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The RpfB-Dependent Quorum Sensing Signal Turnover System Is Required for Adaptation and Virulence in Rice Bacterial Blight Pathogen *Xanthomonas oryzae* pv. *oryzae*

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***Xanthomonas oryzae* pv. *oryzae*, the bacterial blight pathogen of rice, produces diffusible signal factor (DSF) family quorum sensing signals to regulate virulence. The biosynthesis and perception of DSF family signals require components of the *rpf* (regulation of pathogenicity factors) cluster. In this study, we report that RpfB plays an essential role in DSF family signal turnover in *X. oryzae* pv. *oryzae* PXO99A. The production of DSF family signals was boosted by deletion of the *rpfB* gene and was abolished by its overexpression. The RpfC/RpfG-mediated DSF signaling system negatively regulates *rpfB* expression via the global transcription regulator Clp, whose activity is reversible in the presence of cyclic diguanylate monophosphate. These findings indicate that the DSF family signal turnover system in PXO99A is generally consistent with that in *Xanthomonas campestris* pv. *campestris*. Moreover, this study has revealed several specific roles of RpfB in PXO99A. First, the *rpfB* deletion mutant produced high levels of DSF family signals but reduced extracellular polysaccharide production, extracellular amylase activity, and attenuated pathogenicity. Second, the *rpfB/rpfC* double-deletion mutant was partially deficient in xanthomonadin production. Taken together, the RpfB-dependent DSF family signal turnover system is a conserved and naturally presenting signal turnover system in *Xanthomonas* spp., which plays unique roles in *X. oryzae* pv. *oryzae* adaptation and pathogenesis.**

Xanthomonas oryzae pv. *oryzae* is a causal agent of bacterial blight disease of rice. *X. oryzae* pv. *oryzae* invades through either wounds or hydathodes, propagates in the epithem and then moves to the xylem vessels in which it activates multiplication, resulting in blight disease symptoms on leaves (Niño-Liu et al. 2006; Shen and Ronald 2002). A range of virulence factors are produced by *X. oryzae* pv. *oryzae* that are necessary for its pathogenicity, including exopolysaccharide (EPS), extracellular enzymes, iron-chelating siderophores, the membrane-bound yellow pigment xanthomonadin, and the type III secretion–dependent effectors (Hu et al. 2007; Jha et al. 2007; Rai et al. 2015; Rajeshwari et al. 2005; Ray et al. 2000). A previous study has shown that *X. oryzae* pv. *oryzae* produces diffusible signal factor (DSF) family quorum sensing (QS) signals, including DSF (*cis*-11-methyl-2-dodecenoic acid), BDSF (*cis*-2-dodecenoic acid), and CDSF (*cis*-11-methyldodeca-2,5-dienoic acid) via the synthase RpfF to regulate virulence factor production (He et al. 2010). The two-component system comprising the sensor kinase RpfC and the response regulator RpfG plays essential roles in the perception and transduction of DSF family signals in *Xanthomonas* spp. (Barber et al. 1997; He et al. 2006a, 2010; Slater et al. 2000). RpfC is a dually functional protein, which uses a conserved phospho-relay mechanism to transduce DSF family signals to its receptor RpfG and, meanwhile, negatively controls DSF family signal production through a domain-specific protein-protein interaction with RpfF (He et al. 2006a). A high-resolution transcriptional analysis has revealed that mutation of *rpfC* is associated with a substantial increase (sixfold) in transcript level of *rpfF*, which suggests that RpfC probably regulates DSF synthetic gene expression at transcriptional level (An et al. 2013). RpfG contains a receiver domain for signal transduction and a HD-GYP domain for cyclic diguanylate monophosphate (c-di-GMP) degradation (Dow et al. 2003; Ryan et al. 2006; Slater et al. 2000). Through controlling the intracellular level of c-di-GMP, the congenital ligand of the global transcription factor Clp, RpfG modulates the regulatory activity of Clp, which, in turn, positively regulates virulence factor production (He et al. 2007; Ryan et al. 2006; Tao et al. 2010). The *rpfF* mutant of an Indian *X. oryzae* pv. *oryzae* isolate, which is defective in DSF production and growth under low iron conditions, is also deficient in virulence (Chatterjee and Sonti 2002). Null mutation of the genes encoding RpfC, RpfG, or Clp substantially affects EPS synthesis and virulence in *X. oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola* (Qian et al. 2013; Tang et al. 1996; Zhang et al. 2013).

Signal turnover systems are essential components of many genetic regulatory mechanisms (Hochstrasser 1996). QS is one of the most important regulatory systems in numerous species of bacteria. The QS process generally consists of two phases, i.e., how bacterial cells enter the QS phase and how bacterial cells exit the QS phase, which is also termed as quorum quenching (QQ) (Dong et al. 2007). Studies in the past two decades have elaborated the first phase in detail; however, the QS turnover system is less understood. Acyl homoserine lactones (AHL) belong to a group of most extensively studied QS signals produced by many gram-negative bacterial species, and a number of enzymes with AHL degradation activity have been identified, mostly in non-AHL-producing bacteria, fungi, and

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mammals (Dong et al. 2007; Draganov et al. 2005; Du et al. 2014; Zhang et al. 2002). In *Agrobacterium tumefaciens*, a naturally occurring QS signal turnover system comprising an AHL degradation enzyme, AttM (also designated as BlcC), and a negative transcription factor, AttJ (also designated as BlcR), was identified to control bacterial cells exiting from the QS phase (Zhang et al. 2002). In *Pseudomonas aeruginosa* PAO1, three AHL acylases encoded by *pa2385*, *pa1032*, and *pa0305* are involved in the degradation of the AHL signals it produces (Huang et al. 2003, 2006; Sio et al. 2006; Wahjudi et al. 2011). In addition to AHL degradation enzymes, a group of bacterial isolates that are able to degrade DSF family signals have been identified, and *carAB* carried by *Pseudomonas* sp. strain G contributes to DSF degradation (Newman et al. 2008). Previous studies have shown that DSF in the supernatants of *Xanthomonas campestris* pv. *campestris* accumulates in the early stationary phase and its level subsequently declines rapidly (Barber et al. 1997; Wang et al. 2004). In *X. oryzae* pv. *oryzae* KACC10331, the BDSF level in the supernatant decreased dramatically in the stationary phase (He et al. 2010). All these findings suggest that *Xanthomonas* spp. deploy a naturally occurring signal turnover system for DSF family signals.

