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Expression of *colSR* Genes Increased in the *rpf* Mutants of *Xanthomonas oryzae* pv. *oryzae* KACC10859

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The *rpf* genes and *colS*$_{XOO1207}$/*colR*$_{XOO1208}$ were known to require for virulence of *Xanthomonas oryzae* pv. *oryzae* (Xoo). In Xoo KACC10331 genome, two more *colS/colR* genes, *colS*$_{XOO3534}$ (*raxH*)/*colR*$_{XOO3535}$ (*raxR*) and *colS*$_{XOO3762}$/*colR*$_{XOO3763}$ were annotated. The *colS*$_{XOO3534}$/*colR*$_{XOO3535}$ were known to control AvrXa21 activity and functions of *colS*$_{XOO3762}$/*colR*$_{XOO3763}$ were unknown in Xoo. To characterize the relationship between *rpf* and *colS*/*colR* genes, expression of *colS*/*colR* genes in Rpf mutants of Xoo were analyzed with quantitative reverse transcription PCR (qRT-PCR). Expressions of all three *colS*/*colR* genes increased in the *rpfF* mutant in which DSF synthesis is defective. Expression of *colS*$_{XOO1207}$/*colR*$_{XOO1208}$, *colS*$_{XOO3534}$/*colR*$_{XOO3535}$ and *colS*$_{XOO3762}$/*colR*$_{XOO3763}$ increased 2, 2–7, 3–13 folds respectively. Expression of *colS*$_{XOO3534}$ and *colS*$_{XOO3762}$ also increased 2–4 folds in the *rpfG* mutant in which the signal from DSF is no longer transferred to down-stream. Expression of the other *colS*/*colR* genes was not significantly changed in the *rpfG* mutant compared to the wild type. Since RpfF and RpfG are responsible for DSF synthesis and signal transfer from DSF to down-stream to regulate virulence gene expression, these results suggest that the DSF and DSF-mediated signal regulate negatively three *colS*/*colR* genes in Xoo.

Keywords **:** ColS/ColR, Rpf, virulence regulation, *Xanthomonas oryzae* pv. *oryzae*

Plant pathogenic bacteria must be virulent and able to suppress host resistance to cause a disease on host plant. As bacterial two-component system (TCS), which perceives outside signals and regulates responses inside bacterial cell, *rpfC*/*rpfG* and *colS*$_{XOO1207}$/*colR*$_{XOO1208}$ are required for virulence of *Xanthomonas oryzae* pv. *oryzae* (Xoo) (Slater et al., 2000; Subramoni et al., 2012). The Rpf (regulation of pathogenicity factors) system is known to regulate virulence by the cell-cell communication in *X. campestris* pv. *campestris* (Xcc) (Barber et al., 1997; He et al., 2007; Slater et al., 2000; Tang et al., 1991). Among *rpf* genes that were identified as a cluster (*rpfA-I*) (Tang et al., 1991), RpfF are responsible for diffusible signal factor (DSF) production (Barber et al., 1997; Wang et al., 2004), and RpfC and RpfG comprise a two-component system, which senses the DSF signal and transfers it to signal cascades involved in virulence (Slater et al., 2000). Virulence factor production and biofilm dispersal are controlled by cyclic di-GMP and Clp, which are on downstream of RpfG (He et al., 2007; Ryan et al., 2007). The *rpf* genes and functions of the core *rpf* genes, *rpfB*, *rpfC*, *rpfF*, *rpfG*, are well conserved in Xoo (Chatterjee and Sonti, 2002; Jeong et al., 2008).

The *colS/colR* genes, which encode a two-component system, were originally identified from the root-colonizing bacterium *Pseudomonas fluorescens* and found to be involved in the capacity of the bacterium to colonize plant roots (Dekkers et al., 1998). Subsequently, *colS/colR* genes were reported to regulate different biological responses to transposition of transposon and various stresses including phenol and heavy metals (Hu and Zhao, 2007; Kivistik et al., 2006). The *colS/colR* genes are required for virulence of several important plant pathogens including Xoo (Subramoni et al., 2012; Yan and Wang, 2011; Zhang et al., 2008).

Mutation of *colS*$_{XOO1207}$/*colR*$_{XOO1208}$ decreased virulence of Xoo. These mutations also caused growth defect in iron-limiting condition and deficiency in elicitation of hypersensitive response on non-host tomato (Subramoni et al., 2012). Another *colS/colR* genes, *colS*$_{XOO3534}$ (*raxH*)/*colR*$_{XOO3535}$ (*raxR*), were known to control avrXa21 activity

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in Xoo pXO99A (Burdman et al., 2004; Lee et al., 2008). These previously published results indicate two virulence regulation systems, Rpf and ColS/ColR, control virulence in Xoo. To characterize the relationship between *rpf* and *colS/colR* genes for virulence regulation, expression of *colS/colR* genes in Rpf mutants of Xoo were analyzed with quantitative reverse transcription PCR (qRT-PCR).

