Identification of seven *Xanthomonas oryzae* pv. *oryzicola* genes potentially involved in pathogenesis in rice

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Xanthomonas oryzae pv. *oryzicola* (*Xoc*) causes bacterial leaf streak (BLS) in rice, an emerging and destructive disease worldwide. Identification of key virulence factors is a prerequisite for understanding the pathogenesis of *Xoc*. In this study, a Tn5-tagged mutant library of *Xoc* strain RS105 was screened on rice, and 27 Tn5 mutants were identified that were either non-pathogenic or showed reduced virulence in rice. Fourteen of the non-pathogenic mutants were also unable to elicit the hypersensitive response (HR) in tobacco and were designated Pth[−]/HR[−] mutants; 13 mutants showed attenuated virulence and were able to induce an HR (Vir[−]/HR⁺). Sequence analysis of the Tn5-tagged genes indicated that the 14 Pth[−]/HR[−] mutants included mutations in *hrcC*, *hrcT*, *hrcV*, *hpaP*, *hrcQ*, *hrpF*, *hrpG* and *hrpX*. The 13 Vir[−]/HR⁺ mutants included *tal-C10c-like* (a transcriptional activator-like TAL effector), *rpfC* (regulator of pathogenicity factors), *oxyR* (oxidative stress transcriptional regulator), *dsbC* (disulfide isomerase), *opgH* (glucan biosynthesis glucosyltransferase H), *rfaA* (glucose-1-phosphate thymidyltransferase), *amtR* (aminotransferase), *purF* (amidophosphoribosyltransferase), *thrC* (threonine synthase), *trpA* (tryptophan synthase alpha subunit) and three genes encoding hypothetical proteins (*Xoryp_02235*, *Xoryp_00885* and *Xoryp_22910*). Collectively, the 27 Tn5 insertions are located in 21 different open reading frames. Bacterial growth and *in planta* virulence assays demonstrated that *opgH*, *purF*, *thrC*, *trpA*, *Xoryp_02235*, *Xoryp_00885* and *Xoryp_22910* are candidate virulence genes involved in *Xoc* pathogenesis. Reduced virulence in 13 mutants was restored to wild-type levels when the cognate gene was introduced *in trans*. Expression profiles demonstrated that the seven candidate virulence genes were significantly induced *in planta*, although their roles in *Xoc* pathogenesis remain unclear.

Received 13 April 2011
Revised 17 October 2011
Accepted 7 November 2011

INTRODUCTION

Xanthomonas oryzae pv. *oryzicola* (*Xoc*) is the causal agent of bacterial leaf streak (BLS), one of the most destructive diseases in high-yield hybrid rice (*Oryza sativa* L.) (Zhao *et al.*, 2004; Chen *et al.*, 2005; Zou *et al.*, 2006; Wang *et al.*, 2007). The pathogen enters rice leaves through stomata or wound sites and colonizes intercellular spaces in the mesophyll, resulting in water-soaked interveinal lesions that develop into translucent streaks (Chen *et al.*, 2005; Niño-Liu *et al.*, 2006;

Zou *et al.*, 2006). Unfortunately, efficient, environmentally friendly strategies to control BLS are lacking.

BLS pathogenesis is totally different from the vascular disease bacterial blight (BB) caused by *X. oryzae* pv. *oryzae* (*Xoo*). *Xoo* invades rice plants through water pores or wound sites on leaves, then colonizes the vascular tissues and propagates in the xylem, generating tannish-grey to white lesions along the leaf veins, which leads to yield loss (Ou, 1985). The differences in tissue specificity and symptom development between BLS and BB are presumably due to unique pathogenicity genes that differ in these two bacterial pathogens.

In the last decade, the rapid development of functional and comparative genomics has facilitated the identification of

Abbreviations: BLS, bacterial leaf streak; HR, hypersensitive response; LPS, lipopolysaccharide; p.i., post-infection; T2SS/T3SS, type II/III secretion system; *Xcc*, *Xanthomonas campestris* pv. *campestris*; *Xoc*, *Xanthomonas oryzae* pv. *oryzicola*; *Xoo*, *X. oryzae* pv. *oryzae*.

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

pathogenicity-related genes. The whole genome sequences of *Xoo* strains KACC10331 (Lee *et al.*, 2005), MAFF311018 (Ochiai *et al.*, 2005) and PXO99^A (Salzberg *et al.*, 2008), and *Xoc* strain BLS256 (<http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=Xoc>) have revealed differences between *Xoc* and *Xoo* (Wren, 2000; Wang *et al.*, 2007). One distinguishing feature between the two pathogens is the prevalence of more transcription activator-like (*tal*) effector genes of the *avrBs3/pthA* family in *Xoc* than in *Xoo* (Yang & White, 2004; Chen *et al.*, 2005; Zou *et al.*, 2005; Niño-Liu *et al.*, 2006); this may contribute to a wider host range for *Xoc* than for *Xoo* (Makino *et al.*, 2006). Furthermore, some of the type II and type III secretion system (T2SS and T3SS, respectively) effectors differ in the two pathogens. For example, the T3SS effectors XopO, XopAF, XopAJ and XopAK (<http://www.xanthomonas.org/t3e.html>) and the T2SS effector extracellular protease EcpA (unpublished data) are present in *Xoc* but not *Xoo*. Previous studies also compared the role of pathogenicity-related genes between these two pathovars, including the *hrp* (hypersensitive response and pathogenicity) (Zou *et al.*, 2006), *tal* (Yang & White, 2004; Chen *et al.*, 2005; Zou *et al.*, 2005), *rpf* (regulatory pathogenicity factors) (Tang *et al.*, 1996; Zhao *et al.*, 2011), EPS (extracellular polysaccharide) and LPS (lipopolysaccharide) (Wang *et al.*, 2007; Kim *et al.*, 2009) synthesis genes. However, it remains unclear what novel virulence factors are required by *Xoc* to determine pathogenesis in rice.

Previous studies have utilized suppression-subtractive hybridization, transcriptional profiling and proteomic analyses to compare virulence genes in *Xoo* and *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Soto-Suárez *et al.*, 2010; Gonçalves *et al.*, 2010; Hsiao *et al.*, 2011; Zhao *et al.*, 2011). To further understand the *Xoc*-rice pathosystem, we recently constructed a Tn5 mutant library of *Xoc* (Zou *et al.*, 2011). In this report, we screened the Tn5 mutants in the library and identified 27 genes involved in *Xoc* pathogenesis, including seven candidate genes that contribute to virulence in rice.

