**CATAAT**GCCGCG**C**GCCCTTTCGGGCAGCGTGTCCACTCTTATTC**CGAGG**
-10 ↑ transcriptional start site reporter con-

CCATACCTCT**GTG**AGCAATACCTCTTCGAAACTGGATTCGATCGCGCAAG
struction start codon mutagenesis plasmid construction

CCAAAGCCAAGCTGCTGGACGAACTGCAGAAGCTGGAAGAACAGGAGAAA
ACCGAGCGCGCGAGCGAGGCATCCTCTGCGCATGCCGCCATCGTTTCGCT
GCTCGAACAGTTCGCCGGTCACTTCAACACCAAGCAGCGCAACGACATTG
CCGCCTACCTGGGTACCAGCGCGGCCCGCAAGAACGTGGTCAAGAGCGGC
CGCAGCGAAGTGAAGCCCAAGTACGAACTGCCGCACACTGGCGAAACCTG
mutagenesis plasmid construction

GTCCGGCCGTGGCCGCACCCCGAAGGCCTTCGCTGCCTGGGAAGGCTCGG
TGTCCTACAAGGAATGGAAGGCCAAGAACCCGGACCTCAAGTTCCCGCTG
GTTCGCGAG**TAA**GGCCAAGGCGCATAAGTCCGTGCCGCGTTGCACGGTCG
stop codon
GCTGCGTCCTTCGCTTGGCGAACCGCGCAGCTTGTGGGTAGAATCCAAGG
ACATGGGGCGGTAGCTCAGCTGGGAGAGCGTCGCGTTCGCATCGCGAAGG
TCGAGGGTTCGATCCCCTTCCGCTCCACCAATAGAATCAAGGGCTTAGCC
GATGACCAGGCCCTTTTTCTTTGGTAGATTCAATTTATTGC**GCCAATTTT**
complementation
GCGCCACTCGGGCTGTCGAAGCCTTGGACGCAACACTTGAGGAGCAGTGA
plasmid construction

Fig. 1. Analyses of the *xrvA* gene of *Xoo*. (a) Primer extension analysis of *xrvA*. The end-labelled primer was annealed to total RNA isolated from the wild-type strain carrying pGXN3400 and extended by AMV reverse transcriptase. Lanes 1, 2 and 3, primer extension products at annealing temperatures of 78 °C, 68 °C and 58 °C respectively; lanes T, G, C, A, sequencing ladder generated using the same end-labelled primer. The sequence is given on the right; the asterisk indicates the base (C) which defines the limit of the primer extension. (b) *xrvA* and its flanking sequences. The transcriptional start site, the proposed −35 region, −10 region, SD sequence and start codon, and sequences representing the positions of the primers used for construction of complementation plasmid pLAFRJ*xrvA*, are marked in bold; sequences representing the positions of the primers used for construction of the *xrvA* reporter plasmid pGUS*xrvA* and the plasmid pK18MobGII*xrvA* for generating the *xrvA* mutant are underlined.

for details). A complemented strain named GXC2088 was also constructed by introducing the recombinant plasmid pLAFRJ*xrvA*, which carries the wild-type *xrvA* gene (Table 1), into the mutant GXN1280 (see Methods for details). Our previous aCGH (array-based comparative genome hybridization) analyses showed that the pLAFR6 derivative has only one copy in *Xcc* cells (He *et al.*, 2007). Since plasmid pLAFRJ*xrvA* and pLAFR6 derivatives contain the same replication origin, and *Xcc* and *Xoo* are closely related, it is most likely that the complemented strain GXC2088 harbours a single copy of plasmid pLAFRJ*xrvA* and thus possesses only one functional copy of the *xrvA* gene. Plasmid pLAFRJ*xrvA* was also introduced into the wild-type strain 13751, resulting in strain GXO3098 (Table 1), which harbours an extra *xrvA* copy carried by the plasmid in addition to the original chromosomal one. All of these strains grew similarly to the wild-type in rich medium OB and minimal medium XOM2 (data not shown).

