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Genome-wide Screening to Identify Responsive Regulators Involved in the Virulence of *Xanthomonas oryzae* pv. *oryzae*

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Two-component systems (TCSs) are critical to the pathogenesis of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). We mutated 55 of 62 genes annotated as responsive regulators (RRs) of TCSs in the genome of *Xoo* strain PXO99A and identified 9 genes involved in *Xoo* virulence. Four (*rpfG*, *hrpG*, *stoS*, and *detR*) of the 9 genes were previously reported as key regulators of *Xoo* virulence and the other 5 have not been characterized. Lesion lengths on rice leaves inoculated with the mutants were shorter than those of the wild type and were significantly restored with gene complementation. The population density of the 5 mutants in planta was smaller than that of PXO99A at 14 days after inoculation, but the growth curves of the mutants in rich medium were similar to those of the wild type. These newly reported RR genes will facilitate studies on the function of TCSs and of the integrated regulation of TCSs for *Xoo* pathogenesis.

Keywords **:** response regulator, two-component regulatory system, *Xanthomonas*

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Living organisms have well-developed signal transduction pathways that rapidly respond to various external changes. In both prokaryotes and eukaryotes, these systems separately or co-operatively regulate the expression of genes associated with most biological processes. The two-component system (TCS) is a dominant signaling pathway in bacteria and is typically composed of two proteins; a membrane-bound histidine kinase (HK), which functions as a sensor, and the other is a cytoplasmic responsive regulator (RR), which typically functions as a transcription factor. The perception of extracellular stimuli by HKs activates RRs via phosphorylation and alternate gene expression (Stock et al., 2000). Therefore, mechanism of the TCS signaling in bacteria has been considered relatively simple compared to the complex signaling by multiple proteins in eukaryotes. However, recent findings acknowledge that more complex mechanisms exist owing to the diverse phosphotransmission pathways and cross or joint regulation by TCSs (Buelow and Raivio, 2010). It might be an evolutionary consequence that bacteria are able to respond to diverse environmental challenges with a limited number of genes for TCSs. Successful reprogramming through simple and more complex pathways involving TCSs surprisingly guarantees the survival of bacteria in diverse conditions. The role of TCSs is also crucial in pathogenic bacteria, which immediately face exclusive conditions in hosts after infection and cannot survive the competition without rapid reprogramming of gene expression by signal pathways such as TCSs. Study of pathogenic bacterial TCSs, therefore, provides critical information to understand microbe-host interactions, but few are functionally defined. In this study, we attempted to identify all RR genes contributing to the virulence of a phytopathogenic bacterium, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), using 55 RR gene knock-out mutants. We believe that the new RR genes identified from our experiments will extend the knowledge of the TCSs function in *Xoo* pathogenesis and provide essential clues in

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the study of the signaling network of TCSs to understand the integrated systems for full fitness of the bacteria in host conditions.

Xoo is a causal agent of a bacterial leaf blight disease in the staple crop plant rice. The pathovar is commonly responsible for loss of 10 to 20% of rice production. To date, genome sequencing of 44 *Xoo* strains has been completed, and the information is available in the National Center for Biotechnology Information (NCBI) genome database (https://www.ncbi.nlm.nih.gov/). According to the database, the *Xoo* genome is generally predicted to possess just over 4,000 genes encoding proteins; around 100 of them are annotated as HK and RR genes for TCSs. First, we chose a representative *Xoo* strain isolated from the Philippines (PXO99A) and selected 62 RR genes in the genome for generation of knock-out mutant strains based on the NCBI and another database, P2CS (Prokaryotic Two-Component System, http://www.p2cs.org/) (Table 1). PXO99A has more RR genes in the genome than the other two representative *Xoo* strains (MAFF 311018 and

