Identification of 17 HrpX-Regulated Proteins Including Two Novel Type III Effectors, XOC_3956 and XOC_1550, in *Xanthomonas oryzae* pv. *oryzicola*

Xiao-bo Xue1, Li-fang Zou1,2, Wen-xiu Ma1, Zhi-yang Liu1, Gong-you Chen1,2*

1 School of Agriculture and Biology, Shanghai Jiao Tong University/Key Laboratory of Urban (South) by Ministry of Agriculture, Shanghai, China, 2 State Key Laboratory of Microbial Metabolism, School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai, China

**Abstract**

The function of some hypothetical proteins, possibly regulated by key *hrp* regulators, in the pathogenicity of phytopathogenic bacteria remains largely unknown. In the present study, *in silico* microarray data demonstrated that the expression of 17 *hrp*X-regulated protein (Xrp) genes of *X. oryzae* pv. *oryzicola* (*Xoc*), which causes bacterial leaf streak in rice, were either positively or negatively regulated by HrpX or/and HrpG. Bioinformatics analysis demonstrated that five Xrps possess a putative type III secretion (T3S) signal in the first 50 N-terminal amino acids, six xrp genes contain a PIP-box-like sequence (TTCGB-NX-TTCGB, 9≤X≤25) in the promoter regions, and two Xrps have both motifs. Twelve Xrps are widely conserved in *Xanthomonas* spp., whereas four are specific for *X. oryzae* (Xrp6) or Xoc (Xrp8, Xrp14 and Xrp17). In addition to the regulation by HrpG/HrpX, some of the 17 genes were also modulated by another *hrp* regulator HrpD6. Mutagenesis of these 17 genes indicated that five Xrps (Xrp1, Xrp2, Xrp5, Xrp8 and Xrp14) were required for full virulence and bacterial growth *in planta*. Immunoblotting assays and fusion with N-terminally truncated AvrXa10 indicated that Xrp3 and Xrp5 were secreted and translocated into rice cells through the type-III secretion system (T3S), suggesting they are novel T3S effectors. Our results suggest that Xoc exploits an orchestra of proteins that are regulated by HrpG, HrpX and HrpD6, and these proteins facilitate both infection and metabolism.

**Introduction**

Bacterial leaf streak (BLS) of rice, which is caused by *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), is a destructive plant disease in Asia. The pathogen infects rice through leaf stomata or wounds and colonizes intercellular spaces in the mesophyll, resulting in water-soaked interveinal lesions that develop into translucent streaks [1]. The infection routes and the symptoms caused by *Xoc* differ from those incited by the closely-related pathogen, *X. oryzae* pv. *oryzae* (*Xoo*). *Xoo* enters rice leaves through hydathodes or wounds, propagates in the intercellular spaces of the underlying epidermis, and then spreads throughout the plant in the xylem, where it presumably interacts with xylem parenchyma cells [2,3,4]. The *Xoc*-rice pathosystem is an important working model to elucidate how pathogens evade the plant host immune system [2,5]. The complete genome sequence and comparative functional genomic studies of three *Xoo* strains like KACC10331 [6], PXO99α [7], MAFF311018 [8] and *Xoc* strain BLS256 [9] have furthered our understanding of *Xanthomonas*-rice interactions. However, it remains unclear whether the numerous hypothetical proteins, maybe possibly regulated by HrpX (Xrps) annotated in *X. oryzae*, are involved in virulence.

The type III secretion system (T3S) is a pathogenicity determinant machine for Gram-negative pathogenic bacteria in host plants [10,11]. The Xanthomonad T3S is encoded by the *hrp*-*hpa* genes [5,12] and secretes a repertoire of effector proteins (T3SEs) into plant cells to trigger plant disease development [13–16]. These T3SEs may function to overcome PAMP- (pathogen-associated molecular pattern) triggered immunity (PTI) and Effector-triggered immunity (ETI), or promote effector-triggered susceptibility (ETS) [17–19]. In *X. oryzae*, T3SEs are classified into two types: transcriptional activator-like effectors (TALEs) [6,20,21] and NTAEs (non-TAL effectors); the latter group is also known as the *Xop* (*Xanthomonas* out protein) effectors [16,22]. Some of the NTAEs are Xrps originally annotated in the genomes of *Xanthomonas* spp. [16], implying that some Xrps may be uncharacterized T3SEs.

The expression of genes coding for the T3S and effectors is generally plant-inducible and regulated by a key *hrp* regulatory factor, HrpX [12,14,23,24]. HrpX is an AraC-type transcriptional regulator that controls the expression of genes in the HrpX regulon by binding the Pip (plant-inducible promoter)-box; this is a conserved cis-element with the consensus TTCGB-N15-TTCGB (‘B’ refers to any base except adenine) [25–28]. The Pip-box is normally followed by a −10 box that is located at 30–32 bp further downstream [29]. T3SEs also contain secretion signals in the first 30 N-terminal amino acids, which are characterized by one or more of the following: Ψ20% Ser and Pro [22,26,30]; more
than five Ser residues [13,31,32]; an aliphatic amino acid (Ile, Leu, or Val) or Pro at the third or fourth position; and a lack of negatively charged amino acids within the first 12 residues [33]. However, it is important to note that genes in the HrpX regulon may not be T3SEs, for example, HrpX-regulated proteins from Xoo recently identified by 2D-difference gel electrophoresis (2-DIGE) did not function as T3SEs [34]. The transcription and translation of HrpX regulon candidates have been examined using several reporter systems, such as calmodulin-dependent adenylate cyclase (Cya) of Bordetella pertussis [22,30,31,35], gusA [36–40] and avrulence proteins (e.g., AvrB31 and AvrXa10) lacking the T3S signal sequence [24,38,41].

The expression of HrpX is regulated by HrpG, which belongs to the OmpR family of two-component signal transduction response regulators [42,43]. HrpG regulates the expression of hrpX and hrpD operon and also controls the expression of several proteins that function as cell wall degrading enzymes (CWDEs), which are secreted by the type II secretion system (T2SS) [14,44,45]. Recently, a novel hrpG regulator, HrpD6, was identified and shown to be regulated by HrpG and HrpX; HrpD6 regulates the expression of hpa2, hpa1, hpaB, hrcC, and hrcT [12]. However, it remains unclear whether XrpS in Xanthomonas are regulated by HrpG or HrpD6.