METHODS

Bacterial strains, culture media and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Xoc* strains were grown at 28 °C in NB (1 g yeast extract, 3 g beef extract, 5 g polypeptone, 10 g sucrose; all l⁻¹), NA (NB with 15 g agar l⁻¹), NY (NB without beef extract and sucrose), MMX minimal medium [5 g glucose, 2 g (NH₄)₂SO₄, 1 g trisodium citrate dihydrate, 4 g K₂HPO₄, 6 g KH₂PO₄, 0.2 g MgSO₄·7H₂O; all l⁻¹]. *Xoc* broth cultures were incubated at 28 °C at 200 r.p.m. *Escherichia coli* strains were grown in LB (Luria-Bertani) medium (5 g yeast extract, 10 g NaCl, 10 g tryptone, 15 g agar; all l⁻¹) at 37 °C (Miller, 1972). Antibiotics were added at the following concentrations (µg ml⁻¹) when required: kanamycin (Kan), 25; rifampicin (Rif), 50; ampicillin (Ap), 100; spectinomycin (Sp), 50; and streptomycin (Sm), 50.

DNA manipulation. These were performed as described previously (Sambrook *et al.*, 1989). The conjugation between *Xoc* and *E. coli* strains containing plasmids was performed as described by Turner

(2004). Kits for isolating genomic and plasmid DNA and for purifying DNA from agarose gels were purchased from Axygen. Restriction enzymes and DNA ligases were used according to the manufacturer's instructions (TaKaRa). PCR primers were purchased from Jinsite and are listed in Supplementary Table S1 (available with the online version of this paper).

Hypersensitive response (HR) and pathogenicity assays. Assays for HR and pathogenicity were performed as described previously (Zou *et al.*, 2006). Briefly, *Xanthomonas* cells were grown in NB broth with appropriate antibiotics at 28 °C and 200 r.p.m. for 16 h, when cells approached the exponential phase of growth. Bacterial cells were then harvested by centrifugation, washed twice and resuspended in sterile water to OD₆₀₀ 0.3 (approx. 1 × 10⁸ c.f.u. ml⁻¹). Bacteria were then infiltrated into tobacco leaves (cv. Xanthi) using a needleless syringe or inoculated into leaves of adult rice plants (cv. Shanyou63, susceptible to BLS, 2 months old) using leaf-needling for lesion length measurement or a needleless syringe for evaluating water-soaking symptoms in rice seedlings (2 weeks old). All plants were grown and maintained in a greenhouse as described previously (Wang *et al.*, 2007). Plant phenotypes were scored 24 h post-inoculation (p.i.) for HR in tobacco, 3 days p.i. for water-soaking symptoms in rice seedlings, and 14 days p.i. for lesion lengths in adult rice. Five leaves were inoculated for each independent experiment and each treatment was repeated at least three times.

Southern blot assay. Genomic DNA of *Xoc* strains was extracted as described by Leach *et al.* (1990). DNA samples were digested with *Pst*I for 4 h and separated in 1.2 % agarose gels in TAE buffer for 6 h at 80 V. Subsequently, the digested DNA was transferred to a Hybond-N⁺ nylon membrane and hybridized with a 500 bp PCR probe amplified from Tn5 and labelled with digoxigenin (PCR DIG probe synthesis kit, Roche). Hybridization was detected using the DIG luminescence detection kit (Roche). The wild-type *Xoc* strain RS105 was used as a negative control. Southern hybridization experiments were conducted twice to confirm the results.

Determination of the sequences flanking the transposon by TAIL-PCR. Genomic DNA sequences flanking the Tn5 insertion in selected mutants were determined by thermal asymmetrical intercalated PCR (TAIL-PCR) (Yuan *et al.*, 2009; Zou *et al.*, 2011). For TAIL-PCR, two sets of nested primers specific to the two ends of the transposon and six arbitrary degenerate primers were used (Supplementary Table S1). TAIL-PCR products were gel-purified, cloned into PMD18-T (TaKaRa) and sequenced. DNA sequences were analysed by evaluating homology to the *hrp* clusters of *Xoc* RS105 (GenBank accession nos AY875714 and AY875714), the genome sequence of *Xoc* strain BLS256 (<http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=Xoc>; and GenBank accession no. CP003057, Ye *et al.*, 2006).

Complementation of the tested mutants. For complementation analysis, DNA fragments containing the intact genes or operons with native promoters were amplified by PCR using genomic DNA of *Xoc* RS105 as a template (primer sets listed in Supplementary Table S1). After sequence verification, the amplified DNA fragments were cloned into the pHM1 vector at *Hind*III, *Kpn*I, *Sal*I or *Eco*RI sites to create recombinant plasmids (Table 1). Recombinant plasmids were transformed into the corresponding Tn5 insertion mutants by electroporation (Zou *et al.*, 2006), and the transformants harbouring the recombinant plasmids were screened on NA plates with appropriate antibiotics. Selected transformants were verified by PCR amplification using the corresponding primer pairs (Supplementary Table S1).

Determination of bacterial growth ability. To determine their ability to multiply *in planta*, *Xanthomonas* suspensions (1 × 10⁸ c.f.u. ml⁻¹) were infiltrated into the intercellular spaces of fully expanded leaves of rice (2 weeks old, cv. Shanyou63) with a needleless syringe at

