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Virulence Reduction and Differing Regulation of Virulence Genes in *rpf* Mutants of *Xanthomonas oryzae* pv. *oryzae*

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To define the functions of the *rpf* genes in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which regulates pathogenicity factors in *Xanthomonas campestris* pv. *campestris* (*Xcc*), marker-exchange mutants of each *rpf* gene were generated. When the mutants were inoculated on a susceptible cultivar, the lesion lengths caused by the *rpfB*, *rpfC*, *rpfF*, and *rpfG* mutants were significantly smaller than those caused by the wild type, whereas those caused by the *rpfA*, *rpfD*, and *rpfI* mutants were not. Several virulence determinants, including extracellular polysaccharide (EPS) production, xylanase production, and motility, were significantly decreased in the four mutants. However, the cellulase activity in the mutants was unchanged. Complementation of the *rpfB* and *rpfC* mutations restored the virulence and the expression of the virulence determinants. Expression analysis of 14 virulence genes revealed that the expression of genes related to EPS production (*gumG* and *gumM*), LPS (*xanA*, *xanB*, *wxoD*, and *wxoC*), phytase (*phyA*), xylanase (*xynB*), lipase (*lipA*), and motility (*pilA*) were reduced significantly in the mutants *rpfB*, *rpfC*, *rpfF*, and *rpfG*. In contrast, the expression of genes related to cellulase (*eglxob*, *clsA*), cellobiosidase (*cbsA*), and iron metabolism (*fur*) was unchanged. The results of this study clearly show that *rpfB*, *rpfC*, *rpfF*, and *rpfG* are important for the virulence of *Xoo* KACC10859, and that virulence genes are regulated differently by the Rpfs.

Keywords* : EPS, Exo-enzymes, motility, virulence reduction, *rpf

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a causal agent of the economically important bacterial blight disease of rice. In this disease, infection of the pathogen through the epitheme is followed by movement to xylem vessels and then growth of the pathogen. More than a dozen *Xoo* genes are required for the disease to develop in infected rice leaves. Genes related to the production of extracellular polysaccharide (EPS; *gumG* and *gumM*, Ray et al., 2000), lipopolysaccharide (LPS; *xanA* and *xanB*, Köplin et al., 1992; *wxoD* and *wxoC*, Patil et al., 2004), extracellular enzymes (*xpsF* and *xpsD*, Ray et al., 2000; *phyA*, Chatterjee et al., 2003; *eglxob*, Hu et al., 2007; *xynB*, Rajeshwari et al., 2005; *clsA*, *cbsA*, *lipA*, Jha et al., 2007), motility (*pilA*, Ryan et al., 2007), and iron metabolism (*fur*, Subramoni et al., 2005) are important virulence and pathogenicity factors. How these genes are regulated coordinately during disease development is a major question for understanding the pathogenesis of this organism.

In *Xanthomonas campestris* pv. *campestris* (*Xcc*), the *rpf* genes regulate virulence and pathogenicity factors by a cell-cell communication mechanism utilizing a small diffusible factor (DSF) (Barber et al., 1997; Slater et al., 2000). The signal from the DSF, which is produced by the *rpfB* and *rpfF* gene products and has been characterized as *cis*-11-methyl-2-dodecenoic acid (Wang et al., 2004), is transferred to a two-component regulatory system consisting of the *rpfC* and *rpfG* gene products (He et al., 2006). The RpfC and RpfG two-component system positsively regulates pathogenicity factors such as EPS, LPS, and extracellular enzymes (Dow et al., 2006).

The roles of the *rpf* genes in the regulation of virulence or pathogenicity factors in *Xoo* have not been characterized clearly. A mutation in *rpfC* had no effect on the extracellular enzyme factors, but affected EPS synthesis and virulence (Tang et al., 1996), whereas a mutation in *rpfF* resulted in defective DSF production but did not affect the production of EPS or xylanase, an important virulence factor that is one of the extracellular enzymes (Chatterjee and Sonti, 2002). In the genome of *Xoo* KACC10331 (Lee et al., 2005), the core *rpf* genes are highly conserved (Lee et al., 2006). To clarify the roles of the *rpf* genes in the pathogenicity of *Xoo*, *rpf* mutants were generated, and the expression of virulence factors and virulence genes were analyzed in the mutants.

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We report that mutations in the core *rpf* genes *rpfB*, *rpfC*, and *rpfF*, and that *rpfG* reduced the EPS levels, the xylanase activity, and the motility and virulence of *Xoo*. Gene expression profiles of the *rpf* mutants indicate that different virulence-related genes are regulated differently in *Xoo*.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. *X. oryzae* pv. *oryzae* strains were grown in Nutrient Broth (NB) and Peptone Sucrose Broth (PSB) at 28°C. For solid media, 16 g of agar per liter were added. *E. coli* strains were cultured in Luria-Bertani medium at 37°C. The Xoo and *E. coli* strains used are listed in Table 1, and the plasmids constructed are summarized in Table 2. The antibiotics used in this study were 50 μg/ml kanamycin (Km), 50 μg/ml streptomycin (Sm), 100 μg/ml ampicillin (Ap), 20 μg/ml cephalexin (Cp), 50 μg/ml spectinomycin (Sp), and 50 μg/ml gentamicin (Gm).

Molecular techniques and transformation. Standard methods (Sambrook et al., 1989) were used for DNA manipulations. Total RNA was extracted from *Xoo* cells grown in NB using TRIzol® Reagent (Invitrogen, Inc.). Enzymes used for cDNA synthesis were purchased from Qiagen. DNA and RNA concentrations were measured using the Qubit™ fluorometer (Invitrogen, Inc.). The Gene Pulser Xcell™ system (Bio-Rad) was used for *E. coli* and *Xoo* transformation with a voltage pulse of 3.0 kV, a capacitance of 25 μF, and a resistance of 200 Ω.