The *rpfB* gene, genetically located immediately upstream of the *rpfFCG* operon (Supplementary Fig. S1), was initially predicted to be involved in DSF biosynthesis in *X. campestris* pv. *campestris* (Barber et al. 1997; Slater et al. 2000). RpfB in a Korean *X. oryzae* pv. *oryzae* strain, KACC10859, was reported to be associated with virulence factor production and pathogenesis on rice; however, this study did not present any molecular mechanism in detail (Jeong et al. 2008). Subsequent studies demonstrated that *rpfB* was not required for DSF biosynthesis in *X. campestris* pv. *campestris* or *Xylella fastidiosa* but was likely involved in DSF processing (Almeida et al. 2012). This research team also pointed out that the previously generated *rpfB* mutations in *X. campestris* pv. *campestris* apparently have a polar effect on the downstream *rpfF* gene, giving rise to the DSF-deficient phenotype (Almeida et al. 2012). Recently, RpfB in *X. campestris* pv. *campestris* has been characterized as a fatty acyl-CoA ligase (FCL) displaying high activity on short- (C8), medium-, and long-chain fatty acids, exclusive of DSF and BDSF, in an in vitro biochemical analysis, while it is able to counteract the RpfF thioesterase activity by catalyzing uptake and activation of the free fatty acids to produce acyl-CoAs that can be utilized to restore membrane lipid synthesis in vivo (Bi et al. 2014). Our latest results have revealed that *X. campestris* pv. *campestris* deploys a regulatory system, consisting of at least RpfB and the transcriptional regulator Clp, to control DSF family signal turnover in vivo (Zhou et al. 2015a). Moreover, the *rpf* cluster, RpfB, and Clp were identified in an even wider range of bacterial genomes by BLAST analysis of the nonredundant database in National Center for Biotechnology Information (Zhou et al. 2015a). These findings suggest that RpfB-dependent signal turnover might be a conserved mechanism in bacteria.

In the present study, we aimed to elucidate the biological functions of *rpfB* and its regulatory mechanism in the rice pathogen *X. oryzae* pv. *oryzae*. Our results showed that the FCL activity of RpfB was required for DSF and BDSF signal turnover and expression of *rpfB* was growth phase–dependent and negatively controlled by the RpfC/RpfG-mediated DSF signaling cascade in *X. oryzae* pv. *oryzae*, which generally resembled the DSF family signal turnover system in *X. campestris* pv. *campestris*. However, results of the present study also revealed several specific phenotypes of the *rpfB* deletion mutant and the *rpfB/rpfC* double-deletion mutant of *X. oryzae* pv. *oryzae*, implying that RpfB plays unique roles in controlling *X. oryzae* pv. *oryzae* pathogenesis.

RESULTS

RpfB is required for DSF and BDSF signal turnover in *X. oryzae* pv. *oryzae*.

Our previous study suggested that the RpfB-dependent QS signal turnover system likely existed in various bacterial species, such as *X. campestris* pv. *campestris*, *X. oryzae* pv. *oryzae*, *Xylella fastidiosa*, and *Stenotrophomonas maltophilia* (Zhou et al. 2015a). To investigate the DSF family QS signal turnover activity of RpfB in *X. oryzae* pv. *oryzae*, the *rpfB* in-frame deletion mutant Δ*rpfB*, and the *rpfB/rpfC* double-deletion mutant Δ*rpfB*Δ*rpfC* were generated in *X. oryzae* pv. *oryzae* wild-type strain PXO99A, in the present study. Deletion of *rpfB* in PXO99A and the DSF overproduction mutant Δ*rpfC* significantly boosted the production of DSF and BDSF (Supplementary Figs. S2 and S3). Complementation by a single copy of *rpfB* in Δ*rpfB* (the resultant strain called Δ*rpfB*::*rpfB* hereafter) or Δ*rpfB*Δ*rpfC* (the resultant strain called Δ*rpfB*Δ*rpfC*::*rpfB*) fully restored DSF and BDSF production to their respective parental strain's level. Overexpression of RpfB in Δ*rpfB* (the resultant strain called Δ*rpfB*[*rpfB*]) completely abolished the production of DSF and BDSF. However, expression of RpfB with an E to A mutation on the 365th residue, which is a conserved residue crucial for the catalytic activity of FCL (Zhou et al. 2015a), in the mutant Δ*rpfB* (the resultant strain called Δ*rpfB*[E365A]) completely impaired DSF family signal turnover activity of RpfB.

DSF family signal molecules are synthesized through the fatty acid synthetic pathway intracellularly in *X. campestris* pv. *campestris* and *Burkholderia cenocepacia* (Bi et al. 2012; Deng et al. 2015; Zhou et al. 2015b). Therefore, cellular levels of DSF and BDSF by the *rpfB* mutants of PXO99A were determined. Deletion of *rpfB* in either the wild-type strain PXO99A or the DSF overproduction strain Δ*rpfC* markedly increased cellular levels of DSF and BDSF (Fig. 1), which could be fully restored by *rpfB* complementation.