The virulence of these *Xoo* strains was tested on the hybrid rice cultivar Teyou 63 by the leaf-clipping inoculation method (Kauffman *et al.*, 1973). Starting inoculum levels were either 10^6 c.f.u. ml^{-1} (an OD_{600} of 0.001, data not shown) or 10^8 c.f.u. ml^{-1} (an OD_{600} of 0.1, Fig. 2). Although the mutant strain GXN1280 could still cause obvious bacterial leaf blight symptoms, the symptoms were significantly less severe than those caused by the wild-type (Fig. 2a). At 14 days after inoculation and at both initial inoculum densities, the mean lesion lengths caused by the

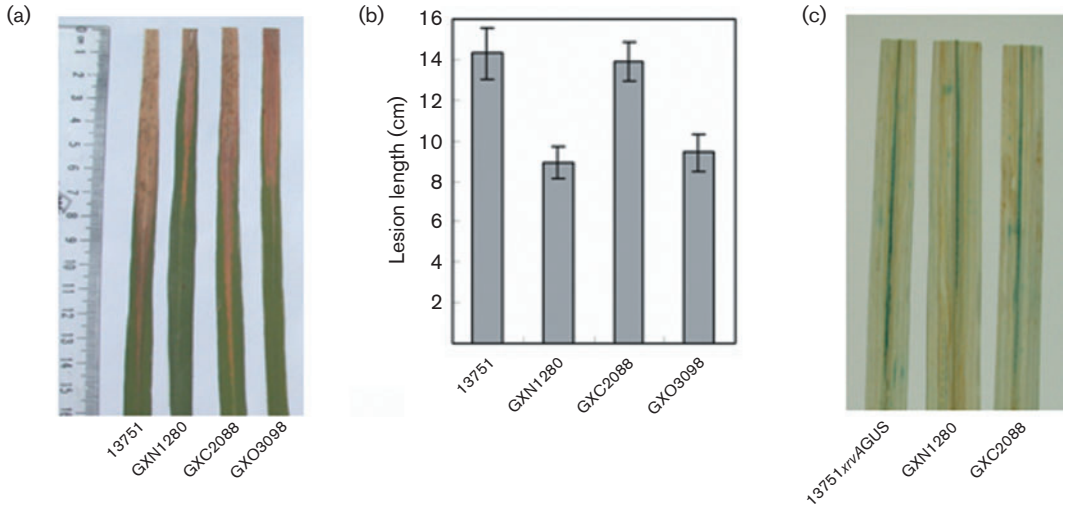


Fig. 2. XrvA is required for the virulence of *Xoo*. (a) The symptoms caused by *Xoo* strains in inoculated leaves of hybrid rice cultivar Teyou 63 inoculated at 10^8 c.f.u. ml^{-1}. Photographs were taken 14 days post-inoculation. (b) Lesion lengths caused by *Xoo* strains inoculated at 10^8 c.f.u. ml^{-1}. Data are the means ± SD from three repeats, each using 30 leaves. (c) Spread of bacteria within the rice vascular system visualized by histochemical staining for GUS activity. The *xrvA* mutant strain GXN1280 and the complemented strain GXC2088 as well as the wild-type reporter strain 13751*xrvA*GUS were inoculated into the leaves of rice Teyou 63. At 14 days after inoculation, the infected leaves were stained using an *in situ* GUS staining method. Initial inoculum concentration was 10^8 c.f.u. ml^{-1}.

mutant were reduced by approximately 37 % compared to the wild-type (P=0.01 by t test) (Fig. 2b). The mean lesion lengths caused by the complemented strain GXC2088 were however not significantly different from those caused by the wild-type (Fig. 2b). The mean lesion lengths caused by the overexpression strain GXO3098 were also reduced by approximately 35 % compared to the wild-type (P=0.01 by t test) (Fig. 2b). The wild-type harbouring the vector pLAFRJ showed similar symptoms to the wild-type (data not shown). These findings demonstrated that *xrvA* is required for the full virulence of *Xoo*, but suggested that increasing the copy number of *xrvA* reduced the virulence of the pathogen, which was consistent with the outcome of the initial experiments.