Mutagenesis of *rpf* genes. To generate *rpf* knockout mutants, insertional mutagenesis of *Xoo* was performed as described (Lee et al., 2004) with an EZ-Tn5™ <KAN-2> Transposome™ insertional kit (Epicentre Technologies Co.). Using appropriate restriction enzymes, each *rpf*

Table 1. Bacterial strains used in this study

Strain	Relevant characteristics[a]	Source
Escherichia coli		
DH5α	F⁻ *gyrA96*(Nal^r) *recA1 relA1 endA1 thi-1 hsdR17* ($r_K^- m_K^+$) *glnV44 deoR* Δ*(lacZYA-argF)U169* [Φ80dΔ*(lacZ)M15*]	RBC Real Biotech
Xanthomonas oryzae pv. *oryzae*		
KACC10859	Wild-type strain, Cp^r	RDA, South Korea
CBNUXO01 (*rpfA*::Tn5)	KACC10859 *rpfA*::Tn5, Km^r	This study
CBNUXO02 (*rpfB*::Tn5)	KACC10859 *rpfB*::Tn5, Km^r	This study
CBNUXO03 (*rpfC*::Tn5)	KACC10859 *rpfC*:: Tn5, Km^r	This study
CBNUXO04 (*rpfD*:: Tn5)	KACC10859 *rpfD*::Tn5, Km^r	This study
CBNUXO05 (*rpfF*:: Tn5)	KACC10859 *rpfF*::Tn5, Km^r	This study
CBNUXO06 (*rpfG*:: Tn5)	KACC10859 *rpfG*::Tn5, Km^r	This study
CBNUXO07 (*rpfI*:: Tn5)	KACC10859 *rpfI*::Tn5, Km^r	This study
CBNUXO08 (*rpfB*^{c})	KACC10859 *rpfB* complemented with pML122*rpf*B, Gm^r	This study
CBNUXO09 (*rpfC*^{c})	KACC10859 *rpfC* complemented with pML122*rpf*C, Gm^r	This study

[a]Cp^r, Cephalexin resistance; Km^r, kanamycin resistance; Gm^r, gentamycin resistance

Table 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pUC18	pMB1 ori, Ap^r	Invitrogen
pML122	OriV, OriT, Gmr, *p*Nm (*npt*II), broad host-range expression vector	Labes et al., 1990
pUCrpfA	pUC18 carrying the *rpfA* gene, disrupted by a Tn5 insertion	This study
pUCrpfD	pUC18 carrying the *rpfD* gene, disrupted by a Tn5 insertion	This study
pUCrpfF	pUC18 carrying the *rpfF* gene, disrupted by a Tn5 insertion	This study
pUCrpfG	pUC18 carrying the *rpfG* gene, disrupted by a Tn5 insertion	This study
pUCrpfI	pUC18 carrying the *rpfI* gene, disrupted by a Tn5 insertion	This study
pUCrpfB	pUC18 carrying the *rpfB* gene, disrupted by a Tn5 insertion	This study
pUCrpfC	pUC18 carrying the *rpfC* gene, disrupted by a Tn5 insertion	This study
pMLrpfB	pML122 carrying a *Hin*dIII-*Bam*HI fragment from pUC*rpfB*	This study
pMLrpfC	pML122 carrying a *Hin*dIII-*Bam*HI fragment from pUC*rpfC*	This study

Table 3. RT-PCR primers used in this study

Gene	Primer	Primer sequence[a]
16S rRNA	rRNA-F	5'-AATGGGCGCAAGCCTGATC-3'
	rRNA-R	5'-TTTGTCACCGGCGGTCTCC-3'
eglXob	eglXob-F	5'-CTCGCAACAGCCCGGCGTAC-3'
	eglXob-R	5'-GATTGCAGGTGACCGTCTTGC-3'
lipA	lipA-F	5'-GAACATCCACGAGTGCTTCA-3'
	lipA-R	5'-CAGAATTTCGATGCGGGTAT-3'
wxoC	wxoC-F	5'-ATCGAATTTGGATGCAGGTC-3'
	wxoC-R	5'-TCATTAGCACCGTTTGGTGA-3'
wxoD	wxoD-F	5'-CAGACCTTGGGTGGTTATGC-3'
	wxoD-R	5'-GGAACCGCACTATCTGGAAA-3'
clsA	clsA-F	5'-CTGACACCGACTGGAACAAG-3'
	clsA-R	5'-GTTGTTGGGGAAGTTGCTGT-3'
cbsA	cbsA-F	5'-TCTTCGGATCGTCAACATCA-3'
	cbsA-R	5'-GTGTAATTGGCGGTGTTGGT-3'
phyA	phyA-F	5'-GCTTTCGGCGTTATCAAGTC-3'
	phyA-R	5'-GTTGCACGCAAACAAGGTAA-3'
xynB	xynB-F	5'-AACGAGCGTTAGGGATGGT-3'
	xynB-R	5'-GTATCGGTAACGACGCGAAT-3'
gumM	gumM-F	5'-GATTGCGTTAGGTGGCTTTC-3'
	gumM-R	5'-CGCACTCTCACGACACAGAT-3'
gumG	gumG-F	5'-CGTTGTTCTTCATCGCCATA-3'
	gumG-R	5'-ATTCCCCGAATGAAATAGGC-3'
xanA	xanA-F	5'-TTACTTCATTGCGCATCACC-3'
	xanA-R	5'-GTACGCGTTTCCAGCAGTG-3'
xanB	xanB-F	5'-CAATGAAGAACACCGCTTCA-3'
	xanB-R	5'-GTCACCAACTTCCCCTTGCTC-3'
fur	fur-F	5'-ATGGAAACCCACGACCTGCG-3'
	fur-R	5'-TCAGCGCGGACGCTTCTTGC-3'
pilA	pilA-F	5'-TTTCCCGGCTTACCACGATTA-3'
	pilA-R	5'-CATTCGCTGCTGTCGAAAC-3'
rpfB	rpfB-F	5'-GAACGCTGGAAGAAGGTCAC-3'
	rpfB-R	5'-TCCAATAGCCCTTCATCACC-3'
rpfC	rpfC-F	5'-GATGTCGAGGACAGCGGTAT-3'
	rpfC-R	5'-CTGCAACTCGAACCAGAACA-3'
rpfF	rpfF-F	5'-TCATGCTTGAAGGCAATCTG-3'
	rpfF-R	5'-GCAACGACTTCTCGCCTAAC-3'
rpfG	rpfG-F	5'-CTCGAACGCATGTCTCATGT-3'
	rpfG-R	5'-ACCTGGATGAAACGGTTCTG-3'
rpfI	rpfI-F	5'-TCATGACGAAACCAAAGTGC-3'
	rpfI-R	5'-TCCAGCAATCGGTAGAGACC-3'

[a]All sequences were designed based on the *Xoo* KACC10331 genomic sequence in GenBank (accession number AE013598)

gene was inserted into pUC19 from a BAC library clone (C4) containing the complete *rpf* gene cluster of the KACC10331 strain. Tn5 was inserted into each *rpf* gene in pUC19 by mixing the plasmids with Transposome™ (20 ng/μl) and transforming the resulting plasmids into *E. coli* (DH5α). Clones containing the Tn5 insertion in the middle of an *rpf* gene were selected by restriction screening, and the location of each Tn5 insertion was confirmed by nucleotide sequencing. The selected plasmids were introduced into *Xoo* KACC10859 by electroporation, and homologous recombinants were selk,ected by screening for Km^R and Ap^S transformants. The marker exchange of the target genes was confirmed by PCR. Expression of the mutated *rpf* gene in the corresponding mutant was checked by RT-PCR with primers designed from the flanking region of Tn5 insertion (Table 3).