In this study, bioinformatic and genetic approaches were used to characterize 17 Xrp-coding genes from in silico data. Different transcriptional profiles of these genes in the wild-type strain Xoo RS105, hpgG (RAhpgG), and hrpX (RAhrpX) mutants [12] were compared. Two Xrp proteins, XOC_3956 and XOC_1550, were identified as new T3SEs in Xoo.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1 in File S1. Escherichia coli was grown at 37°C in Luria-Bertani medium [46]. Xanthomonas strains and other derivatives were grown in NB, NA, NAN, NAS [47], XOM3 [48] or with rice suspension cells [49] before resuspension in rice cells at centrifugation at 5,000 rpm. Each strain was washed once in rice suspension cells [48]. Antibiotics were added at the following concentrations (µg/ml) when required: kanamycin (Kan), 25; rifampicin (Rif), 50; ampicillin (Ap), 100; and spectinomycin (Sp), 50.

Microarray design

An oligonucleotide microarray was designed at the Shanghai Biotechnology Corporation (Shanghai, China). Each slide contained six arrays, and each array contained approximately 15,000 spots (our probes were represented in triplicate). For Xoo BLS256, the genome sequence was also available from the NCBI database as accession AAC001000001 (G1:49721669). Up to five candidate probes per target (sense orientation) were designed with the Agilent eArray web tool, using temperature-matching methodology, a preferred probe melting temperature of 80°C, no 3'bias, and a target length of 60 bp. Shorter probes were extended to 60 bp using the Agilent linker.

RNA isolation and microarray execution

Xoo strain RS105 and the hrpG and hrpX mutants (RAhrpG and RAhrpX, respectively) [12] were cultured overnight in NB broth at 28°C in a shaking incubator and collected the following day via centrifugation at 5,000 rpm. Each strain was washed once in rice suspension cells [49] before resuspension in rice cells at OD₆₀₀ = 0.6. Strains were then incubated for 16 h at 60 rpm at 25°C. RNA was extracted from 1 ml of co-cultivated cells using TRIzol® Reagent (Invitrogen, Shanghai, China) as described by the manufacturer. All RNA was quantified using an Eppendorf BioSpectrometer kinetic (Eppendorf, Shanghai, China) and checked for quality using an RNA 6000 Nano Kit and a 2100 Bioanalyzer (Agilent Technologies). Fluorescent labeling of total RNA was performed as described previously [49] using the following array design on a single 6×15 k format slide: 1, WT rep 1 (Cy5) and RAhrpG rep 1 (Cy3); 2, RAhrpG rep 2 (Cy3) and WT rep 2 (Cy5); 3, WT rep 3 (Cy3) and RAhrpG rep 3 (Cy5); 4, WT rep 1 (Cy3) and RAhrpX rep 1 (Cy5); 5, RAhrpX rep 2 (Cy3) and WT rep 2 (Cy5); 6, WT rep 3 (Cy3) and RAhrpX rep 3 (Cy5). This design incorporated a dye-swap and balanced labeling of all samples. Levels and efficiencies of labeling were estimated using a spectrophotometer. Microarray hybridization, washing and scanning were performed in the JHII Sequencing and Microarray Facility as described previously [50]. Microarray images were imported into Agilent Feature Extraction (FE) (v.9.5.3) software and aligned with the appropriate array grid template file (021826_D_F_20081029). Intensity data and quality control (QC) metrics were extracted using the recommended FE protocol (GE2-v5_95_Feb07). Entire FE datasets for each array were loaded into GeneSpring (v.7.3) software for further analysis.

Microarray analysis

Data were normalized using default settings for two-channel arrays and transformed to account for dye-swaps. Data from each array were normalized using the Lowess algorithm to minimize differences in dye incorporation efficiency. Unreliable data flagged as absent in all replicate samples by the FE software were discarded. Gene lists with significant change were generated from combined replicate datasets for each pares, RAhrpG/RS105 and RAhrpX/RS105, using volcano plot filtering if the ratio is lower than 0.55 or higher than 1.75 with the P value less than 0.05 (Student’s t test).

DNA manipulation and plasmid construction

DNA manipulation was performed following standard procedures [46]. Biparental conjugal transfer of plasmids from E. coli to Xoo was performed as described previously [51]. PCR amplification was performed with primers (Table S2 in File S1) and genomic DNA of Xoo RS105; the genome sequence of Xoo BLS256 was used as a reference (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=Xoc). All plasmids constructs were confirmed by restriction enzyme digestion and sequencing.

To construct transcriptional fusions of xrp genes with the promoterless gusA (β-glucuronidase) gene, a 500-bp region upstream of each xrp ORF was PCR-amplified with primer sets pXrpX-F/pXrpX-R (X refers to 17 xrp genes; 1 to 17) (Table S2 in File S1). The PCR products were fused in-frame with gusA in pUF034GUS (Table S1 in File S1), resulting in pXrpXGUS (X refers to 17 xrp genes; 1 to 17). Recombinant plasmids were introduced into the wild-type strain RS105, and the hrpG, hrpX and hrpD6 mutants (see reference 12 for RAhrpG, RAhrpX, and RAhrpD6) (Table S1 in File S1) using biparental conjugation as described above.

To generate transcriptional fusions of xrp genes with c-Myc tags, fragments containing native promoters and corresponding xrp ORFs were PCR-amplified with the xrpX-F/xrpX-R primer pairs (X refers to 17 xrp genes, Table S2 in File S1) using RS105 genomic DNA as the template. After sequencing for confirmation, PCR products were cloned into pUF034Myc in-frame at suitable enzyme sites, generating pXrpXMyc (X refers to 1 to 17 xrp genes) (Table S1 in File S1). The recombinant plasmids were introduced into wild-type RS105 and the hrcV mutant (RAhrcV) by the biparental conjugation method.
Chimeric fusions were also generated between a variant of AvrXa10 lacking 28 amino acids at the N-terminus and xrp3 and xrp5. The truncated form of AvrXa10 was designated AvrXa10Δ28 and has been described previously [26]. The sequences obtained from xrp3 and xrp5 included approximately 300 bp upstream of the translational start site and the first 150 nucleotides. These regions of xrp3 and xrp5 were obtained using primer pairs xrp3-N-F/xrp3-N-R and xrp5-N-F/xrp5-N-R, respectively (Table S2 in File S1). The PCR products were ligated into pHblueAvrXa10Δ28 at SacI and PdiI sites. The constructs were then cloned into pPHMI at the SacI site, resulting in pXrp3/AavrXa10Δ28 and pXrp5/AavrXa10Δ28 (Table S1 in File S1). Recombinant plasmids were introduced into Xoo PXO99A and P∆hrcU by electroporation.