Differences in lesion lengths caused by the different *Xoo* strains may be associated with differential pathogen spread in the vascular system. To investigate this, we compared the distribution of cells of the mutant strain GXN1280 and the complemented strain GXC2088 in the infected leaves. The suicide plasmid pK18MobGII (Katzen *et al.*, 1999) used to create the *xrvA* mutant strain GXN1280 carries a *gusA* gene. Both strains GXN1280 and GXC2088 express β-glucuronidase (GUS), allowing the bacteria to be visualized within the plant tissue. Histochemical staining of inoculated rice leaves for GUS activity showed that the mutant GXN1280 spread slowly compared with the complemented strain GXC2088 in the vascular system of the infected rice leaves (Fig. 2c). Parallel experiments were carried out with the wild-type reporter strain 13751*xrvA*GUS, which carries the *xrvA* promoter-*gusA* reporter plasmid pGUS*xrvA* (Table 1). This strain had a similar spread to that of the complemented strain (Fig. 2c). These findings revealed a correlation between the bacterial spread in rice leaves and the symptoms produced by *Xoo*. However, the total bacterial counts for the wild-type, *xrvA* mutant, complemented mutant, and overexpression strain GXO3098 within the infected leaves were not significantly different during the 14 days post-inoculation (P=0.05 by t test) (data not shown).

To determine whether *xrvA* has any effect on the elicitation of an HR, we tested the HR-inducing capability of the *xrvA* mutant in the non-host castor-oil plant. The *hrcV* mutant of *Xoo*, strain 13751hrcV (Table 1), was used as a negative control, as this strain is defective in the type III secretion system apparatus and cannot elicit an HR. The results showed that the *xrvA* mutant strain GXN1280 and overexpression strain GXO3098 elicited an HR that was delayed compared to that caused by the wild-type and the complemented strain GXC2088 (Fig. 3). As expected, the *hrcV* mutant elicited no visible HR. These observations suggest that *xrvA* is not absolutely essential for HR induction by *Xoo*, but contributes to the rapidity of the response.

XrvA regulates EPS production, DSF synthesis and glycogen accumulation

The influence of mutation of *xrvA* on virulence raises the issue of whether XrvA acts to regulate the synthesis of any of the known virulence factors of *Xoo*. To answer this

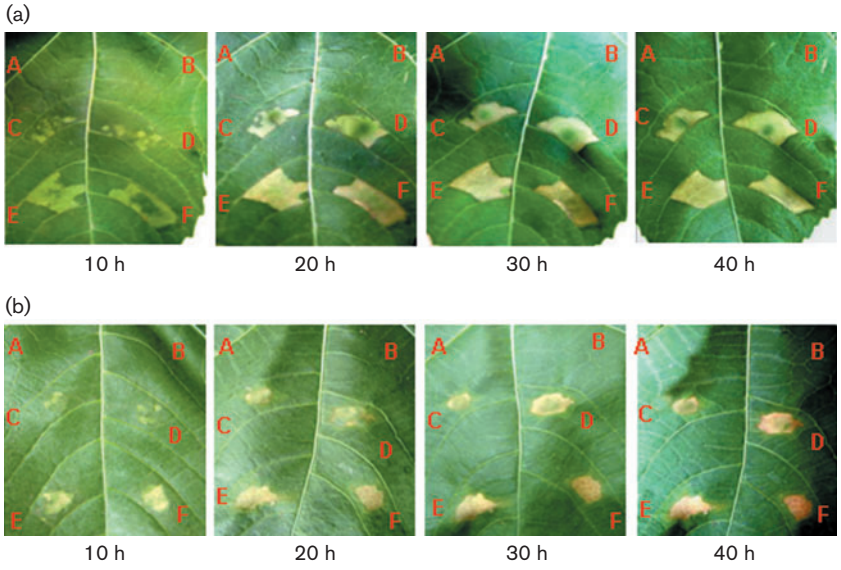


Fig. 3. Elicitation of the HR on the non-host castor-oil plant by strains of *Xoo*. Approximately 20 µl bacterial culture suspension in 10 mM sodium phosphate buffer was infiltrated into the leaf mesophyll tissue with a 1 ml blunt-end plastic syringe at an inoculum level of 5×10^8 c.f.u. ml^{-1} (a) or 5×10^7 c.f.u. ml^{-1} (b). Photographs of the same leaf were taken 10, 20, 30 and 40 h after infiltration, as indicated below each picture. Similar results were obtained in four independent experiments. A, double-distilled H_2O (negative control); B, 13751hrcV (type III secretion system-deficient strain; negative control); C, *xrvA* mutant strain GXN1280; D, overexpression strain GXO3098; E, wild-type strain 13751; F, complemented strain GXC2088.

