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DNA MICROARRAY BASED EXPRESSIONAL PROFILING OF *HRPXO* DEPENDENT UP-REGULONS IN *XANTHOMONAS ORYZAE* PV. *ORYZAE*

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ABSTRACT

Thirty-mer oligonucleotides for microarray analysis were designed from the annotated open reading frames (ORFs) in the whole genome sequence of *X. oryzae* pv. *oryzae* KACC10331. Mutant *hrpXo::TN* and wild-type strain KACC10859 were cultured in *hrp* inducing medium (XOM2), and cDNAs, which were synthesized from the total RNA samples from both strains, were hybridized on a DNA microarray. The microarray data showed that 210 genes were down-regulated more than 2-fold in *hrpXo*::TN, while 115 genes were up-regulated more than 2-fold. The HrpXo regulons differently included 54 hypothetical genes: type III secretory genes (11 *hrp* genes); genes encoding type III secretory effectors (*xopP* genes and *avr*/*pthA*); genes encoding type II secretory effectors (6 proteases, a lipase, a polygalacturonase, and 4 cellulases); 7 iron-uptake genes; 6 *pil* genes encoding fimbrial assembly membrane proteins; and 14 transposon genes. Significant plant-inducible promoter (PIP) sequences were newly identified on HrpXo regulons. The validity of the expressional profiles was further confirmed by reverse transcription (RT)-PCR.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*), a causal agent of bacterial blight (BB), is economically the most important bacterial pathogen in rice plants (*Oryza sativa* L.) worldwide. The hypersensitive response and pathogenicity (mediated by the *hrp* gene) and the production of extracellular polysaccharides (EPS), extracellular enzymes, and lipopolysaccharides (LPS) are known to be critical factors in pathogenesis (Chan and Goodwin 1999; Shen and Ronald 2002). Additionally, various virulence genes have been newly identified from the *Xoo* genome by transposon mutagenesis (Park *et al*. 2007; Wang *et al*. 2008).

The *hrp* genes encode proteins involved in the type III secretion system (T3SS), which is involved in the secretion of effector proteins from bacteria to plant (Büttner and Bonas 2002; Grulebeck *et al*. 2005; Keyu *et al*. 2005). The *hrp* gene cluster in *Xoo* is composed of 27 genes, from *hrpa*2 to *hrp*F (Lee *et al*. 2005), and the expression of these genes is regulated by 2 regulators, *hrpG* and *hrpX*, which are separate from the *hrp* gene cluster (Oku *et al*. 1995; Tsuge *et al*. 2005). Hrpg belongs to the OmpR family and activates the expression of *hrp*X, an AraC-like transcription activator that controls *hrp* genes along with some effector proteins (Tsuge *et al*. 2006; Wengelnik and Bonus 1996; Wengelnik *et al*. 1999). The HrpX regulons of *Xanthomonas* species have been found to include a consensus sequence motif termed the plant-inducible promoter region (PIP) box (TTCGC-N15-TTCGC) around the promoter regions (Furutani *et al*. 2006; Koebnik *et al*. 2006; Tsuge *et al*.2005). In *Xoo*, an additional conserved *cis*-regulatory element, a -10 box-like sequence that resembles the binding motif of RNA polymerase, was found downstream of the PIP box (Furutani *et al*. 2006). By using PIP box sequences, numerous candidate genes belonging to Hrp regulons, other than the genes in the *hrp* cluster, have been identified from the *Xanthomonas* genome database (da Silva *et al*. 2002; Furutani *et al*. 2006; Koebnik *et al*. 2006). It has been demonstrated that HrpXo regulates the transcriptional expression of genes associated with type II secretory proteins such as cysteine proteases (Furutani *et al*. 2004). Recently, it was

reported that polygalacturonase (PG) Wang[a)] *et al*.2008; and extracellular proteases (Wei *et al*. 2007) in *X. campestris* pv. *campestris* are regulated by HrpX. These reports suggest that HrpX can potentially function in regulating the expression of other genes in addition to the *hrp* genes. A useful *hrp*-inducing medium, XOM2, was established for cultivation of Xoo and has greatly contributed to elucidating the *in vitro* functional roles of *hrpXo* (Tsuge *et al*. 2002).

Xanthomonas genomes, including the genomes of Xoo, *X. axonopodis* pv. *citri*, and *X. campestris* pv. *campestris*, have been completely sequenced (da Silva *et al.* 2002; Lee *et al*. 2005; Ochiai *et al*. 2005), and our knowledge of the sequences provides a powerful insight into the general and specific features of pathogenicity-related genes in *Xanthomonas* genomes. On the basis of the sequence information, DNA microarrays of the *Xanthomonas oryzae* pathovars *oryzae* and *oryzicola* have been constructed as biosensors, which can be used for high-throughput genome-wide gene expression analysis to study the regulation of *hrp* genes (Seo *et al*. 2008). In addition, DNA microarrays of the phytopathogenic bacteria *Pseudomonas syringae*, *Ralstonia solanacearum*, *Xanthomonas axonopodis*, *Xanthomonas campestris*, and *Xylella fastidiosa* have been used to probe genome-wide expression related to pathogenesis and to assess the genomic diversity of meaningful bacterial isolates by (Astua-Monge *et al*. 2005; Guidot *et al*. 2009; He *et al.* 2007; He *et al.* 2006; Koide *et al*. 2006; Lu *et al*. 2005; Wang *et al*. 2006). DNA microarray chip technology promises to allow monitoring of the entire genome on a single chip, thereby providing researchers with a better picture of the simultaneous interactions among thousands of genes (de Morgan *et al*. 2010).