YFP was used as a reporter to investigate the subcellular localization of selected T3SEs. The complete ORFs encoding xrp5 and xrp3 were then transferred into Arabidopsis (Ecotype Col-0) mesophyll protoplasts by PEG-calcium fusion as described previously [53].

Quantitative real-time PCR (qRT-PCR)

The expression of selected xrp genes was assayed by qRT-PCR with corresponding primer pairs (Table S2 in File S1). All primers were designed with Beacon Designer 7 software. RNA was extracted from Xanthomonas strains as described previously [47] using TRIzol® Reagent (Takara, Dalian, China) and the manufacturer’s recommendations. cDNA synthesis was conducted with AMV random primers purchased from Takara. Prior to synthesis of the first-strand cDNA, total RNAs were digested with RNase-free DNase I (TaKaRa) to remove potential traces of genomic DNAs. qRT-PCR was performed on the Applied Biosystems 7500 qRT-PCR System using SYBR Premix ExTaq™ (Takara). PCR conditions included the following parameters: denaturation at 95°C for 30 s; 40 cycles at 95°C, 5 s; and 60°C, 34 s. Experiments were performed at least three times in triplicate. Internal controls included gviB (XOC_0096, DNA gyrase B subunit) and tpoD (XOC_2329, RNA polymerase sigma factor 70) [28,54].

β-glucuronidase (GUS) activity assays

For GUS activity assays, Xoo strains were preincubated in 5 ml NB broth at 28°C for 16-20 h until the OD600 was 0.6. An aliquot (100 μl) of this culture was transferred into 5 ml fresh NB broth. Bacterial cells were collected, washed twice, and resuspended in XOM3 to an OD600 of 2.0. After incubation at 28°C for 6 h, 1 ml of sonic buffer [40 mM Tris-HCl, pH 7, 20 mM β-mercaptoethanol, 10 mM EDTA, and 2% Triton X-100] was added into 1 ml of the bacterial culture. This mixture was frozen in liquid nitrogen and then thawed in at 37°C for 5 min. This procedure was repeated five times, and the mixture was centrifuged (12,000 rpm) at 4°C for 15 min. Then, 90 μl 4-methylumbelliferylone-β-glucuronide (4-MUG) (Sigma, Shanghai, China) [55] was added into 10 μl supernatant and incubated at 37°C for 1 h. The reaction was terminated by adding 1 ml of 2 M Na2CO3. GUS activity was measured at 415 nm with the Modulus™ Single Tube Multifunction Tester (YuanPingHao, Beijing, China) [56]. One unit was defined as 1 nM of 4-methyl-umbelliferone (4-MU) produced per min per OD600 of bacterial cells as described [56].

Three independent experiments were performed, and similar results were obtained.

Mutant construction and complementation studies

To generate non-polar mutations in the 17 xrp genes, two fragments flanking each gene were PCR-amplified from Xoo RS105 genomic DNA with primer pairs xrp3-F/xrp3-R and xrp3II-F/xrp3II-R (X refers to the 17 xrp genes; 1 to 17, Table S2 in File S1); the results confirmed that the PCR products were smaller than those obtained in RS105 (data not shown). For complementation studies, constructs designated pXrpXMyc (X refers to 1 to 17, Table S1 in File S1) were introduced into the corresponding xrp mutants by biparental conjugation. Mutants containing xrp genes in trans were verified by colony-PCR and named CRMxrpX (X refers to 1 to 17, Table S1 in File S1).

Bacterial virulence and growth in planta

Rice cultivar Shanyou 63 (two weeks old) was used to evaluate the virulence of Xoo RS105 and derivatives. Bacterial cells were adjusted to 1×108 cfu/ml and infiltrated into newly-expanded leaves with a needleless syringe at three locations per leaf. Three leaf discs (0.5 cm in diameter) were excised with a cork borer from each infiltrated area. After sterilization in 70% ethanol and 30% hypochlorite, the discs were macerated using a sterile mortar and pestle in 1 ml of distilled water, diluted and plated to determine cfu/cm2. Serial dilutions were spotted in triplicate on NA plates with appropriate antibiotics. The plates were incubated at 28°C for 3-4 days until single colonies could be counted. The bacterial population (cfu/cm2 of leaf area) was then estimated, and the standard deviation was calculated using colony counts from three triplicate spots of three samples obtained at each time point. All rice cultivars were grown in a greenhouse maintained at 25°C with a 12-h photoperiod. Experiments were repeated at least three times.

Near-isogenic lines of rice cultivar IRBB10 were used to assay the pathogenicity of Xoo PXO99A and derivatives as described previously [47]. Plant responses were scored 14 dpi for lesion lengths. Experiments were repeated at least three times.

Type III secretion assays

The pXrpXMyc plasmids were transformed into the wild-type RS105 and RΔhrcV for detection of secreted proteins. Xoo strains were preincubated in NB medium, washed twice, and suspended at OD600 = 2.0 with sterilized water. An aliquot (40 μl) of the bacterial suspension was inoculated into 1 ml of XOM3 medium (pH 6.0), adjusted to OD600 = 1.0, and incubated at 28°C for 6 h with the appropriate antibiotics. Supernatant fractions were separated using a 0.22 μm filter, and the supernatant fraction (50 ml) was reduced to 5 ml by vacuum evaporation. Proteins were precipitated with 12.5% trichloroacetic acid at 4°C for 16 h, centrifuged at 3000×g for 15 min, and then washed briefly with acetone and air-dried. The protein pellet was resuspended in SDS buffer containing dithiothreitol (DTT) [38]. Proteins were separated on 10% SDS-PAGE gels and transferred to membranes for immunoblotting using primary antibody anti-c-Myc (Huaan, Hangzhou, China). Primary antibodies were recognized by anti-
rabbit secondary antibodies (Huaan) and visualized by autoradiography with the Western-Light chemiluminescence system (Transgene, Beijing, China). Experiments were repeated at least twice and Hpa1 was used as a positive control [59].

