Comparative Transcriptome Profiling Reveals Different Expression Patterns in *Xanthomonas oryzae* pv. *oryzae* Strains with Putative Virulence-Relevant Genes

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Abstract

*Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the causal agent of rice bacterial blight, which is a major rice disease in tropical Asian countries. An attempt has been made to investigate gene expression patterns of three *Xoo* strains on the minimal medium XOM2, PXO99 (P6) and PXO86 (P2) from the Philippines, and GD1358 (C5) from China, which exhibited different virulence in 30 rice varieties, with putative virulence factors using deep sequencing. In total, 4,781 transcripts were identified in this study, and 1,151 and 3,076 genes were differentially expressed when P6 was compared with P2 and with C5, respectively. Our results indicated that *Xoo* strains from different regions exhibited distinctly different expression patterns of putative virulence-relevant genes. Interestingly, 40 and 44 genes involved in chemotaxis and motility exhibited higher transcript alterations in C5 compared with P6 and P2, respectively. Most other genes associated with virulence, including exopolysaccharide (EPS) synthesis, *hrp* genes and type III effectors, including *Xanthomonas* outer protein (Xop) effectors and transcription activator-like (TAL) effectors, were down-regulated in C5 compared with P6 and P2. The data were confirmed by real-time quantitative RT-PCR, tests of bacterial motility, and enzyme activity analysis of EPS and xylanase. These results highlight the complexity of *Xoo* and offer new avenues for improving our understanding of *Xoo*-rice interactions and the evolution of *Xoo* virulence.

Introduction

The gram-negative plant pathogenic *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the causal agent of bacterial blight disease on rice [1]. Bacterial blight is the most serious bacterial disease of rice in tropical Asian countries where high-yielding rice cultivars are often highly susceptible, and it has the potential to reduce rice yields by as much as 50% [2]. The complete genome sequences have been published for Japanese race 1 [3], Korean race 1 [4], and PXO99a, a 5-azacytidine-resistant derivative of the Philippines’ race 6 [5]. These genomes have helped to elucidate the molecular interactions between a pathogen and a monocotyledonous plant and have greatly advanced the understanding of the molecular interactions between rice and *Xoo*. Several virulence-related factors have been identified, such as the hypersensitive response and pathogenicity (*hrp*) genes [6], type III (T3) effectors [7,8], genes associated with the production of exopolysaccharides (EPS), and genes associated with motility and extracellular enzymes [9,10].

The type II (T2S) and type III (T3S) secretion systems are important for the virulence of *Xoo*. The T3S system, encoded by *hrp* genes, plays an important role in interactions between *Xoo* and rice by injecting T3 effectors into plant cells, whereas the T2S system may play a role in the secretion of other virulence factors, such as extracellular enzymes like xylanase [11]. The T3S system is transcriptionally induced in certain minimal media and in plants [12], and the ompR-type response regulator HrpG, which is activated by unknown plant signals, controls the genome-wide regulon, including *hrps*, T3 effectors and putative virulence genes [13].

The collection of T3 effectors in *Xanthomonas* are designed as *Xanthomonas* outer proteins (Xop). Sixteen and 18 candidate Xop effectors were identified in *Xoo* strains MAFF311018 and PXO99a, respectively. Among them, XopZPXO99 was demonstrated to contribute to the virulence of *Xoo* strains [14,15]. Besides Xop genes, there is another important type T3 effector, the transcriptional activator-like (TAL) effectors in *Xoo*, which contain a central repeat domain in which amino acids 12 and 13 [known as the repeat variable diresidue (RVD)] of each repeat, and have been shown to transcriptionally activate the corresponding host genes for host disease susceptibility or resistance by recognizing and binding specific DNA sequences within the promoters of host target genes with RVDs [16–20].

The transcriptional regulation of putative virulence-relevant genes is critical to *Xoo* for infection and proliferation in rice varieties. Although the complete genome sequences of three *Xoo* strains from Asia and a draft genome sequence from Africa have been analyzed [3–5,21], so far only microarray analysis has revealed that a greater number of *Xoo* genes are differentially
expressed in XOM2 relative to PSB [22], and little is known about the transcriptome patterns of different strains. Analysis and comparisons of gene expression profiles in different strains will provide new insight into the pathogen's virulence strategies. Here, we report the transcriptional expression profiling of genes involved in the virulence of three Xoo strains, one from China and two from the Philippines, induced on XOM2 minimal media.

Materials and Methods

Bacterial Culture and Isolation of Total RNA

Xoo strains PXO99 (Philippine race 6, P6), PXO86 (Philippine race 2, P2), and GD1358 (China race 5, C5) were used for this experiment. Cells were grown at 28°C with shaking at 200 rpm in nutrient-rich PSB (10 g/liter of peptone, 10 g/liter of sucrose, 1 g/liter of L-glutamic acid, monosodium salt) until OD600 equaled 2.0, washed twice, and immediately transferred into XOM2 for 16 h. XOM2 consists of 0.18% xylose sugar, 670 μM D. L-methionine, 10 mM sodium L(+)-glutamate, 14.7 mM KH2PO4, 40 μM MnSO4, 240 μM Fe (III) EDTA and 5 mM MgCl2, pH 6.5 [Tsuge et al., 2002]. Bacterial cells were washed twice prior to being harvested and snap-frozen in liquid nitrogen. Total RNA was extracted from each Xoo sample with RiboPure™-Bacteria kit and quantified by Qubit RNA assay kit (both from Applied Biosystems). The RNA integrity was checked with Agilent 2100 Bioanalyzer (Agilent Technologies).