Table 1. Strains and plasmids used in this studyAp^r, ampicillin resistant; Km^r, kanamycin resistant; Rif^r, rifampicin resistant; Sp^r, spectinomycin resistant; Sm^r, streptomycin resistant.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>E. coli</i>		
DH5 α	F [−] ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>endA1 deoR recA1 hsdR17</i> (r _K [−] m _K [−]) <i>phoA supE44 λ[−] thi-1 gyrA96 relA1</i>	Clontech
S17- λ pir	<i>recA thi pro hsdR[−] M⁺</i> , RP4::2-Tc::Mu:KmTn7, λ pir, Tp ^r , Sm ^r	This lab
<i>X. oryzae</i> pv. <i>oryzicola</i>		
RS105	Wild-type, the causal agent of rice bacterial leaf streak, Rif ^r	This lab
R Δ <i>hrcV</i>	RS105 <i>hrcV</i> knockout mutant, Rif ^r	Wang <i>et al.</i> (2009)
CMXoc1304	MXoc1304 containing pC1304, Km ^r Sp ^r	This work
CMXoc0236	MXoc0236 containing pC0236, Km ^r Sp ^r	This work
CMXoc0028	MXoc0028 containing pC0028, Km ^r Sp ^r	This work
CMXoc0525	MXoc0525 containing pC0525, Km ^r Sp ^r	This work
CMXoc0104	MXoc0104 containing pC0104, Km ^r Sp ^r	This work
CMXoc0091	MXoc0091 containing pC0091, Km ^r Sp ^r	This work
CMXoc0350	MXoc0350 containing pC0350, Km ^r Sp ^r	This work
CMXoc0065	MXoc0065 containing pC0065, Km ^r Sp ^r	This work
CMXoc1232	MXoc1232 containing pC1232, Km ^r Sp ^r	This work
CMXoc0729	MXoc0729 containing pC0729, Km ^r Sp ^r	This work
CMXoc0761	MXoc0761 containing pC0761, Km ^r Sp ^r	This work
CMXoc0852	MXoc0852 containing pC0852, Km ^r Sp ^r	This work
CMXoc0601	MXoc0601 containing pC0601, Km ^r Sp ^r	This work
Plasmids		
pMD18-T	pUC <i>ori</i> , cloning vector, Ap ^r	TaKaRa
pHM1	Broad-host range cos <i>parA</i> IncW derivative of pRI40, Sp ^r Sm ^r	This lab
pC1304	pHM1 expressing <i>Xoryp_04700</i> under its native promoter; Sp ^r	This work
pC0236	pHM1 expressing <i>Xoryp_02235</i> under its native promoter; Sp ^r	This work
pC0028	pHM1 expressing <i>Xoryp_18935</i> under its native promoter; Sp ^r	This work
pC0525	pHM1 expressing <i>Xoryp_18770</i> under its native promoter; Sp ^r	This work
pC0104	pHM1 expressing <i>Xoryp_10705</i> under its native promoter; Sp ^r	This work
pC0091	pHM1 expressing <i>Xoryp_13880</i> under its native promoter; Sp ^r	This work
pC0350	pHM1 expressing <i>Xoryp_14510</i> under its native promoter; Sp ^r	This work
pC0065	pHM1 expressing <i>Xoryp_00885</i> under its native promoter; Sp ^r	This work
pC1232	pHM1 expressing <i>Xoryp_10980</i> under its native promoter; Sp ^r	This work
pC0729	pHM1 expressing <i>Xoryp_21665</i> under its native promoter; Sp ^r	This work
pC0761	pHM1 expressing <i>Xoryp_22910</i> under its native promoter; Sp ^r	This work
pC0852	pHM1 expressing <i>Xoryp_03285</i> under its native promoter; Sp ^r	This work
pC0601	pHM1 expressing <i>Xoryp_05420</i> under its native promoter; Sp ^r	This work

three locations per leaf. Three leaf discs (0.8 cm in diameter) were harvested with a cork borer from each infiltrated area. After sterilization in a solution containing 70 % ethanol and 30 % sodium hypochlorite, the leaf discs were homogenized in 1 ml sterile distilled water. Diluted homogenates were plated on NB agar supplemented with rifampicin (wild-type *Xoc*) or spectinomycin plus kanamycin (for complementing strains) to determine the c.f.u. cm^{−1}. Bacterial c.f.u. were counted after incubation at 28 °C for 3 days, and the c.f.u. per cm² leaf area was calculated.

To investigate bacterial growth *in vitro*, NB, NY and MMX media were used. *Xanthomonas* cells were pre-incubated in 5 ml NB medium for 16–20 h at 28 °C, 200 r.p.m. until OD₆₀₀ 0.6. Bacterial cells were then collected by centrifugation, washed twice and resuspended to OD₆₀₀ 0.05 in 20 ml NB, NY and MMX media. For each time point, 200 μ l each culture was removed and the OD₆₀₀ was measured. The data presented are representative of three independent experiments.

Rice suspension cells. *Oryza sativa* subsp. *indica* cv. Shanyou63, which is susceptible to *Xoc* strain RS105, was used for callus induction. Seeds were dehulled and sterilized in 70 % ethanol for 10 min, transferred to a solution containing 50 % commercial bleach with a few drops of Tween 20 for 30 min, and then soaked in 1 % HgCl₂ for 15 min. Sterilized seeds were washed five times with sterile distilled water and incubated on N6 medium (Chu, 1978) containing 2,4-dichlorophenoxyacetic acid (2,4-D)(5 mg l^{−1}) at 28 °C in darkness. Actively growing calli were then selected and transferred to liquid N6 medium containing 5 mg 2,4-D l^{−1} and 1 mg kinetin (KT) l^{−1}. Rice calli were incubated in the dark and transferred by subculturing at 7 day intervals at a dilution of 1:5 (inoculum:fresh medium). Generally, large amounts of rice suspension cells were obtained after 4–5 weeks of subculturing; at this time, single round rice cells could be observed under the microscope.

Real-time quantitative RT-PCR. Expression of the seven candidate virulence genes of *Xoc* RS105 strain was assayed by real-time quantitative RT-PCR with the corresponding primer pairs (Supplementary Table S1). The *Xoc* strains were pre-incubated in 20 ml NB medium for 16–20 h or until OD₆₀₀ 0.6. At this time, 40 µl was transferred into 20 ml fresh NB, incubated for an additional 16–18 h, suspended in sterile water to OD₆₀₀ 2.0, and washed twice. An aliquot of the washed bacterial cells (40 µl) was then inoculated into 1 ml NB, MMX or rice suspension cells, incubated for 16 h at 25 °C, or infiltrated into rice leaves for 8 h. As a template, total RNA was extracted from each treatment using Trizol reagent as recommended by the manufacturer (TaKaRa). cDNA synthesis was conducted with AMV reverse transcriptase (TaKaRa) and random primers as specified by the manufacturer. Before synthesis of the first-strand cDNA, total RNA was digested with RNase-free DNase I (TaKaRa) to remove potential traces of genomic DNA. Real-time quantitative RT-PCR was performed using the Applied Biosystems 7500 Real-time PCR System and SYBR Premix Ex Taq (TaKaRa). PCR conditions were as follows: denaturation at 95 °C for 30 s and 41 cycles of 95 °C for 5 s and 60 °C for 34 s. The expression level of the 16S rRNA gene was used as an internal standard, and fragments were amplified using the corresponding primers (Supplementary Table S1). The comparative-threshold method was used to calculate the relative mRNA level with respect to the corresponding transcript in bacterial cells grown in NB medium. All real-time quantitative RT-PCR experiments were performed in two independent biological experiments with three replicates in each test.