Results

Screening of T3SE candidates from Xoc

Genome-wide identification of bacterial virulence genes has been greatly facilitated by the availability of microarrays [60,61]. Considering the critical fact that HrpG and HrpX are two key regulators for pathogenicity determinants in Xanthomonads [14,26,28,29,43,44], the expression profiles of genome widely annotated hypothetical proteins in Xoc RS105 and hrpG and hrpX mutants were only evaluated based on the annotated sequence of Xoc BLS256 (AAQN01000001.1) (GI:94721269). The three Xoc strains (RS105, RAΔhrpG, RAΔhrpX) were co-cultivated with rice cells for 16 h at 25°C, and bacterial RNAs were extracted and hybridized with the BLS256 genechip. The expression levels of hypothetical protein genes in the hrpG and hrpX mutants were compared with the wild-type RS105 and taken as the candidates if the ratio was lower than 0.55 or higher than 1.75 with the P value less than 0.05 (Table S3 in File S1). For a comparison, we checked the expression of hpg genes and known T3SE genes (Table S3 in File S1) and found that the expression patterns of these genes in silicon were consistent with those that were experimentally explored previously [12,23,44,56,61]. Totally, there were 247 hypothetical protein genes in the Xoc silico data showed that the expression of xrp15, xrp12, xrp11, xrp10, xrp9 and xrp7 was significantly positively regulated by HrpG and HrpX, which is consistent with our in silicon data (Fig. 1A, Table S3 in File S1); the expression of xrp5 and xrp8 was decreased in the hpg mutant, relative to the wild-type and hpg mutant; the expression of xrp4, xrp3, xrp2, xrp13 and xrp14 was significantly positively regulated by both HrpG and HrpX, but negatively regulated by HrpD6 (Fig. S1 in File S1); this observation is consistent with previous results [12]. As shown in Fig. 1B, the expression of xrp1, xrp2, xrp3, xrp13 and xrp14 was significantly positively regulated by both HrpG and HrpX, which is consistent with our in silicon data (Fig. 1A, Table S3 in File S1); the expression of xrp3 and xrp8 was decreased in the hpg mutant, relative to the wild-type and hpg mutant; the expression of xrp4, xrp5, xrp10, xrp11 and xrp12 was decreased in RAΔhrpX, but increased not only in RAΔhrpG but also in RAΔhrpD6; and the expression of xrp6, xrp7, xrp15 and xrp17 was significantly increased in the hpg mutant. The qRT-PCR results (Fig. 1A) were generally consistent with the expression in silicon (Fig. 1).

To further investigate the expression of these 17 xhp genes, we fused the putative promoter regions (500 bp upstream of the translational start site) to a promoterless gusA gene. The promoters were PCR-amplified from genomic DNA of strain RS105 using the primers listed in Table S2 in File S1. After sequencing these PCR products from RS105, no obvious difference was found in the corresponding regions in BLS256 genome (data not shown). The pxrp-gusA fusions were then transferred into the wild-type RS105, the hpg, hpgX and hpgD6 mutants, respectively, then incubated in XOM3 (a hrpD-inducing medium) [49] at 20°C for 6 h. The GUS expression patterns (Fig. 1C) detected by these 17 xhp promoters was similar to the regulation observed by genechip and qRT-PCR analysis (Figs. 1A, B). However, there were no obvious differences in GUS activities expressed from the xrp4, xrp13, xrp14 and xrp16 putative promoters in RS105, and the hpg, hpgX and hpgD6 mutants (Fig. 1C), possibly because that these four genes localize within adjacent operons where the tested promoters are not real for GUS activity detection (data not shown).

Expression patterns of xhp genes controlled by HrpG, HrpX and HrpD6

To confirm the expression profiles of these 17 xhp genes in RS105, RAΔhrpG, and RAΔhrpX (Fig. 1A), we used quantitative real-time PCR (q-RT PCR) to determine whether the expression was HrpG or HrpX-dependent. Primers (Table S2 in File S1) selected for this experiment were based on the genome of BLS256 (NZ_AAQN01000001.1, GI:353459993) [10]. Since HrpD6 is a newly identified hpg regulatory factor, we also investigated whether the expression of these 17 xhp genes was altered in the mutant RAΔhrpD6. Controls included Hpa1, which contains a T3S signal at the N-terminus and the PIP-box promoter [24,59] and XOC_0618, which is homologous to Xpox [22]. Hpa1 and XOC_0618 were positively regulated by HrpG and HrpX, but negatively regulated by HrpD6 (Fig. S1 in File S1); this observation is consistent with previous results [12]. As shown in Fig. 1B, the expression of xrp1, xrp2, xrp3, xrp13 and xrp14 was significantly positively regulated by both HrpG and HrpX, which is consistent with our in silicon data (Fig. 1A, Table S3 in File S1); the expression of xrp3 and xrp8 was decreased in the hpg mutant, relative to the wild-type and hpg mutant; the expression of xrp4, xrp5, xrp10, xrp11 and xrp12 was decreased in RAΔhrpX, but increased not only in RAΔhrpG but also in RAΔhrpD6; and the expression of xrp6, xrp7, xrp15 and xrp17 was significantly increased in the hpg mutant. The qRT-PCR results (Fig. 1A) were generally consistent with the expression in silicon (Fig. 1). In contrast, the expression of xrp1, xrp2, xrp3, xrp4, xrp5, xrp6, xrp7, xrp10, xrp11, and xrp15 was significantly increased in the hpgD6 mutant (Fig. 1B).

