Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Temuujin and Kang, J Plant Pathol Microbiol 2017, 8:8
DOI: 10.4172/2157-7471.1000416

Research Article Open Access

Expressional Regulation of the Virulence Gene eglXoA Encoding Endoglucanase, Dependent on HrpXo and Cyclic AMP Receptor-Like Protein (Clp) in *Xanthomonas oryzae* pv. *oryza*

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Abstract

An *eglXoA* encoding endoglucanase that clustered with *eglXoB and eglXoC* in *Xanthomonas oryzae* pv. *oryza* genome (accession No. AE013598) is a pathogenicity related gene. RT-PCR showed that the *in trans eglXoA* was transcriptionally regulated by *HrpX*, a type III secretion regulator, and cyclic AMP receptor-like protein in *X. oryzae* pv. *oryzae* (*ClpXo*), which has been known as a global regulator. Western blot analysis showed that *EglXoA* is secreted *via* a type II secretion system and was detected in wild-type strain KACC10859, but not in the mutant strains *hrpX*::Tn5 and *clpXo*::Tn5. In an electrophoretic mobility shift assay, the promoter region of *eglXoA* directly bound to *ClpXo*. The two consensus *eglXoA* upstream regions were found to include putative Clp-binding sites with a perfect TCACA-N block in the left arm and a 2/5 matched block, TGT, in the right arm. *eglXoA*, which encodes endoglucanase, appears to be the first gene of *Xoo* known to be activated by *ClpXo via* direct binding to the promoter region. Molecular interaction between *HrpX* and *ClpXo* shows that *ClpXo* acts as transcription regulator of *hrpX* and binds to promoter region of *hrpX*.

Keywords: *ClpXo*; *eglXoA* gene; Expression; *HrpX*; *Xanthomonas oryzae* pv. *oryzae*

Introduction

Xanthomonas oryzae pv. *oryzae* (*Xoo*), which causes bacterial leaf blight, is the most economically important bacterial disease in rice. Bacterial blight is prevalent in many rice-growing countries [1]. During pathogenesis, plant cell walls act as the first barrier of defense against bacterial invasion and multiplication. Nevertheless, the enzymatic activities of Cell Wall-Degrading Enzymes (CWDEs) may facilitate pathogen invasion into the cells of host plants by digesting the plant cell walls [2-4]. Research regarding CWDEs in phytopathogenic bacteria has mainly focused on enzymatic activities, the identification of genes encoding them, and their roles in virulence [5,6]. The type II secretion system (T2SS) allows most gram-negative bacteria to secrete extracellular hydrolytic enzymes and toxins [7,8], many of which are responsible for pathogenesis in plants, into their surroundings and hosts. CWDEs such as cellulases, pectinases, xylanases, and proteases are secreted by plant pathogens to degrade the components of host cell walls and may play a crucial role in virulence and bacterial nutrition [9,10]. Currently, genes encoding CWDEs such as cellulase and xylanase are thought to play a role in the virulence of *Xoo* [11-14]. The T2SS-related gene cluster consists of 11 genes, *xpsEFGHIJKLMND,* in the *Xoo* genome. Mutations in the *xpsD* and *xpsF* structural genes of the *Xoo* T2SS reduce virulence and cause xylanase accumulation in the periplasmic space [5,12].

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 plants [15]. The *hrp* gene cluster in *Xoo* is composed of 27 genes, from *hrpA*2 to *hrpF* [16], and the expression of these genes is regulated by two regulators, *hrpG* and *hrpX*, which are separate from the *hrp* gene cluster [17]. HrpG belongs to the OmpR family and activates the expression of *hrpX*, an AraC-like transcription activator that controls *hrp* genes along with some effector proteins [17,18]. Moreover, *HrpX*o regulates the transcriptional expression of genes associated with T2SS proteins such as cysteine proteases [19]. Recently, it was reported that polygalacturonase and extracellular proteases in *X. campestris* pv. *campestris* (*Xcc*) are regulated by *HrpX* [20,21]. These reports suggest that *HrpX* can potentially regulate expression of other genes in addition to the *hrp* genes. It has been reported that purified cellulase and lipase induce defense responses in rice that are suppressible by *Xoo* in a T3SS-dependent manner [22]. Therefore, it is plausible that genes encoding CWDEs can participate in diverse virulence functions associated directly or indirectly with the expression of key pathogenicity-related genes, such as *hrp* genes.

Catabolite-Activator Protein (CAP), also called the cAMP receptor protein (CRP)-like protein (Clp), belongs to the CRP/FNR superfamily of transcriptional factors, which is one of the largest groups of bacterial environmental sensors [23-25]. Also, it has been known to be a global transcriptional regulator for the expression of virulence factors in *Xcc*. Clp transcriptionally activates more than 150 genes, including those that encode for extracellular enzymes and the production of exopolysaccharides (EPS), and other macromolecules such as flagellin and Hrp proteins [24]. Clp contains nucleotide- and DNA-binding domains and binds to the promoters of an endoglucanase (*engA*) from Xcc [26].

Recently, 12 genes that encode cellulases, including endoglucanases and exoglucanases, were isolated from the *Xoo* genome and mutated, and novel pathogenicity-related cellulase genes were identified and characterized [14]. Interestingly, the *eglXoABC* genes arranged as a cluster in genome of *X. oryzae* pv. *oryzae.* Of them, it was revealed that transposon insertion mutant of *eglXoA* and *eglXoA* displayed virulence-

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Received July 26, 2017; **Accepted** August 12, 2017; **Published** August 15, 2017