The in vivo activity of RpfB was further analyzed by hourly monitoring the remaining levels of exogenously applied DSF and BDSF, at the initial concentration of 15 and 8 μM, respectively, in the cultures of the following five strains: PXO99A, Δ*rpfB*, Δ*rpfB*::*rpfB*, Δ*rpfB*(*rpfB*), and Δ*rpfB*(E365A). Our results showed that the levels of DSF and BDSF in the culture supernatant of strain Δ*rpfB* gradually increased during growth, which was similar to the trend of strain Δ*rpfB*(E365A) (Supplementary Fig. S4). In contrast, levels of DSF and BDSF in the cultures of strains PXO99A and Δ*rpfB*::*rpfB* declined progressively at a generally parallel rate. Additionally, BDSF and DSF were completely undetectable in the culture supernatant of strain Δ*rpfB*(*rpfB*) after a 2-h incubation. Consistently, immunoblotting analysis using the polyclonal antibody against RpfB derived from *X. campestris* pv. *campestris* revealed that the expression level of RpfB was significantly higher in Δ*rpfB*(*rpfB*) than in PXO99A and Δ*rpfB*::*rpfB*, suggesting that the efficiency in DSF family signal turnover depended on the expression level of RpfB in *X. oryzae* pv. *oryzae*. These findings confirmed that RpfB in *X. oryzae* pv. *oryzae*, similar to its homolog in *X. campestris* pv. *campestris*, was essential for DSF family signal turnover.

The *rpfB* deletion mutants confer pleiotropic phenotypes in *X. oryzae* pv. *oryzae*.

When analyzing the DSF and BDSF signal turnover activity of RpfB in PXO99A, we also observed and characterized some distinct phenotypes caused by *rpfB* deletion. First, *rpfB* single deletion in PXO99A had little effect on *X. oryzae* pv. *oryzae* growth in nutrient agar (NA) broth, since no significant differences in bacterial density (indicated by optical density at a wavelength of 600 nm [OD_{600}]) and viable cell number

(indicated by CFU) were observed between PXO99A and Δ*rpfB*, while the double-deletion mutant Δ*rpfB*Δ*rpfC* had significantly impaired growth after culturing for 72 h in NA broth, compared with its parental strain Δ*rpfC*. Moreover, no viable cells were detected for strain Δ*rpfB*Δ*rpfC* at 96 h after inoculation, which could be fully restored by Δ*rpfB*Δ*rpfC::rpfB*.

Second, distinct colony morphologies for the mutants containing *rpfB* deletion, Δ*rpfB* and Δ*rpfB*Δ*rpfC*, were observed on NA plates. Compared with the wild-type strain PXO99A, the colony of Δ*rpfB* grown on NA had a wrinkled yellow but dry surface (Fig. 2A), while the complementation strain Δ*rpfB*::*rpfB* fully restored its watery colony morphology (Fig. 2A). Interestingly, the double-deletion mutant Δ*rpfB*Δ*rpfC* had a wrinkled oblate surface with pale yellow color (Fig. 2A). Therefore, the production of the yellow pigment xanthomonadin by the *rpfB* mutants was assayed. Consistent with the observations on NA plates, deletion of *rpfB* in the wild-type strain PXO99A had little effect on xanthomonadin production in NA broth (Fig. 2B), while deletion of *rpfB* in Δ*rpfC* reduced xanthomonadin production by 30% (Fig. 2B). In addition, the *rpfC* deletion mutant Δ*rpfC* produced approximately 20% less xanthomonadin than the wild-type strain PXO99A and the *rpfB* deletion mutant Δ*rpfB* (Fig. 2B).

The biosynthesis of xanthomonadin requires the *xanB2* gene, which encodes a chorismatase responsible for the conversion of chorismate to 3-hydroxybenzoic acid (3-HBA) and 4-HBA in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (He et al. 2011; Zhou et al. 2013a and b). The C-terminal YjgF-like domain of XanB2 confers activity for the biosynthesis of 3-HBA, an intermediate in xanthomonadin biosynthetic pathway, and its N-terminal FGFG motif–containing domain is responsible for the biosynthesis of 4-HBA, an intermediate in coenzyme Q_8 biosynthetic pathway (Zhou et al. 2013b). Therefore, transcript levels of *xanB2* in PXO99A, Δ*rpfC*, Δ*rpfC*Δ*rpfB*, and Δ*rpfC*Δ*rpfB*::*rpfB* were compared using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Our results showed that the expression level of *xanB2* in PXO99A was approximately twofold higher than that in Δ*rpfC*, Δ*rpfC*Δ*rpfB,* or Δ*rpfC*Δ*rpfB*::*rpfB*, but deletion of *rpfB* in Δ*rpfC* could not further influence *xanB2* expression (Table 1). Subsequent analysis showed that the levels of 3-HBA and 4-HBA were differentially affected in Δ*rpfC* and Δ*rpfB*Δ*rpfC*. Similar levels of 3-HBA in the culture supernatants of Δ*rpfC*, Δ*rpfB*Δ*rpfC*, and Δ*rpfB*Δ*rpfC*::*rpfB* was detected, which were significantly higher than the level obtained for PXO99A (Table 1). Compared with the wild-type *X. oryzae* pv. *oryzae* strain, the level of 4-HBA in the supernatant of Δ*rpfC* reduced by 32%; however, deletion of *rpfB* in Δ*rpfC* resulted in an increased 4-HBA level, similar to PXO99A (Table 1). Subsequently, we found that Δ*rpfB*Δ*rpfC* produced 20% less coenzyme Q_8 than PXO99A and Δ*rpfC* ($P < 0.05$), which could be fully restored by *rpfB* complementation in Δ*rpfC* (Table 1).

Third, the wrinkled colony surface of Δ*rpfB* (Fig. 2A) implied a reduction in EPS production. To verify this hypothesis, the EPS yields of the *X. oryzae* pv. *oryzae rpfB* mutants in NA broth were measured. Figure 2C shows deletion of *rpfB* in PXO99A resulted in significantly reduced EPS production by 69%, which was fully restored in Δ*rpfB::rpfB*, whereas deletion of *rpfB* in Δ*rpfC* had no additional effect on EPS production.