To further investigate the expression of these 17 xhp genes, we fused the putative promoter regions (500 bp upstream of the translational start site) to a promoterless gusA gene. The promoters were PCR-amplified from genomic DNA of strain RS105 using the primers listed in Table S2 in File S1. After sequencing these PCR products from RS105, no obvious difference was found in the corresponding regions in BLS256 genome (data not shown). The pxrp-gusA fusions were then transferred into the wild-type RS105, the hpg, hpgX and hpgD6 mutants, respectively, then incubated in XOM3 (a hrpD-inducing medium) [49] at 20°C for 6 h. The GUS expression patterns (Fig. 1C) detected by these 17 xhp promoters was similar to the regulation observed by genechip and qRT-PCR analysis (Figs. 1A, B). However, there were no obvious differences in GUS activities expressed from the xrp4, xrp13, xrp14 and xrp16 putative promoters in RS105, and the hpg, hpgX and hpgD6 mutants (Fig. 1C), possibly because that these four genes localize within adjacent operons where the tested promoters are not real for GUS activity detection (data not shown).

xrp1, xrp2, xrp5, xrp8 and xrp14 genes are required for Xoc virulence to rice

The expression patterns of these 17 xhp genes in the hpg, hpgX and hpgD6 mutant backgrounds prompted us to determine whether they are involved in Xoc virulence. Each xhp gene was PCR-amplified using primers derived from the BLS256 genome (Table S2 in File S1). Sequence and BLAST analysis of xhp PCR products from RS105 showed no differences in these xhp genes with the corresponding ORFs in BLS256 (Table 2). All these Xhp proteins
of RS105 showed 100% identity to the homologs of BLS256 (Table 2). Homologs of Xrp2 were not identified in X. axonopodis pv. citri strain 306 (Xoc 306) and X. campesstri pv. campesstri (Xco) ATCC33913. Xrp6 was only identified in Xoc BLS256 and Xoo PX099^a^. Xrp8 had no orthologs in Xoo strains but in other three Xanthomonas species, and Xrp12 was not present in Xoo PXO99^a^.

The c-Myc-tagged constructs were introduced into Xoc RS105 (Fig. 2A), and these five mutants were restored to wild-type levels by the introduction of wild-type (Fig. 2B–G). Virulence and bacterial growth in rice leaves compared to the wild-type (Fig. 2B–G). The above data indicate that xrp1, xrp2, xrp5, xrp8 and xrp14 mutants are required for bacterial virulence and growth in rice.

### Table 1. Protein and nucleotide sequence analysis of 17 xrp genes.

| Protein ID in BL256 | Gene ID in BL256 | Ser and Pro^b^ | Ser | Pro | Leu^b^ | Asp and Glu^c^ | 3rd AA | 4th AA | PIP box-like^d^ | 5'-3' sequence | 10 Box-like |
|---------------------|------------------|----------------|-----|-----|--------|---------------|--------|--------|-----------------|---------------|------------|
| Xrp1                | YP_005627937     | XOC_1601       | 8   | 6   | 2      | 8             | 0      | T      | H               | −96/−75       | TTCGG-N17-TCGG |
| Xrp2                | YP_005630809     | XOC_4584       | 6   | 4   | 2      | 2             | 0      | K      | F               | −106/−85      | TTCGG-N17-TCGG |
| Xrp3                | YP_005630213     | XOC_3956       | 10  | 6   | 4      | 3             | 1      | T      | R               | N             | N          |
| Xrp4                | YP_005630212     | XOC_3955       | 8   | 6   | 2      | 2             | 0      | R      | H               | N             | N          |
| Xrp5                | YP_005627898     | XOC_1550       | 8   | 7   | 1      | 7             | 2      | G      | E               | N             | N          |
| Xrp6                | YP_005629702     | XOC_3440       | 6   | 5   | 1      | 4             | 1      | V      | E               | N             | N          |
| Xrp7                | YP_005630808     | XOC_4583       | 9   | 2   | 7      | 6             | 1      | S      | L               | N             | N          |
| Xrp8                | YP_005628761     | XOC_2462       | 9   | 6   | 3      | 6             | 1      | G      | L               | −81/−56       | TTCGA-N15-TCGG |
| Xrp9                | YP_005628272     | XOC_1951       | 11  | 3   | 8      | 8             | 0      | L      | R               | −114/−69      | TTCGG-N15-TCGG |
| Xrp10               | YP_005626937     | XOC_0560       | 6   | 2   | 4      | 1             | 0      | V      | P               | N             | N          |
| Xrp11               | YP_005629396     | XOC_3130       | 8   | 8   | 0      | 4             | 1      | I      | Q               | N             | N          |
| Xrp12               | YP_005630266     | XOC_4010       | 5   | 2   | 3      | 8             | 1      | A      | L               | N             | N          |
| Xrp13               | YP_005627233     | XOC_0860       | 5   | 1   | 4      | 5             | 1      | L      | A               | −240/−211     | TTCGG-N15-TCGG |
| Xrp14               | YP_005626509     | XOC_0084       | 10  | 3   | 7      | 4             | 2      | D      | D               | −61/−26       | TTCGG-N15-TCGG |
| Xrp15               | YP_005629104     | XOC_2829       | 2   | 1   | 1      | 5             | 1      | I      | E               | −121/−90      | TTCGG-N15-TCGG |
| Xrp16               | YP_005629103     | XOC_2828       | 5   | 3   | 2      | 5             | 0      | A      | P               | −362/−331     | TTCGG-N15-TCGG |
| Xrp17               | hrpFB            |                 |     |     |       |               |        |        |                 |               | N          |

*aNumber of Ser and Pro residues in the N-terminal 50 amino acids.
*bNumber of Leu residues in the N-terminal 50 amino acids.
*cNumber of Asp and Glu residues in the N-terminal 12 amino acids.
*dIncludes an imperfect PIP-box like sequence (TTGCXB-NxTCGG,B/A/T/C/G) in the respective promoter.

**Novel T3SE Proteins in Xoc**

In the complementation studies mentioned above, a c-Myc tagged hrpF gene was fused in-frame at the C-termini of the 17 Xrp proteins (see Table S1 in File S1), which facilitated subsequent expression studies. The c-Myc-tagged constructs were introduced into Xoc RS105 and RAavrV [62] and incubated in hrp-inducing medium XOM3 [40] for 6 h. The supernatants (SN) and total extracts (TE) of bacterial cells were used to investigate whether the Xrps were expressed by Western blotting using a c-Myc specific polyclonal antibody (Huaan, Hangzhou, China). With the exception of Xrp4, Xrp6, Xrp9, Xrp10 and Xrp13, the other 12 Xrp proteins were not found in the SN or TE of Xoc.