Citation: Temuujin U, Kang HW (2017) Expressional Regulation of the Virulence Gene eglXoA Encoding Endoglucanase, Dependent on HrpXo and Cyclic AMP Receptor-Like Protein (Clp) in *Xanthomonas oryzae* pv. *oryza*. J Plant Pathol Microbiol 8: 416. doi: 10.4172/2157-7471.1000416

deficient phenotype, but not in the *eglXoC*. However, little is known about expressional regulation of *eglXo* genes in the pathogenesis of *Xoo*. The goal of this study was to elucidate expressional regulation of *eglXoA*. We demonstrate that *HrpX* and *ClpXo* act as regulators of expression of *eglXoA*. Furthermore, the electrophoretic mobility shift assay (EMSA) showed that *ClpXo* binds to the promoter region of *eglXoA*.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type strain *Xoo* KACC10859 was obtained from the Korean Agricultural Culture Collection (KACC) at the National Institute of Agricultural Biotechnology, Suwon, Korea. *Xoo* strains were cultured at 28°C on peptone sucrose agar (PSA: peptone, 10 g/L; sucrose, 10 g/l; and agar, 15 g/L) or XOM2 medium [27]. *E. coli* was grown in Luria-Bertani (LB) broth (Difco, Detroit, MI, USA) at 37°C for 18 h. Antibiotics were added at the following final concentrations for *E. coli* and *Xoo*, respectively: ampicillin, 80 μg/mL and 50 μg/mL; gentamycin, 50 μg/mL and 20 μg/mL; and kanamycin, 50 μg/mL and 20 μg/mL.

Pathogenicity assays

Inoculums (approximately 1×10^6 cells/ml) prepared from wild-type and mutant strains of *Xoo* were grown on PSA for 3 days. Pathogenicity assays were performed on 60-days-old leaves of a susceptible rice cultivar (Milyang 23) by the leaf-punching method by Temujin et al. [14]. Pathogenicity was observed at 14 days post inoculation.

Northern blot analysis

Xoo strains were first incubated in NB to OD600=1.0 and then collected by centrifugation. The pelleted cells were washed twice with XOM2 media and resuspended in XOM2 to OD_{600}=0.5 and cultured in XOM2 to OD_{600}=1.0. The total RNAs were extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and electrophoresed. Probes were labeled with the DIG Northern Starter Kit (Roche) according to the manufacturer's protocol. Hybridization signals were detected on exposure of the samples to X-ray film (Fujifilm, Tokyo, Japan).

Reverse transcription (RT)-PCR analysis

The transposon mutants and wild-type *Xoo* KACC10859 were cultured in nutrient broth (NB) until OD_{600}=0.5, pelleted by

Bacterial strains, plasmids and PCR primers	Characteristics	References
Bacterial strains		
E. coli BL21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdSλ DE3=λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	Lab collection
Xanthomonas oryzae pv. *oryzae* strains		
KACC10859	Wild type strain, Korean race 1	KACC
eglxoA::Tn5	Transposon insertion in *eglXoA* of KACC10859, Km^r	This study
clpXo::Tn5	Transposon insertion in *clpXo* of KACC10859, Km^r	This study
hrp X::Tn5	Transposon insertion in *hrpX* of KACC10859, Km^r	This study
xps F::Tn5	Transposon insertion in *xpsF* of KACC10859, Km^r	This study
CeglXoA	*eglXoA::Tn5* harboring pMLeglXoA, Gm^r Km^r	
Plasmids		
pGEM-TEasy vector	T- cloning vector, Amp^r	Promega
pQE-80L	Overexpression vector, Ampr	Lab collection
pQE-clpXo	pQE-80L harboring clpXo, Ampr	This study
pET15b	Overexpression vector, Ampr	Novagen
pET-eglXoA	pET15b harboring *eglxo*A, Amp^r	This study
pML122	Broad-host-range vector, Gm^r	Lab collection
pMLeglxoA	pML122 harboring *eglxo*A	This study
Primers for RT-PCR		
eglXoA	F: 5'-GCA TCC ATC GAG AGA AAC CAC-3' R: 5'-CAA TAG CGT GAA CTG CCT TC-3'	This study
hrpX	F: 5'-AGG AGC AGT TTC GCG AAC TC-3' R: 5'-TCT GCG TCC TGC TCA TCC AA-3'	This study
xpsF	F: 5'-GTT GCG CAA GAA GCC GTT CG-3' R: 5'-GTG CCACAT CCA GGC TTT CG-3'	This study
clpXo	F: 5'-GGT TGT GAC TAC GAC GGT AC-3' R: 5'-GCT TCC GGC TCT TTG GAA AG-3'	This study
16S rDNA	F: 5'-TCG TGA TCG CGACCG TAA CC-3' R: 5'-GTT GAG CTC CTC CAC CTT CT-3'	This study
Primer for probes used in EMSA		
hrpX	Probe 1: F: 5'-CTT ACA TAA CGG GCA TGT GGG-3' Probe 2: F: 5'-CTG CCG CTC ATC ATT AAG CCA-3' Probe 3: F: 5'-GAC GTG CTC GTT TGA GAA CAG-3' R: 5'- CAA CGC AGA GAT CGC TGC AAA-3'	This study
eglXoA	Probe 1: F: 5'- GTG CTC ATC TGA AAA CTC CGG -3' Probe 2: F: 5'- CGC AGA GAA AGG ATC GAT AGC -3' Probe 3: F: 5'- ACG CAG CAG CCG ATC ACC CTG -3' R: 5'- CAG GCC AGC GGT TTC CTT CTT -3'	This study
Km^r: Kanamycin Resistant; Amp^r: Ampicillin Resistant; KACC: Korea Agricultural Culture Collection		

Table 1: Bacterial strains, plasmids and PCR primers used in the study.

centrifugation at 3,000 g for 10 min, and washed with distilled water. The bacterial cells were suspended in 5 mL of XOM2 medium and additionally cultured in a shaking incubator (180 rpm) at 28°C for 36 h. Total RNA was extracted by Trizol Reagent according to manufacturer's instructions and treated with RNase-free water. Then, DNase I (Promega, Madison, WI, USA) was used to remove potential traces of DNA according to the manufacturer's instructions. The cDNA synthesis and PCR were conducted using a SuperScript First-Strand RT-PCR kit (Invitrogen) with the RT-PCR primers listed in Table 1 under the following conditions: 1 cycle of 1 min at 94°C; 30 cycles of 30 sec at 94°C, 30 secs at 60°C, 1 min at 72°C; and a final extension cycle of 10 min at 72°C. PCR products were visualized in agarose gels by staining with ethidium bromide.