question, we examined the effect of *xrvA* mutation on the production of EPS, extracellular enzymes and DSF as outlined in Methods. The *xrvA* mutant strain GXN1280 and overexpression strain GXO3098 produced about twofold less EPS compared to the wild-type, while the complemented strain GXC2088 produced a wild-type amount of EPS (Table 2). In parallel, bacterial colonies growing on OB agar plates were assessed for the production of the intracellular polysaccharide glycogen by iodine vapour staining. The wild-type strain 13751 and the complemented strain GXC2088 gave a similar yellowish colour in this assay (data not shown). However, the *xrvA* mutant strain GXN1280 and overexpression strain GXO3098 stained dark brown, indicating that these two strains contained excess glycogen (data not shown). The amount of intracellular glycogen was then measured quantitatively. As shown in Table 2, the *xrvA* mutant accumulated almost twofold more glycogen than the wild-type. The level of glycogen in strain GXO3098 was also significantly higher than that in the wild-type ($P=0.01$ by *t* test), whereas the complemented strain GXC2088 produced the wild-type level of glycogen (Table 2). These data indicate that *xrvA* acts to positively regulate EPS production but negatively regulate intracellular glycogen accumulation in *Xoo*.

Table 2. Quantification of extracellular polysaccharide and intracellular glycogen produced by *Xoo* strains

Data are the means ± SD from triplicate measurements for a representative experiment. The same letters indicate no significant differences; different letters mean significantly different ($P=0.01$ by *t* test). The experiment was repeated three times, and similar results were obtained.

Strain	Extracellular polysaccharide (g l^{-1})	Intracellular glycogen (g l^{-1})
13751 (wild-type)	17.3 ± 0.013^A	3.9 ± 0.016^A
GXN1280	9.8 ± 0.031^B	7.6 ± 0.014^B
GXC2088	17.1 ± 0.006^A	4.2 ± 0.030^A
GXO3098	11.1 ± 0.041^B	6.8 ± 0.041^B

The relative levels of the DSF signal molecule in culture supernatants of the various *Xoo* strains were bioassayed by measuring the restoration of endoglucanase activity to the *rpfF* mutant strain 8523 of *Xcc*. This strain cannot make DSF and is deficient in endoglucanase production; addition of exogenous DSF restores endoglucanase production (Barber *et al.*, 1997; Slater *et al.*, 2000). Addition of the DSF preparations from the wild-type and the complemented strain led to a similar significant increase in the endoglucanase production by the mutant 8523, while the preparation from the *xrvA* mutant strain gave a lesser effect (Fig. 4). Addition of the DSF preparation from strain GXO3098 also led to an increase in endoglucanase production, but the effect was significantly weaker ($P=0.05$ by *t* test) than that seen with the wild-type (Fig. 4). These findings demonstrate that *xrvA* has an influence on DSF production in *Xoo*.

Although XrvA regulates EPS and DSF production and glycogen accumulation, further experiments revealed that *xrvA* mutation has no effect on the production of the extracellular enzymes amylase, endoglucanase, protease and xylanase, or on biofilm formation, cell motility, or cell adhesion to inert surfaces (data not shown).

XrvA regulates the expression of a number of virulence genes in *Xoo*

To investigate whether XrvA plays any role in regulating the expression of other known virulence genes such as

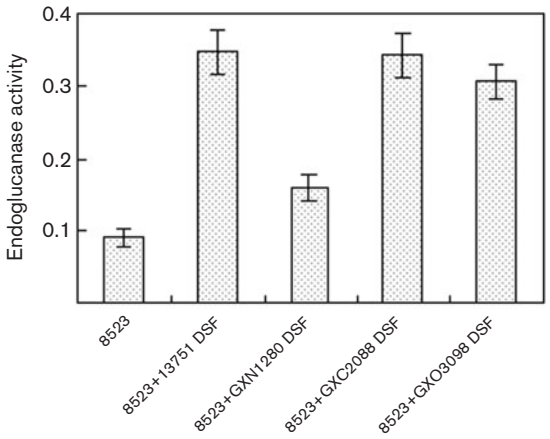


Fig. 4. XrvA regulates DSF biosynthesis of *Xoo*. DSF extracted from culture supernatants of different strains of *Xoo* was bioassayed by measuring the restoration of endoglucanase activity to the *rpfF* mutant strain 8523 of *Xcc*. Three replicates were done for each experiment and each experiment was repeated three times. Endoglucanase activity is expressed as units per OD_{600} unit per ml. Means ± SD from one experiment are plotted. Similar results were obtained in all experiments.