**Xrp3 and Xrp5 are novel T3SEs**

In the complementation studies mentioned above, a c-Myc tag was fused in-frame at the C-termini of the 17 Xrp proteins (see Table S1 in File S1), which facilitated subsequent expression studies. The c-Myc-tagged constructs were introduced into Xoc RS105 and RAavrV [62] and incubated in hrp-inducing medium XOM3 [40] for 6 h. The supernatants (SN) and total extracts (TE) of bacterial cells were used to investigate whether the Xrps were expressed by Western blotting using a c-Myc specific polyclonal antibody (Huaan, Hangzhou, China). With the exception of Xrp4, Xrp6, Xrp9, Xrp10 and Xrp13, the other 12 Xrp proteins were not found in the SN or TE of Xoc.
28 amino acid residues at the N-terminus (avrXa10). Xrp3 and Xrp5 were fused with a truncated AvrXa10 that lacked and Xrp5 may be translocated into host cells. To further Ser/Pro residues, respectively (Fig. 4A, B), indicating that Xrp3 amino acid residues of Xrp3 and Xrp5 contain a total of 10 and 8 translocated into plant cells [24,38]. The first 50 N-terminal signal, and investigate whether the fused effector can be candidate effector with an Avr protein that lacks the secretion T3SE secretion via the T3S is to fuse the N-terminus of a contain a T3S signal [22,33]. One strategy for determination of least five Ser residues in their N-terminal sequences are deemed to Xrp1 and Xrp2 may be secreted via other systems. Xrp1 and Xrp2 were detected in the SN fractions of both the wild-type, but not in the T3S mutant (Fig. 3). These data indicate that Xrp3 and Xrp5, like Hpa1, were are shown in Fig. 3). However, Xrp3 and Xrp5, like Hpa1, were detected in SNs of the wild-type, but not in the T3S mutant (Fig. 3). The above data indicate that the N-terminal portions of Xrp3 and Xrp5 enable the N-terminal truncated AvrXa10 to be secreted through the T3S and translocated into rice cells for HR induction.

### Table 2. Conservation of 17 Xrps of X. oryzae pv. oryzicola RS105 in other Xanthomonas species.

| Xoc | RS105 | Description | Xoc BLS256 | Xoo | Xac 306 | Xcv 85-10 | Xcc ATCC33913 |
|-----|-------|-------------|------------|-----|--------|----------|--------------|
| Xrp1 | cysteine protease | XOC_1601 (100a) | PXO_04730 (83) | XOO1385 (84) | XOO1487 (83) | XCC2853 (88) | XCV3013 (87) | XCC2693 (83) |
| Xrp2 | hypothetical protein | XOC_4584 (100) | PXO_03859 (82) | XOO14169 (82) | XOO4426 (82) | N | XCV0093 (54) | N |
| Xrp3 | hypothetical protein | XOC_3956 (100) | PXO_03076 (88) | XOO0633 (89) | XOO0696 (89) | XAC3685 (88) | XCV3806 (86) | XCC3645 (79) |
| Xrp4 | hypothetical protein | XOC_3955 (100) | PXO_03077 (85) | XOO0634 (85) | XOO0697 (85) | XAC3684 (78) | XCV3805 (80) | XCC3644 (60) |
| Xrp5 | hypothetical protein | XOC_1550 (100) | PXO_04764 (93) | XOO1359 (89) | XOO1457 (89) | XAC2878 (81) | XCV3033 (82) | XCC2715 (80.8) |
| Xrp6 | hypothetical protein | XOC_3440 (100a) | PXO_01952 (93) | N | N | N | N | N |
| Xrp7 | glucuronate isomerase | XOC_4583 (100) | PXO_03860 (96) | XOO4170 (96) | XOO4427 (95) | XAC4241 (92) | XCV4357 (92) | XCC4417 (86) |
| Xrp8 | hypothetical protein | XOC_2462 (100) | N | N | N | XAC2155 (91) | XCV2099 (91) | XCC2200 (86) |
| Xrp9 | hypothetical protein | XOC_1951 (100) | PXO_00529 (98) | XOO2257 (99) | XOO2488 (96) | XAC2517 (96) | XCV2699 (92) | XCC2382 (84) |
| Xrp10 | hypothetical protein | XOC_0560 (100) | PXO_04113 (90) | XOO3892 (91) | XOO4113 (90) | XAC3866 (94) | XCV3805 (94) | XCC3811 (81) |
| Xrp11 | hypothetical protein | XOC_3130 (100) | PXO_01766 (85) | XOO1798 (85) | XOO1902 (85) | XAC1364 (85) | XCV1420 (85) | XCC1318 (83) |
| Xrp12 | S-(hydroxymethyl) glutathione dehydrogenase | XOC_4010 (100) | N | XOO0585 (96) | XOO0635 (95) | XAC3747 (92) | XCV3866 (94) | XCC3703 (90) |
| Xrp13 | Protease | XOC_0860 (100) | PXO_04349 (96) | XOO3388 (97) | XOO3806 (96) | XAC0795 (79) | XCV0845 (80) | N |
| Xrp14 | cytochrome P450 B1-1 | XOC_0084 (100) | N | N | N | XAC3170 (29) | N | N |
| Xrp15 | NDP-hexose isomerase | XOC_2828 (100) | PXO_00206 (94) | XOO2849 (96) | XOO2998 (98) | XAC1687 (92) | XCV1723 (93) | XCC1670 (86) |
| Xrp16 | transferase | XOC_2828 (100) | PXO_00206 (94) | XOO2848 (94) | XOO2997 (94) | XAC1686 (88) | XCV2006 (83) | N |
| Xrp17 | hypothetical protein | HrpFB | 100a | N | N | N | N | N |

| Xoc | RS105 | Description | Xoc BLS256 | Xoo | Xac 306 | Xcv 85-10 | Xcc ATCC33913 |
|-----|-------|-------------|------------|-----|--------|----------|--------------|
| Xrp18 | hypothetical protein | XOC_3440 | N | N | N | N | N |

aParentheses indicate percent amino acid identity. N, no homologous gene was identified.