Overexpression and purification of *EglXoA* and *ClpXo*

eglXoA was amplified from genomic DNA of *Xoo* using a forward primer (*eglXoA*-F: 5′-CAGAATCTCATATGTCCAACCGCACCAC-3′) containing an *Nde*I restriction site (underlined) at the start codon of the ORF and a reverse primer (*eglXoA*-R: 5′-CTGCTCGAGTCAATTTTGATTCACCAAC-3′) containing an *Xho*I restriction site after the stop codon. The PCR amplicons were double digested with *Nde*I and *Xho*I, ligated into the pET-15b expression vector (Novagen) containing a 6× His tag upstream of a thrombin cleavage site and the multiple cloning site, and transformed into *E. coli* BL21 (DE3) pLysS, yielding the recombinant clone pET-eglA. To construct the *clpXo* overexpression vector, *clpXo* was amplified with the forward primer containing a *BamH*I restriction site (underlined) at the start codon of the ORF *clpXo*-F: (5′-GGATCCATGAGCTCAGCAAAC-3′) and the reverse primer *clpXo*-R: (5′-AAGCTTTTAGCGCGTGCCGTA-3′) containing a *Hind* III restriction site spanning the stop codon. The PCR product was digested with *BamH*I and *Hind* III, ligated in the pQE-80L vector, and then transformed into *E. coli* BL21 (DE3) pLysS, yielding recombinant clone pQE-*ClpXo*. For protein overexpression of *EglXoA* and *ClpXo* in *E. coli*, clones pET-*eglXoA* and pQE-*ClpXo* were grown in 1 L of LB liquid medium containing ampicillin at 37°C until OD_{600}=0.5 and overexpression was induced by adding 0.5 mM IPTG for 3 h. To purify *EglXoA*, the bacterial cells were pelleted and suspended in 200 mL buffer (20 mM Tris, 5 mM imidazole, pH 8.0), sonicated for 2 min at 20 kHz and the acoustic power ranged between 35 W and 95 W, and centrifuged at 6,500 g for 15 min. The supernatant was loaded on a column packed with nickel-nitrilotriacetic acid (Ni-NTA) equilibrated with buffer solution (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 8.0 M urea, pH 8.0). The column was first washed with the same buffer containing 50 mM imidazole and the fusion protein was then eluted using a 250-mM imidazole in the same buffer. To purify *ClpXo*, the bacterial cells were pelleted and suspended in 200 mL buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0), sonicated 6 times, and centrifuged at 6,500 g for 15 min. The pelleted cells were washed with 0.5% Triton X-100 in 1 × PBS and solubilized by the buffer. The mixture was centrifuged at 6,500 g for 15 min, and the supernatant purified using an Ni-NTA column as for *EglXoA*.

Western blot analysis

The 30 N-terminal amino acids (NH2-LILYQKNAKAAELSKKILGLQAQDLPGNLA) corresponding to residues 98 to 127 in *EglXoA* were deduced from nucleotide sequence data and synthesized, and antiserum against the peptide was commercially produced (Peptron, Daejeon, Korea). To isolate, extraction of extracellular secreted proteins from bacterial cells, *Xoo* strains were precultured in 5 mL NB media for 3 days and pelleted and then suspended in 30 mL XOM2 liquid media and cultured additionally to OD_{600}=0.8. The bacterial cells were harvested by centrifugation at 15,000 rpm for 20 min and further filtrated by membrane filter (0.45 μm pore size) to remove remnant cells. The supernatant was precipitated by 30% ammonium sulfate addition on ice for 30 min. After centrifugation at 15,000 rpm for 30 min, protein precipitates were washed with ester and suspended in 1/30 original volume of 50 mM Tris-HCl (pH 8.0) and resuspended in 2x Laemmli buffer. Protein samples were boiled for 5 min and separated by 10% of sodium dodecyl sulfate SDS-PAGE by CBB and Silver staining methods. For western blot analysis, protein samples from different *Xoo* strains were electrophoresed in 12% SDS-PAGE gels and transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Little Chalfont, UK). The blotted membrane was incubated at room temperature for 2 h in a blocking solution and then hybridized with anti-EglXo antibody. After 3 washes with the washing solution, the PVDF membrane was incubated with anti-rabbit secondary antibody HRF (GE Healthcare) for 1 h according to the manufacturer's protocol. The signals on the membrane were detected by exposure to X-ray film (Fujifilm).

Electrophoretic mobility shift assay

The DNA probes used for the EMSA were prepared by PCR amplification of the desired regions of the *eglXoA* promoter under the following conditions: 1 cycle of 4 min at 94°C; 30 cycles of 30 secs at 94°C, 30 secs at 58°C, 1 min at 72°C; and a final extension cycle of 10 min at 72°C, using 5′-end biotin-labeled synthetic oligonucleotides as the primers in Table 1. The amplicons were purified from agarose gels and used for gel-shift experiments. The EMSA reaction mixture (10 μL) contained ca. 1.0 pmol of biotin-labeled probe and various amounts of *ClpXo* in a 5 × binding buffer containing 1 μg of nonspecific competitor DNA poly d(I-C) (Panomics, Inc). Following incubation at room temperature for 5 min, the DNA-protein complexes were resolved by electrophoresis in a 6% non-denaturing polyacrylamide gel in 0.5 × TBE buffer (40 mM Tris-Cl, pH 8.3, 45 mM boric acid, 1 mM EDTA). The gel was transferred onto a positively charged Hybond N^+ nylon membrane (GE Healthcare) for 30 min at 300 mA. After UV cross-linking, biotinylated probes in the membrane were detected corresponding to protocol provided by the EMSA Gel Shift Kit (Panomics, Inc).