In this study, our aim was to identify the genome-wide *hrpXo* regulons by using an Xoo DNA microarray. The *Xoo* microarray was constructed using 30-mers derived from annotated open reading frames (ORFs) in the genome sequence of Xoo KACC10331. The transcriptional profiles of the *hrpXo* mutant were compared with those of the wild-type strain KACC10859. The resultant *hrpXo* regulons included different genes, including T3SS genes, T3SS effector genes, type II secretory system (T2SS) effector genes such as genes encoding proteases and cellulases, as well as genes involved in the uptake of iron, fimbrial-assembly genes, and metabolism-related genes.

MATERIALS AND METHODS

Bacterial strains and culture

Wild type strain, *X. oryzae* pv. *oryzae* KACC10859 was obtained from the Korean Agricultural Culture Collection (KACC) at the National Academy of Agricultural Science (NAAS), Suwon, Korea. Transposon mutant, *hrpXo::TN* were a gift from Dr. Cho of National Academy of Agricultural Science, Suwon. *Xoo* strains were cultured on pepton sucrose agar (PSA) medium (Tsuchiya *et al*. 1982) at 28°C for 3 days. Antibiotic, kanamycin were added to final concentration (20 mg/liter) 20 for mutant, *hrpXo::TN*.

Induction of *hrpXo* gene expression

Method of Tsuge *et a*l. (2002) was applied to modification for inducing hrpXo gene expression. Wild type and mutant, *hrpXo::TN* strains were grown on PSA for 3 days and the bacterial cells were washed twice distilled water (DW) and resuspended in DW at an optical density of 2.0 /ml at 600 nm. The bacterial cells were harvested by centrifugation at 6,000 rpm and suspended in 5 ml of XOM2 medium and incubated with shaking for 18 h at 25 C. The cells were used for total RNA extraction.

Fabrication of DNA microarray

Fifty-mer oligonucleotides were designed from the respective ORFs in the genomic sequence data of *X. oryzae* pv. *oryzae* KACC10331 by employing the following criteria: melting temperatures were normalized within 2°C; the G+C content of the designed oligonucleotides was restricted to 50 ± 5%, matching the 63.7%

G+C content of *X. oryzae* pv. *oryzae* KACC10331; "no sequence homology" to other regions of the genome was restricted to a maximum of 35 bp, with no exact sequence matches of more than 15 bp. Finally, 3,057 oligonucleotides were directly synthesized on CombiMatirx CustomArray™ 12K Microarray (Ghindilis *et al*. 2007)according to the manufacturer's protocol (Macrogen, Korea).

cDNA labeling and hybridization

The experiments were performed according to the standard CombiMatrix protocol described in detail at). Total RNA from mutant *hrpXo::TN* and the wild-type strain KACC10859, which were grown on XOM2 media, was purified by using the RNeasy mini kit in accordance with the manufacturer's instructions (Qiagen, Valencia, CA, U.S.A.). Labeled cDNA was synthesized by random primed reverse transcription reactions in the presence of Cy3/Alexa 546 or Cy5/Alexa 647 by using SuperScript II (Invitrogen, USA). One microgram of RNA was mixed with 2 μg of random primers, heated to 80°C for 10 min, and cooled on ice for 2 min. Unlabeled deoxynucleoside triphosphates (10 mM each of dATP, dTTP, dGTP, and dCTP), 200 units of reverse transcriptase, 5× reverse transcription buffer, and 1 μl of SUPERase-In RNase inhibitor (Ambion, U.S.A.) were added to a final volume of 20 μl. The samples were incubated at 42°C for 2 h. The reaction was stopped by the addition of 3.5 μl of stop-solution (0.5 M NaOH, 50 mM EDTA), and the RNA was degraded by incubation at 65°C for 15 min. The mixture was neutralized by adding 5 μl of 1 M Tris-HCl (pH 7.5), and 21.5 μl of 1× TE buffer was added to a final volume of 50 μl. After purification of the cDNA sample by using a MinElute PCR purification kit (Qiagen, Germany), 16 μl of the sample was heated to 80°C for 10 min and cooled on ice for 2 min. Four microliters of 10 mM dTTP, either 2.5 μl of terminal deoxynucleotidyl transferase, and 2.5 μl of 10× tailing buffer were added to a final volume of 25 μl. The samples were incubated at 37°C for 30 min, heated to 95°C for 10 min, and then immediately cooled on ice for 2 min. Five microliters of 6× ligation mix (Cy3/Alexa 546 or Cy5/Alexa 647) and 2 μl of T4 DNA ligase (Genishpere, U.S.A.) were added to a final volume of 32 μl. The sample was incubated at room temperature for 30 min, and 14.5 μl of 1× TE buffer was added to a final volume of 50 μl.

For hybridization, 2× hybridization buffer (6× SSPE, 0.05% Tween-20, 20 mM EDTA, 5× Denhardt's solution, 1 mg Salmon sperm DNA, and 0.05% SDS) was added to the CombiMatirx CustomArray™ 12K Microarray assembly and prehybridized at 65°C for 10 min, and 10 μl of the labeled cDNA was added to a pre-warmed microarray and then incubated at 65°C overnight in a dark humidified chamber. After hybridization, the microarrays were washed with pre-warmed washing buffer A (6× SSPE), B (3× SSPE), and C (0.5× SSPE) at 42°C for 15 min each and finally washed with PBS buffer.

Image scan

ElectraSenseTM Reader (http://www.combimatrix.com/ products electrasense.htm) and ElectraSenseTM 12K microarrays were used for all electrochemical assays according to the standard CombiMatrix protocol. The microarrays were assembled with special ElectraSenseTM caps to create hybridization chambers. After hybridization, the microarrays were washed and incubated with HRP-Avidin solution in BSA Peroxidase Stabilizer (1:1000) for 45 min. Finally, the hybridization chambers were filled with TMB solution, and the microarrays were inserted into the ElectraSenseTM Reader for detection.