RESULTS

Identification of Tn5 mutants involved in *Xoc* pathogenicity and the HR

To identify key virulence genes or factors involved in *Xoc* pathogenicity, we previously generated a library of Tn5 insertion mutants (Zou *et al.*, 2011), and 27 mutants showed altered pathogenicity or virulence compared with the wild-type strain RS105. To confirm the attenuated pathogenicity in rice and HR induction in tobacco, we reinoculated these 27 mutants to rice seedlings (2-week-old seedlings of cv. Shanyou63). The phenotype of lesions and lesion lengths were scored 3 and 14 days p.i. and compared with the wild-type RS105 (Fig. 1a, Table 2). In subsequent experiments, the mutants were individually inoculated to adult rice (2 months old) by leaf-needling and infiltrated into tobacco for a more precise assessment of pathogenicity and ability to induce the HR, respectively. Fourteen mutants were non-pathogenic in rice and defective in the ability to trigger an HR in tobacco (designated Pth[−]/HR[−]) (Table 2). The remaining 13 mutants showed reduced virulence in rice, but retained the ability to elicit an HR in tobacco (Vir[−]/HR⁺) (Fig. 1, Table 2). With the exception of Mxoc0729, the Vir[−] mutants generated significantly smaller lesion lengths than the wild-type ($P=0.01$, t test) (Fig. 1b). Thus, the results indicated that the 27 mutants could be divided into two groups: Pth[−]/HR[−] and Vir[−]/HR⁺ (Table 2).

Sequence analysis of disrupted genes

To further characterize the 27 Tn5 insertion mutants, we first evaluated whether each mutant contained a single Tn5 insertion. Genomic DNA of the mutants and wild-type was

digested with *Pst*I and hybridized with the 500 bp probe derived from Tn5. Southern blot analysis indicated that each mutant contained a single Tn5 insertion (Fig. 2a).

It has been confirmed that the ability of *Xoc* to trigger an HR in non-host tobacco and to be pathogenic in rice is determined by the *hrp* clusters that encode components of the T3SS (Zou *et al.*, 2006; Li *et al.*, 2011a, b). To determine whether or not the 14 Pth[−]/HR[−] mutants contain Tn5 insertions in the *hrp* gene cluster, we employed long PCR to amplify the 5 kb *hrp*-*hrc*-*hpa* regions of *Xoc* RS105 (Supplementary Table S1) and the 14 Pth[−]/HR[−] mutants. After sequencing the 14 amplified fragments (data not shown), we demonstrated that Tn5 insertions mapped to *hrcC* (mutants Mxoc0397 and Mxoc1472), *hrcT* (Mxoc0529), *hrcV* (Mxoc1114, Mxoc1042, Mxoc1021 and Mxoc1481), *hpaP* (Mxoc0536), *hrcQ* (Mxoc0139 and Mxoc0665), *hrpF* (Mxoc0548), *hrpG* (Mxoc1546) and *hrpX* (Mxoc0707 and Mxoc1314) (Table 2, Fig. 3a). Sequence analysis also indicated that the transposon had inserted at different locations within single *hrp* genes (e.g. see *hrpV*; Fig. 3a).

To locate the Tn5 insertion in the 13 Vir[−]/HR⁺ Tn5 mutants (Fig. 1, Table 2), we employed a TAIL-PCR technique, as described previously (Yuan *et al.*, 2009; Zou *et al.*, 2011), and amplified regions flanking the transposon in these mutants. After sequencing the flanking fragments, the transposon insertions were mapped and oriented by aligning the sequences with the completed genome of *Xoc* BLS256 strain as the reference. The results are presented in Table 2 and Fig. 3(b–n). The genes disrupted by Tn5 could be divided into two groups. Group I includes six genes with known functions in other phyto bacteria (Fig. 3, Table 2), including the *tal-C10c-like* gene, which encodes a TAL effector (<http://www.xanthomonas.org/t3e.html>) in the mutant Mxoc0729; *rpfC* (*Xoryp_10980*), a regulatory pathogenicity factor identified in *Xcc* (He & Zhang, 2008) in Mxoc1232; *oxyR* (*Xoryp_04700*), an oxidative stress transcriptional regulator characterized in the phytopathogen *Xylella fastidiosa* (Toledo *et al.*, 2011) in Mxoc1304; *dsbC* (*Xoryp_18770*) encoding a disulfide isomerase involved in *Xcc* virulence (Jiang *et al.*, 2008) in Mxoc0525; *rfbA* (*Xoryp_18935*), a glucose-1-phosphate thymidyltransferase required for mycobacterial growth (Qu *et al.*, 2007) in Mxoc0028; and *amtR* (*Xoryp_18935*), an aminotransferase associated with virulence in *Pseudomonas syringae* (Park *et al.*, 2010), in Mxoc0091. It is important to note that *oxyR*, *rfbA* and *amtR* have not been previously reported in the virulence of *Xanthomonas* species.

Group II contains seven genes not previously reported in the virulence of phytopathogenic bacteria but are highly conserved in *Xanthomonas* species (Fig. 3, Table 2). This group includes *opgH* (*Xoryp_03285*), which encodes glucan biosynthesis glucosyltransferase H in Mxoc0852; *purF* (*Xoryp_05420*) an amidophosphoribosyltransferase in Mxoc0601; *thrC* (*Xoryp_10705*) a threonine synthase in Mxoc0104; *trpA* (*Xoryp_14510*) a tryptophan synthase alpha subunit in Mxoc0305, and the other three genes,

Fig. 1. Pathogenicity tests in rice (cv. Shanyou63, 2 weeks old) by infiltration of candidate virulence-reduced mutants of *Xoc* at OD₆₀₀ 0.3 (approx. 1×10^8 c.f.u. ml⁻¹) with needleless syringes (a) and by inoculation of the same candidates at the same concentration in rice (cv. Shanyou63, 2 months old) by using the leaf-needling method (b). Water-soaked symptoms were photographed 3 days p.i. in rice seedlings. The lesion length in adult rice was measured 14 days p.i. The T3SS-deficient mutant RΔhrcV and the wild-type RS105 were used as the controls. In (b), values are the means $\pm$ SD from three repeats, each with five leaves; hatched bars, mutant strains; black bars, complemented strains. ** $P=0.01$; * $P=0.05$; paired, two-tailed Student *t* test for each mutant relative to the wild-type. 1, Mxoc1304; 2, Mxoc0236; 3, Mxoc0028; 4, Mxoc0525; 5, Mxoc0104; 6, Mxoc0091; 7, Mxoc0350; 8, Mxoc0065; 9, Mxoc1232; 10, Mxoc0729; 11, Mxoc0761; 12, Mxoc0852; 13, Mxoc0601; 14, RΔhrcV; 15, RS105.