Results

eglXoA is essential for the virulence and its expression is non-polar

The virulence of the *eglXoA* mutant strain assayed on leaves of a susceptible rice variety, Milyang 23. The degree of pathogenicity was checked in 14 days after inoculation. Figure 1, shows the disease symptom of brown stripes was observed on rice leaf inoculated with the wild-type strain. In contrast, the mutant strains *eglXoA*::*Tn5* was virulence deficient. Mutation was confirmed by complementation with the entire sole *eglXoA* gene. The complemented *X. oryzae* pv. *oryzae* strain *CeglXoA*::*Tn5* was constructed by introducing the recombinant plasmid pML*eglxoA* containing the entire wild-type *eglXoA* gene into the mutant *eglXoA*::*Tn5* (Table 1). Consequently, *CeglXoA*::*Tn5* recovered virulence and produced disease lesions similar to those of the wild-type strain KACC10859 (Figure 1), suggesting that *eglXoA* is essential for the virulence.

The *eglXoA* was located upstream *eglXoB* and it was observed long intergenic regions of 682 bp between *eglXoA* and *eglXoB* and 649 bp between *eglXoB* and *eglXoC* (Figure 1). The transcriptional linkage in the gene cluster was analyzed by RT-PCR. Two cDNA products synthesized from total RNA samples of *eglXoA*::*Tn5* and wild-type

strain KACC10859 were used as template. The primer pair targeting *eglXoABC* genes amplified RT-PCR products of the predicted length in the wild-type RNA sample, while mutation in *eglXoA* did not affect transcriptional expression of *eglXoB* and *eglXoC*, as they continued to generate RT-PCR products (Figure 2). These results reasonably assumed that each *eglXoA, eglXoB,* and *eglXoC* is monocistronically transcribed without polar expressional fashion dependent on a promoter.

eglXoA expression is regulated by *HrpX* and *ClpXo*

In this experiment, the *in trans* transcriptional regulation of *eglXoA* was investigated with regard to *HrpX* and *ClpXo*. The total RNA samples were extracted from the mutants and wild-type strain KACC10859 after cultivation in *hrp*-inducing XOM2 medium. RT-PCR analysis was performed by using primer pairs targeting *eglXoA*. The primer set targeting 16S rRNA, which was used as a positive control, amplified relatively intense RT-PCR bands from the wild-type strain as well as the *hrpX*::*Tn5* and *clpXo*::*Tn5* mutants. On the other hand, the primer pair targeting *eglXoA* amplified the RT-PCR band only in the wild-type strain KACC10859 RNA sample and not in those of the *hrpX*::Tn5 and *clpXo*::Tn5 mutants (Figure 3A). These results indicate that transcriptional expression of *eglXoA* is regulated by *hrpX* and *clpXo*.

An N-terminal peptide consisting of 30 amino acids corresponding to residues 98 to 127 of the *EglXoA* protein (357 amino acids) was inferred from the nucleotide sequence of *eglXoA* (1,074 bp), which was artificially synthesized, and polyclonal antibodies were raised against

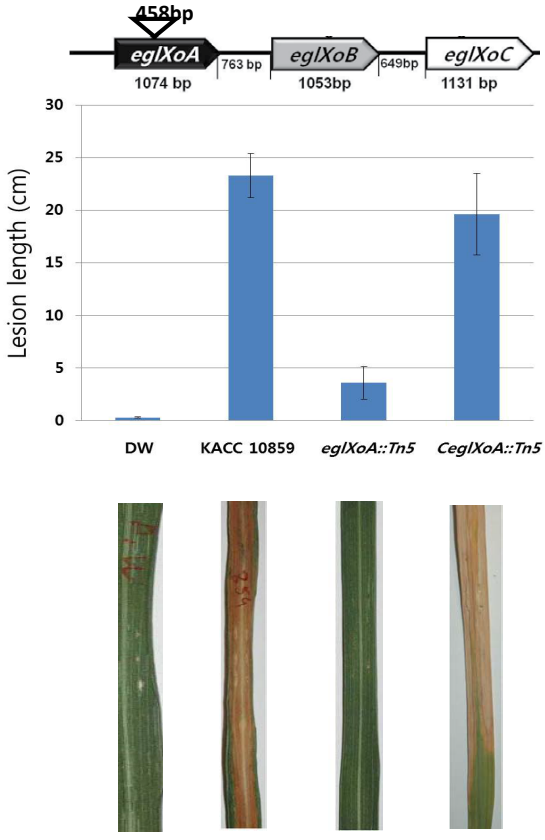

Figure 1: Pathogenicity assay of *eglXoA* in a gene cluster encoding endoglucanases of *Xanthomonas oryzae* pv. *oryzae*. The mutant strain *eglXoA*::*Tn5*, wild type strain KACC10859 and complement strain pMLeglA were inoculated on leaves of rice variety, Milyang 23 using leaf-punching method and pathogenicity was checked after 14 days of inoculation. Distilled water (DW) was used as negative control.

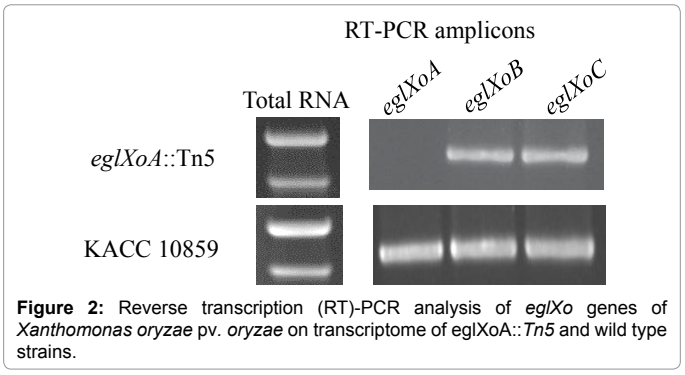

Figure 2: Reverse transcription (RT)-PCR analysis of *eglXo* genes of *Xanthomonas oryzae* pv. *oryzae* on transcriptome of eglXoA::*Tn5* and wild type strains.