Reverse transcriptase-PCR analysis

The mutant strain *hrpXo*::*TN* and wild-type strain KACC10859 were cultured in 5 ml of the *hrp*-inducing medium XOM2 (Tsuge *et al*., 2002) and were extracted with an RNeasy kit (Qiagen, Valencia, CA, U.S.A.), which was followed by DNase I (Promega, U.S.A.) treatment. The RNA concentration was determined using a NanoDrop photometer (NanoDrop, Wilmington, DE, U.S.A.). Primer sets designed from the 67 genes were used for reverse transcriptase

(RT)-PCR analysis to verify the *hrpX*-regulated genes appearing on the DNA microarray. After the quality of the RNA was confirmed on a 1% agarose gel, it was added to a 20-μl reaction mixture containing 20 pmol of downstream primer, 4 μl of 5× RT buffer, 2 μl of dNTPs (10 mM), 10 units of RNase inhibitor, and 10 units of reverse transcriptase, and incubated in a PTC-225™ thermocycler (MJ Research, USA) under the following conditions: 42°C for 30 min. After the cDNA synthesis reaction, 10 μl of each cDNA was PCR-amplified by HQ Taq Polymerase (JK Biotech. Korea) using the gene-specific primer sets in Table 1 under the following conditions: 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1.5 min. The PCR amplicons were visualized by ethidium bromide staining after 1% agarose gel electrophoresis. As a positive control, 16S rRNA was used. The primer set was as follows: 16S_F, 5′-GGAGGAACATCAGTGGCGAAGG-3′ and 16S_R, 5′-CCGAAGGCACCAATCCATCTCTG-3′.

RESULTS AND DISCUSSION

Microarray data analysis

In this study, we aimed to perform genome-wide identification of *hrpXo* regulons in *Xoo* by using DNA microarray analysis. The XOM2 media was formulated to induce the expression of *hrp* genes, including *hrpX*, and has been used to study *hrpX*-dependent expression in the *Xoo* genome (Furutani *et al*. 2004; Tsuge *et al*. 2002). Therefore, we employed this medium to induce expression of the *hrpX* gene. Labeled cDNAs were synthesized from the total RNA from *hrp*Xo::TN and the wild type strain KACC10859, which were cultured in XOM2, and the synthesized cDNAs were hybridized with the Xoo DNA microarray plate with 3 duplications. Oligonucleotides consisting of 30 bases were designed from the annotated ORFs in the whole genome sequence data of Xoo KACC10331, but ORFs with length less than 50 bp and those with repetitive sequences, such as transposable elements, were excluded from this study. Finally, individual oligonucleotides designed from the 3,017 ORFs were synthesized on the microarray. The expression profile of the wild-type strain KACC10859 differed from that of the *hrp*Xo::TN mutant strain (Fig. 1 A), and the differences were observed in the 3 duplicated samples. Fig. 1B shows a scatter plot of the up- and down-regulated genes in *hrp*Xo::TN, when compared with the expression in the wild-type strain. A large number of genes were down-regulated. In total, 210 genes were down-regulated more than 2-fold in the *hrp*Xo::TN mutant, while 115 genes were up-regulated, suggesting that numerous *hrp*Xo-regulated genes are distributed in the Xoo genome. The oligonucleotides are directly synthesized on individually addressable microelectrodes in a semiconductor matrix, and therefore, a relatively fixed quantity can be provided for microarray analysis.

In a previous study, the *hrp*-inducing XVM2 medium, which provides an environment similar to the host intercellular components, was extensively used for inducing virulence genes in different *Xanthomonas* species (Wengelnik *et al*. 1996). A DNA microarray consisting of 279 potential pathogenicity-related virulence genes from *X. axonopodis* pv. *citri* was applied for comparison between the transcriptomes from *X. axonopodis* pv. *citri* colonies cultured in XVM2 and nutrient broth media (Gustavo *et al*. 2005). When *X. axonopodis* pv. *citri* was grown in XVM2 medium, 31 genes, including those encoding cell wall degradation enzymes, Hrps, and avirulence proteins were profiled to be up-regulated, indicating that the artificial media can induce the expression of pathogenicity-related genes. The XOM2 medium was modified for Xoo with the same objective and mainly used for studying *hrpX*-regulated genes (Tsuge *et al*. 2002). In addition, microarray analysis to compare the global gene expression in representative strains of Xoo and *X. oryzae* pv. *oryzicola* (*Xoc*) grown in PSB and XOM2 has been performed (Seo *et al*. 2008). It was found that 247 Xoo genes and 39 Xoc genes, including bacterial-motility and signal-transduction related genes, *hrp*, and *rax*