Table 1. Sixty-two candidate RR genes in the PXO99A genome

Strains	Locus tag No. of mutated gene	Family	P2CS description	References or source
RR1	PXO_RS 08445	CheY	Response Regulator	in this study
RR2	PXO_RS 08330	CheB	Response Regulator	in this study
RR3	PXO_RS 08270	RpfG	Response Regulator	RpfG (He et al., 2010), in this study
RR4	PXO_RS 07450	OmpR	Response Regulator	in this study
RR5	PXO_RS 09090	PleD-VieA	Response Regulator	in this study
RR6	PXO_RS 08775	PleD	Response Regulator	in this study
RR7	PXO_RS 08715	RpfG	Response Regulator	
RR8	PXO_RS 10565		Hybrid	in this study
RR9	PXO_RS 10155	NarL	Response Regulator	in this study
RR10	PXO_RS 11120	PrrA	Response Regulator	in this study
RR11	PXO_RS 11220	unclassified	Response Regulator	in this study
RR12	PXO_RS 12150/ PXO_RS 13080	CheY	Response Regulator	in this study
RR13	PXO_RS 11975/ PXO_RS 12905	CheY	Response Regulator	VemR(*Xcc*) (Tao and He, 2010), in this study
RR14	PXO_RS 11965/ PXO_RS 12895	NarL	Response Regulator	CitB (Sahebi et al., 2015)
RR15	PXO_RS 11880/ PXO_RS 12810	CheV	Response Regulator	in this study
RR16	PXO_RS 11855/ PXO_RS 12785	PleD-VieA	Response Regulator	PdeR (Yang et al., 2012), RavR(*Xcc*) (He et al., 2009), in this study
RR17	PXO_RS 15740		Hybrid	in this study
RR18	PXO_RS 15515	CheY	Response Regulator	
RR19	PXO_RS 15395	NarL	Response Regulator	in this study
RR20	PXO_RS 15100	CheY	Response Regulator	in this study
RR21	PXO_RS 14975	CheY	Response Regulator	in this study
RR22	PXO_RS 14970	CheY	Response Regulator	in this study
RR23	PXO_RS 14605		Hybrid	in this study
RR24	PXO_RS 14600		Hybrid	in this study
RR25	PXO_RS 14595		Hybrid	in this study
RR26	PXO_RS 14460	unclassified	Response Regulator	in this study
RR27	PXO_RS 18055	OmpR	Response Regulator	HrpG (Tsuge et al., 2006), in this study
RR28	PXO_RS 18925	OmpR	Response Regulator	ColR (Subramoni et al., 2012)
RR29	PXO_RS 19035	OmpR	Response Regulator	in this study
RR30	PXO_RS 19370		Hybrid	