Xoryp_02235, *Xoryp_00885* and *Xoryp_22910*, which encode hypothetical proteins in Mxoc0236, Mxoc0065 and Mxoc0761, respectively.

Since the genome sequence of *Xoc* RS105 is unavailable, we used the genome of *Xoc* BLS256 as a reference. To determine whether RS105 and BLS256 vary in the sequence of the genes identified in this study, 12 primer pairs (Supplementary Table S1; the *tal-C10c-like* gene was not included) were designed and used to amplify the Tn5-disrupted genes in the mutants; the wild-type RS105 was used as a control. The results showed that the PCR-amplified fragments in the mutants were 1221 bp larger than fragments amplified from the wild-type RS105 (Fig. 2b). Further analysis indicated no differences between RS105 and BLS256 in these 12 genes (data not shown). An exception was the *tal-C10c-like* gene. Since more than 20 *tal* genes have been identified in *Xoc*

strain RS105 (Zou *et al.*, 2005), we chose to amplify the *tal-C10c-like* gene mutated in Mxoc0729; this gene was annotated as *Xoryp_21665* in *Xoc* BLS256. For this, a primer specific to Tn5 (0729F) and another primer homologous to *Xoryp_21665* were used (Supplementary Table S1). The results confirmed that the *tal-C10c-like* gene was mutated in Mxoc0729 (Fig. 2b) and indicated that this gene is present in both RS105 and BLS256. Collectively, the data suggest that there are no obvious sequence differences in these 13 genes in strains RS105 and BLS256.

Complementation analysis of 13 *Xoc* Tn5 mutants

It has been demonstrated that transposon insertions can cause polar effects on downstream genes (Wang *et al.*, 2007). To exclude the possibility that the reduced virulence observed for group II mutants resulted from polar effects

Table 2. Tn5-inserted mutants of *X. oryzae* pv. *oryzicola* strain RS105

Pth⁻, No pathogenicity in rice; HR⁻, no HR induction in tobacco; HR⁺, HR induced in tobacco. *Xoo*, *X. oryzae* pv. *oryzae*; *Xcc*, *X. campestris* pv. *campestris*; *Xac*, *X. axonopodis* pv. *citri*; *Xcv*, *X. campestris* pv. *vesicatoria*.

Mutants	Rice lesion length (cm)*	HR induction	ORFs in BLS256†	Inserted position‡	Putative product	Homologues in other <i>Xanthomonas</i> species (similarity/identity; %)				Reference or source	
						Xoo	PXO99 ^A	Xcc 8004	Xac 306		Xcv85-10
MXoc1472	Pth [−]	HR [−]	<i>Xoryp_21900</i>	903/1824	HrcC	99/98		92/84	97/95	98/96	Cho <i>et al.</i> (2008)
MXoc0529	Pth [−]	HR [−]	<i>Xoryp_21905</i>	702/831	HrcT	99/98		93/86	98/97	98/96	Cho <i>et al.</i> (2008)
MXoc1114	Pth [−]	HR [−]	<i>Xoryp_21950</i>	493/1923	HrcV	99/99		98/95	99/97	99/98	Wang <i>et al.</i> (2009)
MXoc1042	Pth [−]	HR [−]	<i>Xoryp_21950</i>	932/1923	HrcV						
MXoc1021	Pth [−]	HR [−]	<i>Xoryp_21950</i>	944/1923	HrcV						
MXoc1481	Pth [−]	HR [−]	<i>Xoryp_21950</i>	1124/1923	HrcV						
MXoc0536	Pth [−]	HR [−]	<i>Xoryp_21955</i>	236/645	HpaP	97/95		72/59	91/87	93/90	Furutani <i>et al.</i> (2009)
MXoc0139	Pth [−]	HR [−]	<i>Xoryp_21960</i>	12/954	HrcQ	98/95		82/73	93/89	96/93	Cho <i>et al.</i> (2008)
MXoc0665	Pth [−]	HR [−]	<i>Xoryp_21960</i>	912/954	HrcQ						
MXoc0548	Pth [−]	HR [−]	<i>Xoryp_22025</i>	1455/2409	HrpF	99/98		81/67	89/81	97/93	Zou <i>et al.</i> (2006)
MXoc1546	Pth [−]	HR [−]	<i>Xoryp_16910</i>	12/750	HrpG	99/98		86/76	94/88	93/88	Jiang <i>et al.</i> (2009)
MXoc0707	Pth [−]	HR [−]	<i>Xoryp_16900</i>	212/1527	HrpX	99/99		95/92	99/98	99/98	Jiang <i>et al.</i> (2009)
MXoc1314	Pth [−]	HR [−]	<i>Xoryp_16900</i>	231/1527	HrpX						
MXoc0729	2.2 ^A	HR ⁺	<i>Xoryp_21665</i>	1402/3387	TAL effector (tal-C10c)	87/85		N	93/90	N	Yang & White (2004)
MXoc1232	0.8 ^C	HR ⁺	<i>Xoryp_10980</i>	1462/2178	RpfC	99/97		94/88	73/60	97/93	He & Zhang (2008)
MXoc1304	0.6 ^C	HR ⁺	<i>Xoryp_04700</i>	536/942	Oxidative stress transcriptional regulator (OxyR)	99/97		99/94	98/96	99/96	Toledo <i>et al.</i> (2011)
MXoc0525	0.4 ^C	HR ⁺	<i>Xoryp_18770</i>	325/798	Disulfide isomerase (DsbC)	99/99		96/95	98/98	99/98	Jiang <i>et al.</i> (2008)
MXoc0028	1.3 ^B	HR ⁺	<i>Xoryp_18935</i>	665/888	Glucose-1-phosphate thymidyltransferase(RfbA)	99/99		98/96	99/97	98/96	Qu <i>et al.</i> (2007)
MXoc0091	0.6 ^C	HR ⁺	<i>Xoryp_13880</i>	489/1112	Aminotransferase (AmtR)	99/99		90/84	97/92	96/93	Mann & Ploux (2011)

Table 2. cont.