Pathogenicity Assays and Growth Curve of Xoo

Thirty rice varieties (Figure 1A) were used to evaluate the pathogenicity of Xoo strains P6, P2 and C5. The plants were grown in the screenhouse of the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China in the summer of 2011. For evaluating bacterial blight resistance, seeds of the rice varieties were sown in the seedling nursery and 30-day-old seedlings were transplanted in the screenhouse with 9 plants in each row at spacing of 20×17 cm. At the tillering stage (plant age was 65 days), four to five of the uppermost leaves of each plant were inoculated with Xoo strains by the leaf-clipping method [23]. Inoculum of each race was prepared by suspending the bacterial mass in sterile water at a concentration of 108 cells ml−1. Five central plants of each line were inoculated with each race for three replications. The lesion lengths (LL) were measured on all inoculated leaves 2 weeks after inoculation when lesions became obvious and stable in the susceptible variety IR24, and the average LL of each plant was calculated according to its three longest lesions. Evaluation of the resistance level of each variety was based on the average LL of 15 plants. LL of each variety was calculated according to its three longest lesions. The growth curve of Xoo was produced according to the method described by Song et al. [24]. Five inoculated leaves of Hanyou715 and Hanyou533 from three plants were collected at 1, 2, 3, 4 and 5 days post-inoculation (dpi). The collected leaves were immediately frozen in liquid nitrogen, and then kept at −70°C. For each time point, the bacterial populations were determined by grinding three leaves separately, plating the resulting extract on potato sucrose agar media containing 200 μM azacytidine, and counting colony-forming units (CFUs) after 40 h at 28°C.

Library Preparation and Illumina Sequencing

The ribosomal RNA (rRNA) was removed from 3 μg of total RNA with Ribo-Zero™ Magnetic kit for Gram-Negative Bacteria (EpiconceptBio) by the manufacturer’s instructions. The RNA library was constructed according to the TruSeq™ RNA Sample Preparation kit (Illumina) with minor modification. Briefly, RNA was fragmented and the first cDNA strand was synthesized by using the random hexamers and SuperScript II Reverse Transcriptase (Invitrogen), then RNA template was removed and a replacement strand was synthesized to generate double-stranded (ds) cDNA. After end repair and 3‘ end adenylation, the indexed adapter was ligated with the dsDNA. Fragments of 300~350 bp were excised and enriched by PCR for 12 cycles. The yield and size distribution of PCR products were checked by QUBIT and Agilent 2100 Bioanalyzer respectively. The produced libraries were performed cluster generation on cBot and sequenced on HiSeq 2000 platform (illumina) with 100 bp paired-end reads by CapitalBio Corporation, Beijing, China. Illumina Casava (version 1.7) was used for basecalling, then all the sequencing data was processed by removing sequencing adapters for further analysis.

Analysis of Illumina Sequencing

The analysis of RNA-seq sequencing data was performed as described by Zhao et al. [25] (Figure S1). All the tags mapped to reference sequences by Burrows-Wheeler Aligner [26,27] with a maximum of five nucleotide mismatch. For gene expression analysis, the value of reads per kilo bases per million reads (RPKM) [28] was calculated. DEGseq [29] was applied to identify differentially regulated genes between two samples using the two classes unpaired MA-plot-based method to detect and visualize gene expression difference with significant P-values less than 0.001. The whole genome sequence of Xoo was downloaded from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), and coding regions were annotated according to the annotated protein data sets of Xoo strain PXO999®.

Validation of Expression Patterns of DEGs Using Quantitative Real-time RT-PCR

To validate the results of the Illumina sequencing experiment, a subset of differentially expressed genes (DEGs) were verified by quantitative real-time RT-PCR (qRT-PCR). An independent set of cell cultures of the three Xoo strains were cultured following the same protocol as for the Illumina analysis. QRT-PCR followed the methods described by Swarbrick et al. [30]. The sequence of each gene was obtained from the X. oryzae pv. oryzae PXO99® database (http://www.ncbi.nlm.nih.gov), and the sequences from each gene were used for designing primers by Primer 5 software (http://frodo.wi.mit.edu/) (Table S1). RNA samples from three independent replicates for each treatment were pooled before cDNA synthesis. Thirty-three Xoo genes were tested in 50 μl reactions using the SYBR® Green PCR Master Mix kit (Applied Biosystems, CA, USA) following the manufacturer’s protocol. The correlation coefficient between the qRT-PCR and RNA-Seq results was calculated.

Motility Analysis

Fresh colonies from PS agar plates were stabbed into swarm plates composed of 0.03% (wt/vol) Bactopeptone, 0.03% yeast extract, and 0.3% agar. The inoculated cells were cultured at 28°C and examined for bacteria swarming away from the inoculation site at 12, 24, 48 and 72 h after inoculation [31]. This study was repeated three times for reproducibility.
Quantitative Determination of EPS and Xylanase Activity

The fresh colonies of Xoo strains were grown at 28°C with shaking at 200 rpm in nutrient-rich PSB until the OD600 equaled 2.0. They were then washed twice and immediately transferred into XOM2. After growth for 16 h, the bacterial cultures were collected and supernatants were prepared by centrifugation at 5000 rpm for 10 min. The extracellular xylanase was measured using 4-O-methyl-D-glucurono-D-xylan-Ramezol Brilliant Blue R (RBB-xylan; Sigma Co.) according to the methods described by Biely et al. [32]. The production of EPS was determined according to the methods described by He et al. [33].