Complementation of mutants. *rpfB* and *rpfC* genes that were amplified by PCR using the primers *rpfB*-C-F (5'- GTGTTCGCCACGTCGGTAAAA-3') and *rpfB*-C-R (5'-TTACGTTTTCGGCGCGTCCCG-3') or *rpfC*-C-F (5'-ATGAAGTCTCCACTGACATG-3') and *rpfC*-C-R (5'-CTATTCGCTCCGGGGGG-3') were inserted into the T&A Cloning Vector (RBC Real Biotech Co.). The *rpfB* and *rpfC* genes were then excised and inserted into pML122 (Labes et al., 1990) using the appropriate restriction enzymes, and the resulting plasmids were introduced into the *rpfB* and *rpfC* mutant strains by electroporation.

Xylanase assay. Xylanase activity was determined by using 4-O-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (RBB-Xylan; Sigma Co.) as described (Biely et al., 1988). *Xoo* KACC10859 and the *rpf* mutants were cultured in NB for 72 hours, after which the optical density of the bacterial cultures was adjusted to 1.0 at 600 nm with NB. One ml of each culture supernatant was incubated at 26°C for 30 min with 480 μl of an assay mixture containing 5 mg of RBB-xylan per ml and 25 mM MES (pH 5.8). After 30 min, the reactions were stopped by the addition of 960 μl of ethanol. After incubation at room temperature for 40 min, the assay mixtures were centrifuged for 5 min at 15,000×*g* and the absorbances of the supernatants at 590 nm were measured.

Cellulase assay. Cellulase activities were assayed as described (Chatterjee et al., 1995). *Xoo* KACC10859 and the mutants were cultured in NB medium for 72 h, after which the optical density of the cultures was adjusted to 1.0 at 600 nm with NB. Thirty microliters of culture supernatant were placed in a hole in the assay agar medium, which contained 0.1% carboxymethyl cellulose, 50 mM sodium phosphate (pH 7.0), 0.8% agarose, and 0.02% sodium azide, and the plate was incubated for 20 h at 28°C. The incubated plates were stained with 0.1% Congo Red for 10 min and then washed several times with 1 M NaCl. After washing, the cellulase activity was determined by measuring the diameter of the clear zone around the hole.

Measurement of exopolysaccharide (EPS). A single colony of each mutant and the wild type was inoculated in

Table 4. Cellulase activities of *Xoo* KACC10859 and null mutant strains

Strain	Halo[a] diameter (mm)
KACC10859 (Wild type)	13.3±1.3 a
CBNXO02 (*rpfB*::Tn5)	12.8±1.5 a
CBNXO03 (*rpfC*::Tn5)	12.9±1.4 a
CBNXO05 (*rpfF*::Tn5)	13.0±1.2 a
CBNXO06 (*rpfG*::Tn5)	13.1±1.5 a
CBNXO07 (*rpfI*::Tn5)	12.8±1.4 a
CBNXO08 (*rpfB* complemented with pML122*rpfB*)	13.1±0.9 a
CBNXO09 (*rpfC* complemented with pML122*rpfC*)	12.9±1.1 a

[a]Means with the same letter are not significantly different at the 5% level based on Duncan's multiple range test.

40 ml of NB medium and incubated for 72 h at 28°C with agitation. The optical density of the bacterial cultures was adjusted to 1.0 at 600 nm with NB. The culture supernatants were transferred to new 50-ml tubes and supplemented with 1.0% potassium chloride (w/v; final concentration). Two volumes of absolute ethanol were added to each solution, and the tubes were placed at –20°C overnight. The precipitated crude EPS was collected by centrifugation for 30 min at 83,000×*g*. The EPS pellets were dried at 55°C for 12 h and the dry weight of each was measured.

Motility assay. Ten microliters of bacterial suspension were placed on Peptone Sucrose soft agar containing 0.3% agar, and the plates were incubated at 28°C for 48 h. After two days, the diffusion of the bacteria on the plate was observed.

Virulence assay. Using the scissor-clip method (Kauffman et al., 1973), bacterial suspensions (10^8 CFU ml^{-1}) of *Xoo* KACC10859 and the *rpf* mutants were inoculated on plants of the rice line Milyang 23ho that had been grown in a greenhouse for six weeks. The lesion lengths were measured 14 days after inoculation. The averages and standard deviations of the lesion lengths on 20 rice leaves per test were calculated.

Expression analysis of virulence and pathogenicity genes. Real time RT-PCR was performed with SYBR® Premix Ex *Taq*™ (TaKaRa Bio Inc.) using a Smart Cycler® II system (TaKaRa Bio Inc.). One microgram of RNA isolated from bacterial cells cultured in NB was used for cDNA synthesis with a QuantiTect® Reverse Transcription Kit (Qiagen). The cDNA was treated with DNase-free RNase for 10 min at 42°C and then immediately used as a template for PCR. The sequences of all of the primers used in this study are listed in Table 3. PCR was carried out with an initial denaturation step at 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 5 sec, primer annealing at 56°C for 15 sec, and extension at 72°C for 20 sec. The