gumB, *hrpG*, *hrpX*, *rpfB*, *rpfC*, *rpfF* and *rpfG*, we employed Northern hybridization analysis to determine the transcript levels of these genes in the *xrvA* mutant. As outlined above, *hrpG*, which encodes the master regulator of the *hrp* regulon in xanthomonads, is expressed in minimal media but is repressed in rich media (Wengelnik *et al.*, 1996). It has been shown that for *Xoo*, the expression of the *hrp* genes including the regulatory gene *hrpX* is induced in XOM2 medium (Tsuge *et al.*, 2002). Therefore, the strains were grown in XOM2 medium for analysis of the transcripts of *hrpG* and *hrpX*, and in OB medium for analyses of the other genes. The results showed that the transcript levels of *gumB*, *hrpG*, *hrpX*, *rpfC*, *rpfF* and *rpfG* were lower in the *xrvA* mutant GXN1280 than in the wild-type (Fig. 5a–f), although the transcript level of *rpfB* was unaltered (Fig. 5g). The transcript levels of all the tested genes in the complemented strain GXC2088 were almost the same as in the wild-type (Fig. 5). In strain GXO3098, the transcript levels of *gumB*, *hrpX*, *rpfC*, *rpfF* and *rpfG* were lower than in the wild-type (Fig. 5a, c, d, e, f). These results suggest that *xrvA* plays roles in regulating the expression of the virulence genes *gumB*, *hrpG*, *hrpX*, *rpfC*, *rpfF* and *rpfG* in *Xoo*.