Results

Pathogenicity Testing of Xoo Strains

To evaluate the pathogenicity of P6, P2 and C5, 30 rice varieties, including 29 recently developed varieties in China, and a cultivar susceptible to all Philippine Xoo races from the International Rice Research Institute (IRRI), IR24, were inoculated at the tillering stage. Also included were CBB23, Xinhuangzhan, and Hua201S-1, which carry the bacterial blight resistance gene Xa23, a single completely dominant resistance gene identified from wild rice species of Oryza rufipogon [34,35]; Xa21 from the wild rice strain XF10450 [36] and O. longistaminata [37], and Xa7 from
Only four varieties were resistant to the three Xoo strains. Among them, three varieties including CBB23, Xinhuanzhan, and XF10450, exhibited a typical hypersensitive reaction (HR) with less than 0.5 cm LL. The LL of Hua201S-1 inoculated with P6, P2, and C5 was 3.0±1.1 cm, 3.1±0.9 cm, and 0.4±0.4 cm, respectively. Except for Teqing, which was moderately resistant to P6 and P2 and susceptible to C5, the other 25 varieties displayed a moderate or high susceptibility to the three strains with LLs ranging from 12.1 to 43.5 cm.

The 30 rice varieties were placed in three groups depending on the LL phenotypes exhibited after inoculation (Figure 1 A). Group I, which showed LL ranging from 0.2±0.1 to 28.1±5.9 cm, 0.3±0.1 to 30.6±4.1 cm, and 0.1±0.1 to 30.7±3.3 cm against P6, P2, and C5, respectively, consisted of 12 varieties: CBB23, Xinhuanzhan, XF10450, Huanghuazhan, Xinliangyou341, Hanyou75, 389A, IR24, Ganhui2833, Hanyou119, 3233, and 9067. There were no significant differences among LLs within the same variety when inoculated with the three Xoo strains. Group II, with LL ranging from 3.0±1.1 to 43.5±2.1 cm, 3.1±0.9 to

![Figure 2. Overview of mRNA-seq data and mapping to the Xanthomonas oryzae pv. oryzae (Xoo) PXO99A genome.](image-url)

(A) Total number of mRNA-seq reads mapped in each Xoo strain library. (B) Histogram presentation of gene ontology classifications. The results are summarized in three categories: cellular components, molecular functions, and biological processes. The right y-axis indicates the number of genes in a category. The left y-axis indicates the percentage of a specific category of genes in that main category. (C) Comparison of transcription measurements by Illumina sequencing and qRT-PCR assays. The correlation coefficient (R2) between the two datasets is 0.8927.

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42.0±3.6 cm and 0.4±0.4 to 32.1±2.4 cm against P6, P2 and C5, respectively, also consisted of 12 varieties: Hua2018, Yunguang20, Tianyou20, Tianyou145, Yunjing30, Hanyou73, Liangfengyou339, Bohanyou131, CDR22, Hanyou715, Hanyou113, and Hanyou53. The LLs of the same variety were significantly longer when inoculated with two of the Xoo strains than when infected by the third strain. Eleven of the varieties had significantly longer LLs when infected by P6 or P2 than when infected by C5. The exception was the variety Liangyou1013 (Figure 1A). Bacterial growth curve analysis indicated that the growth of P2 and P6 increased by more than three-fold compared with C5 in Hangyou715 36 hr post-inoculation, and the growth of P2 and P6 increased by more than 20-fold compared with C5 in Hanyou53 36 h post-inoculation (Figure 1B, C).

Group III had six varieties. The LLs caused by one strain were significantly longer than when infected by the other two strains. The LLs of Wuhuashandao and Yunzijing20 when infected by P6 were significantly longer than when infected by P2 and C5, and the LLs of Yunguang101 and Hanyou79 when infected by P2 were significantly longer than when infected by P6 and C5. However, the LLs of Teqing and Hanyou715 when infected with C5 were significantly longer than when infected by P6 and P2.

Our results indicated that the virulence of P2 was similar with that of P6 when infecting 21 rice varieties, and the LLs of five and four varieties were significantly longer and shorter against P6 than against P2, respectively. However, there was no significant difference between the virulence of C5 and P6 when infecting 14 rice varieties, C5 was significantly weaker than P6 when infected by the third strain. Eleven of the varieties had significantly up- and down-regulated genes in C5 compared with P6 and P2, which exhibited similar expression patterns. Nineteen TCSs differentially expressed in P2 compared with P6. Among them there were two and 17 significantly up- and down-regulated genes, respectively. Forty-four genes differentially expressed in C5 compared with P6; 11 and 33 were significantly up- and down-regulated genes in C5, respectively. Similarly, of the 44 TCSs differentially expressed in C5 compared with P2, 11 and 33 were significantly up- and down-regulated genes in C5, respectively (Figure 3A).

The TCS transcriptome profiling of three Xoo strains revealed two interesting aspects. First, the expression pattern of the RpfC/RpfG two-component regulatory system associated with quorum sensing (QS) was different between P2, P6 and C5 (Table 1). Genetic and genomics evidence suggest that Xoo might use the difusible signal factor (DSF) QS system to regulate virulence factor production [33]. QS is a complicated bacterial group behavior for producing, sensing and responding to multifarious chemical signals, which increases their chances of survival and propagation, and it provides bacterial pathogens an obvious competitive advantage over their hosts in pathogen-host interactions [42]. The RpfC/RpfG two-component regulatory system is implicated in sensing and responding to DSF perception and signal transduction [43,44]. RpfC negatively controls DSF biosynthesis by binding to rpfF at a low cell density [33]. Several GGDEF, EAL and HD-GYP domain proteins of X. campestris pv. campesiris (Xcc) are hypothesized to compose a network of signal transduction systems for response to different environmental cues to modulate the level of the second messenger cyclic di-GMP [45]. Our results indicated that a TCS regulatory protein with a HD-GYP domain (PXO_00476) had significantly down-regulated expression in C5 compared with P6 and P2. Moreover, cyclic di-GMP phosphodiesterase A (PXO_00058) had significantly up-regulated expression in C5 compared with P6 and P2.