Mutants	Rice lesion length (cm)*	HR induction	ORFs in BLS256†	Inserted position‡	Putative product	Homologues in other <i>Xanthomonas</i> species (similarity/identity; %)				Reference or source
						Xoo PXO99 ^A	Xcc 8004	Xac 306	Xcv85-10	
MXoc0852	0.8 ^C	HR ⁺	<i>Xoryp_03285</i>	1092/1938	Glucans biosynthesis glucosyltransferase H (OpGH)	99/99	96/92	99/98	99/98	This study
MXoc0601	1.3 ^B	HR ⁺	<i>Xoryp_05420</i>	396/1467	Amidophosphoribosyltransferase (PurF)	99/99	99/99	99/99	99/99	This study
MXoc0104	1.2 ^B	HR ⁺	<i>Xoryp_10705</i>	1198/1305	Threonine synthase (ThrC)	99/99	94/93	99/98	98/97	This study
MXoc0350	1.0 ^{BC}	HR ⁻	<i>Xoryp_14510</i>	475/807	Tryptophan synthase, alpha subunit (TrpA)	99/99	94/90	98/96	98/97	This study
MXoc0236	0.4 ^C	HR ⁺	<i>Xoryp_02235</i>	-249/2709	Hypothetical protein	99/99	98/96	98/96	99/98	This study
MXoc0065	0.5 ^C	HR ⁺	<i>Xoryp_00885</i>	26/474	Conserved hypothetical protein	98/98	78/69	90/86	90/87	This study
MXoc0761	1.7 ^{AB}	HR ⁺	<i>Xoryp_22910</i>	837/981	Conserved hypothetical protein	97/96	88/80	95/91	95/91	This study
RS105	2.4 ^A	HR ⁺								

*Significant ($P=0.01$, t test) reduction in virulence by different mutants is shown by different letters.†The genome sequence of *Xoc* BLS256 strain (<http://cmr.jvri.org/tigr-scripts/CMR/GenomePage.cgi?org=Xoc>) is used as the reference.

‡Position is given relative to the first nucleotide of the open reading frame.

outside the mutated loci, we constructed plasmids containing *tal-C10c-like*, *rpfC*, *oxyR*, *dsbC*, *rfbA*, *amtR*, *opgH*, *trpA*, *thrC*, *purF*, *Xoryp_02235*, *Xoryp_00885* and *Xoryp_22910* and their native promoters in pHM1 (Table 1); the constructs were designed based on the maps of the Tn5-disrupted genes in Fig. 3. Thirteen genes with the corresponding promoters were amplified from genomic DNA of the wild-type RS105 with the primer pairs listed in Supplementary Table S1. These PCR products were first cloned into pMD18-T for sequence verification and then subcloned into pHM1 at *Hind*III, *Kpn*I, *Sal*I or *Eco*RI sites, generating pC1304, pC0236, pC0028, pC0525, pC0104, pC0091, pC0350, pC0065, pC1232, pC0729, pC0761, pC0852 and pC0601 (Table 1). After the recombined plasmids were individually transferred into the corresponding mutants, the complemented strains (CMxoc1304, CMxoc0236, CMxoc0028, CMxoc0525, CMxoc0104, CMxoc0091, CMxoc0350, CMxoc0065, CMxoc1232, CMxoc0729, CMxoc0761, CMxoc0852 and CMxoc0601; see Table 1) and the wild-type were inoculated into rice (1×10^8 c.f.u. ml⁻¹) by the leaf-needling method. The mean lesion lengths in rice, measured at 14 days p.i., demonstrated that mutants harbouring the corresponding genes restored virulence to the wild-type level (Fig. 1b, Table 2), suggesting that the 13 genes are candidate virulence factors in *Xoc*. It is important to note that the mutation in the *tal-C10c-like* gene resulted in a slight, but statistically insignificant, role in virulence.

Of these 13 candidate virulence genes, seven have unknown functions. To confirm the role of these seven candidate virulence genes in the growth of *Xoc* in rice, these seven mutants, the complemented strains and the wild-type RS105 were inoculated to rice seedlings at 1×10^8 c.f.u. ml⁻¹ by infiltration. The bacterial population in the infiltrated areas was counted daily for 4 days. Growth of the complemented strains was restored to wild-type levels, and multiplication of the mutants was significantly lower than the wild-type at each time point (Fig. 4). These results demonstrate that all the seven candidate virulence genes, *opgH*, *trpA*, *thrC*, *purF*, *Xoryp_02235*, *Xoryp_00885*, and *Xoryp_22910*, are required for bacterial growth *in planta*.

To investigate whether the mutations in the seven genes mentioned above were lethal, growth was compared in a nutrient-rich medium (NB), NY media (NB without beef extract and sucrose) and a minimal medium, MMX. The seven mutants defective in *opgH*, *trpA*, *thrC*, *purF*, *Xoryp_02235*, *Xoryp_00885* and *Xoryp_22910* displayed colonies similar to the wild-type on NB and NY agar (data not shown). Mutants Mxoc0104 (*thrC*), Mxoc0350 (*trpA*), and Mxoc0601 (*purF*) did not grow in MMX medium, while Mxoc0236, Mxoc0065, Mxoc0761 and Mxoc0852 were prototrophic and grew as well as the wild-type (data not shown). Growth of these mutants was further investigated by adjusting the bacterial concentration to OD₆₀₀ 0.05 in liquid NB, NY and MMX medium. In NB (data not shown) and NY broth, there were no obvious differences in growth among these seven mutants compared with the wild-type over a 40 h period (Fig. 5a). However, mutant Mxoc0852 (*opgH*) was significantly

Fig. 2. Southern blot analysis (a) and PCR verification (b) of Tn5 insertions in candidate virulence-reduced mutants of *Xoc*. Genomic DNA of the mutants was digested with *Pst*I, and probed with a 500 bp fragment amplified by PCR from the Tn5 transposon. To confirm that Tn5 is inserted in the target of the mutant, the corresponding primer pairs (Supplementary Table S1), based on the genome sequence of *Xoc* BLS256, for the entire genes and their promoter regions were explored to amplify the corresponding DNA fragments from the wild-type RS105 and the Tn5-tagged mutants. The PCR-amplified products in the tested mutants should be 1221 bp larger than those in the wild-type. For detection of the Tn5-inserted *tal-C10c-like* (*Xoryp_21665*) in Mxoc0729, one primer from *Xoryp_21665* and another from the Tn5 transposon (Supplementary Table S1) were used. Mxoc0729 gave rise to a corresponding PCR product, but the wild-type did not. Marker, λ -EcoT14 DNA ladder. 1, Mxoc1304; 2, Mxoc0236; 3, Mxoc0028; 4, Mxoc0525; 5, Mxoc0104; 6, Mxoc0091; 7, Mxoc0350; 8, Mxoc0065; 9, Mxoc1232; 10, Mxoc0729; 11, Mxoc0761; 12, Mxoc0852; 13, Mxoc0601; R, RS105; 15, *Eco*T14; 16, DL2000.

impaired with respect to growth in MMX compared with the wild-type (Fig. 5b). Mutants Mxoc0104 (*thrC*), Mxoc0350 (*trpA*) and Mxoc0601 (*purF*) did not grow in MMX (Fig. 5b). Growth of the complemented strains CMxoc0104, CMxoc0350 and CMxoc0601 was identical to the wild-type in MMX (data not shown), indicating that the growth could be restored by *thrC*, *trpA* and *purF*, respectively, *in trans*.