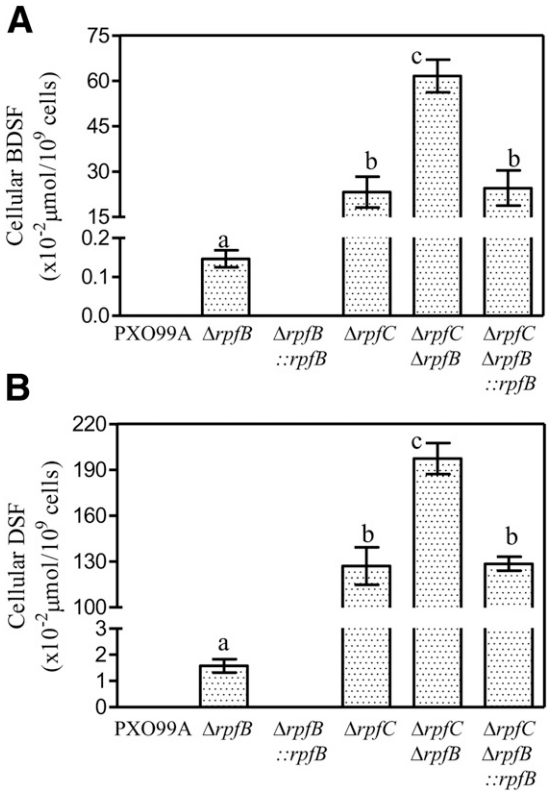

Fig. 1. A, Cellular levels of *cis*-2-dodecenoic acid diffusible signal factor (BDSF) and **B,** DSF in *Xanthomonas oryzae* pv. *oryzae* strains. PXO99A indicates *X. oryzae* pv. *oryzae* wild-type strain PXO99A; Δ*rpfB* indicates the *rpfB* in-frame deletion mutant; Δ*rpfB*::*rpfB* indicates the Δ*rpfB* mutant complemented by a single copy of *rpfB*; Δ*rpfC* indicates the *rpfC* in-frame deletion mutant; Δ*rpfB*Δ*rpfC* indicates the *rpfB/rpfC* double-deletion mutant; Δ*rpfB*Δ*rpfC::rpfB* indicates the *rpfB/rpfC* double-deletion mutant complemented by a single copy of *rpfB*. Data are expressed as the means ± standard deviation of three independent assays. Different letters indicate significant differences between treatments according to least significant difference at $P = 0.05$.

The *rpfB* gene is required for full virulence of *X. oryzae* pv. *oryzae* in rice.

To evaluate the role of RpfB in pathogenesis, PXO99A, Δ*rpfB*, and Δ*rpfB*::*rpfB* were inoculated in two susceptible rice cultivars IRBB3 and IR24 by the leaf-clipping method. Δ*rpfB* was consistently less virulent than the wild-type strain PXO99A on both cultivars (Fig. 3A and C). However, mutants Δ*rpfB*, Δ*rpfC*, and Δ*rpfB*Δ*rpfC* caused a parallel length of lesion on rice cultivar IR24, while inoculation of Δ*rpfC* or Δ*rpfB*Δ*rpfC* led to further reduced lesion length on rice cultivar IRBB3 compared with Δ*rpfB* (Fig. 3A to C). These findings suggested that the *rpfB* gene was associated with *X. oryzae* pv. *oryzae* pathogenesis; however, different rice cultivars may have evolved specific sensitivity to *X. oryzae* pv. *oryzae* mutants.

Extracellular enzymes, including protease, amylase, and cellulase, are important virulence determinants produced by *Xanthomonas* spp. (Hu et al. 2007; Zhu et al. 2011). To verify whether the virulence-attenuated *rpfB* mutants had any defects in extracellular enzyme production, we measured the activity of these enzymes in the *rpfB* mutants. The mutant Δ*rpfB* displayed as much protease activity or cellulase activity as the wild-type strain PXO99A (data not shown). The extracellular amylase activity by Δ*rpfB* or Δ*rpfC* was reduced by approximately 30%, but deletion of *rpfB* in Δ*rpfC* did not lower amylase activity any further (Fig. 2D).

Expression of RpfB is suppressed by the DSF signaling cascade via the global transcription regulator Clp in *X. oryzae* pv. *oryzae*.

In *X. campestris* pv. *campestris*, the RpfC/RpfG-mediated DSF signaling cascade was demonstrated to negatively regulate *rpfB* expression, which relied on the direct interaction between the transcription factor Clp and the *rpfB* promoter (Zhou et al.

2015a). Alignment analysis showed that the promoter of *rpfB* in *X. oryzae* pv. *oryzae* was 65.7% identical to that in *X. campestris* pv. *campestris* (197 of 300 nt) (Supplementary Fig. S5), implying that *X. oryzae* pv. *oryzae* might deploy a similar regulatory system as *X. campestris* pv. *campestris*, to control *rpfB* expression. Therefore, the regulatory mechanism controlling *rpfB* expression in *X. oryzae* pv. *oryzae* was carefully studied in the present work. The expression pattern of *rpfB* in PXO99A during growth in NA broth was studied at the mRNA level by qRT-PCR and the protein level by immunoblotting analysis. Similar to the results obtained for *X. campestris* pv. *campestris*, a growth phase–dependent expression pattern was observed for the *rpfB* gene in *X. oryzae* pv. *oryzae*. The *rpfB* transcript increased progressively, with the maximal level detected at 36 h after inoculation followed by a gradual decrease (Fig. 4A), which was consistent with the RpfB protein level in PXO99A during growth (Fig. 4B).