Validation of Expression Patterns by qRT-PCR

To validate the Illumina sequencing results, qRT-PCR was used to independently assess expression levels for 33 genes involved in motility, the T3S system and T3 effectors (genes and primer sets used are shown in Table S1; the results of qRT-PCR are shown in Figure S3). RNA samples that were used in the Illumina sequencing experiment as well as RNA samples extracted from three additional replicate sets of cultures were used as templates. There was a good correlation between the qRT-PCR and the mRNA-seq results (correlation coefficient was 0.8297) (Figure 2C). Although the amplitude of gene expression fold change between the two techniques is different, as might be expected since qRT-PCR is not a reliable measure of quantitative differences, the general trend of gene expression is consistent.

Differential Expression of Two-component Systems between Xoo Strains

Two-component systems (TCSs) are widespread signal transducers in prokaryotes that serve as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions [40]. They typically consist of a membrane-bound histidine kinase that senses a specific environmental stimulus and a corresponding response regulator that mediates the cellular response [41]. In this study, 55 transcripts associated with TCSs were identified in three Xoo strains (Table S5), and DGE analysis revealed that the transcriptome profile of C5 was considerably different from P6 and P2, which exhibited similar expression patterns. Nineteen TCSs differentially expressed in P2 compared with P6. Among them there were two and 17 significantly up- and down-regulated genes, respectively. Forty-four genes differentially expressed in C5 compared with P6; 11 and 33 were significantly up- and down-regulated genes in C5, respectively. Similarly, of the 44 TCSs differentially expressed in C5 compared with P2, 11 and 33 were significantly up- and down-regulated genes in C5, respectively (Figure 3A).

Mapping of mRNA-seq Reads and Statistical Testing to Detect Differentially Expressed Genes

To compare the transcriptome profile of Xoo strains with different virulence levels, RNA sequencing libraries were constructed for P6, P2 and C5. Each library generated about 28.6 to 29.2 million reads, which were mapped to the PXO99A genome sequence (NCBI Reference Sequence: NC_010717.1) with 78.9%, 80.1% and 81.6% matched reads to NCBI annotated gene sequence (NCBI Reference Sequence: NC_010717.1) with 78.9%, 80.1% and 81.6% matched reads to NCBI annotated gene regions, respectively (GEO database: accession number GSE44215). There were 3,083 protein-coding genes predicted in the genome of PXO99A by genome analysis [39]. In total, 4,781 transcripts were identified in this study. With a threshold of more than five reads mapped to the CDS regions of a given gene in each sample, a total of 4,605, 4,503 and 4,467 protein-coding genes were detected in P6, P2 and C5, respectively (Figure 2A).

The R-package DEGseq [29] was used to identify DEGs. The list of genes with significantly different expression levels between two strains was refined using the criterion of P value <0.001 in t tests, resulting in 1,151, 3,076 and 3,112 DEGs when P6 was compared with P2, P6 was compared with C5, and P2 was compared with C5, respectively (Table S2, S3, S4). And all the DEGs between each of the comparisons shared 782 genes in common (Figure S2).

Gene ontology (GO) assignments were used to classify the function of the DEGs. Based on sequence homology, the DEGs can be categorized into 39 functional groups (Figure 2B). In the three main categories, cellular component, molecular function and biological process, of GO classifications, “cell part”, “catalytic” and “metabolic process” terms are dominant, respectively. We also noticed a high-percentage of genes from categories of “cell”, “binding” and “cellular process” as well as a few genes from “reproduction”, “reproductive process” and “viral reproduction”.

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Second, significant up-regulation of the phoPQ-regulated protein (PXO_01585) was detected in P6 and P2 when compared with C5 (Table 1). This protein is not only required for AvrXA21 activity, but also controls virulence through the regulation of hrpG gene expression [46]. It also regulates numerous cellular activities in Salmonella and other species as a master regulator of virulence [47,48]. AvrXA21 requires a regulatory TCS called RaxRH to regulate expression of 10 rax (required for AvrXA21 activity) genes. Our results indicated that raxH (PXO_04467), raxH2 (PXO_02837), raxR (PXO_04469) and raxR2 (PXO_02836) were significantly up-regulated in P6 and P2 compared with C5. Additionally, raxA (PXO_04478), raxB (PXO_04477), raxC (PXO_02621), raxP (PXO_02134), raxQ (PXO_02135) and raxST (PXO_04479) were also significantly up-regulated (Table 1). However, there was no significant difference in the expression of the phoPQ-regulated protein and the rax genes, except for raxR and raxST between P6 and P2. This suggests the expression pattern of genes involved in AvrXA21 activity and hrpG expression exhibited by C5 was different from P6 and P2.

A set of Genes Possibly Related to Chemotaxis and Bacterial Motility had Significantly Up-regulated Expression Levels in C5

The number of DEGs involved in chemotaxis and motility were differentially expressed between the three strains and exhibited interesting expression patterns (Table S6; Figure 3B). These genes mainly included chemoreceptors, chemotaxis proteins, twitching motility proteins, flagellar motor proteins, pilus biogenesis proteins and pilus assembly proteins. Of the differentially expressed genes, 38 genes had down-regulated expression levels in P2 when compared with P6. In addition, 40 genes had up-regulated expression levels and four genes had down-regulated expression levels in C5 when compared with P6. Finally, 44 genes had up-regulated expression levels and three genes had down-regulated expression levels in C5 when compared with P2. In general, the structural genes encoding motility systems are clustered within large transcriptional units allowing co-regulation of their expression [49]. We found that the expression of the chemoreceptor glutamine deamidase CheA (PXO_00032) increased greater than 2.89-fold in C5 when compared with P6 and P2 (Figure 4A). Consistent with this finding, 15 and 17 genes involved in the encoding and synthetic metabolism of chemotaxis proteins, including CheD (PXO_00056) (Figure 4B), were up-regulated in C5 compared with P6 and P2, respectively. Additionally, many pil genes involved in bacterial movement [50], including pilG (PXO_01602), pilH (PXO_01603), pilL (PXO_01607), pilV (PXO_01321), pilX (PXO_01323), pilY1 (PXO_01324) and pilZ (PXO_00049), were also up-regulated in C5 compared with P6 and P2. In addition, four genes encoding flagellar motor proteins, including MotA (PXO_03068), MotB (PXO_03067), MotC (PXO_00026) and MotD (PXO_00027) (Figure 4C, D), and two genes encoding twitching motility proteins, including P01994 and P01993, were significantly up-regulated in C5.