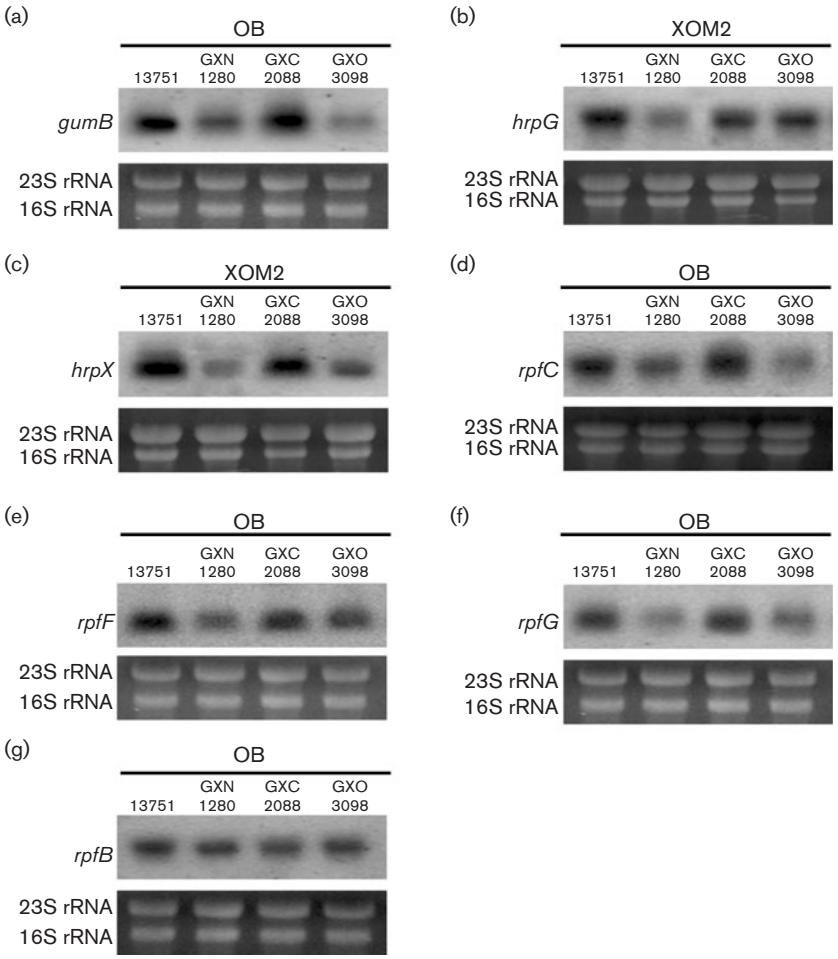


Fig. 5. Northern hybridization analyses of the transcript levels of virulence-associated genes in *Xoo* strains 13751 (wild-type), GXN1280 (*xrvA* mutant), GXC2088 (complemented *xrvA* mutant) and GXO3098 (wild-type with an additional copy of *xrvA*) grown in OB (rich) or XOM2 (minimal) medium. The ethidium bromide gel pictures show the loading controls for the RNA samples. Northern analysis was performed for *gumB* (a), *hrpG* (b), *hrpX* (c), *rpfC* (d), *rpfF* (e), *rpfG* (f) and *rpfB* (g).

The expression of *xrvA* is enhanced in minimal medium and expressed by bacteria inside the host

The above results demonstrated that *xrvA* regulates the expression of a number of virulence genes including the key *hrp* regulatory genes *hrpG* and *hrpX*. As described above, the *hrp* genes of *Xanthomonas* are induced in minimal media and repressed in rich media. We hypothesized that the expression of *xrvA* may also be induced in a minimal medium. To verify this, we compared the transcript levels of *xrvA* in the wild-type strain 13751 grown in the nutrient-rich medium OB and in the *hrp*-inducing minimal medium XOM2 by Northern hybridization. The result showed that the transcript level of *xrvA* was significantly elevated under *hrp*-inducing conditions, i.e. when the bacteria were grown in XOM2 medium (Fig. 6a).

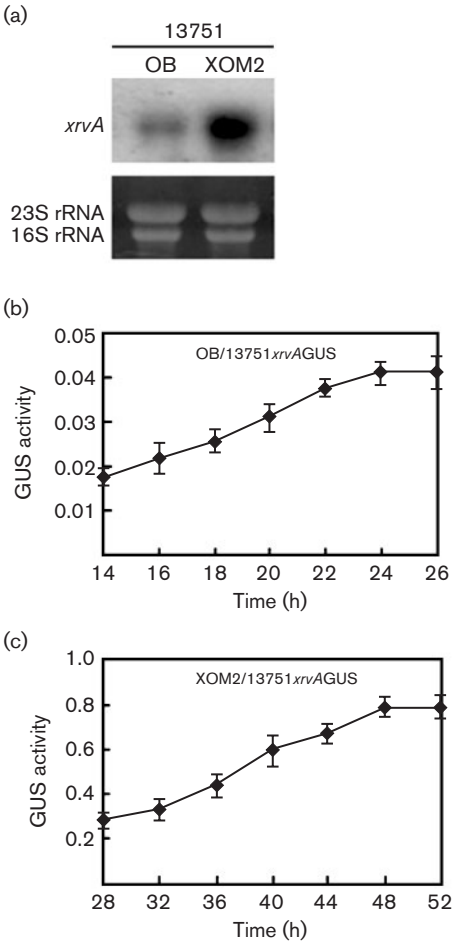


Fig. 6. The expression of *xrvA* is induced in minimal medium. (a) Northern hybridization analysis of the transcript levels of *xrvA* in bacteria grown in OB (rich) or XOM2 (minimal) medium. The ethidium bromide gel picture shows the loading control for the RNA samples. (b, c), Analysis of the expression of *xrvA* in the wild-type reporter strain 13751*xrvA*GUS grown in either OB (rich) or XOM2 (minimal) medium by measurement of the reporter GUS activity (expressed as units per OD_{600} unit per ml).

These experiments were extended by examining the expression of *xrvA* in the wild-type using the *xrvA* promoter-*gusA* reporter plasmid pGUS*xrvA* (Table 1). The result showed that expression of *xrvA* was nearly 20 times higher in the *hrp*-inducing XOM2 medium than in the rich medium OB (Fig. 6b, c), which is consistent with the result from the Northern hybridization analyses (Fig. 6a). Moreover, histochemical GUS staining disclosed that *xrvA* was also expressed when the pathogen was present inside the rice leaves (Fig. 2c).