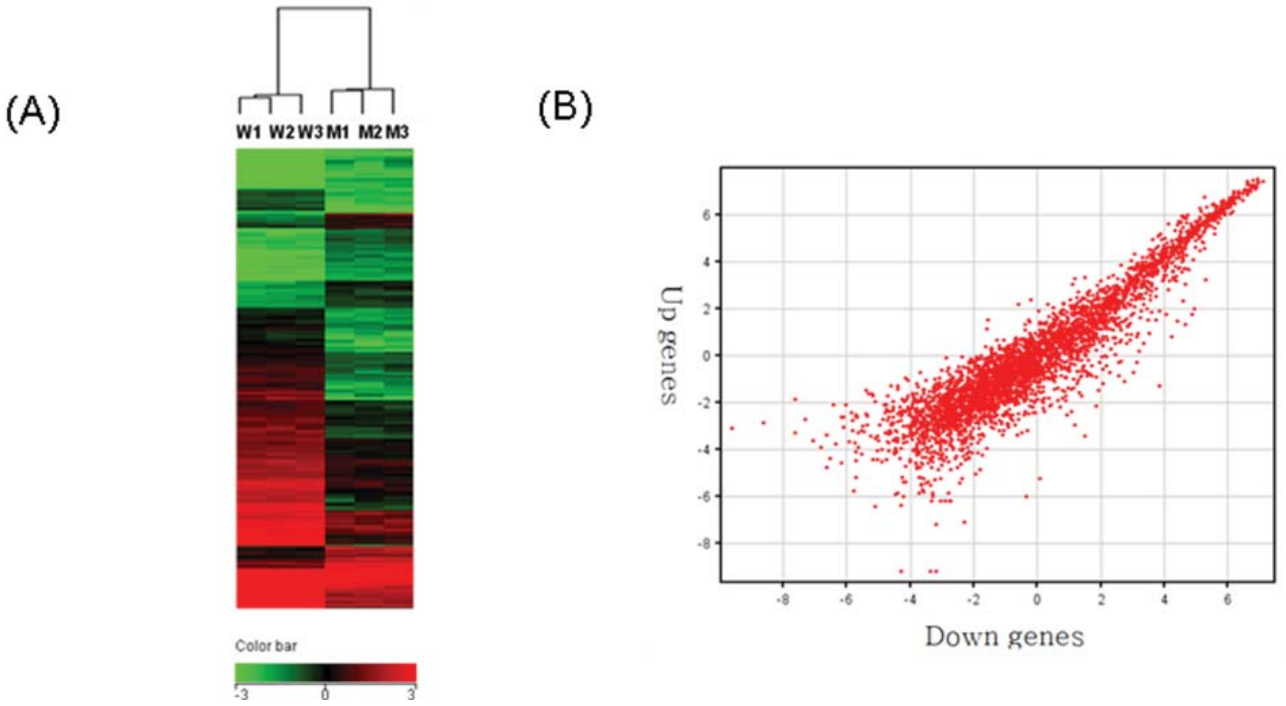

Fig 1. Expression profiling (A) of the *hrpX*::TN mutant and *X. oryzae* pv. *oryzae* KACC10859 on DNA microarray, and scatter plot (B) of *hrpX* regulatory genes in the *hrpX*::TN mutant compared to wild-type strain. M: mutant strains; W: wild-type strains. The scale indicates the relative level of expression of each gene, where red indicates expression and green indicates repression.

genes, are up-regulated in XOM2. Nevertheless, it was reasonably considered that the artificial XOM2 medium can provide environmental signals for inducing different genes, such as metabolism-related genes as well as *hrp* genes, under different media conditions. However, we aimed to determine how *hrpX* induces target genes by comparing the expressional profiles of the *hrpX* mutant and wild-type strains under the same induction medium, XOM2. Thus, we believe that the down-regulated genes on the mutant microarray are dependent on *hrpX* expression, although their expression intensities are different.

To date, different *hrpX*-induced genes have been identified by the presence of a PIP box, a *cis*-regulatory element with a motif [TTCGC-N_{15}-TTCGC] that exists in promoter regions of HrpX regulons. The PIP box has been used as effective marker for screening HrpX regulons (Furutani *et al*. 2006; Koebnik *et al*. 2006). Genome-wide screening for PIP boxes was performed on the whole genomic sequence of Xoo (Furutani *et al*. 2006; Lee *et al*. 2005). On the basis of the PIP box sequences, 17 HrpXo regulons were identified in the genome sequence of Xoo KACC10331. Of these, the cysteine proteinase gene, iron receptor gene, and *hrp* genes were coincident to those observed in the present study. Furthermore, in this study, we investigated the existence of PIP box sequences in the *hrpXo*-regulated genes on a microarray. As shown in Fig. 2, perfect PIP boxes, containing the motif TTCGT- N_{15}-TTCGT, were identified in the promoter regions of 4 *hrp* genes (XOO0095, XOO0096, XOO0094, and XOO0076). Genes encoding cysteine protease contained imperfect PIP boxes (TTCGn-N12-TTCGn in XOO0387, TTCGC-N12-TTCGC in XOO1480, and TTCGT-N_{13}-TTCGT in XOO1487) in their promoter regions. Significant PIP boxes were further identified in other *hrpXo*-regulated genes, including the *xrvA* gene of virulence regulator (XOO2744), a cellulase gene (XOO4036), and 2 *pili* genes (XOO1157, XOO1158), but not in the other *hrpX* regulons in this study. In *Xanthomonas* species, genes with an imperfect PIP box and

Fig. 2. PIP sequences in the promoter regions of *hrp*Xo-regulated genes on the *X. oryzae* pv. *oryzae* microarray. The window boxes indicate consensus sequences in PIP (standard sequence).

genes without a PIP box have been reported to be expressed in an HrpX-dependent manner (Noël *et al.* 2002), suggesting that the PIP box is not an absolute criterion for identifying HrpXo regulons.