Three *colS/colR* genes are conserved in the important plant pathogens. In Xoo KACC10331 genome, three *colS/colR* genes, $colS_{XOO1207}/colR_{XOO1208}$, $colS_{XOO3534}/colR_{XOO3535}$ and $colS_{XOO3762}/colR_{XOO3763}$, have been annotated (Lee et al., 2005). Based on literature and homologous gene search, the three *colS*/*colR* genes were identified to be well conserved in Xcc 8004 (a black rot pathogen of cabbage) and *X. axonopodis* pv. *citri* (Xac) 306 (a citrus canker pathogen of citrus) (Table 1). Nucleotide identity between the *colS/colR* genes of Xoo KACC10331 and Xac 306 or Xcc 8004 were all more than 90%. E-value obtained by BlastN between each *colS*/*colR* genes of Xoo KACC10331 and corresponding genes in either Xac 306 or Xcc 8004 were all 0.0. These indicate that nucleotide sequences of the three *colS*/*colR* genes are well conserved in the three pathogens.

The $colS_{XC\_1050}/colR_{XC\_1049}$, $colS_{XAC3249}/colR_{XAC3250}$ and $colS_{XOO1207}/colR_{XOO1208}$ are known to be all required for virulence in each pathogen. Zhang et al. (2008) showed that $colS_{XC\_1050}/colR_{XC\_1049}$ was involved in virulence, hypersensitive response and tolerance to various stresses in Xcc 8004. The $colS_{XC\_1050}/colR_{XC\_1049}$ positively regulated expression of *hrpC* and *hrpE* operons and expression of $colS_{XC\_1050}/colR_{XC\_1049}$ were not controlled by the key *hrp* regulators HrpG and HrpX. Yan and Wang (2011) showed that $colS_{XAC3249}/colR_{XAC3250}$ were critical for *X. citri* subsp. *citri* (Xac) in virulence, growth *in planta*, biofilm formation, catalase activity, LPS production, and resistance to environmental stress. In Xoo, $colS_{XOO1207}/colR_{XOO1208}$ were required for virulence and hypersensitive response on non-host plant (Subramoni et al., 2012).

Rpf Mutants, complementation and qRT-PCR. Wild type strain, Xoo KACC10859, two Rpf mutant strains, CBNUXO05 (*rpfF*::EZ-Tn*5*), CBNUXO06 (*rpfG*::EZ-Tn*5*) and complement strains, CBNUXO05C (*rpfF*::EZ-Tn*5*/pVSP61-mcs-sp::*rpfF*), CBNUXO06C (*rpfG*::EZ-Tn*5*/pVSP61-mcs-sp::*rpfG*) were used in this study (Table 2). Mutants of *rpf* genes and their biological characteristics including DSF production were published previously (He et al., 2010; Jeong et al., 2008). To complement the mutants, cloning vector, pVSP61-mcs-sp was constructed by modification of pVSP61, which is very stable plasmid vector in Pseudomonads (Loper and Lindow, 1994). Polylinker of pUC9 in pVSP61 was replaced with polylinker of pUC19 at *Eco*RI – *Hind*III sites to increase availability of cloning sites (pVSP61-mcs). Spectinomycin resistance gene was inserted into *Bgl*II site in Km^R gene of the pVSP61-mcs resulting in Kanamycin resistance inactivated and Spectinomycin resistance (pVSP61-mcs-sp).

For complementation of CBNUXO05 (*rpfF*::EZ-Tn*5*), DNA fragments containing *rpfF* including 200 bp of its 5' region were cloned into *Hind*III-*Kpn*I sites with PCR products amplified with primers, rpfF-kpn1-F: 5' AGTGGTACCACATCAGCCGGCGTCAAGC 3' and rpfF-hind3-R: 5' AGTAAGCTTCCGTGAATGCGGGACGCG 3' (pVSP61-mcs-sp::*rpfF*). For complementation of CBNUXO06 (*rpfG*::EZ-Tn*5*), DNA fragments containing *rpfG* including 386 bp of its 5' region were cloned into

Table 1. Conservation of *colS*/*colR* genes in *Xanthomonas oryzae* pv. *oryzae* KACC10331, *Xanthomonas axonopodis* pv. *citri* 306 and *Xanthomonas campestris* pv. *campestris* 8004[a]