In the mutants with disruptions in the genes involved in DSF signaling, including Δ*rpfF*, Δ*rpfC*, Δ*rpfG*, and Δ*clp*, the relative *rpfB* transcript level and RpfB protein level were significantly higher than those in wild-type strain PXO99A, with the highest levels detected in Δ*clp* (Fig. 5A and B). Complementation by a single copy of *clp* in Δ*clp* (the resultant strain called Δ*clp*::*clp*) fully restored RpfB expression to wild-type level (Fig. 5C). Consistently, the DSF and BDSF levels in the culture supernatant of Δ*rpfG* or Δ*clp* mutants were approximately 40 or 75% lower than those in PXO99A and the complementation strain Δ*clp*::*clp* recovered BDSF and DSF levels (Fig. 5D). Subsequent investigation on the interaction between Clp and the *rpfB* promoter in *X. oryzae* pv. *oryzae* was conducted by an electrophoretic mobility shift assay (EMSA). Clp protein was expressed and purified by affinity chromatography (Supplementary Fig. S6), and a putative promoter DNA fragment covering 300 bp upstream of the *rpfB* translational start site, namely, P_{rpfB}, was cloned and labeled with fluorescent FAM for EMSA. Shift bands were observed when purified Clp protein ranging from 120 to 500 ng was applied to the reaction mixture, suggesting the formation of a Clp-P_{rpfB} complex.

Unlike most CRP family proteins, Clp has strong binding affinity to its target DNA in the absence of its ligand, the secondary messenger c-di-GMP, which suppresses the DNA binding activity of Clp allosterically (Chin et al. 2010; Leduc and Roberts 2009). Thus, c-di-GMP at different concentrations was applied to the EMSA reaction mixture containing a saturating amount of Clp (500 ng). The results showed that c-di-GMP at concentrations ranging from 25 to 100 μM partially prevented the formation of the Clp-P_{rpfB} complex, while c-di-GMP at 200 μM completely blocked the complex formation, which demonstrated that the interaction between Clp and P_{rpfB} was modulated by c-di-GMP in vitro. DN*ase* I footprinting analysis was performed to reveal the Clp binding site on the *rpfB* promoter in PXO99A. The results showed that Clp protected a continuous 25-bp region of the antisense strand from the position –91 to –115, relative to the translational start site of *rpfB*. An imperfect palindromic AATGC-N_6-GCATC motif, identical to the Clp binding site in the *rpfB* promoter in *X. campestris*