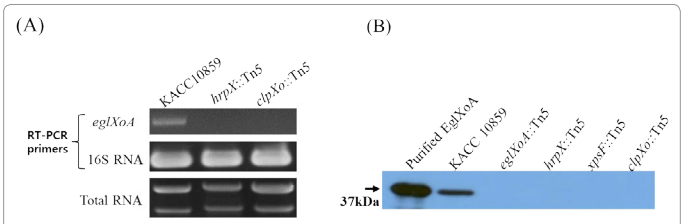

Figure 3: Expression of *eglXoA* dependent on HrpXo and ClpXo. Total RNA samples were extracted from wild-type strain KACC10859, *hrpX*::Tn5, and *clpXo*::Tn5. RT-PCR was conducted using a primer pair targeting *eglXoA*. (A) For western blot analysis, (B) the extracellular proteins were obtained from different *Xanthomonas oryzae* pv. *oryzae* strains cultured in XOM2 medium that were blotted onto PVDF membranes and then probed with an anti-EglXoA antibody.

the peptide. The wild-type strain KACC10859 as well as *xpsF*::*Tn5*, *clpXo*::*Tn5*, and *hrpX*::*Tn5* mutant strains were cultured in XOM2 medium. Their extracellular proteins were isolated, resolved using SDS-PAGE, and then transferred to PVDF membranes. In western blot analysis using anti-*EglXoA* antibodies, the predicted 37-kDa band was detected in the positive controls, the extracellular protein of the wild-type strain, and the purified protein, but not in extracellular proteins of the *xpsF*::*Tn5*, *hrpX*::*Tn5*, *clpXo*::*Tn5*, and *eglXoA*::*Tn5* mutants (Figure 3B). The putative signal peptide consisting of 38 amino acids was observed on deduced sequence of *eglXoA* (Figure 3). Consequently, these findings suggest that *EglXoA* is a secreted protein dependent on T2SS and regulated by *HrpX* and *ClpXo.*

ClpXo binds to the promoter region of *eglXoA*

RT-PCR and western blot analyses showed that *ClpXo* regulates transcriptional expression of *eglXoA* and correspondingly that the gene encoding the protein was not expressed. It has been reported that the Clp protein directly binds to the promoter regions of the endoglucanase (*engA*) and polygalacturonase (*pheA*) genes of *X. campestris,* presumably enhancing transcriptional expression [26,28]. Therefore, it is reasonable to assume that *ClpXo* can also bind to the promoter region of *eglXoA*. EMSA was employed to evaluate the binding between soluble *ClpXo* protein and the promoter region of *eglXoA*. To obtain this protein, *clpXo* (693 bp) was subcloned into plasmid pQE-80L, yielding the recombinant clone pQE-Clp, which was then overexpressed in *E. coli*. The soluble *ClpXo* protein (27.7 kDa) was obtained by purification with a His-binding affinity column.

Promoter regions (probe A, −316/+127; probe B, −263/+127; and probe C, −85/+127) upstream of *eglXoA* were generated by nested PCR (Figure 4A). The EMSA was performed with 50 ng of *ClpXo* protein per reaction. The protein-DNA complex was electrophoresed in a 6% non-denaturing polyacrylamide gel, blotted onto a nylon membrane, and hybridized with biotin-labeled promoter regions. A

probe B-*ClpXo* protein complex was formed, which migrated more slowly than the unbound probe B (Figure 4B). *ClpXo* do not bind to reside in the region −85/+127 and thus the region −256/−85 appears to possess the complete sequence for the binding of *ClpXo*. The *E. coli* CRP-binding site (5′-AAATGTGA-N6-TCACATTT-3′) is 22-bp long, exhibiting perfect 2-fold sequence symmetry, with the bold-faced bases representing the left and right arms for the binding of one subunit of the active CRP dimer. Two putative Clp-binding sites, including a perfect TCACA-N block in the right arm and a 2/5 matched block, TGT, in the left arm, were found from −133 to−153 sequences (Figure 4C).

ClpXo regulates transcriptional expression of *hrpX*

In our results, it was revealed genes, *hrpX* and *clpXo* are closely related to *eglXoA* expression. However, it was wonder how *hrpX* and *clpXo* is interracially associated on each gene expression. To investigate expressional control on both genes, Northern blot hybridization was done by using probes *hrpX* and *clpXo* against total RNA samples that were extracted from the *hrpX*::Tn5 and *clpXo*::Tn5 mutants and wild-type strain. Probe *clpXo* hybridized to RNA samples of wild-type and *hrpX*::Tn5 strains, but not to *clpXo*::Tn5 (Figure 5). However, probe *hrpX* detected hybridized signal on wild-type strain KACC10858, but not on the *clpXo*::Tn5 and *hrpX*::Tn5 mutant strains, suggesting *clpXo* regulates transcriptional expression of *hrpX*. Furthermore, the EMSA was conducted to determine if the promoter region of the *hrpX* gene binds to *ClpXo*, three types promoter regions (-539/+9, -429/+9 and -238/+9) generating probes A, B and C reacted with *ClpXo* protein of 50 ng. The protein-DNA complex showing retarded gel migration observed on probe A and B, but not on probe C (Figures 6A and 6B), suggesting *ClpXo* directly binds to promoter region of *hrpXo*. The putative *ClpXo* binding sequence sites with TGTCG-N-TCACA, including a perfect TCACA block in the right arm and a 4/5 matched block, TGTCG, in the left arm, were found in the −388/−409 sequences (Figure 6C).

Discussion

Novel pathogenicity-related genes including *eglXoA,* which encodes endoglucanase, have been isolated and characterized [14]. Whole genome sequence information of *Xoo* KACC10331 is available on NCBI's GenBank. *eglXoA* (1074 bp), *eglXoB* (1133 bp), and *eglXoC* (1131 bp) are organized as a cluster in the same region of the *Xoo* KACC10331 genome [16]. *EglXoA* was classified into cellulase family 5, which exhibits endo-1,4-glucanase activities, and had identity over 88.8% to endoglucanase genes, *egl1* from *X. campestris* pv. *vesicatoria* [14].