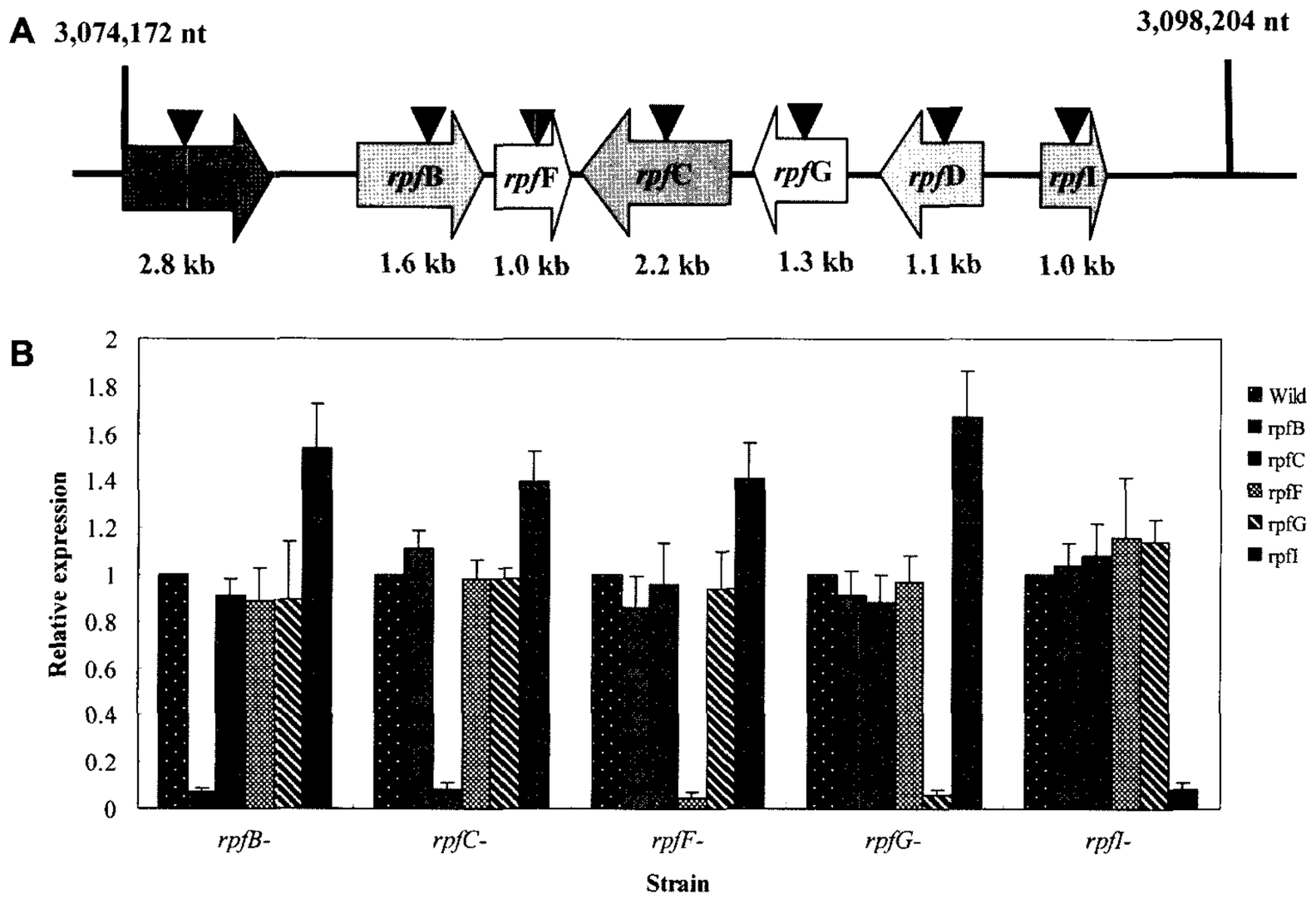

Fig. 1. Schematic representation of mutations in the *rpf* genes of *Xoo* KACC10859 (A). Triangles (▼) indicate the Tn5 insertion site. Expression of the mutated *rpf* gene in the corresponding mutant was analyzed by RT-PCR (B).

quantity of the target mRNA synthesized was calculated using Qubit™ ver 1.01 (Invitrogen) with 16S rRNA as an internal control.

Relative expression of RNA was calculated by SmartCycler® 3.0 software.

Results

Reduction of virulence by mutation of the *rpf* genes. To determine the functions of the *rpf* genes, each gene was inactivated by Tn5 insertion and marker exchange in *Xoo* KACC10859 (Fig. 1A). The mutation of *rpf* gene was confirmed by RT-PCR in the 5 selected *rpf* mutants (Fig. 1B). Expression of the mutated *rpf* genes was decreased significantly in the corresponding mutant (Fig. 1B), which indicates that the mutation of the *rpf* gene by marker exchange was successful. Inoculation of the CBNXO02 (*rpf*B::Tn5), CBNXO03 (*rpf*C::Tn5), CBNXO05 (*rpf*F::Tn5), and CBNXO06 (*rpf*G::Tn5) mutants onto the susceptible rice cultivar Miyang 23ho resulted in lesion lengths significantly smaller than those caused by the wild type (Figs. 2 and 3). In contrast, the lesions caused by CBNXO01 (*rpf*A::Tn5), CBNXO04 (*rpf*D::Tn5), and CBNXO07 (*rpf*I::Tn5) were larger than those caused by the above four mutants (Figs. 2 and 3). Complementation of the *rpfB* and *rpfC* mutants in the strains CBNXO08 (*rpfB* complemented