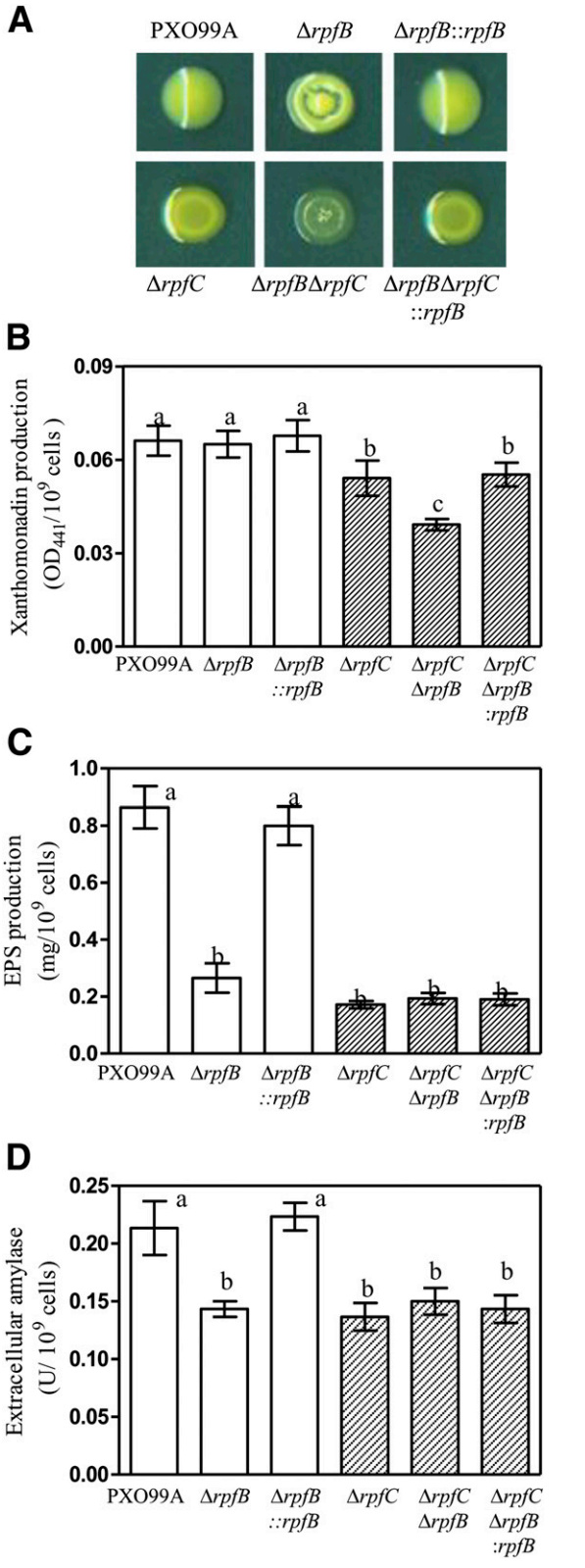

Fig. 2. Effects of *rpfB* on production of xanthomonadin and extracellular polysaccharide (EPS) as well as the activity of extracellular amylase in *Xanthomonas oryzae* pv. *oryzae* strains. **A,** Δ*rpfB* exhibits a wrinkled yellow but waterless colony morphology on nutrient agar (NA) plate, while Δ*rpfB*Δ*rpfC* exhibits a wrinkled oblate but less pigmented colony morphology on NA plate. **B,** Deletion of *rpfB* in PXO99A does not affect xanthomonadin production, while deletion of *rpfB* in Δ*rpfC* reduces xanthomonadin production in NA broth. **C,** Deletion of *rpfB* or *rpfC* in PXO99A affects EPS production in NA broth. **D,** Deletion of *rpfB* or *rpfC* in PXO99A affects extracellular amylase activity in nutrient yeast gycerol broth. PXO99A indicates *X. oryzae* pv. *oryzae* wild-type strain PXO99A; Δ*rpfB* indicates the *rpfB* in-frame deletion mutant; Δ*rpfB*::*rpfB* indicates the Δ*rpfB* mutant complemented by a single copy of *rpfB*; Δ*rpfC* indicates the *rpfC* in-frame deletion mutant; Δ*rpfB*Δ*rpfC* indicates the *rpfB*/*rpfC* double-deletion mutant; Δ*rpfB*Δ*rpfC*::*rpfB* indicates the *rpfB*/*rpfC* double-deletion mutant complemented by a single copy of *rpfB*. Data are expressed as the means ± standard deviation of three independent assays. Different letters indicate significant differences between treatments according to least significant difference at $P = 0.05$.

pv. *campestris* (Zhou et al. 2015a), was identified. Disruption of the Clp binding motif by introducing an AATGC→TTTTT point mutation in the promoter region of *rpfB* in PXO99A resulted in enhanced expression of RpfB and decreased production of DSF and BDSF. Therefore, we concluded that the DSF signaling cascade played essential roles in modulating the activity of RpfB-dependent DSF family signal turnover system in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*.

DISCUSSION

The transposon mutagenesis analysis done in the early 1990s revealed that a cluster of *X. campestris* pv. *campestris* genes, designated *rpfABCDEFG*, was involved in the regulation of EPS and extracellular enzyme production (Tang et al. 1991). Within this cluster, RpfB has been characterized as an FCL that utilizes different fatty acids of variable chain lengths, exclusive of DSF and BDSF, in vitro (Bi et al. 2014) and was subsequently further demonstrated to be essential for DSF family signal turnover in *X. campestris* pv. *campestris* (Zhou et al. 2015a). However, the function of RpfB in the rice pathogen *X. oryzae* pv. *oryzae* remains to be elucidated, although the majority of genes in the *rpf* cluster are highly conserved among most members of the genus of *Xanthomonas* and many closely related species, such as *Xylella fastidiosa* (Almeida et al. 2012; Chatterjee et al. 2008; Zhou et al. 2015a). In this study, we demonstrated that RpfB is required for DSF and BDSF signal turnover in *X. oryzae* pv. *oryzae* by presenting the following lines of evidence. First, deletion of *rpfB* in either the wild-type strain PXO99A or its DSF overproduction mutant Δ*rpfC* resulted in significantly enhanced BDSF and DSF production, which could be fully restored by the *rpfB* complementation strains Δ*rpfB::rpfB* and Δ*rpfB*Δ*rpfC*::*rpfB*, respectively (Fig. 1). Second, overexpression of *rpfB* completely abolished the production of BDSF and DSF. Finally, the *rpfB*-expressing strains, including PXO99A, Δ*rpfB::rpfB*, and Δ*rpfB*(*rpfB*), were capable of scavenging exogenous BDSF and DSF efficiently, whose efficiency largely depended on the expression level of RpfB. Moreover, RpfB was likely to exert its FCL activity to modify DSF and BDSF as well as other free fatty acids by converting them to fatty acyl CoA derivatives that could be used in phospholipid biosynthesis or for degradation in *X. oryzae* pv. *oryzae*, since point mutation on the conserved residue (the 365th amino acid) at its catalytic center completely abolished its DSF family signal turnover activity in vivo (Bi et al. 2014; Zhou et al.2015a). In conclusion, RpfB represents a conserved and naturally occurring QS signal turnover system in *Xanthomonas*.

An earlier study reported that the transcript level of *rpfB* in the *rpfC* mutant 8558 was higher than that of wild-type *X. campestris* pv. *campestris* 8004 (Slater et al. 2000). In addition, our recent study in *X. campestris* pv. *campestris* showed that expression of *rpfB* was regulated by the RpfC/RpfG-mediated DSF signaling system and also identified a Clp binding motif, AATGC-N_6-GCATC, in the promoter region of *rpfB* (Zhou et al. 2015a). Consistent with those findings in *X. campestris* pv. *campestris*, the genetic and biochemical data presented here verified that the components of the DSF signaling cascade, including RpfC, RpfG, and Clp, as well as the DSF synthase RpfF, were involved in the repression of *rpfB* expression in *X. oryzae* pv. *oryzae* (Fig. 5A to C) and that the second messenger c-di-GMP, a ligand of Clp, could effectively reverse this repression. C-di-GMP has been implicated as a ubiquitous second messenger in a wide range of bacteria, influencing various bacterial behavior including cell differentiation, motility, biofilm formation, and production of virulence factors (Aldridge et al. 2003; Cotter and Stibitz 2007; Ross et al. 1987; Ryan 2013; Ryan et al. 2007; Yang et al. 2015). The intracellular level of c-di-GMP is precisely and dynamically balanced by the process of biosynthesis and degradation of c-di-GMP, which is catalyzed by diguanylate cyclases containing the GGDEF domain and phosphodiesterases containing EAL or HD-GYP domains (Ryan 2013). In the genome of PXO99A, a total of 26 genes encoding GGDEF, EAL, or HD-GYP domains were identified, among which, two component systems RpfC/RpfG and PdeK/PdeR have previously been demonstrated to be involved in c-di-GMP degradation (He et al. 2010; Ryan et al. 2007; Yang et al. 2012). Therefore, the regulatory mechanism on DSF family signal turnover in *Xanthomonas* is likely complicated and fine-tuned, involving integration of signals from diverse environmental inputs that remain to be explored.