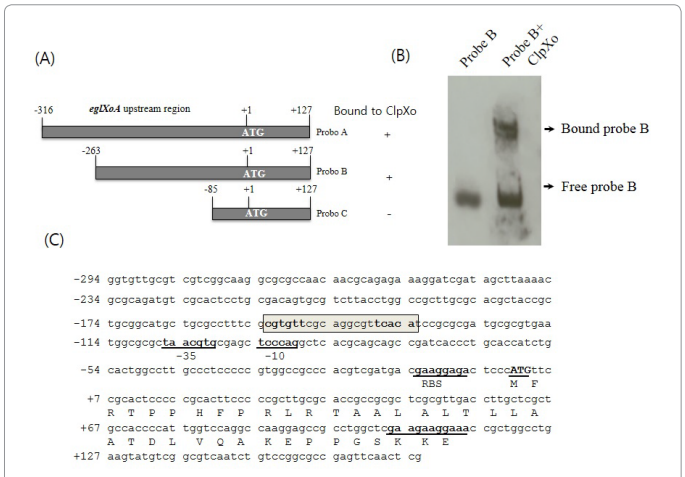

Figure 4: Electrophoretic mobility shift assay (EMSA) evaluating the binding of the *eglXoA* promoter region to the ClpXo protein. Different promoter regions upstream of *eglXoA*, probes A, B, and C, (A) bound to *ClpXo*. The probe B-*ClpXo* complex was retarded in SDS-PAGE, (B) The promoter sequence upstream of *eglXoA,* (C) includes putative ClpXo-binding sites (Boxed sequence), predicted consensus sequence, −10/−35, putative Ribosome-Binding Site (RBS). Amino acids of N-teminal; indicate putative signal peptide.

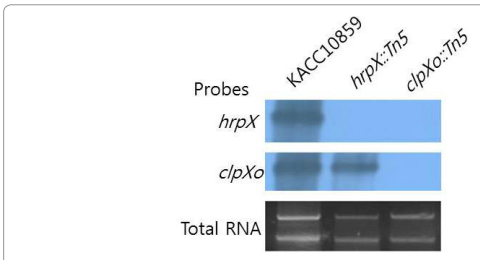

Figure 5: Northern blot profile of *X. oryzae* pv. *oryzae* strains using probes *hrpX* and *clpXo*. Total RNA was extracted from wild-type KACC10859 and mutants *hrpX*::Tn5 and *clpXo*::Tn5. For northern blot analysis, total RNA was loaded in each lane of 1.5% formaldehyde agarose gels, blotted onto nylon membranes, and hybridized with *hrpX* and *clpXo* genes.

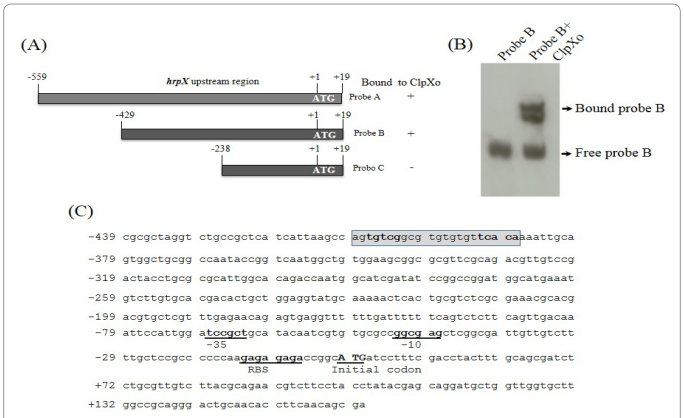

Figure 6: Electrophoretic Mobility Shift Assay (EMSA) for evaluating the binding of *hrpX* promoter region using probe ClpXo. The promoter regions (−539/+19) upstream *hrpX,* (A) was reacted with ClpXo protein (50 ng), the ClpXo protein-DNA complex was electrophoresed in a polyacrylamide gel, and the DNA-protein complex formation was observed as a retardation of migration in the gel, (B) The sequence region upstream hrpX, (C) includes ClpXo-binding sites (boxed sequence), predicted consensus sequence, −10/−35 and putative ribosome-binding site (RBS); capitalized sequence: initial codon (ATG).

Currently, genes encoding CWDEs such as cellulase and xylanase are thought to play a role in the virulence of *Xoo* [12,14,29]. It has been reported that purified cellulase and lipase proteins induce defense responses in rice that are suppressible by *Xoo* in a T3SS-dependent manner [22]. Therefore, cellulase genes may play a role in diverse virulence traits that are directly or indirectly associated with the expression of key pathogenicity-related genes, such as *hrp* genes.

HrpX is the key regulator in the T3SS that controls the expression of *hrp* and some effector genes. Previous studies have revealed that expression of the virulence genes *pghAxc* and *pghBxc*, which encode extracellular polygalacturonase in *Xcc*, are regulated by the T3SS regulator *HrpX* [20]. It was also reported that *hrpX* negatively regulates the α-amylase isozymes in *X. axonopodis* pv. *citri* [29] and extracellular proteases in *Xcc* [21]. In *Xoo*, only a gene encoding the extracellular T2SS enzyme Cysp2, which is related to pathogenicity, has so far been demonstrated to be regulated by *HrpX* [19]. These reports support that *HrpX* is also involved in regulating the expression of some T2SS-related extracellular enzymes. Using RT-PCR, we discovered that transcriptional products of *eglXoA* from *Xoo* did not amplify in the *hrpX*

mutant, showing *HrpX*-dependent expression. This finding strongly indicates that *HrpX* is the key regulator of *eglXoA* expression. The *HrpX* regulons of *Xanthomonas* species include a consensus sequence motif called the PIP box (TTCGC-N15-TTCGC) around the promoter regions [30,31]. However, the PIP box was not observed in *eglXoA*. The PIP box can be an effective marker for screening *HrpX* regulons from the entire genomic sequence database, and several of these regulons are predicted to be involved in the pathogenicity of xanthomonads and *R. solanacearum* [31]. Twelve and 20 candidate genes for *HrpX* regulons, which did not include the genes in *hrp* clusters, were found in *Xanthomonas campestris* pv. *campestris* and *Xanthomonas axonopodis* pv. *citri* [32], respectively. However, genes with an imperfect PIP box and genes without a PIP box have been found to be expressed in an *HrpX*-dependent manner [33]. This regulation by *HrpX* indicates that the signal transduction networks of pathogens are cross-linked and that the T3SS and T2SS may cooperate *via* various regulators to promote virulence of the pathogen in the host. *Xanthomonas* protein secretion (*xps*) genes encode structural proteins that form the T2SS, which is essential for the secretion of T2SS extracellular enzymes [11,12]. Immunoblot analysis using anti-*EglXoA* antibodies in this study provided direct evidence of T2SS/Xps-dependent secretion in the culture media of wild-type *Xoo* and *xps* mutants.