Table 1. Continued

Strains	Locus tag No. of mutated gene	Family	P2CS description	References or source
RR31	PXO_RS 19400	NarL	Response Regulator	in this study
RR32	PXO_RS 19415	OmpR	Response Regulator	TctD(*Xcv*) (Tamir-Ariel et al., 2011), in this study
RR33	PXO_RS 19870	OmpR	Response Regulator	*PhoB*, in this study
RR34	PXO_RS 20355	RpfG	Response Regulator	in this study
RR35	PXO_RS 20535	CheY	Response Regulator	in this study
RR36	PXO_RS 23080		Hybrid	in this study
RR37	PXO_RS 22695	OmpR	Response Regulator	*PhoP* (Lee et al., 2008)
RR38	PXO_RS 22160	VieA	Response Regulator	in this study
RR39	PXO_RS 21800	NtrC	Response Regulator	XibR(*Xcc*) (Pandey et al., 2016), in this study
RR40	PXO_RS 21790	unclassified	Response Regulator	in this study
RR41	PXO_RS 21740	CheY	Response Regulator	in this study
RR42	PXO_RS 21605	OmpR	Response Regulator	in this study
RR43	PXO_RS 21535		Hybrid	StoS (Ikawa et al., 2014; Zheng et al., 2016), in this study
RR44	PXO_RS 23555	NarL	Response Regulator	in this study
RR45	PXO_RS 01340	NtrC	Response Regulator	in this study
RR46	PXO_RS 02255	FrzZ	Response Regulator	in this study
RR47	PXO_RS 02040	OmpR	Response Regulator	in this study
RR48	PXO_RS 02690	NtrC	Response Regulator	in this study
RR49	PXO_RS 03980	LytTR	Response Regulator	in this study
RR50	PXO_RS 04300	CheY	Response Regulator	SreR (Zheng et al., 2016), in this study
RR51	PXO_RS 04305		Hybrid	SreS (Zheng et al., 2016), in this study
RR52	PXO_RS 04760	OmpR	Response Regulator	in this study
RR53	PXO_RS 04595	OmpR	Response Regulator	in this study
RR54	PXO_RS 06090	NarL	Response Regulator	in this study
RR55	PXO_RS 06050	OmpR	Response Regulator	
RR56	PXO_RS 05400	unclassified	Response Regulator	in this study
RR57	PXO_RS 05395	CheY	Response Regulator	in this study
RR58	PXO_RS 05125	OmpR	Response Regulator	DetR (Nguyen et al., 2016), in this study
RR59	PXO_RS 06365	VieA	Response Regulator	in this study
RR60	PXO_RS 06355	CheB	Response Regulator	in this study
RR61	PXO_RS 06785	OmpR	Response Regulator	in this study
RR62	PXO_RS 06935	NtrC	Response Regulator	in this study

Gray cells indicate that the knock-out mutant strain of the RR gene reduced *Xoo* virulence in the present study.

KACC 10331). According to P2CS, PXO99A has 58 RRs with 4 mispredicted TCS proteins; MAFF 311018, isolated in Japan, has 52 RRs with 4 mispredicted TCS proteins; and KACC 10331, isolated in Korea, has 53 RRs with 5 mispredicted TCS proteins. However, 53 is the actual number of RR proteins that PXO99A can produce from 58 RR genes because 10 of the 58 RR genes are duplicates (PXO_RS12150 and PXO_RS13080, PXO_RS11975 and PXO_RS12905, PXO_RS11965 and PXO_RS12895, PXO_RS11880 and PXO_RS12810, PXO_RS11880 and PXO_RS12785) with 100% sequence identity (Table 1). We then selected an additional 9 genes (*RR8*, *RR17*, *RR23*, *RR24*, *RR25*, *RR30*, *RR36*, *RR43*, and *RR51*) annotated as hybrids in the NCBI genome database, but not in the P2CS. With these 62 RR gene (including hybrids) candidates, we successfully generated 55 mutant strains, covering 88.7% of all predicted RR genes in the PXO99A genome (Table 1) using marker-exchange mutagenesis (Lee and Ronald, 2007). We suspect that three (RR7, RR18, and RR30) of the 7 genes that we could not generate knock-out mutants are presumably lethal, while another 4 genes [*CitB* (RR14), *ColR* (RR28), *PhoP* (RR37), and *RaxR* (RR55)] have been previously characterized with knock-out mutants in *Xoo* (Burdman et al., 2004; Lee et al., 2008; Sahebi et al., 2015; Subramoni et al., 2012). CitB, ColR, and PhoP deficiency fully or mildly weakens virulence in *Xoo*, whereas RaxR

is a required gene for a-virulence activity by rice XA21 protein; however, mutation of the genes failed in the present study despite many attempts. We secured more than three independent replicates for each mutant, and three of the strains for each mutant were used in the experiments of this study. The primer sets used for cloning of each gene and restriction enzymes used for insertion of the kanamycin cassette to carry out marker-exchange mutagenesis are listed in Supplementary Table 1.