*hrp*Xo dependent up-regulated genes

Pathogenesis-related genes

Virulence and regulatory genes required for bacterial pathogenicity are commonly found in pathogenicity islands (PAIs) that encode for a type III protein secretion system assembled from *hrp* gene products. During infection, phytopathogenic bacteria use T3SS to deliver effector proteins into the host plant, thereby causing disease symptoms in susceptible hosts and defense responses in resistant hosts. An *hrp* gene cluster containing 27 genes, including *hpa2* and *hrpF*, was identified in the *Xoo* genome. The *Xoo hrp* PAI (31.3 kb) was larger than its counterparts in *Xac* (25.6 kb) and *Xcc* (23.1 kb), owing to the presence of 4 transposed genes (about 6 kb) located between the *hpaB* and *hrpF* genes (Lee, *et al.* 2005). In this result, it was revealed that *hrpXo* regulates the transcriptional expression of *hrpF*, *hrpE*, *hrpD6*, *hpaA*, *hrcV*, *hrpB3*, *hrpB8*, *hrcC*, *hpa1*, and *hpa*2 (Table 1). Previously, microarray experiments were performed to compare global gene expression between *Xoo* and *Xoc* grown in nutrient medium (PSB) and those grown in *hrp*-inducing XOM2 media (Seo *et al*., 2008). The *hpa1* and *hrpE* genes in *X. oryzae* pv. *oryzicola* are specifically induced in *hrp*-inducing XOM2 media, but not in nutrient media, indicating that *hpa1* and *hrpE* are under different or additional regulatory controls from the other *hrp* genes, which is unlikely to be the case in *Xoo*. In contrast, expression of *Xoo hpa1*, *hrpG*, *hrcC*, and *hrpE* were up-regulated in XOM2. However, it is not clear whether the induced expression of the *hrp* genes in XOM2 is due to environmental signals in the media or *hrpXo*. On the basis of our data, we considered that *hrpX* expression is induced by environmental factors in plants or XOM2 media and that the activated HrpX then stimulates the expression of other *hrp* genes such as *hpa1*, *hrpG*, *hrcC*, and *hrpE*. HrpX is known to be a regulator of *hrp* genes and several effector genes (Wengelnik and Bonas 1996).

Furthermore, we identified 3 T3SS effector genes XOO4466 (*XopP*), XOO2127 (*Avr*/*PthA*), and XOO4033 (*XopP*) in this microarray analysis. *Xoo* harbors multiple genes for T3S effectors of the transcription activator-like (TAL) effector family,

called the *avrBs3/pthA* family (Lee *et al*. 2005; Ochiai *et al*. 2005). AvrXa7, AvrXa10, and AvrXa27 are representative TAL effectors that elicit disease resistance in hosts with corresponding resistance genes and thereby function as determinants of race–cultivar specificity (Gu *et al*. 2005; Yang *et al*. 2000). Hrp outer proteins (Hops) from *P. syringae* strains and *Xanthomonas* outer proteins (Xops) have been shown to be T3S effectors. Recently, on the basis of the natural features of N-terminal amino acids of Hop, Xop proteins, and T3S substrates, T3S system-dependent translocation of 60 effector candidates was identified from the genome data of *Xoo* MAFF311018 and screened using *Bordetella pertussis* calmodulin-dependent adenylate cyclase (Cya) reporter for translocation into plant cells (Frutani *et al*. 2008). Finally, 16 T3SS effector genes that are also regulated by hrpX were identified. Of these, XOO4466 and XOO4033, from this study, were included among the effectors.

The *rtx* genes (XOO4535 and XOO1389) have been shown to be up-regulated by HrpXo in *Bradyrhizobium elkanii*, a symbiotic nitrogen-fixing bacterium that produces the enol-ether amino acid rhizobitoxine; the *rtxC* gene is required for the production of rhizobitoxine, which is regarded as a phytotoxin responsible for progressing chlorosis symptoms in soybean (Okazaki *et al*. 2004). RTX toxins are important virulence factors for a variety of human and animal pathogens (Welch *et al*. 1992) and have been found in several plant pathogenic bacteria, including *X. fastidiosa*, *Rhizobium leguminosarum*, and *Erwinia carotovora* (Kuhnert *et al*. 1997; Oresnik *et al*. 1999; Simpson *et al*. 2000). The genes for 2 apparent RTX toxins, *rtxA* and *rtxC*, were identified in the Xoo genome but were not detected in the Xcc or Xac genomes.

The virulence gene *xrvA*, which encodes a histone-like nucleoid-structuring protein, was also revealed to be an *hrpXo* regulon. Recently, it was reported that *xrvA* demonstrates enhanced expression in XOM2 medium and further regulates pathogenicity-related genes such as *hrpG*, *hrpX*, *rpfC*, and *rpfG*, which play an important role in the hypersensitive response and EPS and LPS biosynthesis (Feng *et al*. 2009). The *xrvA* function found in the study by Feng *et al*. seems to be contrary to the result of our microarray analysis, but this is not conclusive because we do not know how virulence genes are interracially associated with other unknown pathogenesis-related genes.

In the present study, it was revealed that 7 iron-receptor proteins, which encode the genes XOO4431, XOO0407, XOO0008, XOO0009, XOO0029, and XOO1368, are regulated by *hrpXo*. In *X. campestris* pv. *campestris*, TonB, ExbB, and ExbD have been reported to play a critical role in Fe^{3+} uptake and induction of a hypersensitive response in pepper (Wiggerich *et al*. 1997; Wiggerich and Puhler 2000). Forty-two putative iron uptake-related genes, including TonB-dependent receptor proteins, were annotated in the genomic sequence of Xoo KACC10331. In a recent report, mutation in a TonB-dependent receptor led to the loss of pathogenicity and also caused a reduction in mobility and extracellular enzymes (Wu *et al*. 2010). A mutation in XOO1368, homologous to bfeA, resulted in reduced pathogenicity (Wang *et al.* 2008), suggesting that sequestration of iron is important for *Xoo* virulence. A 3-gene cluster (*bfeABR*) is also present in the *Xoo* genome. The *bfeA* gene was isolated from *Bordetella pertussis* and *Bordetella bronchiseptica*, obligate respiratory pathogens of mammals that encode a predicted outer-membrane siderophore receptor exhibiting a high degree of similarity to the ferric enterobactin receptors. The extremely limited availability of free iron requires pathogenic bacteria to utilize specialized uptake mechanisms to successfully compete with the host and other microorganisms for iron.