Xanthomonas T3SEs containing up to 20% Ser/Pro residues or at least five Ser residues in their N-terminal sequences are deemed to contain a T3S signal [22,33]. One strategy for determination of T3SE secretion via the T3S is to fuse the N-terminus of a candidate effector with an Avr protein that lacks the secretion signal, and investigate whether the fused effector can be translocated into plant cells [24,38]. The first 50 N-terminal amino acid residues of Xrp3 and Xrp5 contain a total of 10 and 8 Ser/Pro residues, respectively (Fig. 4A, B), indicating that Xrp3 and Xrp5 may be translocated into host cells. To further investigate this possibility, the N-terminal 50 amino acids of Xrp3 and Xrp5 were fused with a truncated AvrXa10 that lacked 28 amino acid residues at the N-terminus (avrXa10). The chimeric proteins Xrp3avrXa10A and Xrp5avrXa10A were constructed using native xpf promoters (see Materials and Methods), resulting in pPXrp3avrXa10A and pPXrp5avrXa10A, respectively (Table S1 in File S1, Fig. 4A, B). The wild-type RS105 harbouring avrXa10 does not trigger an HR in rice line IRBB10, which carries the cognate R gene Xa10 [24]; thus, we utilized Xso strain PXO99A to investigate whether the fused proteins can trigger an HR in IRBB10. The T3S mutant PAhCU was used as a negative control. PXO99A containing pPXrp3avrXa10A or pPXrp5avrXa10A produced an HR in IRBB10, as did PXO99A (pavrXa10) (Fig. 4C). However, HR was not elicited by PXO99A containing pavrXa10A or the empty vector pUFR034 (Fig. 4C).

As predicted, the T3S mutant PAhCU did not elicit symptoms in cultivar IRBB10 (Fig. 4C). We then used immunoblotting to determine whether the translational fusions present in pPXrp3avrXa10A and pPXrp5avrXa10A were expressed in the tested strains. As expected, the chimeric proteins PXrp3avrXa10A and PXrp5avrXa10A, like the wild-type AvrXa10A and the N-terminal truncated AvrXa10A, were detectable in the TEs of both wild-type PXO99A and PAhCU, but not in SN fraction of PAhCU (Fig. 4D). The above data indicate that the N-terminal portions of Xrp3 and Xrp5 enable the N-terminal truncated AvrXa10A to be secreted through the T3S and translocated into rice cells for HR induction.

### Subcellular localization of Xrp3 and Xrp5

The localization of Xrp3 and Xrp5 was examined by utilizing YFP-tagged Xrp3 and Xrp5 proteins. The protocol (see Materials and Methods) utilizes mesophyll protoplasts of Arabidopsis and PEG-calcium-mediated transfection to deliver pXrp3-YFP and pXrp5-YFP (Table S1 in File S1) into plant cells; GUS-NLS-YFP and YFP were used as a reference. Fluorescence confocal microscopy indicated that YFP-tagged Xrp3 proteins are localized throughout the cell, but Xrp5-YFP is targeted to the plasma membrane and cytoplasm. The latter result was clearly different from the YFP control, which was partially retained in the nucleus (Fig. 5). In general, our results suggest that Xrp3-YFP is preferentially targeted the cytoplasm.

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Discussion

In this report, two new T3SEs, Xrp3 (XOC_3956) and Xrp5 (XOC_1550), were identified and shown to be HrpX-dependent based on transcriptional data in silico (Fig. 1A). These two newly-identified T3SEs can be added to the extensive list of Xanthomonas T3SEs at http://www.Xanthomonas.org/t3e.html [16]. Interestingly, xrp3 and xrp5 are highly conserved in Xanthomonads rather
than other plant pathogenic bacteria whose genomes have been completely sequenced (Table 2). Xrp5, but not Xrp3, was important for a full level of virulence in rice (Fig. 2). This is consistent with a previous study that many NTALEs but XopZ in Xoo do not impact bacterial virulence in rice [63].

The in silico and qRT-PCR data (Figs. 1A, B) consistently showed that the expression of 17 xrp genes was regulated by HrpX and/or HrpG at the transcriptional level. However, GUS activity detection showed that the expression of xrp4 (XOC_3955) and xrp6 (XOC_2828) did not display any obvious differences in regulation in WT and mutant strains (Fig. 1C), being due to that they are members of their adjacent operons (data not shown). Giving that the expression of xrp1 (XOC_1601), xrp2 (XOC_4584), xrp3 (XOC_3956), xrp4 (XOC_3955), xrp5 (XOC_1530), xrp6 (XOC_3440), xrp7 (XOC_4583), xrp10 (XOC_0550), xrp11 (XOC_3130) and xrp15 (XOC_2829) was negatively regulated by HrpD6 but either positively regulated by HrpX and/or HrpG or negatively regulated by HrpX (Fig. 1B), HrpG-, HrpX- and HrpD6-mediated regulation in xanthomonads is very complicated to follow the concept that hrpX expression is controlled by HrpG [14,44] and HrpX regulates the expression of hrpD6 [12]. In general, one criterion for identification of HrpX regulon genes is the PIP-box [26,27,43,64]. However, PIP-box-like sequences are absent in the xrp3 and xrp5 upstream regions (Table 1). This is consistent with reports that the expression of some HrpX-regulated genes that lack PIP-box promoters is modulated directly by HrpX and indirectly by HrpG [22]. For example, there is no PIP-box in the promoter regions of hpaJ, XCV_0869 or XCV_3406 genes, but their expression is HrpX-dependent [26]. Thus, there may be an alternative regulatory system in Xanthomonas where HrpX activates genes independently via an unknown regulator(s), like HrpD6.