Expression of seven candidate virulence genes is induced *in planta*

Real-time quantitative RT-PCR was used to assess transcriptional activity of these seven genes in the wild-type

RS105 grown in four conditions: nutrient-rich medium (NB), minimal medium (MMX), rice suspension cells and rice leaves (see Methods). Expression of the seven candidate virulence genes was significantly higher (2.1- to 21.4-fold higher; $P=0.01$, *t* test) when the pathogen interacted with rice suspension cells or rice leaves than when grown in NB and MMX, respectively (Fig. 6). Interestingly, transcription of the seven genes was significantly higher in rice suspension cells than in rice leaves (Fig. 6). The results suggest that the seven genes (*opgH*, *trpA*, *thrC*, *purF*, *Xoryp_02235*, *Xoryp_00885* and *Xoryp_2291*) are potentially involved in virulence, and their expression is dependent on the interaction of *Xoc* with rice.

Fig. 3. Genomic locations and orientations of the Tn5-transposon insertions in pathogenicity-deficient or virulence-reduced mutants of *Xoc*. Each mutant resides in only one insertion. Disrupted genes are shown as empty arrows. Tn5 transposon insertions are shown as inverted triangles. The orientation of the Tn5-transposon in each insertion is represented by an arrow above the triangle. Mutant designations relative to each insertion are shown in (a). Numbers to the left and right show genomic locations in base pairs. Gene symbols or products are labelled below disrupted genes and partial flanking genes. The *hrp* clusters of *Xoc* RS105 (GenBank accession no. AY875714 and AY272885) and the genome sequence of *Xoc* BLS256 (<http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=Xoc>) were used as the references.

DISCUSSION

In the present study, a Tn5-tagged mutant library of *Xoc* was screened, and 21 genes were identified with roles in pathogenicity or virulence. In addition to genes with known functions [e.g. *tal* (*tal-C10c-like*), *rpfC*, *amtR*, *oxyR*, *dsbC* and *rfbA*], seven candidate virulence genes were identified, including *opgH*, *purF*, *thrC*, *trpA*, *Xoryp_02235*, *Xoryp_00885* and *Xoryp_22910*; the latter three genes

encode hypothetical proteins highly conserved in *Xanthomonas* species (Table 2).

Oxidative stress plays a crucial role in plant defence against the invasion and colonization of pathogens (Baker & Orlandi, 1995). *oxyR*, the gene disrupted in mutant Mxoc1304, encodes a transcriptional regulator of oxidative stress (Table 2). OxyR, a LysR-type transcriptional regulator, is a DNA-binding protein that governs bacterial

Fig. 4. Bacterial populations of the seven novel gene mutants, the corresponding complementary stains and the wild-type strain RS105 in rice (cv. Shanyou63). Bacteria were recovered from the inoculated leaves at 1 day intervals for 4 days p.i. by infiltration of the above strains at OD₆₀₀ 0.3 (approx. 1×10^8 c.f.u. ml⁻¹) with needleless syringes, and were homogenized in sterile water. The homogenates were diluted and plated on NA plates with appropriate antibiotics. Bacterial c.f.u. were counted after incubation at 28 °C for 3 days. Data are the mean $\pm$ SD from three repeats, and each experiment was repeated three times. Mutant, $\blacksquare$; complemented mutant, $\bullet$; wild-type RS105, $\blacktriangle$. Tn5 insertions were at Mxoc0852 (a), Mxoc0601 (b), Mxoc0104 (c), Mxoc0350 (d), Mxoc0236 (e), Mxoc0065 (f) and Mxoc0761 (g).

oxidative stress and scavenges reactive oxygen species (ROS) (Lee *et al.*, 2004; Toledo *et al.*, 2011; Zheng *et al.*, 1998; Maddocks & Oyston, 2008). OxyR has been studied

in *E. coli* (Christman *et al.*, 1989), *Xylella fastidiosa* (Toledo *et al.*, 2011), and *Xanthomonas campestris* pv. *phaseoli* (Kullik *et al.*, 1995). The highly conserved N-terminal

Fig. 5. Growth curves of *Xoc* strains in NY (no sugar) medium (a) and MMX minimal medium (b). The wild-type strain RS105 was used as a control. Strains were inoculated into 20 ml liquid medium and incubated at 28 °C with shaking at 200 r.p.m. Aliquots (200 μ l each) were taken in triplicate at intervals, and bacterial growth was determined by measuring OD₆₀₀ against the medium blank. Data presented are means $\pm$ SD from a representative experiment; similar results were obtained in two other independent experiments. Mxoc0104, $\blacklozenge$; Mxoc0350, $\blacksquare$ (dotted line); Mxoc0601, $\blacktriangle$; Mxoc0852, $\circ$; Mxoc0065, $\triangle$; Mxoc0236, $\square$; Mxoc0761, $\times$; RS105, $\blacksquare$ (solid line).

Fig. 6. Expression profiles of the seven novel genes of *Xoc* RS105 strain by real-time quantitative RT-PCR. RNA was isolated from the cultures of the wild-type RS105 grown in NB (black bars) or MMX (white bars), or rice suspension cells (dotted bars) for 16 h and from rice leaves in which the wild-type strain had been infiltrated and allowed to interact for 8 h (grey bars). The relative mRNA level of seven novel genes was calculated with respect to the level of the corresponding transcript in cells cultured in NB medium. Values given are the means $\pm$ SD of triplicate measurements from a representative experiment, and similar results were obtained in two other independent experiments. Bars with the same letter are not significantly different.

DNA-binding domain and C-terminal regulatory domain in OxyR may enable plant-pathogenic bacteria to sense intracellular ROS and activate the transcription of genes involved in scavenging oxidative species. The disruption of *oxyR* in *Xoc* (mutant Mxoc1304) impaired virulence in rice (Fig. 1), which suggests that successful infection and colonization of rice requires *Xoc* to respond appropriately to oxidative stress.