The transcription factor Clp is a member of a conserved global-regulator family that regulates the expression of approximately 300 genes involved in pathogenesis of *Xanthomonas* spp. [23]. Clp is a homologue (45% amino acid sequence identity) of the model transcription factor CRP of *E. coli*. Clp in *Xcc* also influences the expression of a number of genes, especially the genes in the T2SS [23,24]. The *clp* gene in *Xoo* was isolated and characterized [34], and a mutation in *clpXo* resulted in a significant decrease in the production of cellulase, xylanase, and EPS. Moreover, a previous study demonstrated the direct binding of Clp to promoter regions [26]. The data from the present study indicate that *eglXoA* is regulated by *ClpXo*, which is consistent with previous *Xcc* studies that show that Clp is involved in the expression of extracellular enzymes of the T2SS [24,26]. Experimental evidence was provided by EMSA, wherein the *ClpXo-eglXoA* promoter region complex showed gel retardation, indicating that *ClpXo* directly binds to the *eglXoA* promoter region. In sequence analysis, a potential Clp-binding site (GTGTT-N9-TCACA) was identified in the *eglXoA* promoter region. The Clp in *X. campestris* is homologous to the CRP of *E. coli*. It was reported that Clp upregulates the transcription of endoglucanase-encoding *engA* in *X. campestris* by direct binding to the upstream region of Clp. Two consensus Clp-binding sites were determined on the *engA* promoter region by site-directed mutagenesis. In this study, two putative Clp-binding sites, including a perfect TCACA-N block in the left arm and a 2/5 matched block in the right arm, TGT, were found in sequence upstream of *eglXoA*. *ClpXo* bound to the promoter region that possesses the Clp-binding sites, whereas the promoter region that does not contain Clp-binding sites does not result in a DNA-protein complex, assuming that these sites are responsible for *ClpXo* binding to DNA. The transcriptional regulator Clp contains nucleotide- and DNA-binding domains that bind to promoters of target genes; DNA binding of Clp from *X. axonopodis* pv. *citri* is inhibited *in vitro* by cyclic di-GMP [35]. In *X. campestris* pv. *campestris*, Clp induces the expression of genes belonging to the diffusible signal factor (DSF) regulon, which encodes extracellular enzymes, components of T2SS and T3SS, and genes involved in EPS synthesis. Previous findings regarding DSF-dependent quorum sensing, including the transcriptional self-regulation of Clp, depict a detailed DSF signaling model of the regulation of bacterial virulence. Consequently, the cellular level of free Clp increases, then the regulator acts as a positive transcription factor to induce its own gene transcription and virulence gene expression.

Conclusion

In present study, we concluded that two regulatory genes, *hrpX* and *clpXo*, are interracially associated with expression of the endoglucanase gene *eglXoA* of *X. oryzae* pv. *oryzae*. Furthermore, we investigated the interplay between *hrpX* and *clpXo*. In northern blot analysis, *clpXo* mutant inhibited transcriptional expression of *hrpX*, suggesting *ClpXo* acts as transcription regulator of *hrpX* expression. In EMSA, we found that the *hrpX* promoter region directly binds to the *ClpXo* protein (Figure 6B). In conclusion, our results suggest that *ClpXo* leads to dual regulation by binding to the promoter regions of *hrpX* and *eglXoA*. In the future, it would be interesting to evaluate how the binding target sequences of *ClpXo* play a role in regulating expression of *eglXoA*, either as an activator or an enhancer.

References

1. Ezuka A, Kaku H (2000) A historical review of bacterial blight of rice. Bull Natl Inst Agrobiol Resour Japan 15:1-207.
2. Mäe A, Heikinheimo R, Palva ET (1995) Structure and regulation of the *Erwinia carotovora* subspecies carotovora SCC3193 cellulase gene *celV1* and the role of cellulase in phytopathogenicity. Mol Gen Genet 247: 17-26.
3. Saile E, McGarvey JA, Schell MA, Denny TP (1997) Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathol 87: 1264-1271.
4. Vian B, Reis D, Gea L, Grimault V (1996) The plant cell wall, first barrier or interface for microorganism: In site approaches to understanding interactions. In: Nicole M, Gianinazzi-Pearson V (eds). Histology, ultrastructure and molecular cytology of plant-microorganisms interactions. Kluwer Academic Publisher, Dordrecht, Netherlands. pp. 99-115.
5. Kang HW, Park YJ, Lee BM (2009) Functional description and identification of virulence related genes from whole genome sequence of *Xanthomonas oyzae* pv. oryzae KACC10331. Kor J Microbiol 44: 1-9.
6. Liu H, Zhang S, Schell MA, Denny TP (2005) Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol Plant Microbe Interact 18: 1296-1305.
7. Pugsley AP (1993) The complete general secretory pathway in gram-negative bacteria. Microbiol Rev 57: 50-108.
8. Pugsley AP, Francetic O, Possot OM, Sauvonnet N, Hardie KR (1997) Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in gram- negative bacteria- A review. Gene 192: 13-19.
9. Barras F (1994) Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu Rev Phytopathol 32: 201-234.
10. Jahr H, Dreier J, Meletzus D, Bahro R, Eichenlaub R (2000) The endo-beta-1,4-glucanase CelA of *Clavibacter michiganensis* sub sp. michiganensis is a pathogenicity determinant required for induction of bacterial wilt of tomato. Mol Plant Microbe Interact 13: 703-714.
11. Hu NT, Hung MN, Chiou SJ, Tang F, Chiang DC, et al. (1992) Cloning and characterization of a gene required for the secretion of extracellular enzymes across the outer membrane by *Xanthomonas campestris pv. campestris*. J Bacteriol 174: 2679-2687.
12. Rajeshwari R, Jha G, Sonti RV (2005) Role of an in planta-expressed xylanase of *Xanthomonas oryzae pv. oryzae* in promoting virulence on rice. Mol Plant Microbe Interact 18: 830-837.
13. Sun QH, Hu J, Huang GX, Ge C, Fang RX, et al. (2005) Type-II secretion pathway structural gene xpsE, xylanase- and celllulase secretion and virulence in *Xanthomonas oryzae pv. oryzae*. Plant Pathol 54: 15-21.
14. Temuujin U, Kim JW, Kim JK, Lee BM, Kang HW (2011) Identification of novel pathogenicity-related cellulase genes in *Xanthomonas oryzae pv. oryzae*. Physiol Mol Plant Pathol 76: 152-157.
15. Buttner D, Bonas U (2010) Regulation and secretion of Xanthomonas virulence factors. FEMS Microbiol Rev 34:107-133.