Swarm plate analysis displayed that the swimming diameter of C5 was significantly larger than those of P6 and P2, and they tended to form larger swarming colonies at 24, 48, 72 and 96 h post-inoculation (Figure 4E). This confirms that the motility of C5 was significantly stronger as reflected by the enhanced expression levels of many genes encoding chemotaxis proteins, pil proteins and flagellar motor proteins.

Differential Expression of a Gum Gene Cluster Involved in EPS Synthesis

The gum gene cluster involved in EPS synthesis functions as a virulence determinant in Xanthomonas [51]. EPS synthesis in Xcc is directed by genes within the gum cluster, which contains 12 genes and has a major promoter upstream of the first gene, gumB [52]. Similarly, the Xoo gum cluster is composed of 14 ORFs that constitute an operon expressed from a promoter located upstream of gumB, but the cluster also has internal promoters upstream of gumG, gumH and gumM [46,53]. In our study, 12 gum genes were differentially expressed in C5 compared with P6 and P2. Except for the gumB (PXO_01391) up-regulated gene, the other 11 genes from gumC to gumM (PXO_01392-PXO_01403) were all down-regulated (Table S7). By contrast, five genes (gumD, gumH, gumK, gumL, and gumM) were up-regulated in P2 compared with P6. In addition, two genes encoding xylanase (PXO_03864 and PXO_04558) were down-regulated in C5 compared with P6 and P2, and PXO_04558 also had down-regulated expression in P2 compared with P6.

Interestingly, xanA (PXO_003174), xanB (PXO_03173), wxoA (PXO_003160), wxoB (PXO_03161), wxoC (PXO_05411), and wxoE
| Locus ID | P6 vs. P2* | P6 vs. C5 | P2 vs. C5 | Gene Name/Function |
|----------|------------|-----------|-----------|-------------------|
|          | Log2 fold-change | P value | Signature | Log2 fold-change | P value | Signature | Log2 fold-change | P value | Signature |
| PXO_00069 | 0.0842 | 1.49E-06 | True | 0.1886 | 9.48E-24 | True | 0.1044 | 5.94E-09 | True |
| PXO_00070 | 0.1302 | 6.30E-12 | True | −0.1081 | 1.67E-08 | True | −0.2382 | 4.04E-38 | True |
| PXO_00073 | 0.1078 | 0.0008 | True | 0.3191 | 1.10E-19 | True | 0.2113 | 3.77E-10 | True |
| PXO_00084 | −0.1047 | 0.0551 | False | −0.1772 | 0.0018 | False | −0.0725 | 0.1674 | False |
| PXO_00068 | 0.0615 | 0.0128 | False | 0.3166 | 2.69E-31 | True | 0.2551 | 7.03E-23 | True |
| PXO_00067 | −0.0227 | 0.291 | False | 0.1906 | 5.82E-16 | True | 0.2133 | 4.79E-22 | True |
| PXO_00064 | −0.4466 | 3.77E-126 | True | −0.5352 | 1.44E-167 | True | −0.0886 | 1.61E-07 | True |
| PXO_00058 | 0.3422 | 9.37E-73 | True | −1.2474 | 0 | True | −1.5897 | 0 | True |
| PXO_00476 | 0.0542 | 0.163 | False | 0.4295 | 8.98E-23 | True | 0.3753 | 1.81E-19 | True |
| PXO_00466 | 0.724 | 2.01E-230 | True | −1.7169 | 0 | True | −2.4409 | 0 | True |
| PXO_02944 | −0.0315 | 0.1155 | False | 0.0446 | 0.0366 | False | 0.0761 | 0.0001 | True |
| PXO_01585 | −0.0217 | 0.518 | False | 1.4111 | 4.50E-212 | True | 1.4328 | 8.08E-248 | True |
| PXO_04467 | −0.097 | 0.2354 | False | 0.403 | 1.78E-05 | True | 0.5 | 9.12E-09 | True |
| PXO_02837 | −0.014 | 0.7173 | False | 0.4235 | 7.21E-22 | True | 0.4376 | 4.37E-26 | True |
| PXO_02836 | −0.0124 | 0.4796 | False | 0.2808 | 3.10E-47 | True | 0.2932 | 7.58E-58 | True |
| PXO_04469 | 0.2741 | 0.0004 | True | 0.4216 | 4.36E-07 | True | 0.1475 | 0.0736 | False |
| PXO_04478 | −0.0551 | 0.2428 | False | 1.3968 | 7.03E-106 | True | 1.4519 | 7.07E-131 | True |
| PXO_04477 | −0.0533 | 0.1293 | False | 1.2391 | 1.18E-157 | True | 1.2924 | 2.68E-196 | True |
| PXO_02621 | −0.0355 | 0.0845 | False | 0.2162 | 1.48E-21 | True | 0.2518 | 1.96E-32 | True |
| PXO_02134 | −0.0819 | 0.1335 | False | 0.5034 | 2.57E-15 | True | 0.5853 | 4.15E-23 | True |
| PXO_02135 | −0.0918 | 0.0126 | False | 0.7017 | 4.51E-56 | True | 0.7936 | 1.72E-82 | True |
| PXO_04479 | −0.1778 | 2.23E-07 | True | 0.9684 | 8.61E-107 | True | 1.1462 | 7.08E-178 | True |

*The fold change of gene expression is represented by a log2 ratio. A log2 ratio of 0.8 is equivalent to a 1.75 fold relative increase in expression.

b) "True" means significant differential expression (p-value <0.001), and "False" represents the opposite.