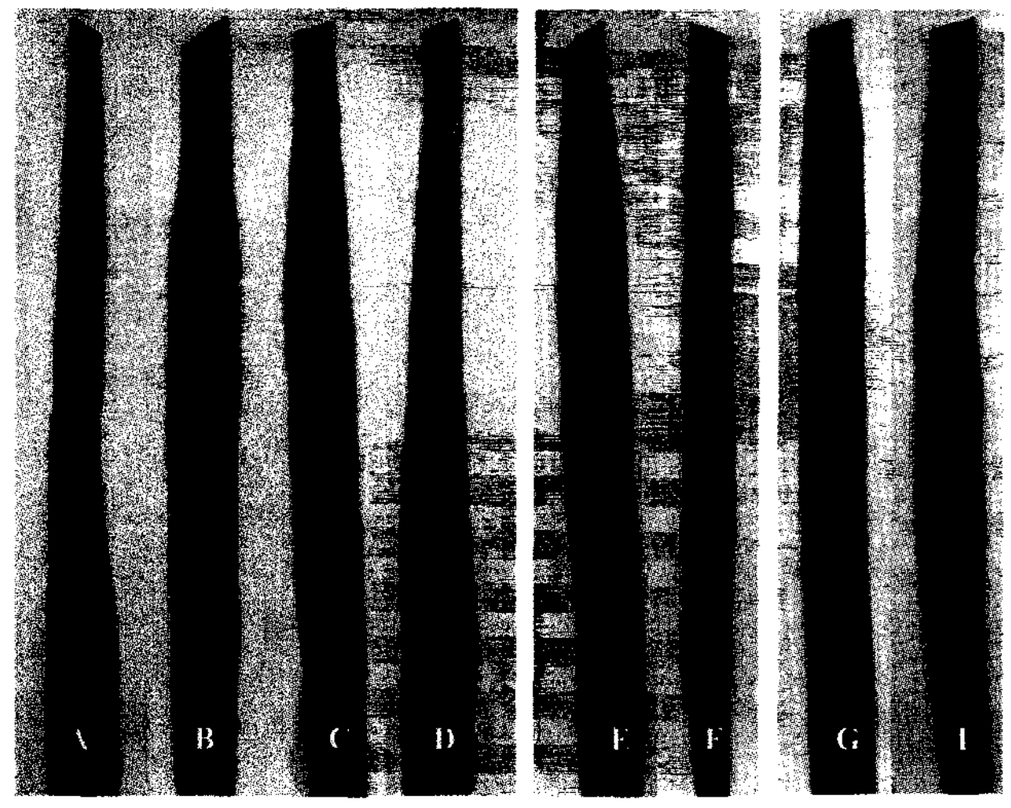

Fig. 3. Lesions on Milyang 23ho leaves inoculated with KACC-10859 (**A**); its null mutant strains CBNXO02 (**E**, *rpfB*::Tn5), CBNXO03 (G, *rpfC*::Tn5), CBNXO05 (**B**, *rpfF*::Tn5), CBNXO-06 (**C**, *rpfG*::Tn5), or CBNXO07 (**D**, *rpfI*::Tn5); or its complementation strains CBNXO08 (**F**, *rpfB* complemented with pML122*rpfB*) or CBNXO09 (**I**, *rpfC* complemented with pML122*rpfC*) at 14 days after inoculation.

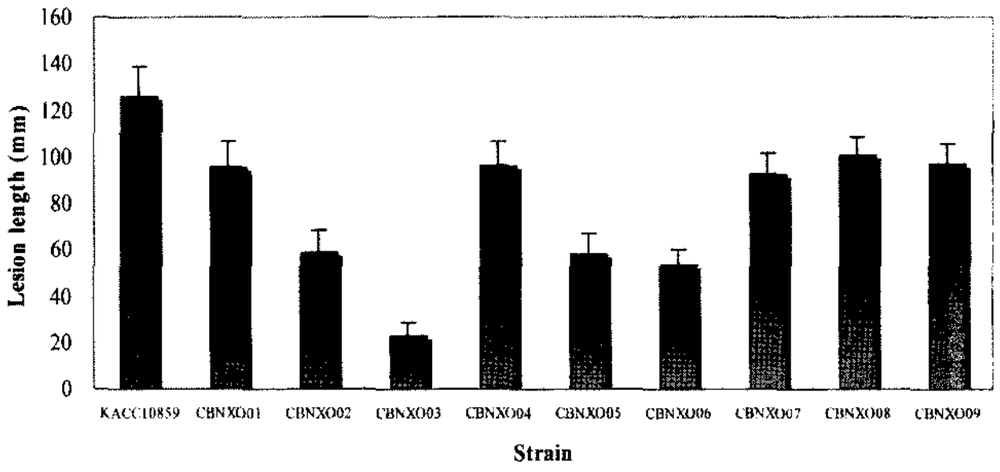

Fig. 2. Lesion lengths on Milyang 23ho leaves inoculated with KACC10859; its null mutant strains CBNXO01 (*rpfA*::Tn5), CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO04 (*rpfD*::Tn5), CBNXO05 (*rpfF*::Tn5), CBNXO06 (*rpfG*::Tn5), or CBNXO07 (*rpfI*::Tn5); or its complementation strains CBNXO08 (*rpfB* complemented with pML122*rpfB*) or CBNXO09 (*rpfC* complemented with pML122*rpfC*) at 14 days after inoculation. Means and standard deviations were calculated from 20 leaves per inoculated strain.

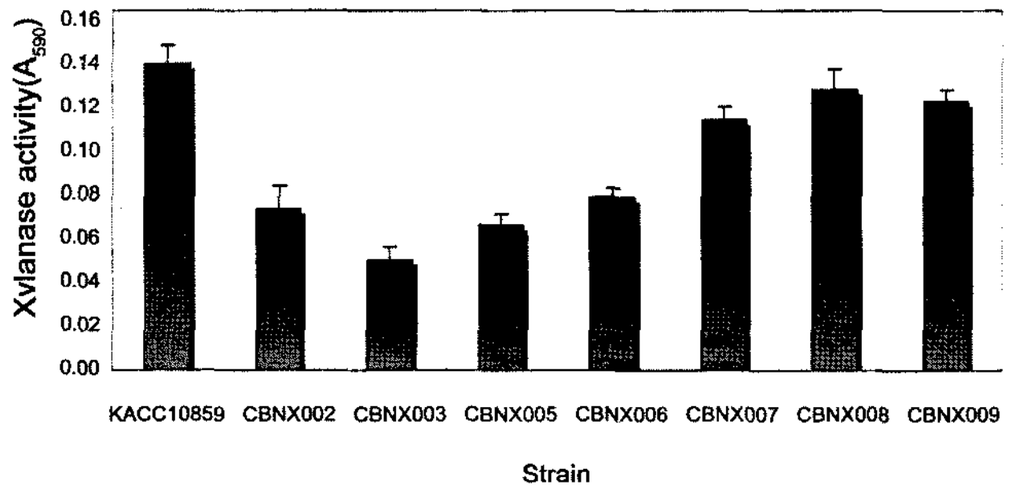

Fig. 5. Xylanase activities of *Xoo* KACC10859; its null mutant strains CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO05 (*rpfF*::Tn5), CBNXO06 (*rpfG*::Tn5), and CBNXO07 (*rpfI*::Tn5); and its complementation strains CBNXO08 (*rpfB* complemented with pML122*rpfB*) and CBNXO09 (*rpfC* complemented with pML122*rpfC*) cultured in nutrient broth. Means and standard deviations were calculated from three independent experiments.