Xoo KACC10331		Xac 306		Xcc 8004	
Gene/Gene ID	Function	Gene/Gene ID	Function	Gene/Gene ID	Function
colS, XOO1207/ *colR*, XOO1208	Virulence, HR on non-host, growth in iron-limiting; Subramoni et al. (2012)	*colS*, XAC3249/ *colR*, XAC3250	Virulence, biofilm formation, resistance to environmental stress; Yan & Wang (2011)	XC_1050/ XC_1049	Virulence, HR, tolerance to various stress; Zhang et al. (2008)
colS (*raxH*), XOO3534/*colR* (*raxR*), XOO3535	AvrXa21; Burdman et al. (2004), Lee et al. (2008)	*colS*, XAC1222/ *colR*, XAC1221	unknown	XC_3125/ XC_3126	unknown
colS, XOO3762/ *colR*, XOO3763	unknown	*colS*, XAC0835/ *colR*, XAC0834	unknown	XC_3451 / XC_3452	unknown

[a]Nucleotide identity covered regions between the *colS*/*colR* genes of Xoo KACC 10331 and Xac 306 or Xcc 8004 were more than 90%. E-values between *colS*/*colR* genes of Xoo KACC10331 and corresponding genes in either Xac 306 or Xcc8004 were all 0.0.

Table 2. Bacterial strains and plasmids used in this study

Strain/Plasmid	Relevant Characteristics*	Source
Xanthomonas oryzae pv. *oryzae*		
KACC10859	Wild-type, Cp^r	RDA, South Korea
CBNOXOO5	*rpfF*::EZ-Tn*5*, Km^r	Jeong et al., 2008; He et al., 2010
CBNUXOO6	*rpfG*::EZ-Tn*5*, Km^r	Jeong et al., 2008; He et al., 2010
CBNUXOO5C	CBNUXOO5/pVSP61-mcs-sp::*rpfF*, Km^r, Sp^r	This study
CBNUXOO6C	CBNUXOO6/pVSP61-mcs-sp::*rpfG*, Km^r, Sp^r	This study
pVSP61	Km^r	Loper and Lindow, 1994
pVSP61-mcs-sp	pVSP61::MCS of Puc19::Sp^r, Sp^r	This study
pVSP61-mcs-sp::*rpfF*	pVSP61-mcs-sp::*rpfF*, Sp^r	This study
pVSP61-mcs-sp::*rpfG*	pVSP61-mcs-sp::*rpfF*, Sp^r	This study

*Cp^r: cephalexin resistance, Km^r: kanamycin resistance, Sp^r: spectinomycin resistance.

*Hind*III-*BamH*I sites with PCR products amplified with primers, rpfG-hind3-F: 5' AGTAAGCTTAAGGACG-GCGGTGACGACG 3' and rpfG-bamh1-R: 5'AGTG-GATCCATCACGCAGCTGACCAGGCG 3' (pVSP61-mcs-sp::*rpfG*). Plasmid pVSP61-mcs-sp::*rpfF* and pVSP61-mcs-sp::*rpfG* were transformed into CBNUXO05 (*rpfF*::EZ-Tn*5*) and CBNUXO06 (*rpfG*::EZ-Tn*5*) with standard electroporation protocol. Mutants and its complementation strains were confirmed by PCR genotyping (supplementary Fig. 1).

RNA was isolated from the bacterial cells cultured in *hrp*-inducing culture conditions (Seo et al., 2008). Wild type, mutant and complement strains were cultured in PS broth (peptone 10 g, sucrose 10 g, L-glutamic acid 1 g per 1 L, pH 7.0) to OD_{600} = 0.2 and the bacterial cells were washed twice with sterilized water and transferred to Xom2 medium (0.18% xylose, 670 uM L-methionine, 10 mM L-glutamic acid, 14.7 mM potassium phosphate (monobasic), 40 μM manganese sulfate, 240 μM Fe(III) EDTA, 5 mM magnesium chloride per 1 L, pH 6.5). After 18 h further culture, bacterial cells were harvested for RNA isolation. Total RNA of each strains was isolated using the RNeasy® Mini kit (Qiagen, Valencia, CA), and residual genomic DNA was removed using the RNase-Free DNase Set (Qiagen, Valencia, CA) and RNeasy® MinElute™ Cleanup kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. DNase I-treated total RNA was measured with a Nano Drop 2000 (Thermo scientific, Wilmington, USA) and 1 μg total RNA was used to synthesize cDNA. cDNA was generated using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. After the reaction, samples were diluted with 190 μL of distilled water and used as a template cDNA for qRT-PCR. qRT-PCR experiments were performed by Rotor-gene Q (Qiagen, Valencia, CA) and using 2× Rotor-gene™ SYBR® Green PCR kit (Qiagen, Valencia, CA) containing 1 μL of diluted cDNA. The constitutively expressed *rrsA* gene coding 16S rRNA was used as an internal control for relative quantification (Subramoni et al., 2012). Gene-specific primers designed and their amplification efficiency was checked with RNA isolated from the wild type strain. Primers with $R^2 > 0.99$ and slope 3.2–3.6 were used (Table 3). qRT-PCR condition included initial heating for 10 min at 95°C, followed by 40 cycles of PCR (95°C, 15 s; 58°C, 30 s; 72°C 30 s). To analyze obtained results, ΔΔCt method was used and quantification results were calculated.