*P2, P6 and C5 represent PXO86, PXO99 and GD1358, respectively.

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were also down-regulated in C5 compared with P6 and P2. However, \textit{xan}\textsubscript{A} (PXO\_03174) and \textit{xan}\textsubscript{B} (PXO\_03173) were up-regulated in P2 compared with P6 and there was no difference in the expression levels of the other genes. In addition, two genes encoding xylanase (PXO\_03864 and PXO\_04558) were significantly down-regulated in C5 compared with P6 and P2. Activity analysis showed that EPS activity in P2 and P6 was significantly higher than that in C5 (Figure 5A), and the xylanase activity in P2 was significantly higher than that in P6 and C5 (Figure 5B).

Jeong et al. (2008) [54] reported that \textit{Rpf}\textsubscript{B}, \textit{Rpf}\textsubscript{C}, \textit{Rpf}\textsubscript{F}, and \textit{Rpf}\textsubscript{G} are important for the virulence of \textit{Xoo} KACC10859, and that some virulence genes revealed the significantly reduced expression of genes related to exopolysaccharide (EPS) production (\textit{gum}\textsubscript{G} and \textit{gum}\textsubscript{M}), lipopolysaccharide (LPS: \textit{xan}\textsubscript{A}, \textit{xan}\textsubscript{B}, \textit{xan}\textsubscript{D}, and \textit{xan}\textsubscript{C}), xylanase (\textit{xyl}\textsubscript{B}), and motility (\textit{pil}\textsubscript{A}) in the mutants \textit{Rpf}\textsubscript{B}, \textit{Rpf}\textsubscript{C}, \textit{Rpf}\textsubscript{F} and \textit{Rpf}\textsubscript{G} [54]. However, our results indicated that the activities of EPS and xylanase were reduced in C5, but that the bacterial motility increased when \textit{Rpf}\textsubscript{B} (PXO\_00067), \textit{Rpf}\textsubscript{C} (PXO\_00069) and \textit{Rpf}\textsubscript{F} (PXO\_00068) were down-regulated and \textit{Rpf}\textsubscript{G} (PXO\_00070) was up-regulated.

**Hrp Genes and T3 Effectors had Significantly Up-regulated Expression Levels in P6 and P2**

Knowledge of \textit{hrp} genes in \textit{Xanthomonas} arises mainly from studies of the \textit{X. campestris} species [55]. \textit{Hrp} genes are essential for pathogenicity in both \textit{Xoo} and \textit{Xoc}. \textit{Hrp}\textsubscript{F} has been found to be a

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Figure 4. The expression patterns of genes associated with motility and swarming motility of \textit{Xanthomonas oryzae pv. oryzae} (\textit{Xoo}) strains PXO99 (P6) and PXO86 (P2) from the Philippines and GD1358 (C5) from China. (A) The expression patterns of \textit{che}\textsubscript{A}. (B) The expression patterns of \textit{che}\textsubscript{D}. (C) The expression patterns of \textit{mot}\textsubscript{C}. (D) The expression patterns of \textit{mot}\textsubscript{D}. The gene expression level (arbitrary units) was normalized using 16sRNA as an internal reference. The gene expression level was quantified by real-time RT-PCR. (E) Swarming motility of \textit{Xoo} on plates. * and ** represent significant difference at \textit{P}<0.05 and 0.001, respectively.

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(PXO\_03158) were also down-regulated in C5 compared with P6 and P2. However, \textit{xan}\textsubscript{A} (PXO\_03174) and \textit{xan}\textsubscript{B} (PXO\_03173) were up-regulated in P2 compared with P6 and there was no difference in the expression levels of the other genes. In addition, two genes encoding xylanase (PXO\_03864 and PXO\_04558) were significantly down-regulated in C5 compared with P6 and P2. Activity analysis showed that EPS activity in P2 and P6 was significantly higher than that in C5 (Figure 5A), and the xylanase activity in P2 was significantly higher than that in P6 and C5 (Figure 5B).

Jeong et al. (2008) [54] reported that \textit{Rpf}\textsubscript{B}, \textit{Rpf}\textsubscript{C}, \textit{Rpf}\textsubscript{F}, and \textit{Rpf}\textsubscript{G} are important for the virulence of \textit{Xoo} KACC10859, and that some virulence genes revealed the significantly reduced expression of genes related to exopolysaccharide (EPS) produc-
putative type III translocon protein required for pathogenicity [56]. HrpG activates the expression of hrpA and hrpX. HrpX encodes a protein belonging to the AraC family of positive transcriptional activators and controls the expression of operons hrpB to hrpF as well as avrXv3 and a number of putative virulence factors. We examined expression of hrp genes and tested the expression of several genes by qRT-PCR (Figure 5C, D, E, F; Figure S3). Interestingly, the expression of 29 genes associated with hrp genes (Table S8), including genes encoding hrpF (PXO_03417), hrpG (PXO_01951) (Figure 5C), the hrpA type III secretion outer membrane pore (PXO_03393) and hrpX (PXO_01953) (Figure 5D), were significantly down-regulated in C5 compared with P6 and P2. However, 14 of 29 genes were up-regulated in P2 compared with P6.