Table 1. Downregulated genes in hrpX: TN mutant strain

Gene ID	Description	Expression folds	Gene ID	Description	Expression folds
XOO0832	holC;DNA polymerase III subunit chi	**-2.02**	XOO1623	rpsT;30S ribosomal protein S20	**-2.58**
XOO4019	type VI secretion system Vgr family protein	**-2.03**	XOO0079	hpaA;HpaA	**-2.59**
XOO0844	protease	**-2.03**	XOO1822	hypothetical protein	**-2.59**
XOO1259	peptidase	**-2.03**	XOO4535	RTS beta protein	**-2.60**
XOO0094	hrp 1	**-2.05**	XOO3277	hypothetical protein	**-2.62**
XOO2522	hypothetical protein	**-2.05**	XOO0094	hrcC;HrcC	**-2.62**
XOO4431	cirA;TonB-dependent receptor	**-2.07**	XOO1157	pilM;fimbrial assembly membrane protein	**-2.62**
XOO1943	nodQ;ATP sulfurylase/adenylylsulfate kinase	**-2.07**	XOO2231	proP;Prop transport protein	**-2.63**
XOO3773	nrtB;permease	**-2.08**	XOO4418	salR;sal operon transcriptional repressor	**-2.66**
XOO2294	phosphatase	**-2.10**	XOO3464	Type I restriction enzyme StySPI specificity protein	**-2.67**
XOO3594	rplA;50S ribosomal protein L1	**-2.13**	XOO1831	histidine kinase-response regulator hybrid protein	**-2.68**
XOO0495	radC;DNA repair protein RadC	**-2.15**	XOO3372	thiG;thiazole synthase	**-2.68**
XOO2348	hypothetical protein	**-2.16**	XOO0019	;hypothetical protein	**-2.69**
XOO3127_	hypothetical protein	**-2.17**	XOO0093	hrpB8;HrpB8	**-2.69**
XOO3184	rplT;50S ribosomal protein L20	**-2.17**	XOO0704	hypothetical protein	**-2.72**
XOO0348	;pseudouridylate synthase	**-2.18**	XOO3238	tpiA;triosephosphate isomerase	**-2.73**
XOO0408	csd2 family	**-2.18**	XOO2744	xrvA;virulence regulator	**-2.75**
XOO4263	icfG;IcfG protein	**-2.19**	XOO1077	Cellulase S	**-2.75**
XOO2561	;hypothetical protein	**-2.20**	XOO3557	rplQ;50S ribosomal protein L17	**-2.76**
XOO3449	uidR;transcriptional regulator uid family	**-2.20**	XOO3269	integral membrane protein	**-2.78**
XOO0869	CRISPR-associated proteins	**-2.21**	XOO2084	tRNA/rRNA methyltransferase	**-2.78**
XOO4265	SpoII AA	**-2.22**	XOO3000	cytochrome C	**-2.83**
XOO1497	cysteine protease	**-2.23**	XOO3255	truA;tRNA pseudouridine synthase A	**-2.85**
XOO0705	oxidoreductase	**-2.23**	XOO1456	hypothetical protein	**-2.85**
XOO2530	Dehydrogenases	**-2.26**	XOO1590	fic;Cell filamentation protein Fic	**-2.85**
XOO0409	TolB protein	**-2.26**	XOO4465	hypothetical protein	**-2.87**
XOO1612	hypothetical protein	**-2.27**	XOO4426	hypothetical protein	**-2.87**
XOO1908	hypothetical protein	**-2.29**	XOO0174	hypothetical protein	**-2.89**
XOO1262	;hypothetical protein	**-2.30**	XOO0411	hypothetical protein	**-2.90**
XOO0407	piuB;iron-uptake factor	**-2.31**	XOO4490	hypothetical protein	**-2.91**
XOO1201	omp21;outer membrane protein	**-2.31**	XOO2127	avirulence protein, Avr/PthA	**-2.92**
XOO0028	kdgK;2-keto-3-deoxygluconate kinase	**-2.31**	XOO0703	rsuA;ribosomal small subunit pseudouridylate synthase	**-2.92**
XOO0747	hypothetical protein	**-2.33**	XOO3561	rpsM;30S ribosomal protein S13	**-2.96**
XOO1606	hypothetical protein	**-2.34**	XOO2746	hypothetical protein	**-2.96**
XOO2499	phy;putative phytase precursor	**-2.35**	XOO0288	transglycolase; epimerase	**-2.99**
XOO2989	methyltransferase	**-2.35**	XOO1531	phuR;outer membrane hemin receptor	**-3.00**
XOO2346	hypothetical protein	**-2.35**	XOO1160	pilP;fimbrial assembly protein	**-3.05**
XOO2147	cycH;C-type cytochrome biogenesis protein	**-2.38**	XOO2986	glycosyltransferase	**-3.08**
XOO2047	hypothetical protein	**-2.38**	XOO1401	acetyltransferase	**-3.09**
XOO3205	hypothetical protein	**-2.38**	XOO1055	hypothetical protein	**-3.10**
XOO1528	short chain dehydrogenase	**-2.38**	XOO2845	tsr;chemotaxis protein	**-3.10**
XOO4482	yojM;superoxide dismutase like protein	**-2.39**	XOO3559	rpsD;30S ribosomal protein S4	**-3.13**