16. Lee BM, Park YJ, Park DS, Kang HW, Kim JG, et al. (2005) The genome sequence of *Xanthomonas oryzae* pathovar oryzae KACC10331, the bacterial blight pathogen of rice. Nuc Acids Res 33: 577-586.

17. Tsuge S, Furutani A, Kaku H (2006) Gene involved in transcriptional activation of the hrp regulatory gene hrpG in *Xanthomonas oryzae pv. oryzae*. J Bacteriol 188: 4158-4162.

18. Wengelnik K, Bonas B (1996) HrpXv, an AraC-type regulator, activates expression of five of the six loci in the hrp cluster of *Xanthomonas campestris pv. vesicatoria*. J Bacteriol 178: 3462-3469.

19. Furutani A, Tsuge S, Ohnishi K, Hikichi Y, Oku T, et al. (2004) Evidence for HrpXo-dependent expression of type II secretory proteins in *Xanthomonas oryzae pv. oryzae*. J Bacteriol 186: 1374-1380.

20. Wang L, Wei R, Chaozu H (2008) Two Xanthomonas extracellular polygalacturonases, *PghAxc* and *PghBxc*, are regulated by type III secretion regulators *HrpX* and *HrpG* and are required for virulence. Mol Plant Microbe Interact 21: 555-563.

21. Wei K, Tang JL (2007) *hpaR*, a putative marR family transcriptional regulator, is positively controlled by *HrpG* and *HrpX* and involved in the pathogenesis, hypersensitive response, and extracellular protease production of *Xanthomonas campestris pv. campestris*. J Bacteriol 189: 2055-2062.

22. Jha G, Rajeshwari R, Sonti RV (2007) Functional interplay between two *Xanthomonas oryzae pv. oryazae* secretion systems in modulating virulence on rice. Mol Plant Microbe Interact 20: 31-40.

23. Chin KH, Lee YC, Tu ZL, Chen CH, Tseng YH, et al. (2010) The cAMP receptor-like protein CLP is a novel c-di-GMP receptor linking cell–cell signaling to virulence gene expression in *Xanthomonas campestris*. J Mol Biol 396: 646-662.

24. He YQ, Zhang L, Jiang BL, Zhang ZC, Xu RQ, et al. (2007) Comparative and functional genomics reveals genetic diversity and determinants of host specificity among reference strains and a large collection of Chinese isolates of the phytopathogen *Xanthomonas campestris pv. campestris.* Genome Biol 8: R218.

25. Körner H, Sophia J, Zumft WG (2003) Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: Exploiting the metabolic spectrum by controlling alternative gene programs. FEMS Microbiol Rev 27: 559-592.

26. Hsiao YM, Liao HY, Lee MC, Yang TC, Seng YH (2005) Clp upregulates transcription of engA gene encoding a virulence factor in *Xanthomonas campestris* by direct binding to the upstream tandem Clp sites. FEBS Lett 579: 3525-3533.

27. Tsuge S, Furutani A, Fukunaka R, Oku T, Tsuno K, et al. (2002) Expession of *Xanthomonas oryzae pv. oryzae* hrp genes in a novel synthetic medium, XOM2. J Gen Plant Pathol 68: 363-371.

28. Hsiao YM, Zheng MH, Hu RM, Yang TC, Tseng YH (2008) Regulation of the *pehA* gene encoding the major polygalacturonase of *Xanthomonas campestris* by *Clp* and *RpfF*. Microbiology 154: 705-713.

29. Hu J, Qian W, He C (2007) The *Xanthomonas oryzae pv. oryzae* eglXoB endoglucanase gene is required for virulence to rice. FEMS Microbiol Lett 269: 273-279.

30. Furutani A, Nakayama T, Ochiai H, Kaku H, Kubo Y, et al. (2006) Identification of novel *HrpXo* regulons preceded by two cis-acting elements, a plant-inducible promoter box and a 10 box-like sequence, from the genome database of *Xanthomonas oryzae pv. oryzae*. FEMS Microbiol Lett 259: 133-141.

31. TsugeS, Terashima S, Furutani A, Ochiai H, Oku H, et al. (2005) Effects on promoter activity of base substitutions in the cis-acting regulatory element of *HrpXo* regulons in *Xanthomonas oryzae pv. oryzae*. J Bacteriol 187: 2308-2314.

32. Da Silva ACR, Ferro JA (2002) Comparison of genomes of two Xanthomonas pathogens with differing host specificities. Nature 417: 459-463.

33. Noe¨l L, Thieme F, Ninnstiel D, Bonas U (2001) cDNA-AFLP analysis unravels a genome-wide hrpG-regulon in the plant pathogen Xanthomonas. Mol Microbiol 41: 1271-1281.

34. Cho JH, Jeong KS, Han JW, Kim WJ, Cha JS (2011) Mutation in *clpxoo4158* reduces virulence and resistance to oxidative stress in *Xanthomonas oryzae pv. oryzae* KACC10859. Plant Pathol J 27: 89-92.

35. Leduc JL, Roberts GP (2009) Cyclic di-GMP allosterically inhibits the CRP-like protein (Clp) of *Xanthomonas axonopodis* pv. citri. J Bacteriol 91: 7121-7122.