The first QQ enzyme, AiiA, that effectively hydrolyzes the lactone ring of AHL-type QS signal molecules was identified in *Bacillus subtilis* 240B1 (Dong et al. 2001). Subsequently, many QQ enzymes, such as AHL lactonase, AHL acylase, and oxidoreductase, have been found in various organisms, including bacteria, fungi, and mammals (Du et al. 2014). However, the majority of the QQ enzymes are heterogeneously expressed to interfere with the QS system in an interspecies manner. In *A. tumefaciens*, a naturally occurring AHL lactonase encoded by *attM* was identified to cleave the self-produced QS signals 3-oxo-octanoylhomoserine lactone (OC8HSL) and γ-butyrolactone (GBL), whose expression was negatively regulated by transcription factor AttJ (Zhang et al. 2002). Although our studies have shown that *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* use a similar regulatory system consisting of at least RpfB and the repressor Clp to modulate DSF family signal level (Zhou et al. 2015a), a fundamental difference between *Xanthomonas* spp. and *A. tumefaciens* has been revealed. Direct repression on *attM* expression by AttJ in *A. tumefaciens* can only be reversed by the presence of γ-butyrolactone, γ-hydroxybutyrate, succinic semialdehyde, γ-aminobutyrate, and salicylic acid, which are accumulated in *A. tumefaciens*–infected plants or their metabolites, independent of any QS signals, including *A. tumefaciens*–produced OC8HSL (Haudecoeur and Faure 2010). However, repression of *rpfB* expression is regulated, at least partially, by the DSF-signaling pathway in *Xanthomonas.*

Table 1. Levels of 4- hydroxybenzoic acid (4-HBA), 3-HBA, and coenzyme Q_8 produced by *Xanthomonas oryzae* pv. *oryzae* strains as well as their expression of *xanB2*[y]

Strains	4-HBA (μM)	3-HBA (μM)	CoQ_8 (μM)	*xanB2*[z]
PXO99A	0.66 ± 0.048a	0.42 ± 0.029a	5.64 ± 0.31a	1.00 ± 0.03a
Δ*rpfC*	0.21 ± 0.024b	0.92 ± 0.042b	5.36 ± 0.14a	0.43 ± 0.06b
Δ*rpfC*Δ*rpfB*	0.60 ± 0.012a	0.86 ± 0.020b	4.30 ± 0.13b	0.36 ± 0.08b
Δ*rpfC*Δ*rpfB*::*rpfB*	0.27 ± 0.015b	1.05 ± 0.028b	5.40 ± 0.08a	0.50 ± 0.12b

[y] Data are the mean ± standard error from three replications. Different letters indicate significant differences between groups according to least significant difference at $P = 0.05$.

[z] Expression level of *xanB2* in the *X. oryzae* pv. *oryzae* strains listed in the first column relative to that of the wild-type strain PXO99A is shown (mean rations of replications ± standard error). Amount of RNA relative to that in PXO99A is equal to 1.0 and was normalized for population size, using 16S rRNA as endogenous control.

As described above, the FCL activity of RpfB toward DSF family signals and its regulatory mechanism are generally consistent in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*. However, the present study also provides evidence that *X. oryzae* pv. *oryzae* has evolved specific features in DSF family signal–mediated adaptation and pathogenesis. First, data presented here showed that PXO99A produces significantly more DSF and BDSF than *X. campestris* pv. *campestris* XC1. The maximal levels of BDSF and DSF in the supernatant of XC1 were previously determined to be 0.03 and 0.11 μM, respectively (Zhou et al. 2015a), whereas they were 0.35 and 1.05 μM, respectively, in the supernatant of PXO99A, which is approximately 10 times higher. Similarly, the deletion mutants, Δ*rpfB*, Δ*rpfC*, and Δ*rpfB*Δ*rpfC* of PXO99A produced and secreted significantly higher levels of BDSF and DSF than the parallel mutants generated in XC1 (Fig. 1) (Zhou et al. 2015a). Recently, Deng et al. (2015) pointed out that the fatty acid carbon chain of DSF was derived from glucose and DSF family signal molecules were generated through the fatty acid synthetic pathway under in planta conditions. In an independent study, carbohydrates, including glucose, sucrose, and fructose as well as starch, were verified as the precursors of BDSF, while leucine and valine were the precursor of DSF in *X. campestris* pv. *campestris* (Zhou et al. 2015b). Moreover, biosynthesis of DSF family signals depends on the classic fatty acid synthesis elongation cycle (Zhou et al. 2015b). Based on these findings, we hypothesize that the excessive production of DSF family signals by the *rpfB* deletion mutant in PXO99A is seemingly a double-edged sword for *X. oryzae* pv. *oryzae*, since the biosynthesis of DSF family molecules is an energy input–required process that consumes large quantities of carbohydrates or amino acids, which probably leads to a global metabolic imbalance and at least partially causes the growth defects of the *rpfB/rpfC* double-deletion mutants of *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* in the late stationary phase (Zhou et al. 2015a). However, metabolic fingerprinting analysis on DSF overproduction mutants may help to elucidate this hypothesis in the future.

Additionally, the present study has reported that hyperproduction of DSF family signal molecules by Δ*rpfC* and Δ*rpfB*Δ*rpfC* led to a reduction in xanthomonadin production by approximately 20 and 40%, respectively (Fig. 2A and B), which was not observed in the parallel mutants of *X. campestris* pv. *campestris* strains XC1 or 8004 (Zhou et al. 2015a). The *xanB2* gene is responsible for the production of biosynthetic intermediates of xanthomonadin and coenzyme Q_8, 3-HBA, and 4-HBA in *Xanthomonas* spp. (Zhou et al. 2013a and b). In addition, 4-HBA may participate in the production of xanthomonadin with alternative chemical structures (Zhou et al. 2013a and b). Transcriptional analysis showed that *xanB2* expression was downregulated in Δ*rpfC* and Δ*rpfB*Δ*rpfC* (Table 1). However, a similar level of *xanB2* transcript was detected in the two xanthomonadin differentially produced mutants, Δ*rpfC* and Δ*rpfB*Δ*rpfC* (Table 1; Fig. 2A and B), which suggested that the expression level of *xanB2* was not directly associated with the actual level of xanthomonadin produced by *X. oryzae* pv. *oryzae* strains. Therefore, we speculated that the levels of 3-HBA and 4-HBA that remained