We also specifically examined the expression levels of T3 effectors. Fifteen Xop genes were significantly down-regulated in C5 when compared with P6 and P2, and 10 Xop genes were down-regulated in P6 when compared with P2 (Table 2). These effectors...
six genes were down-regulated in P6 compared with P2, including *pthxo1* (PXO_03992) increases bacterial populations in plants. However, six genes were down-regulated in P6 compared with P2, including *pthxo1* (Table 2).

### Table 2. Expression profiles of *xop* and *tal* genes in three *Xanthomonas oryzae* pv. *oryzae* strains.

| Locus ID | P6 vs. P2 | P6 vs. C5 | P2 vs. C5 | Gene name |
|----------|-----------|-----------|-----------|-----------|
| PXO_03413 | 0.2477 | 2.76E-11 | 2.75E-107 | *XopF1* |
| PXO_03356 | 0.0368 | 0.2331 | 1.7430 | *XopW* |
| PXO_03901 | 0.0990 | 0.0003 | 0.0550 | *XopQ* |
| PXO_03833 | 0.2146 | 6.85E-34 | 1.9105 | *XopX* |
| PXO_03819 | 0.1194 | 6.94E-17 | 1.5300 | *XopY* |
| PXO_03702 | 0.2793 | 2.69E-42 | 1.3885 | *XopP* |
| PXO_04172 | 0.0505 | 0.1077 | 1.4076 | *XopL* |
| PXO_04866 | 0.2214 | 1.63E-06 | 0.7567 | *XopQ* |
| PXO_02236 | 0.0307 | 0.1122 | 0.4611 | *XopA* |
| PXO_02334 | 0.0029 | 0.9379 | 1.7636 | *XopA* |
| PXO_01625 | 0.3258 | 1.74E-36 | 0.8774 | *XopP* |
| PXO_01620 | 0.0417 | 0.1091 | 2.0899 | *XopP* |
| PXO_02108 | 0.3271 | 1.74E-32 | 1.8881 | *XopP* |
| PXO_02107 | 0.1448 | 1.44E-07 | 3.2766 | *XopP* |
| PXO_02170 | 0.5070 | 0.2176 | 3.7467 | *XopP* |
| PXO_02760 | 0.2747 | 6.32E-98 | 1.9034 | *XopP* |
| PXO_02223 | 0.0561 | 0.3260 | 0.8741 | *XopP* |
| PXO_05718 | 0.0344 | 0.5475 | 0.8400 | *XopP* |
| PXO_05714 | 0.0528 | 0.1675 | 1.0565 | *XopP* |
| PXO_05572 | 0.2600 | 9.03E-07 | 1.3748 | *XopP* |
| PXO_05546 | 0.2838 | 0.0006 | 0.1354 | *XopP* |
| PXO_02269 | 0.0317 | 0.5266 | 1.8221 | *XopP* |
| PXO_06229 | 0.8398 | 0.3118 | 1.5387 | *XopP* |
| PXO_00557 | 0.2610 | 0.0002 | 0.8474 | *XopP* |
| PXO_01085 | 0.6698 | 0.2400 | 0.1058 | *XopP* |
| PXO_02272 | 0.1400 | 0.0004 | 0.3954 | *XopP* |
| PXO_03922 | 0.1242 | 0.0235 | 0.0431 | *XopP* |
| PXO_00318 | 0.2166 | 0.0406 | 0.6454 | *XopP* |
| PXO_00511 | 0.4406 | 1.50E-25 | 0.0699 | *XopP* |
| PXO_00505 | 0.2363 | 1.47E-13 | 1.5025 | *XopP* |
| PXO_00227 | 0.1228 | 0.0024 | 2.8134 | *XopP* |
| PXO_05609 | 0.0551 | 0.5097 | 1.8985 | *XopP* |
| PXO_02172 | 0.1020 | 0.0532 | 0.1076 | *XopP* |
| PXO_06234 | 0.2147 | 0.7091 | 0.6167 | *XopP* |
| PXO_05633 | 0.1844 | 0.7931 | 1.1464 | *XopP* |

*The fold change of gene expression is represented by a log2 ratio. A log2 ratio of 0.8 is equivalent to a 1.75 fold relative increase in expression.

*iTrue* means significant differential expression (p-value <0.001), and *False* represents the opposite.

*P2, P6 and C5 represent PXO86, PXO99 and GD1358, respectively.

**Discussion**

Bacterial blight occurs in most rice-growing areas of the world, and *Xoo* isolates from within and across Asia, Africa, and Australia show a great diversity of genotypes based on the polymorphisms of transposable elements, predominantly insertion sequences (IS), avirulence genes, rep/box elements, and other markers [57]. The great diversity of strains within *Xoo* undoubtedly reflects adaptation of the pathogen to the diversity of host genotypes as well as the diverse environmental conditions in which rice is grown. Our transcriptome profiling analysis revealed some interesting aspects.
of different Xoo strains from the Philippines and China with putative virulence-relevant genes in vitro.

First, a large set of genes associated with the expression of Hrp genes and T3 effectors were significantly up-regulated in P6 and P2. This finding suggests that hpm genes and genes encoding T3 effector expression may differ for these strains in rice varieties. In phytopathogenic bacteria, the T3S system is encoded by hpm genes and can elicit HR on non-host or resistant host plants and for pathogenesis on susceptible hosts [55]. More and more evidence demonstrates that Hrp proteins and TAL effectors play key roles in host immunity responses or facilitate nutritional or virulence processes in the pathogen. These may trigger a resistance response in plants that contains TAL effector recognition features (such as AvrXa27 and AvrXa10), some of which are critical for virulence (such as PthoXo1, PthoXo6 and PthoXo7), and others of which appear to have more moderate or contextual functions in virulence [56]. The genomic sequences of the published Philippine (PX099), Japanese (MAFF311010) and Korean (KACC10331) strains contain 19, 17 and 15 TAL effector genes, respectively, and the African strain BAI, may contain eight TAL effector genes [21]. However, the X. oryzae strains in the United States lack TAL effectors and exhibit weak pathogenicity and a severely limited range of host cultivars compared with the Asian and African Xoo strains [59].