Expression of *rpfF* and *rpfG* genes in its mutant and complement strains. Expression of *rpfF* and *rpfG* in its mutants and complement strains was checked to confirm the mutation and complementation works. The *rpfF* and *rpfG* was expressed about 11 fold and 100 fold less in its mutant strain, CBNUXO05 (*rpfF*::EZ-Tn*5*) and CBNUXO06 (*rpfG*::EZ-Tn*5*) than in the wild type strain respectively (Table 4). Expression of *rpfF* in the complement strain, CBNUXO05C (*rpfF*::EZ-Tn*5*/pVSP61-mcs-sp::*rpfF*) was similar to the wild type strain while *rpfG* was expressed in the complemented strain, CBNUXO06C (*rpfG*::EZ-Tn*5*/pVSP61-mcs-sp::*rpfG*), was about 4 fold higher than in the wild type (Table 4). Forward and reverse primer of *rpfF*- and *rpfG*-specific primers were designed from outside of both end of EZ-Tn*5* insertion site. Since EZ-Tn*5* contains transcription terminator, expression level of the two genes must be near 0 in the mutant strains. When the PCR products were checked by gel electrophoresis after qRT-PCR, no proper-size band or very faint band were appeared in triplicate lanes. Although results of qRT-PCR showed the low expression level of *rpfF* and *rpfG* in its mutant strains, these results indicate that RpfF and RpfG

Table 3. Primers used for quantitative RT-PCR

Gene ID/Gene	Product	Sequence (5' – 3')	Source
rrsA	16S ribosomal RNA	F: CCCTAAACGATGCGAACTGGATGT R: AGTTTCAGTCTTGCGACCGTACTC	Subramoni et al., 2012
rpfF, XOO2869	RpfF	F: GAGCTGCCACACCATCATCG R: GGCGGAGTACAGATTGCCTTCT	This study
rpfG, XOO2871	Response regulator	F: TTTCATCACGCTCATCTCGTCGT R: TCTCGAACGCATGTCTCATGTGG	This study
colS, XOO1207	Two-component system sensor protein	F: TACAAGCGCAAACAGAATCG R: TTGTTACGGGGTCCGAATTA	This study
colR, XOO1208	Two-component system regulatory protein	F: AGCTTGTTGTCCAGCGAGTC R: ATCGTGCTCGATCTCAACCT	This study
colS, XOO3534	Two-component system sensor protein, RaxH	F: GATAGCGAGATGCGGATGAT R: ATCTGCAACTGGTCCTGGAG	This study
colR, XOO3535	Two-component system regulatory protein, RaxR	F: AAGGATCAGGGCGTCGTAGT R: GCTGCTGGTCATTGAAGACA	This study
colS, XOO3762	Two-component system sensor protein	F: ACGAACGACACCAGATACCC R: CTCACGGTAGCGTTGCCTAT	This study
colR, XOO3763	Two-component system regulatory protein	F: ACGGCTTGGTCAGGTAGTCATC R: CAAGTCCACGCCGGTGTTGAT	This study

Table 4. Effect of *Xanthomonas oryzae* pv. *oryzae* KACC10859 *rpfF* and *rpfG* mutations on expression of expression of *colS/colR*, *rpfF* and *rpfG*