Virulence of the 55 mutant strains was examined after inoculation on leaves of Dong-jin rice plants (a japonica variety, compatible with PXO99A). For the inoculation test, plants were inoculated with a large population (10^7 cfu/ml) of mutant strain using a clipping method (Kauffman et al., 1973). Lesion lengths on leaves inoculated by the 9 mutant strains (RR3, RR13, RR27, RR35, RR39, RR43, RR51, RR54, and RR58) were significantly reduced compared to those on leaves inoculated by other mutants and PXO99A (Fig. 1, Supplementary Fig. 1, 2). Among them, RR3, RR27, RR43, and RR58 are deficient mutants of RpfG, HrpG, StoS, and DetR proteins, respectively. A TCS mediated by RpfC/G is involved in signal sensing and the transduction of diffusible signal factor and is important for *Xoo* virulence (Jeong et al., 2008). Both *rpfC* and *rpfG* knock-out mutants decrease production of several virulence determinants including exopolysaccharide, lipopolysaccharide, and extracellular enzymes except cellulase in *Xoo*. RR3, a knock-out mutant strain of the *rpfG* gene (Table 1), also showed reduced virulence in our test (Supplementary Fig. 2). HrpG was first identified in *Xanthomonas campestris* pv. *vesicatoria* (Wengelnik et al., 1996) and is an RR protein well-known as a representative virulence factor in *Xanthomonas* spp. HrpG belongs to the OmpR family, is the key regulator of pathogenicity (*hrp*) genes in susceptible rice plants, and is responsible for hypersensitive responses in resistant plants. HrpG regulates all genes required for the type III secretion system (T3SS), genes encoding T3SS effectors, and the genes encoding type II secretion system substrate with a transcriptional activator, HrpX (Guo et al., 2011). The critical role of HrpG and regulation of gene expression in *Xoo* pathogenicity were also investigated (Tsuge et al., 2006). The *PXO_RS18055* (*hrpG*) knock-out mutant (RR27) also reduced lesion length in our virulence test (Supplementary Fig. 2). *PXO_RS21535* encodes a hybrid protein containing PAS domain sensor HK, which was previously published as StoS (stress tolerance-related oxygen sensor) in *Xoo* (Ikawa et al., 2014). The knock-out mutant has decreased stress tolerance to high osmolality, sodium, and H_2O_2 and shows mildly reduced virulence. The RR43 strain in our inoculation results also showed mild attenuation in lesion length on inoculated leaves compared to PXO99A (Supplementary Fig. 2). DetR, recently published by our group as a key regulator of *Xoo* virulence, controls exopolysaccharide synthesis, ROS detoxification, and iron homeostasis (Nguyen et al., 2015). Defects in the DetR of *Xoo* strongly reduced virulence (Supplementary Fig. 2).