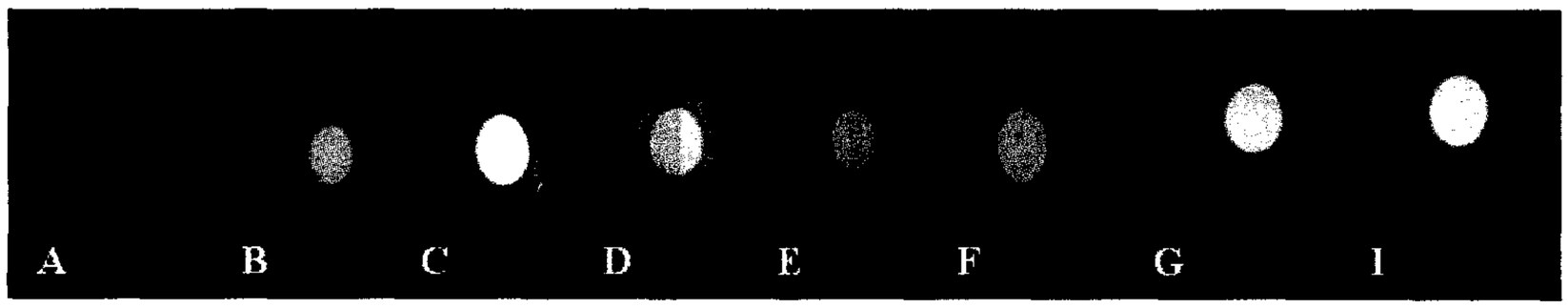

Fig. 4. Motilities of *Xoo* KACC10859 (**A**); the null mutant strains CBNXO02 (**B,** *rpfB*::Tn5), CBNXO03 (**C**, *rpfC*::Tn5), CBNXO05 (**D**, *rpfF*::Tn5), CBNXO06 (**E**, *rpfG*::Tn5), and CBNXO07 (**F**, *rpfI*::Tn5); and its complementation strains CBNXO08 (**G**, *rpfB* complemented with pML122*rpfB*) and CBNXO09 (**I**, *rpfC* complemented with pML122*rpfC*).

with pML122*rpfB*) and CBNXO09 (*rpfC* complemented with pML122*rpfC*) restored the virulence of the strains (Figs. 2 and 3). These results indicate that inactivating the genes encoding RpfB, RpfC, RpfF, or RpfG causes significantly reduced virulence in *Xoo* KACC10859. The *rpfC* mutation had the most effect on the virulence (Fig. 3).

Xylanase and cellulase activities, EPS levels, and motility of the *rpf* mutants. Several virulence-related phenotypes were examined in the *rpf* mutants. The xylanase activity, EPS, and motility were greatly reduced in CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO05 (*rpfF*::Tn5), and CBNXO06 (*rpfG*::Tn5), as compared to the wild type, but the virulence in the complementation strains CBNXO08 (*rpfB* complemented with pML122*rpfB*) and CBNXO09 (*rpfC* complemented with pML122*rpfC*) was at the level of the wild type (Figs. 4, 5, and 6). The xylanase activity, EPS production, and motility of CBNXO07 (*rpfI*::Tn5) were similar to those of the wild type (Figs. 4, 5, and 6). Colonies of CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO05 (*rpfF*::Tn5), and CBNXO06 (*rpfG*::Tn5) were less mucoid and shiny than those of KACC10859 (wild type), CBNXO01 (*rpfA*::Tn5), CBNXO04 (*rpfD*::Tn5), or CBNXO07 (*rpfI*::Tn5) (Fig. 7). These results indicate that the mutations in *rpfB*, *rpfC*, *rpfF*, *and rpfG* reduce the expression of important

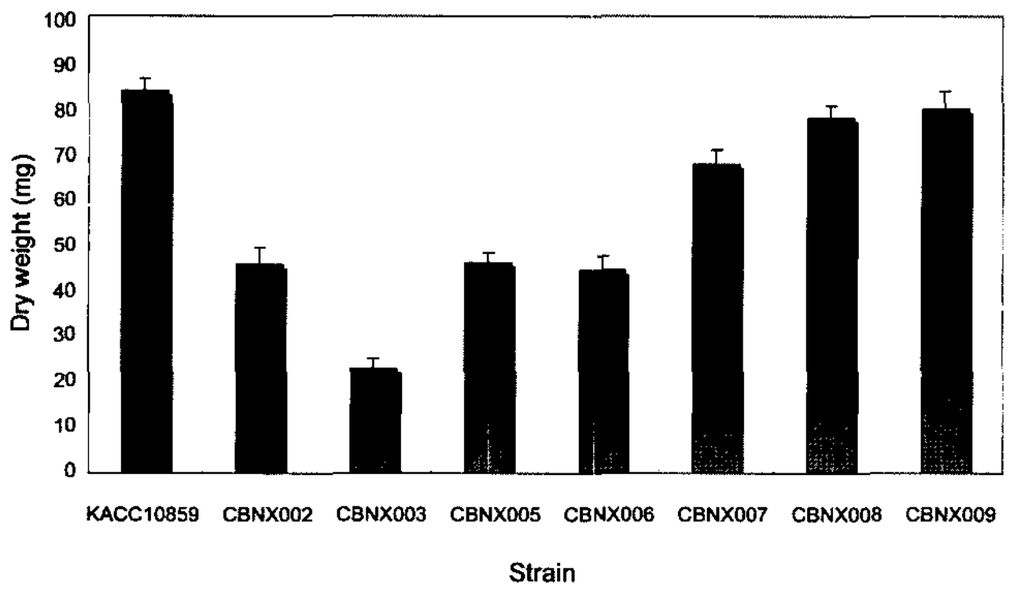

Fig. 6. EPS dry weights of *Xoo* KACC10859; its *rpf* null mutant strains CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO05 (*rpfF*::Tn5), CBNXO06 (*rpfG*::Tn5), and CBNXO07 (*rpfI*::Tn5); and its complementation strains CBNXO08 (*rpfB* complemented with pML122*rpfB*) and CBNXO09 (*rpfC* complemented with pML122*rpfC*) cultured in nutrient broth. EPS was precipitated from 40 ml of culture of an optical density of 1.0 at 600 nm and dried. Means and standard deviations were calculated from three independent experiments.