XOO2263	hypothetical protein	**-3.01**	XOO4527	oxidoreductase	**-3.16**
XOO0947	thiC;thiamine biosynthesis protein ThiC	**-3.01**	XOO0420	hypothetical protein	**-3.16**
XOO3584	rplC;50S ribosomal protein L3	**-3.02**	XOO4466	XopP, Nucleoside hydrolases	**-3.20**
XOO1994	brf;bacterioferritin	**-2.40**	XOO2022	serine peptidase	**-3.21**
XOO03911	prnA;tryptophan halogenase	**-2.42**	XOO2667	hypothetical protein	**-3.22**
XOO1069	Transglutaminase	**-2.44**	XOO2246	;hypothetical protein	**-3.25**
XOO3281	metallopeptidase	**-2.44**	XOO0077	hrpD6;HrpD6	**-3.31**
XOO0084	hrcV;HrcV	**-2.44**	XOO3735	vanR;transcriptional regulator gntR family	**-3.33**
XOO3570	rplE;50S ribosomal protein L5	**-2.46**	XOO4355	Dipeptidyl aminopeptidases	**-3.36**
XOO2688	hypothetical protein	**-2.52**	XOO1541	hypothetical protein	**-3.43**
XOO2042	ldp;dihydrolipoamide dehydrogenase	**-2.53**	XOO3479	hcp;hypothetical protein	**-3.47**
XOO2705	efP;elongation factor P	**-2.54**	XOO4532	hypothetical protein	**-3.59**
XOO3566	rpsE;30S ribosomal protein S5	**-2.54**	XOO0008	tonB;TonB protein	**-3.63**
XOO0699	;hypothetical protein	**-2.55**	XOO3410	methyltransferase	**-3.64**

Type II secretory system related genes

We found that *hrp*Xo regulates T2SS effector genes, including 4 cellulase genes (XOO4036, XOO1077, XOO2699, and XOO4019), 6 protease genes (XOO0007, XOO1497, XOO0387, XOO1480, XOO1487, and XOO0844), a polygalacturonase gene (XOO2699), and a lipase gene (XOO0526). Cell-wall degrading enzymes, such as cellulases, pectinases, xylanases, and proteases, are secreted by plant pathogen cells to break down the components of host cell walls and may play a crucial role in virulence and bacterial nutrition (Barras *et al.* 1994; Shen and Ronald 2002). Currently, genes encoding cell-degrading enzymes (CDEs) such as cellulase and xylanase are considered to play a role in the virulence of Xoo (Hu *et al*. 2007; Rajeshwari *et al*. 2005; Ray *et al*. 2000; Sun *et al*. 2005; Wang *et al*. 2008). Moreover, this study showed that *hrp*Xo regulates the transcriptional expression of cellulase genes in *Xoo*. It has been reported that purified cellulase and lipase proteins induce defense responses in rice that are suppressible by *Xoo* in a T3S-dependent manner (Jha *et al*. 2007). Therefore, cellulase genes are considered to participate in diverse virulence functions associated directly or indirectly with the expression of the main pathogenicity-related genes, such as *hrp* genes. In the case of other *Xanthomonas* species, 2 pathogenicity-related genes (*pghAxc* and *pghBxc*) encoding functional polygalacturonase (PG) from *X. campestris* pv. *campestris* 8004 are regulated by HrpX (Wang[a)], *et al*. 2008). It was also reported that *hrpX* negatively regulates the α-amylase isozymes in *X. axonopodis* pv. *citri* (Yamazaki *et al.* 2008) and extracellular proteases in *X. campestris* pv. *campestris* (Wei *et al*. 2007). In a previous study conducted using SDS-PAGE and RT-PCR analysis, *hrpXo* was shown to regulate the expression of genes associated with T2S proteins, i.e., 2 cysteine proteases (Cysp1 and Cysp2) (Furutani *et al*. 2004), which shows good agreement with our results. In other phytopathogenic bacteria, it is known that the TTSS effectors encoding cysteine proteases, such as XopD, AvrXv4, AvrPphB, and AvrRpt2, proteolyze specific host targets and are indirectly recognized by R proteins as a consequence of their action in plant cells (Andrew and Mary 2004). Thus, it can be reasonably assumed that *hrp*Xo-dependent cysteine proteases may function as T3SS effectors or indirectly control the recognition of the host plant.

Bacterial mobility

On the basis of the microarray data obtained in this study, it was revealed that *hrpXo* regulates *pilMNEGIP*, which encodes fimbrial as asembly proteins that are known as type IV pili. Type IV pili are flexible, filamentous structures protruding from the cell surface of gram-negative bacteria (Shi and Sun 2002; Strom andLory 1993). It has been revealed that type IV pili promote the attachment of bacterial pathogens