It should be emphasized that the location of xrp6 (XOC_3440) and xrp17 (hrpFB) is within the hrp-hrc-hpa cluster, xrp6 is a putative

Figure 2. Virulence evaluation of 17 xrp mutants of X. oryzae pv. oryzicola in rice. (A) Lesion lengths of 17 xrp mutants derived from the wild-type strain RS105. Lesion lengths were measured as the ratio of lesion length caused by an xrp mutant compared to the wild-type RS105. The hrcV mutant was used as a negative control. Xoc strains (~1×10⁸ cfu/mL) were inoculated to rice cv. Shanyou 63 (two-months old) by leaf-needling [58]. Lesion lengths were scored 14 days post inoculation. Data are the mean ± standard deviation (SD) of three replicates, and the data shown are representative of three independent experiments. Asterisks at the top of columns indicate significant differences by the Student’s t test (*P<0.05). Panels B through G show the population dynamics of Xoc RS105 and derivatives in rice leaves. Leaf discs (0.5 cm in diameter) were excised from the inoculated areas, homogenized in sterile water, diluted and plated on nutrient agar (NA) plates. Panels: (B), xrp1 mutant, RΔxrp1; (C), xrp2 mutant, RΔxrp2; (D), xrp3 mutant, RΔxrp3; (E), xrp5 mutant, RΔxrp5; (F), xrp8 mutant, RΔxrp8; and (G) xrp14 mutant, RΔxrp14. Similar results were obtained in two other independent experiments.
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ORF between _hpg_ and _hpx_, and _xrp17_ is _hpg_ upstream of _hpf_. These two ORFs have not been annotated in other _Xanthomonas_ genomes (Table 1). Our data suggest that _xrp6_ and _xrp17_ are not involved in _Xoc_ virulence (Fig. 2A); however, the expression of these two ORFs was negatively regulated by HrpX, but not affected by HrpG (Fig. 1B), and the gene product of _xrp17_ was not detectable in the SN of the WT and mutant strains (data not shown). It remains unclear whether _xrp6_ or _xrp17_ function in the regulation of the _hpg-hrc-hpa_ cluster.

New _Xoc_ virulence factors identified in this study included _xrp1_ (XOC_1601), _xrp2_ (XOC_4584), _xrp8_ (XOC_2462), and _xrp14_ (XOC_0084) (Fig. 2). _Xrp1_ (XOC_1601) encodes a putative cysteine protease, which is highly conserved in _Xoo_, _Xac_, _Xcc_, and _Xcv_ (Table 2); this protein shows 84% identity with the T2SS protein CysP2 in _Xoo_ [14], _XOC_1601 is controlled by DSF-mediating QS (quorum-sensing) in _Xoc_ RS105, involved in extracellular protease activity, cell motility, antioxidative ability and EPS biosynthesis [65]. However, _Xrp1_ is not related to _XopD_, _AvrXv4_, _AvrPphB_ or _AvrRpt2_, which have cysteine protease functions that alter plant immunity [66–71]. Given that _Xrp1_ is involved in _Xoc_ virulence in rice (Fig. 2) and T3S-independent secretion (Fig. 3), we assume that this cysteine protein may be secreted via the T2SS, like the homolog of CysP2 in _Xoo_ [14]. _Xrp1_ could potentially degrade a component of the plant cell walls, and the proteolysis of host substrates may be employed by the pathogen to alter plant physiological processes.

_Xrp2_ (XOC_4584) is conserved in _Xoo_, _Xac_ and _Xcv_, but not in _Xoc_ or _Xcc_ (Table 2); database searches provided no functional clues regarding _Xrp2_ function. The expression of _Xrp2_ is _Hpg_ and _Hpx_-dependent, but upregulated by HrpD6, like the expression of _Xrp1_ (Fig. 1). Thus, in addition to regulating _hpg_ gene expression [12], _HrpD6_ may also regulate other virulence factors that are not secretable via T3S. We found that _Xrp8_ (XOC_2462) is highly conserved in _Xoc_, _Xac_ and _Xcc_, but has no homolog in _Xoo_ strains PXO99A, MAFF31018 or KACC10331 (Table 2); thus, this virulence factor (Fig. 2F) may potentially be required for full virulence of _Xoc_, _Xac_ and _Xcv_ in plants.

_Xrp1_ (XOC_0084) is present in _Xoc_ BLS256 and _Xac_ 306 (Table 2) and encodes a putative gene belonging to the cytchrome P450 family [9]. The P450 family proteins use heme iron to oxidize molecules, often making them more water-soluble [72]. It is interesting to note that there are three Ser and seven Pro residues at the N-terminus of _Xrp1_ (Table 1), indicating that _Xrp1_ is a potential T3SE. However, the protein is undetectable in the SN of the wild-type strain and T3S mutant (Fig. 3), suggesting that the involvement of _Xrp1_ in _Xoc_ virulence is worthy of further investigation.

In _Xanthomonas_ spp., T3SSEs have been identified based on sequence similarities identified from genome sequence data [16,22], avirulence reporter fusion assays [38], Cya-fusion approaches [22] and functional assays of T3S-dependent expression and secretion [73]. It has been demonstrated that the fusion expression of an N-terminally truncated _avr_ gene (avrXa10) with the first 50 N-terminal amino acids of a T3SE is an important tool for identifying _Xoc_ T3SSEs by utilizing the _Xoo-_rice pathosystem [24,47,74]. However, it may be important to utilize the highly sensitive Cya assay to identify _Xoc_ T3SSEs [22,33,75]. In _R. solanacearum_, 72 T3SSEs have been characterized [76]. To date, approximately 26 NTALES and 29 TALEs have been confirmed
Figure 5. Subcellular localization of Xrp3 and Xrp5 in plant cells. The subcellular localization of Xrp3 and Xrp5 was examined by fusing these proteins with yellow fluorescence protein (YFP) and evaluating their expression in Arabidopsis mesophyll cells. Images were generated by fluorescence at 525–550 nm (YFP, yellow) and 610–700 nm (chloroplasts, red) using excitation at 514 nm. Expression was driven by the CaMV 35S promoter. PEG-calcium-mediated transfection was used to deliver DNA into protoplasts, and photos were taken 12 h after transformation. Controls included YFP, which localizes in both cytoplasm and nucleus, and GUS-NLS-YFP, which localizes in the nucleus. Bars correspond to 10 μm. The experiment procedure was performed as described previously [54] and repeated three times with similar results.

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Supporting Information

File S1 File containing supporting Figure S1 and Table S1–S3.

Author Contributions

Conceived and designed the experiments: GC XX. Performed the experiments: XX LZ WM. Analyzed the data: XX ZL. Contributed reagents/materials/analysis tools: LZ GC. Wrote the paper: XX GC.
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