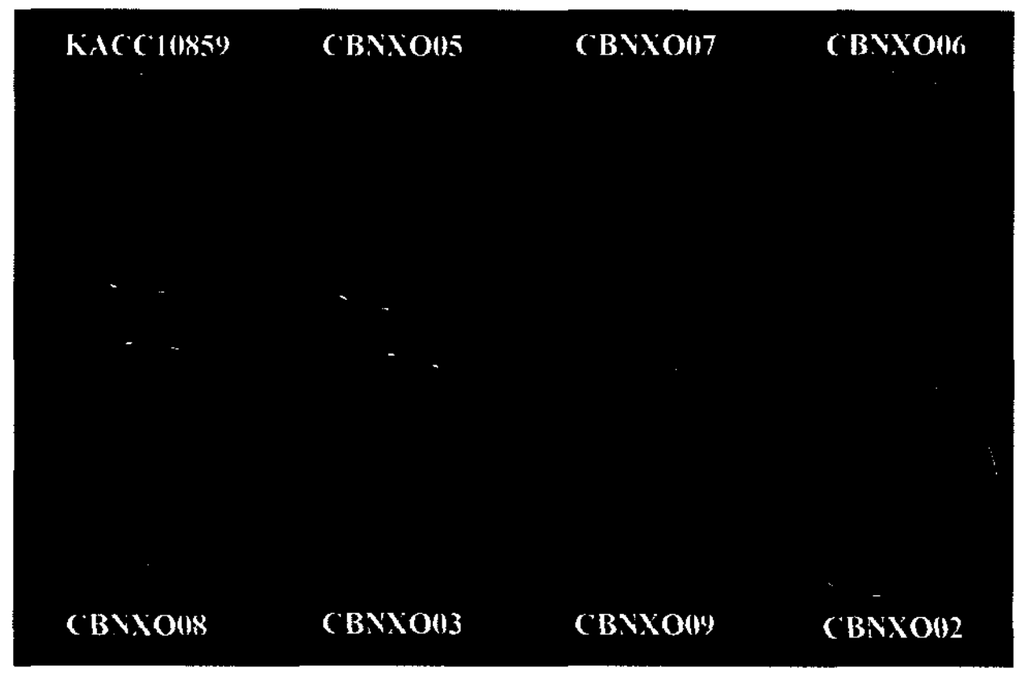

Fig. 7. Colony morphologies of *Xoo* KACC10859; the *rpf* null mutant strains CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO05 (*rpfF*::Tn5), CBNXO06 (*rpfG*::Tn5), or CBNXO07 (*rpfI*::Tn5); or the complementation strains CBNXO08 (*rpfB* complemented with pML122*rpfB*) or CBNXO09 (*rpfC* complemented with pML122*rpfC*). The bacterial strains were cultured for four days on PS agar plates.

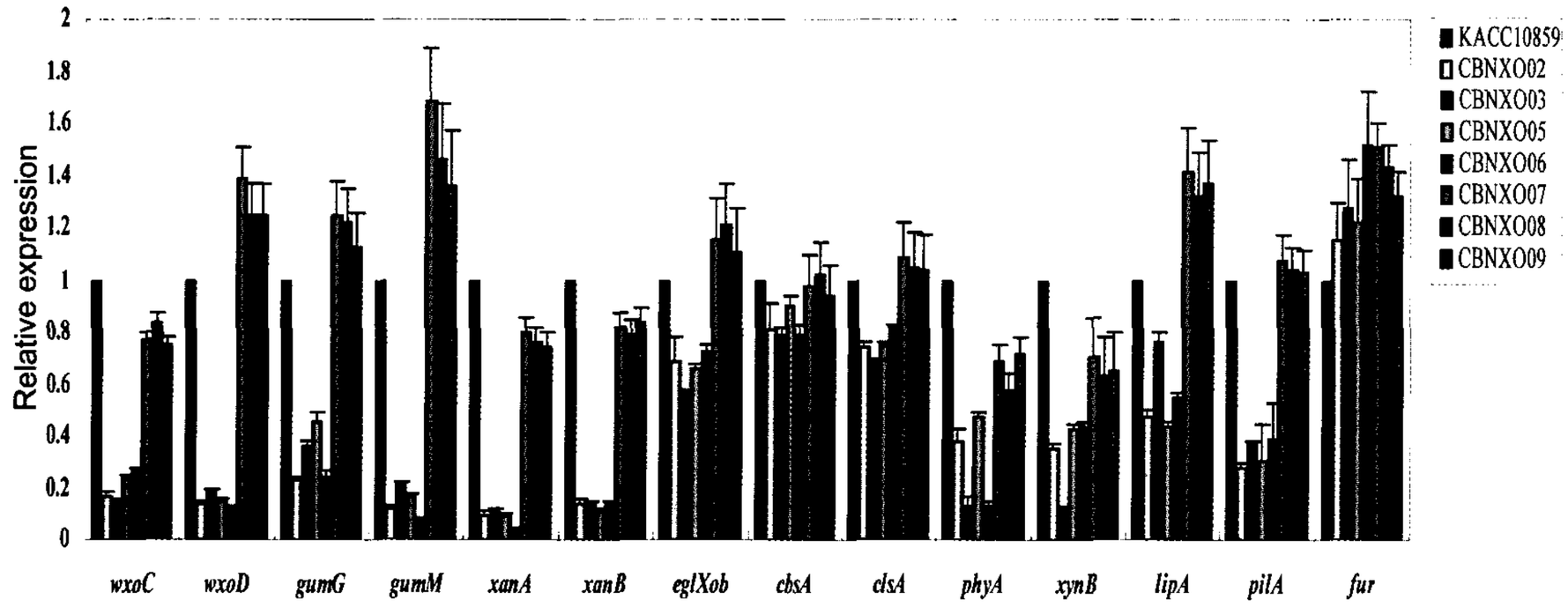

Fig. 8. Expression of virulence-related genes in *Xoo* KACC10859; the *rpf* null mutant strains CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO05 (*rpfF*::Tn5), CBNXO06 (*rpfG*::Tn5), or CBNXO07 (*rpfI*::Tn5); or the complementation strains CBNXO08 (*rpfB* complemented with pML122*rpfB*) or CBNXO09 (*rpfC* complemented with pML122*rpfC*) cultured in nutrient broth. The genes that were analyzed were *gumG, gumM, wxoD*, and *wxoC* (EPS synthesis); *xanA* and *xanB* (LPS synthesis); *phyA*, *eglXob*, *xynB clsA*, *cbsA*, and *lipA* (extracellular enzymes); *pilA* (movement in the vascular system); and *fur* (iron metabolism and growth).

virulence factors, including the xylanase activity, EPS levels, and motility. In contrast, the cellulase activities of the *rpf* mutants and the complementation strains were similar to those of the wild-type.