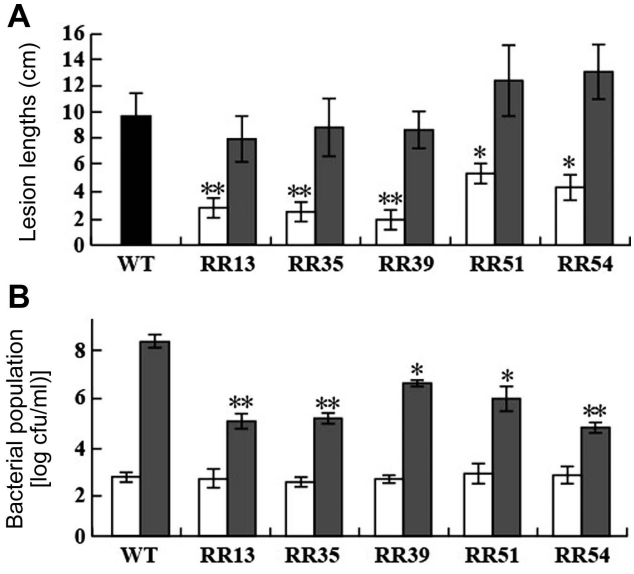

Fig. 1. Virulence phenotypes of PXO99A (WT) and RR mutant strains. (A) Lesion lengths caused by PXO99A (WT, black), RR mutant (white), and the complementary (gray) strains on Dong-jin rice leaves at 14 days after infection (DAI). Rice leaves were infected with 10^7 cfu/ml of bacterial suspension by the scissor clipping method. Bars are mean ± SD (n = 20). * or ** indicate that the lesion length of each mutant was significantly different from that of PXO99A by Duncan's test ($P < 0.05$). (B) Bacterial population at 0 (white) and 14 (gray) DAI. Immediately after infection (0 DAI) and at 14 DAI, 1-cm^2 and 25-cm^2 samples from the infected site of three leaves were used to extract the bacteria population at 0 and 14 dpi, respectively. Bars are mean ± SD (n = 3). * and ** indicate that the population of mutants was significantly smaller than that of PXO99A by Duncan's test ($P < 0.05$). C) All experiments were repeated three times with high consistency, and the results from one experiment are shown.

The other 5 genes (*RR13*, *RR35*, *RR39*, *RR51*, and *RR54*) are new RRs that have not yet been investigated in *Xoo*. The RR13 mutant has no RR protein encoded in *PXO_RS11975* or *PXO_RS12905*. Lesion lengths of the RR13 mutant strain (2.6 ± 1.4 cm) were reduced compared with those of PXO99A (9.8 ± 1.9 cm) (Fig. 1). The RR13 gene is a homolog of *vemR* identified in *Xanthomonas campestris* pv. *campestris* (*Xcc*) ATCC33913. The amino acid sequence of the *Xoo* RR13 protein has 100% identity with 96% query coverage. Only 5 amino acids at the C-terminus are different between the full-length *Xoo* and *Xcc* proteins.

VemR is involved in exopolysaccharide production and mobility to regulate *Xcc* pathogenicity. Our results indicated that the homolog of *Xcc* VemR also regulates virulence in *Xoo*. *PXO_RS20535* (RR35) encodes an orphan RR protein belonging to the CheY family. The lesion length on leaves inoculated with RR35 mutant strain was 2.6 ± 1.5 cm (Fig. 1), while that of PXO99A was 9.8 ± 1.9 cm (Fig. 1). The *RR35* gene is well-conserved in all *Xanthomonas* spp., but has not yet been characterized. Suppression of *PXO_RS21800* gene expression (RR39) exhibited a reduction of lesion lengths on inoculated leaves (Fig. 1). RR39 is a homolog of *Xcc* XibR (*Xanthomonas* iron binding regulator) with a 93% identity in a 99% query coverage. XibR is required for fine-tuning and regulating the expression of iron-regulated genes and virulence-associated function (Pandey et al., 2016). Our results indicated that signaling by the homolog of the *Xcc* XibR is also important in *Xoo* virulence. With regard to StoS (RR43 in this study, Supplementary Fig. 2), there is another report concluding that *Xoo* StoS and SreKRS (salt response kinase, regulator, sensor) orchestrate the behavior of *Xoo* in rice by regulating virulence factor expression (Zheng et al., 2016). However, the absence of StoS or SreK is not reported to weaken the virulence of *Xoo* (Zheng et al., 2016). The *sreKRS* genes in an operon are annotated in NCBI as a PAS-domain S-box protein, a response regulator, and a hybrid HK/RR, respectively. Although SreK was not included in our candidates, SreR and SreS correspond to RR50 and RR51, respectively. In our inoculation test, RR50 did not show any significant change in lesion length. However, the lesion lengths of RR51 were reduced by half compared to those of PXO99A (Fig. 1), which is similar to the results of RR43 shown in Supplementary Fig. 2. It is not obvious if Zheng et al. (2016) tested the SreS mutant for *Xoo* virulence; however, our results strongly indicate that the hybrid (SreS) contributes to *Xoo* virulence. *PXO_RS06090* (RR54), located with a cognate HK gene (*PXO_RS06085*) in an operon of the PXO99A genome, encodes an RR protein belonging to the NarL family. It is well-conserved in all *Xanthomonas* spp. The lengths of lesions on leaves inoculated by RR51 were reduced (4.1 ± 1.7 cm) compared to those of PXO99A, indicating that TCS mediated by the RR is involved in *Xoo* virulence (Fig. 1). Except these 5, we have another questionable RR, published as PdeR (Yang et al., 2012). Yang et al. (2012) reported that deletion of *pdeR* and the cognate HK gene, *pdeK*, attenuates virulence and decreases secretion of exopolysaccharide in *Xoo* (Yang et al., 2012). Indeed the homologs of *Xoo pdeR* and *pdeK* were published previously in *Xcc* with different names, RavR and RavS (He et al., 2009). Deletion of RavR and RavS similarly reduces *Xcc* virulence. However, RR16 (*pdeR* deletion mutant) in our study did not show a conclusive change in virulence (Supplementary Fig. 2). The PXO99A genome has two copies of the gene at PXO_RS11855 and PXO_RS12785, with 100% identity. One possible explanation is that our mutant (RR16) still has one of these two without a disruption.