DISCUSSION

This work adds to the inventory of genes that contribute to the full virulence of *Xoo*. We have presented genetic evidence to demonstrate that the *xrvA* gene of *Xoo* plays an important role in full virulence and normal HR elicitation. Disruption of *xrvA* led to a significant reduction in virulence, a delay in HR elicitation, a decrease in EPS and DSF production and an increase in glycogen accumulation. Recently, screening of a transposon mutant library of a Korean *Xoo* strain, KACC10331, in rice also showed that Tn*5* insertion in the *xrvA* gene (XOO2744) led to reduced virulence; however, the mutant was not characterized in further detail (Wang *et al.*, 2008). The deduced protein encoded by *xrvA* possesses an H-NS domain. These findings are consistent with a body of work that has established a role for *hns* in the virulence of several bacteria such as *E. coli*, *Er. amylovora*, *Er. chrysanthemi*, *Sal. typhimurium* and *A. pleuropneumoniae* (Müller *et al.*, 2006; Hildebrand *et al.*, 2006; Nasser *et al.*, 2001; Harrison *et al.*, 1994; Dalai *et al.*, 2009). The effect of mutation of *hns* on the ability of *Er. amylovora* and *Er. chrysanthemi* to elicit an HR on non-host plants was not assessed, however (Hildebrand *et al.*, 2006; Nasser *et al.*, 2001). Mutation of *xrvA* in *Xoo* reduced virulence by about 37 % compared to the wild-type. BLASTP analyses showed that there is an XrvA homologue encoded by the ORF XOO3363 in *Xoo* strain KACC10331 (48 % identity), XOO_3168 in *Xoo* strain MAFF311018 (48 % identity), and PXO_01852 in *Xoo* strain PXO99A (46 % identity). Whether there is a functional redundancy between XrvA and its homologue in *Xoo* needs to be further investigated.

The *xrvA* mutant GXN1280 and the *xrvA* overexpression strain GXO3098 showed a significant reduction in lesion length compared to the wild-type strain but the bacterial populations of these mutants in rice leaves were not significantly different from that of the wild-type. A similar phenomenon was found for a *Xoo rpfC* mutant, which showed reduced virulence in rice but similar growth as the wild-type strain *in planta* (Tang *et al.*, 1996). These findings may reflect a more marked contribution of virulence factors to bacterial spread in the tissue rather than to the overall bacterial load.

Although the *Xoo xrvA* mutant produces less EPS than the wild-type, the mutant accumulates more intracellular

glycogen. Diverse regulatory effects of *hns* on polysaccharide synthesis have been reported for bacteria from the *Enterobacteriaceae*. For example, *hns* mutants of *E. coli* are mucoid due to increased production of capsular polysaccharide (Sledjeski & Gottesman, 1995). This increased capsule synthesis results from the increased expression of the gene *rcsA*, which encodes a positive regulator of the *cps* genes and is negatively regulated by H-NS (Sledjeski & Gottesman, 1995). *Er. amylovora* synthesizes two major extracellular polysaccharide species: the complex heteropolymer amylovoran and the fructose-containing homopolymer levan. *Er. amylovora* carries two copies of *hns*; one is located on the chromosome and the other on the plasmid pEA29. Mutations in either *hns* gene led to increased levan production in the presence of sucrose. Interestingly, a difference was observed in the function of the two *hns* genes in regulation of amylovoran synthesis. Mutation of the chromosomal *hns* gene caused an increase of EPS production, whereas loss of the plasmid-borne *hns* gene had no effect (Hildebrand *et al.*, 2006). Nevertheless, overexpression of the *hns* gene from the *Er. amylovora* plasmid resulted in decreased production of both amylovoran and levan compared to wild-type (Hildebrand *et al.*, 2006). In *Er. chrysanthemi*, the production of extracellular polysaccharides, which are required for the efficient bacterial colonization of plants, was also negatively controlled by *hns* (Nasser *et al.*, 2001).