Virulence gene expression in the *rpf* mutants. The expression of genes encoding proteins related to EPS (*gumG* and *gumM*), LPS (*xanA*, *xanB*, *wxoD*, *wxoC*), extracellular enzymes (*phyA*, *eglxob*, *xynB*, *clsA*, *cbsA*, *lipA*), motility (*pilA*), and iron metabolism (*fur*), all of which are virulence and pathogenicity factors, were analyzed by RT-PCR in *Xoo* KACC10859 and the *rpf* mutants cultured in NB medium. The expression of genes encoding EPS (*gumG* and *gumM*), LPS (*xanA*, *xanB*, *wxoD*, *wxoC*), phytase (*phyA*), xylanase (*xynB*), lipase (*lipA*), and a motility factor (*pilA*) were reduced significantly in CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO05 (*rpfF*::Tn5), and CBNXO06 (*rpfG*::Tn5), but not in CBNXO07 (*rpfI*::Tn5) (Fig. 8). The expression levels of these genes were restored to those of the wild type in the complementation strains CBNXO08 ($rpfB^c$) and CBNXO09 ($rpfC^c$) (Fig. 8). In contrast, the expression of genes related to cellulase (*eglxob*, *clsA*), cellobiosidase (*cbsA*), and iron metabolism (*fur*) was unchanged in these *rpf* mutants (Fig. 8). These results indicate that several genes related to EPS (*gumG* and *gumM*), LPS (*xanA*, *xanB*, *wxoD*, *wxoC*), phytase (*phyA*), xylanase (*xynB*), lipase (*lipA*), and motility (*pilA*) are regulated by Rpfs in *Xoo* KACC10859 cultured in NB. However, the genes related to cellulase (*eglxob*, *clsA*), cellobiosidase (*cbsA*), and iron metabolism (*fur*) are not controlled by Rpfs. The colony morphology, EPS levels, xylanase and cellulase activities, and motility of the *rpf* mutants support these results: the EPS level, xylanase activity, and motility of CBNXO02 (*rpfB*::Tn5), CBNXO03 (*rpfC*::Tn5), CBNXO05 (*rpfF*::Tn5), and CBNXO06 (*rpfG*::Tn5) were reduced, but those of the CBNXO07 (*rpfI*::Tn5) strain and the cellulase activities of all of the *rpf* mutants were unchanged.

Discussion

In this study, mutations in *rpfB*, *rpfC*, *rpfF*, and *rpfG* significantly reduced the virulence of *Xoo* KACC10859, but mutations in *rpfA*, *rpfD*, and *rpfI* did not. A comparison of these *rpf* genes and their homologs in whole genomes of other Xanthomonads and *Xylella fastidiosa* (*Xf*), which is closely related to Xanthomonads, showed that *rpfB*, *rpfC*, *rpfF*, and *rpfG* are highly conserved (Lee et al., 2006). The role of *rpfE*, a gene that is well conserved in all Xanthomonads and *Xylella fastidiosa* (*Xf*), remains to be determined, as several attempts to obtain an *rpfE* mutant were unsuccessful. In *Xcc*, in which the roles of Rpfs have been studied extensively, the above four genes have been reported to be core genes in the regulation of pathogenicity: *rpfB* and *rpfF* produce DSFs, and *rpfC* and *rpfG* transfer signals from the DSFs to virulence or pathogenicity genes (Barber et al., 1997; Slater et al., 2000). The results of this study clearly show that *rpfB*, *rpfC*, *rpfF*, and *rpfG* are important for virulence in *Xoo* KACC10859.

Expression profiles of virulence genes and the phenotypes of the *rpf* mutants showed that different virulence genes are regulated differently by Rpfs in *Xoo*. The reduced expression of genes related to EPS, LPS, phytase, xylanase, lipase, and motility in the *rpfB*, *rpfC*, *rpfF*, and *rpfG* mutants suggests that the DSFs produced by RpfB and RpfF are required for the expression of virulence genes, and signal transfer by RpfC and RpfG is also required. In *Xcc*, the roles of Rpfs in the regulation of EPS and extracellular enzyme production are well documented (Barber et al., 1997; He et al., 2006; Slater et al., 2000). Tang et al. (1996) reported that EPS production was reduced in an *rpfC* mutant of *Xoo*, and Chatterjee and Sonti (2002) reported that EPS and xylanase production were normal in an *rpfF* mutant, even though the virulence of the mutant was reduced. Analysis of the colony morphology, xylanase activity, and gene expression in the *rpf* mutants in our study clearly showed that EPS production and xylanase were reduced in both the *rpfC* and *rpfF* mutants. The differences between these results and those of Chatterjee and Sonti could be due to different culture media and *Xoo* strains, although the results cannot be compared directly because no data were presented in Chatterjee and Sonti's paper. High concentrations of sucrose in the medium, such as in the PS medium used by Chatterjee and Sonti, have been reported to result in high background in assays of some extracellular enzyme activities (He et al., 2006). NB, which does not contain sucrose, results in a more consistent optical density for *Xoo*, and was used in this study for assays of extracellular enzymes and EPS. The amounts of EPS and extracellular enzymes produced are also known to vary greatly depending on the *Xcc* strain. He et al. (2006) reported that one *rpfF* mutant strain of *Xcc* produces more EPS and protease than another wild-type *Xcc* strain.

There was no difference in the cellulase activities of *Xoo* KACC10859 and the *rpf* mutants. The expression of two cellulase genes, *eglxob* and *clsA*, was normal in the *rpf* mutants cultured in NB, which supports the cellulase activity results. Hu et al. (2007) suggested that *eglxob* is regulated through a PIP (plant inducible promoter) box *in planta*.

The results of this study suggest that different virulence genes are regulated differently in *Xoo* KACC10859. One group of virulence genes, including genes related to EPS, LPS, phytase, xylanase, lipase, and motility, is regulated by Rpfs, but some genes related to cellulase, cellobiase, and

iron metabolism are not regulated by Rpfs. Recently, it has been reported that more regulatory factors downstream of the signal transduction from a two-component system consisting of *rpfC* and *rpfG* regulate different sets of genes in *Xcc* (He et al., 2007). More study is needed of the signal transduction pathway downstream of *rpfC* and *rpfG* and the regulation of the genes not regulated by Rpfs, including the regulation mechanism *in planta*, to better understand the regulation of virulence gene expression in *Xoo*.

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