We next generated complementary strains for the new 5 gene knock-out mutants (Supplementary Table 2). It is to test if the reduced virulence of the 5 mutant strains was caused by unexpected expression changes of the neighboring genes, not caused by the target genes. To generate complementary strains, the full-lengths of *RR13*, *RR35*, *RR39*, *RR51*, and *RR54* genes followed by the sequence for 6 His residues was cloned into a pBBR1MCS-5 vector and introduced into the 5 knock-out mutant strains (RR13, RR35, RR39, RR51, and RR54), respectively. After confirmation of protein expression with western blot analysis (Supplementary Fig. 3), the complementary strains were inoculated on the leaves of Dong-jin rice plants. We then observed a significant restoration in virulence of the complementary strains (Fig. 1A). This result revealed that the observed decrease in virulence of the 5 knock-out mutants was not due to a polar effect. Furthermore, the bacterial population in inoculated rice leaves was also monitored at 0 and 14 days after inoculation (DAI, Fig. 1B). We harvested rice leaves immediately (0 days) after inoculation and 14 DAI and extracted the bacteria by the 1 h shaking-incubation of chopped rice leaves. Whereas the population density of PXO99A at 14 DAI reached 8.2 ± 0.5 cfu/ml in the log phase, the population of mutants only reached 6.4 ± 0.3 cfu/ml even in the most virulent strain RR39 (Fig. 1B). Finally, we established growth curves of the 5 knock-out mutant strains to determine if the reduced virulence was caused by a defect of bacterial growth in normal conditions and was not directly associated with pathogenicity in host conditions (Supplementary Fig. 4). The population of the mutant strains was measured in peptone sucrose broth (PSB) medium every 12 h for 4 days using colony counting. We found no difference in growth between PXO99A and the 5 mutants. This result suggests that the RRs and/or TCSs mediated by the RRs are most likely specific to *Xoo* virulence.

Judging from our results and from previous reports, *Xoo* possesses at least 12 RRs (or 13 if PedR is included) that are closely associated with pathogenicity. Seven (RpfG, RR3; CitB, RR14; PdeR, RR16; HrpG, RR27; ColR, RR28; PhoP, RR37; DetR, RR58) have been previously characterized, and we identified 5 novel RRs (RR13; *Xcc* VemR homolog; RR35, *PXO_RS20535*; RR39, *Xcc* XibR

homolog; RR51, *sreS*; RR54, *PXO_RS06090*) in the present study. Although the 12 (or 13) RR genes cannot be asserted to be all that is required for *Xoo* virulence, we suspect it is likely. Our findings extend the knowledge of RRs (or TCSs mediated by the new RRs) required for *Xoo* virulence and will facilitate further studies to elucidate the network between RRs for full fitness of *Xoo* in host conditions. We are now in the progress of transcriptome analysis with all 12 RR knock-out mutants.

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