As described above, HrpG and HrpX are two key *hrp* regulators in *Xanthomonas*. HrpG, which is predicted to be a member of the OmpR response regulator family of two-component signal transduction systems, regulates the expression of *hrpX*, encoding an AraC-type transcriptional activator, which then activates the expression of other *hrp* operons. Recently, it has been demonstrated that the *trh* and *phoP* genes in *Xoo* positively regulate expression of *hrpG*, although it is not known whether the regulation is direct or indirect (Tsuge *et al.*, 2006; Lee *et al.*, 2008b). The *trh* gene encodes a putative transcriptional regulator (Tsuge *et al.*, 2006) and *phoP* encodes a putative response regulator of two-component regulatory systems (Lee *et al.*, 2008b). Whether *trh* and *phoP* influence the expression of other XrvA targets such as *gum* and *rpf* has not been reported, however. It will be of interest to further study the functional relationship (if any) between *xrvA*, *trh* and *phoP* in regulating *hrpG* expression in order to better understand *hrp* regulatory mechanisms.

The virulence of *Xanthomonas* also depends upon cell-to-cell signalling mediated by DSF. The RpfC/RpfG two-component system couples the DSF sensing to intracellular regulatory networks through a second messenger, cyclic di-GMP, and a global regulator, Clp. Protein–protein interaction between the DSF synthase RpfF and the sensor RpfC may act as a post-translational mechanism to modulate the biosynthesis of DSF (reviewed by Dow, 2008; He & Zhang, 2008). Mutation of *xrvA* significantly reduced the DSF production and decreased the transcript levels of *rpfF*, *rpfC* and *rpfG*. Further studies are required to understand whether XrvA regulates these genes directly or indirectly. Nevertheless, to our knowledge, this is the first report of a gene that is involved in regulation of the expression of *rpfC*, *rpfF* and *rpfG* in *Xanthomonas* spp. It has been established that DSF signalling regulates many different cellular processes in xanthomonads, including the production of the extracellular enzymes endoglucanase, endomannanase and protease, production of EPS and biofilm formation (Dow, 2008). However, our work showed that mutation of *xrvA* did not significantly influence either the production of extracellular enzymes or biofilm formation by *Xoo in vitro* (data not shown). This may reflect the fact that inactivation of *xrvA* reduced but did not completely abolish the synthesis of DSF and expression of the *rpf* genes (Figs 4 and 5). Whether *xrvA* has a more pronounced influence on DSF biosynthesis and hence virulence when bacteria are *in planta* is not known.

Interestingly, in this work, the *xrvA* mutant strain GXN1280 and the *xrvA* overexpression strain GXO3098 showed similar phenotypes in virulence, HR elicitation, EPS production and DSF biosynthesis. A similar phenomenon for virulence has been observed in *Sal. typhimurium*, in which a mutation in the *hns* gene led to reduced virulence, and introduction of the *E. coli hns* gene into the wild-type of the pathogen also resulted in attenuation of virulence (Harrison *et al.*, 1994). The reduced expression of *hrpG*, *hrpX*, *rpfC*, *rpfF*, *rpfG* and *gumB* in the *xrvA* mutant strain GXN1280, and the decreased expression of *hrpX*, *rpfC*, *rpfF*, *rpfG* and *gumB* in the *xrvA* overexpression strain GXO3098 may partially explain the weakened virulence, delayed HR, and reduced EPS and DSF production of GXN1280 and GXO3098. However, the expression level of *hrpX*, *rpfC*, *rpfF*, *rpfG* and *gumB* appeared to be different in GXN1280 and GXO3098, and the expression of *hrpG* in GXO3098 was not significantly different from that in the wild-type. The molecular basis for these alterations in patterns of gene expression in strains either lacking or overexpressing XrvA is unknown. It is possible that there is an optimal cellular level of XrvA required for expression of virulence genes, and higher levels are inhibitory. Alternatively XrvA may differentially affect the expression of subordinate regulators with opposite regulatory influences on the expression of the virulence-related genes. Much work needs to be done to understand the regulatory mechanisms by which XrvA controls these genes in *Xoo*. Furthermore, the occurrence of homologues of XrvA in other *Xanthomonas* spp. warrants study of the role of this H-NS domain protein in the virulence of these pathogens to their diverse host plants.

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