strain	Fold expression change ± standard deviation*							
	XOO1207	XOO1208	XOO3534	XOO3535	XOO3762	XOO3763	*rpfF*	*rpfG*
CBNUXO05 (*rpfF*::EZ-Tn*5*)	2.11±0.14^{a**}	1.90±0.13^{a}	7.53±0.87^{a}	2.95±0.11^{a}	13.31±0.55^{a}	3.45±0.20^{a}	0.09±0.01^{b}	1.44±0.15^{b}
CBNUXO05C (*rpfF*::EZ-Tn*5*/ pVSP61-mcs-sp::*rpfF*)	1.23±0.04^{b}	1.06±0.09^{b}	1.25±0.15^{c}	1.12±0.15^{c}	1.58±0.15^{bc}	1.32±0.23^{b}	1.06±0.13^{a}	0.93±0.12^{c}
CBNUXO06 (*rpfG*::EZ-Tn*5*)	1.04±0.05^{b}	1.12±0.13^{b}	4.24±0.04^{b}	1.78±0.12^{b}	2.13±0.13^{b}	1.34±0.09^{b}	0.92±0.06^{a}	0.01±0^{d}
CBNUXO06C (*rpfG*::EZ-Tn*5*/ pVSP61-mcs-sp::*rpfG*)	1.02±0.08^{b}	1.15±0.02^{b}	1.33±0.04^{c}	1.09±0.04^{c}	1.21±0.04^{c}	1.03±0.01^{b}	0.88±0.05^{a}	4.88±0.19^{a}

*The fold expression change (mutant or complemented mutant/wild type) was calculated using $2^{-\Delta\Delta Ct}$ with three replicates.
**Means with the same letter are not significantly different by Turkey's HSD test using SAS 9.2.

were non-functional in the respective mutant while those in the complemented strains were similar to the wild-type strain.

Expression of *colS/colR* genes was increased in *rpfF* and *rpfG* mutants. Expressions of $colS_{XOO1207}/colR_{XOO1208}$ about 2 fold increased in CBNUXO05 (*rpfF*::EZ-Tn*5*), while expression of both genes was not different in CBNUXO06 (*rpfG*::EZ-Tn*5*) (Table 4). Although we do not know biological significant of 2 fold increase of this two-component system in the *rpfF* mutant yet, qRT-PCR results suggest that expression $colS_{XOO1207}/colR_{XOO1208}$ is influenced directly by DSF rather than signal through RpfG. In Rpf virulence regulation system, RpfF are responsible for the production of DSF (Barber et al., 1997; Wang et al., 2004) and RpfG transfers signal from RpfC that sense the DSF signal to its downstream (He et al., 2007; Ryan et al., 2007; Slater et al., 2000).

Expressions of $colS_{XOO3534}$ (*raxH*)/$colR_{XOO3535}$ (*raxR*) highly increased in both *rpfF* and *rpfG* mutants (Table 4). Coding sensor gene, $colS_{XOO3534}$ was expressed about 7 and 4 fold higher in the *rpfF* mutant and the *rpfG* mutant, respectively, while its cognate coding regulator gene, $colR_{XOO3535}$, was expressed about 3 and 2 fold higher than in the wild type. These results suggest that DSF and signal from DSF through RpfG regulate negatively the expression of $colS_{XOO3534}/colR_{XOO3535}$ in the wild strain. Since $colS_{XOO3534}/colR_{XOO3535}$ has been proved to control of *avrXa21*

(Lee et al., 2008; Burdman et al., 2004), DSF may suppress avirulence activity by suppression of the expression of two genes for promoting virulence.

Expression of $colS_{XOO3762}$/$colR_{XOO3763}$ was increased about 13 and 3 folds, respectively in CBNUXO05 (*rpfF*::EZ-Tn*5*) and expression of $colS_{XOO3762}$ was also increased about 2 folds in CBNUXO06 (*rpfG*::EZ-Tn*5*) comparing to wild type strain and expression of its cognate regulator, $colR_{XOO3763}$, was not changed significantly in CBNUXO06 (*rpfG*::EZ-Tn*5*). These results suggest DSF regulates negatively this two-component system in the wild type. Biological function of positive regulation of $colS_{XOO3762}$ is not clear, since function of $colS_{XOO3762}$/$colR_{XOO3763}$ is not known.

In this study, expression of three two-component system genes, two of them are known to control virulence and avirulence, increased significantly in the *rpfF* mutant, CBNUXO05 (*rpfF*::EZ-Tn*5*), and expression of $colS_{XOO3534}$ (*raxH*)/$colR_{XOO3535}$ (*raxR*) and $colS_{XOO3762}$ also increased in the *rpfG* mutant, CBNUXO06 (*rpfG*::EZ-Tn*5*). Overall these results indicate DSF and downstream of DSF signal regulate negatively three *colS/colR* genes in Xoo. Although biological function of these regulations by DSF is unclear yet and further detail work on this area is needed, we think that these regulations may be a part of hierarchal control of pathogenicity, which is needed for pathogen to successfully cause a disease on host plant.

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