Two genes with known functions, *rfbA* and *amtR*, which were identified in the Vir⁻ mutants Mxoc0028 and Mxoc0091, encode glucose-1-phosphate thymidyltransferase and an aminotransferase, respectively. *RfbA* catalyses the conversion of glucose 1-phosphate to dTDP-rhamnose (Lerouge & Vanderleyden, 2002); the latter is a component of the O-antigen of LPS in Gram-negative bacteria including *Xanthomonas*. In *Mycobacterium tuberculosis*, *rfbA* was identified as a virulence factor that is essential for mycobacterial growth and cell morphology (Qu *et al.*, 2007). Lipopolysaccharides are well-documented virulence determinants in mammalian pathogens (Lerouge & Vanderleyden, 2002) and also function in the *Xcc* infection process (Newman *et al.*, 2002). However, a function for *rfbA* in LPS production by *Xoc* has not yet been demonstrated. Regarding the aminotransferase encoded by *amtR*, the final product catalysed by the enzyme flows into the tricarboxylic acid (TCA) cycle (Mailloux *et al.*, 2007). Bacterial pathogens utilize intermediates of the TCA cycle as carbon sources when there is insufficient hexose (Magne *et al.*, 1997; Oh *et al.*, 2002; Tang *et al.*, 2005). The

reduced virulence and growth observed for the Mxoc0091 mutant (Figs 1 and 4) may be due to an impaired ability to acquire nutrients in rice.

In Gram-negative bacteria, two independent metabolic pathways are involved in disulfide bond formation in extracytoplasmic proteins. One is the DsbA/DsbB oxidation pathway and the other is the isomerization/reduction pathway (Messens & Collet, 2006; Łasica & Jagusztyn-Krynicka, 2007). DsbA is essential for multiple processes in mammalian pathogens including toxin production, adhesion, motility, extracellular enzyme production and the T3SS (Urban *et al.*, 2001; Stenson & Weiss, 2002; Ha *et al.*, 2003; Tinsley *et al.*, 2004; Hiniker & Bardwell, 2004). *dsbB* mutants of *Xcc* exhibited reductions in virulence, the HR, cell motility and bacterial growth *in planta* (Jiang *et al.*, 2008). Furthermore, the *dsbB* mutation impaired the T2SS, T3SS and flagellar assembly (Jiang *et al.*, 2008). In the present study, the *dsbC* mutant (Mxoc0525) was significantly reduced with respect to virulence and growth in rice (Figs 1 and 4). *dsbC* encodes a disulfide isomerase belonging to the isomerization/reduction pathway. Periplasmic DsbC functions as a disulfide bond isomerase to reduce misfolded proteins (Hiniker *et al.*, 2005; Lee *et al.*, 2008). The mutation in the *dsbC* genes had multiple effects on extracellular enzymes and cell motility in *Erwinia carotovora* (Vincent-Sealy *et al.*, 1999) and impaired extracellular enzyme formation in *Pseudomonas aeruginosa* (Urban *et al.*, 2001). It remains unclear whether DsbC could function similarly in *Xoc*, and this warrants further study.

The most compelling group of genes identified in this study are the seven candidate virulence factors (Table 2). *opgH* encodes glucan biosynthesis glucosyltransferase H which is associated with cell-surface adhesion of *Streptococcus mutans* (Cross *et al.*, 2007). OpgH in *Xoc* is 96% identical to OpgH in *Xcv* (Minsavage *et al.*, 2004), which is highly homologous to the previously reported pathogenicity-related HrpM protein of *P. syringae* pv. *syringae* (Mukhopadhyay *et al.*, 1988; Penaloza-Vazquez *et al.*, 2010) and OpgH of *E. chrysanthemi* (Cogez *et al.*, 2001). *PurF* is generally considered to be a key enzyme in the purine *de novo* biosynthetic pathway (Shimaoka *et al.*, 2007). The mutation in *purF* is considered to render the organism auxotrophic as determined by an inability to grow on MMX minimal medium (Qian *et al.*, 2005). It may not function directly to reduce virulence *in planta*, but instead may impact survival due to the subsequent auxotrophy. *thrC*, which encodes a threonine synthase, is required for the production of the toxic intermediate homoserine in *Cryptococcus neoformans* and *Saccharomyces cerevisiae*. In these two organisms, *thrC* mutants suppressed serum sensitivity, which is a consequence of homoserine accumulation. Serum survival is critical for dissemination, an important virulence determinant in *S. cerevisiae* (Kingsbury & McCusker, 2010). The protein product of *trpA*, which encodes a tryptophan synthase alpha subunit, converts indole-3-glycerol phosphate into tryptophan, an essential amino acid for *Salmonella typhimurium* (Kriechbaumer

et al., 2008). Thus, the *trpA* mutation in *Xoc* may attenuate virulence by limiting nutrient availability for the pathogen. However, the actual functions of OpgH, PurF, ThrC and TrpA in *Xoc* pathogenicity warrant further investigation.

The three remaining genes encode hypothetical proteins. The translational product of *Xoryp_02235* has a recognizable AcCoA-syn- α domain typical of acetyltransferase (GNAT) family proteins in *X. campestris* pv. *vasculorum* (NCPB702) (data not shown) and is 96–99 % identical to the homologue in other xanthomonads (Table 2). *Xoryp_022910* encodes a conserved hypothetical protein without any identified conserved domains; similarly, it is 80–96 % identical to homologues in other *Xanthomonas* species. *Xoryp_00885* also encodes a hypothetical protein of unknown function that is highly conserved in *Xanthomonas* species (Table 2). Interestingly, *Xoryp_02235* and *Xoryp_00885* are highly induced (~20-fold) *in planta* compared with expression in NB and MMX minimal medium (Fig. 5). The transcriptional activation of these genes suggests a potential role in the *Xoc*–rice interaction, a theory that will be investigated in future studies.

ACKNOWLEDGEMENTS

We are grateful to Dr Ze-Chun Yuan, Southern Crop Protection and Food Research Centre of Agriculture and Agri-Food, Canada, and Dr Carol Bender, Oklahoma State University, USA, for their editing of the manuscript. This work was supported by the State Key Basic Research and Development Project of China (2012CB114003), the Natural Science Foundation of China (31071656) and PhD Programs Foundation of Ministry of Education of China (20100073110045).

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Edited by: I. K. Toth