Although Illumina data alone were not sufficient to decipher the complicated and repetitive nature of the TAL effector coding sequences in P2 and C5, our results indicate that the expression patterns of hpm genes and T3 effectors were significantly different between Xoo strains from China and the Philippines. Together with the phenotypes of rice varieties, growth curves in Hangyou715 and Hanyou53, and expression patterns of the pathogen, we speculated that the increased virulence of P6 and P2 compared with C5 when infecting some rice varieties was due to the differentially up-regulated expression of hpm genes and T3 effector genes, which might promote Xoo multiplication in rice plants. The finding that three TAL effectors targeting the OsSWEET family of sucrose transporters conferred an increased virulence to the weakly pathogenic USA X. oryzae strain supported our speculation [60].

So far, whether the divergence of T3 effectors of Xoo potentially correlate with the geographic origin and diversity of rice cultivars remains a mystery. Answering this question will be facilitated by the determination of the full genome sequence of more Xoo strains.

Second, a large set of genes encoding chemotaxis and proteins involved in bacterial motility were significantly up-regulated in C5. Motility over solid surfaces is an important bacterial mechanism that allows complex social behaviors and pathogenesis. In some plant-pathogen systems, flagella-driven chemotaxis plays a role in the early interactions with host plants, and motility enables foliar pathogens to reach internal sites in the leaves [61]. Moreover, the bacterial protein flagellin has been found to be a plant elicitor, and plants have a sensitive perception system for this protein [62,63]. Even though the genes encoding Hrp proteins and T3 effectors were significantly down-regulated in C5, there was no significant difference in the LLs of 14 rice varieties infected by C5, P6 and P2. We hypothesize that this is due to C5’s stronger motility, which might compensate for the weaker expression levels of Hrp proteins and T3 effectors, and allows C5 to exhibit similar virulence levels with P2 and P6 in some rice varieties. Verdier et al. (2012) recently reported that the plant genetic background affected the level of virulence enhancement by Xoo TAL effectors [60]. This also provides a clue to why there was no significant difference in virulence levels among P2, P6 and C5 when they infected some rice varieties.

Our analysis of the Xoo transcriptomes based on deep transcriptome sequencing led to remarkable insights into the transcriptional landscape of this important model plant pathogen from different countries, and it offers new avenues for improving our understanding of the Xoo-rice pathogenic mechanism and the evolution of Xoo virulence. Further understanding of the roles of bacterial motility and TAL effectors in diverse plant genetic backgrounds would shed light or provide further insights into their roles in interaction with the host, especially when we include the differing or diverse genetic background of the rice varieties and the environment or ecosystem where the crop is grown.

Supporting Information

Figure S1 The analysis workflow for RNA-seq data based on mapping reads to the reference genome sequence, quantifying the gene expressed, identifying DEGs and categorizing DEGs by Gene Ontology. (PPT)

Figure S2 The Venn diagram of all the DEGs between each of the comparisons (P6 vs. C5, P6 vs. P2, P2 vs. C5). P6, P2 and C5 indicate Xanthomonas oryzae pv. oryzae strain PXO99, PXO86, and GD1388, respectively. (PPT)

Figure S3 The expression patterns of 33 genes in three Xanthomonas oryzae pv. oryzae strains, PXO99 (P6) and PXO86 (P2) from the Philippines and GD1358 (C5) from China. (XLS)

Table S1 Primer sequences for real-time PCR analysis of differentially regulated genes in Xanthomonas oryzae pv. Oryzae. (XLS)

Table S2 Differentially expressed genes in Xanthomonas oryzae pv. oryzae strains from the Philippines, PXO99 (P6) and PXO86 (P2), when PXO86 is compared with PXO99. (XLS)

Table S3 Differentially expressed genes in Xanthomonas oryzae pv. oryzae strains when strain GD1358 (C5) from China is compared with strain (P6) from the Philippines. (XLS)

Table S4 Differentially expressed genes in Xanthomonas oryzae pv. oryzae strains when strain GD1358 (C5) from China is compared with strain PXO86 (P2) from the Philippines. (XLS)

Table S5 Differential gene expression profiles of the genes related to the two-component systems (TCS) in three Xanthomonas oryzae pv. oryzae strains, PXO99 (P6) and PXO86 (P2) from the Philippines and GD1358 (C5) from China. (XLS)

Table S6 Differential gene expression profiles of the genes related to chemotaxis and bacterial motility in three Xanthomonas oryzae pv. oryzae strains, PXO99 (P6) and PXO86 (P2) from the Philippines and GD1358 (C5) from China. (XLS)

Table S7 Differential gene expression profiles of gums genes and gene involved in synthesis of exopolysaccharides and xylanase in three Xanthomonas oryzae pv. oryzae strains, PXO99 (P6) and PXO86 (P2) from the Philippines and GD1358 (C5) from China. (XLS)
Table S8: Differential gene expression profiles of htp genes in three *Xanthomonas oryzae* pv. *oryzae* strains, PXO99 (P6) and PXO86 (P2) from the Philippines and GD1536 (CS) from China.

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Author Contributions
Conceived and designed the experiments: YZ. Performed the experiments: FZ. LH. Analyzed the data: ZD. Wrote the paper: YZ. CVC. ZL.
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