to the specific receptors of host cells during colonization (Bieber *et al.* 1998). Such attachment is an essential event for the initiation of infection. Limited to colonizing the water-conducting xylem vessels of plants, phytopathogenic bacteria develop biofilms that contribute to the blockage of sap flow and thereby cause plant stress and disease (Maxwell *et al*. 2003; Meng *et al*. 2005). How bacteria are disseminated in the xylem vessels from feeding sites is an important question. Generally, it is known that type IV pili in bacteria play an important role in twitching and social gliding motility for cell movement (Mattick 2002). On the basis of colony morphologies, there is also strong evidence for a functional role of *pil* genes in twitching motility in the phytopathogenic bacteria *Ralstonia solanacearum* and *X. fastidiosa* (Liu *et al*.2002; Meng *et al*.2005). The distribution of pili genes in the *Xoo* genome was analyzed according to the *Xoo* sequence information released on the NCBI website. Interestingly, 26 pili-related genes were present in different regions of the *Xoo* genome, including *pilA, pilZ, pilH, pilMNOPQ, pilTU, pilDCE, pilBRS, pilGHIJL, pilF, pilE1Y1*, *pilX*, and *pilV* (Lee *et al*. 2005). *Xoo* genome analysis suggests that a number of pili gene orthologs may encode proteins involved in the biogenesis and function of type IV pili. However, the functional roles of other pili genes have not been proved in the Xoo genome. It was recently reported that Xoo *pilQ* plays a critical role in pathogenicity, twitching motility, and biofilm production (Lim, *et al*. 2008). It was previously reported that 14 pili genes (*pilIJKLMNOPQRSTUV*) are involved in type IV pilus biogenesis in *E. coli* (Sakai andKomano 2002). The genes *pilS, pilV*, and *pilU* encode the major pilin, minor pilin, and prepilin peptidase, respectively. The C-terminal segments of *pilV* genes are under the control of multiple DNA inversions of shufflon and determine recipient specificity in liquid mating. The *pilL* and *pilN* gene products are outer-membrane lipoproteins, and the *pilQ* gene product is a cytoplasmic ATPase and is essential for thin pilus formation (Saiki *et al.* 2001). The remaining *pil* genes are likely to encode structural proteins that function in the establishment of the pilin transport apparatus and the basal body of the thin pilus. Most of these *pil* gene products contain signal sequences or trans-membrane domains, suggesting that they are transported to the peri-plasmic space, inner membrane, and outer membrane.

Verification of microarray results

The effect of the *hrpXo* mutation on the expression of different genes was validated by using RT-PCR. The RT-PCR 20-mer primers were generated from 12 T3SS-related *hrp* genes, 4 T2SS-related cysteine protease-encoding genes, 3 cellulase genes, and 6 type-IV pili genes. As shown in Fig. 2, no, or very weak, amplification was obtained from the genes in the *hrpXo*::Tn5 mutant, although the magnitude of expression was somewhat different to those observed in the microarray analysis. As expected, all *hrp* gene primers tested amplified RT-PCR bands with reduced intensity in the *hrpXo*::Tn5 mutant, compared with the intensities for the wild-type strain. In addition, 4 genes encoding cysteine proteases and cellulases (XOO4036) did not amplify an RT-PCR band in the *hrpXo*::Tn5 mutant, indicating that their transcriptional expression is absolutely dependent on *hrpXo*. Furthermore, RT-PCR analysis also verified that when compared with the wild-type strain, the *hrpXo* mutant visually represents low transcriptional expression of *pilE*, *pilG*, *pilI*, and *pilP*, but not *pilM* and *pilN*. The result confirmed that the microarray result is largely available.

In conclusion, DNA microarray analysis of *X. oryzae* pv. *oryzae* revealed that in addition to regulating the *hrp* genes, the *hrpX* regulon regulates numerous new genes possessing different functions. Furthermore, the potential interactive roles between the *hrpX* regulons and the host plant should be elucidated for pathogenesis mechanisms.

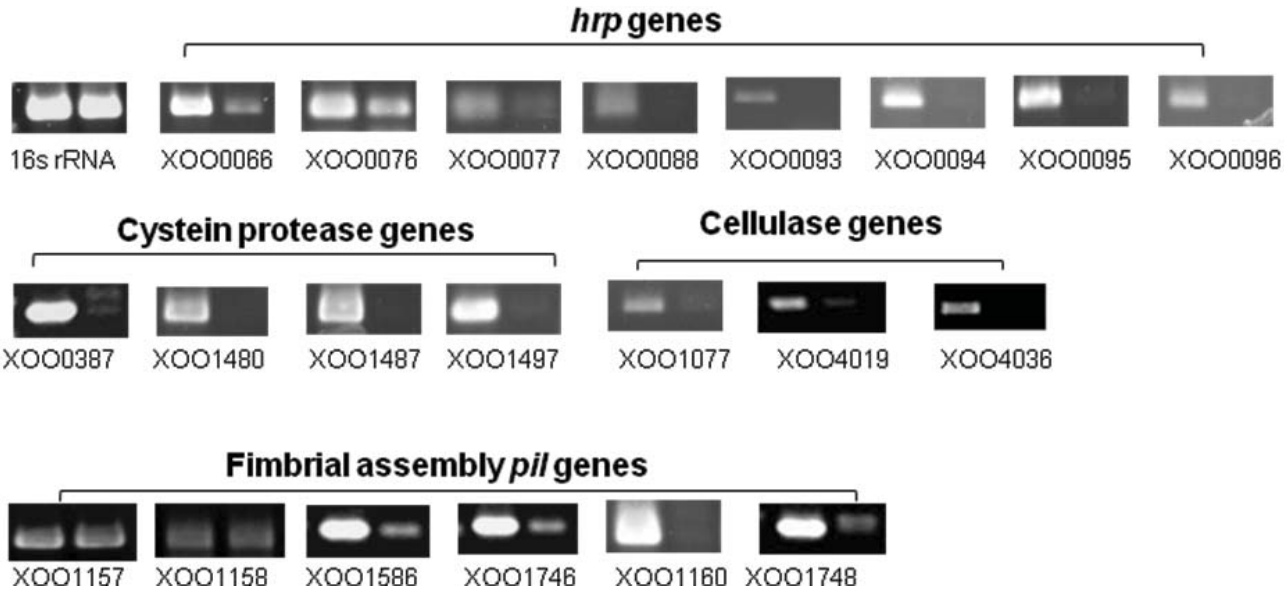

Fig.3. Verification of microarray data by RT-PCR. Total RNAs from wild-type strain KACC10859 and the *hrpX*:: TN mutant were used as PCR templates with primer pairs in Table 2. Amplicons were visualized after agarose gel electrophoresis and ethidium bromide staining.

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