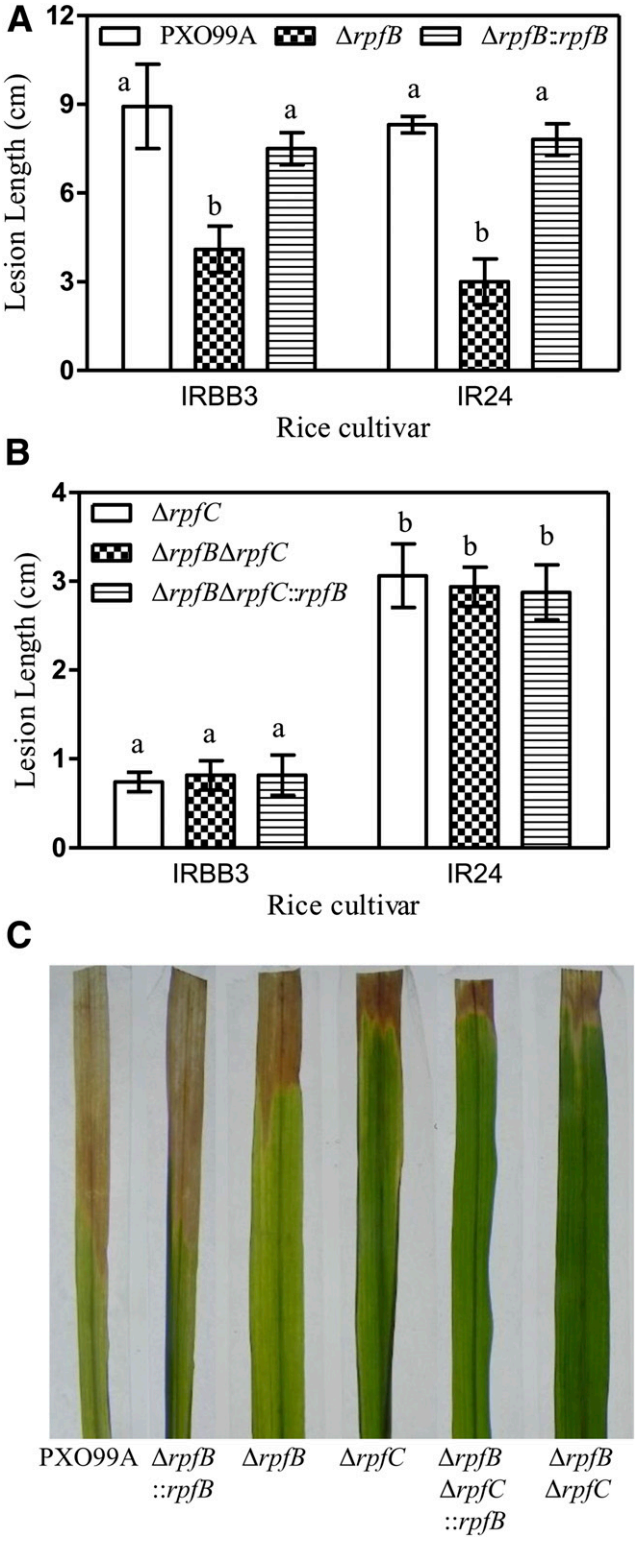

Fig. 3. Deletion of *rpfB* impairs virulence of *Xanthomonas oryzae* pv. *oryzae* on rice cultivars. **A,** Lesion length caused by *X. oryzae* pv. *oryzae* wild-type strain PXO99A, mutant strain Δ*rpfB* and complementation strain Δ*rpfB::rpfB*. **B,** Lesion length caused by *X. oryzae* pv. *oryzae* mutant strains Δ*rpfC* and Δ*rpfB*Δ*rpfC* as well as the complementation strain Δ*rpfB*Δ*rpfC::rpfB*. Virulence of the *X. oryzae* pv. *oryzae* strains was assayed by measuring lesion length after introducing bacteria into the vascular system of rice cultivars IRBB3 and IR24 by leaf clipping. **C,** Representative pictures of lesions on rice cultivar IRBB3 leaves inoculated with *X. oryzae* pv. *oryzae* strains after 2 weeks postinoculation. PXO99A indicates *X. oryzae* pv. *oryzae* wild-type strain PXO99A; Δ*rpfB* indicates the *rpfB* in-frame deletion mutant; Δ*rpfB*::*rpfB* indicates the Δ*rpfB* mutant complemented by a single copy of *rpfB*; Δ*rpfC* indicates the *rpfC* in-frame deletion mutant; Δ*rpfB*Δ*rpfC* indicates the *rpfB/rpfC* double-deletion mutant; Δ*rpfB*Δ*rpfC::rpfB* indicates the *rpfB/rpfC* double-deletion mutant complemented by a single copy of *rpfB*. Data are expressed as the means ± standard deviation of three independent assays, each comprising 10 leaves. Different letters indicate significant differences between treatments according to least significant difference at $P = 0.05$.

in the supernatants might indirectly reflect the in vivo biosynthetic activity of xanthomonadin in *X. oryzae* pv. *oryzae*. The increased level of 3-HBA in the supernatants of Δ*rpfC* and Δ*rpfC*Δ*rpfB* observed in the present study indicated that 3-HBA–induced production of xanthomonadin was likely suppressed by *rpfC* deletion (Table 1). In addition, deletion of *rpfB* in Δ*rpfC* facilitated 4-HBA accumulation in *X. oryzae* pv. *oryzae* (Table 1). The likely reason behind this observation is that the double-deletion mutant Δ*rpfC*Δ*rpfB* has certain defects in 4-HBA–dependent xanthomonadin biosynthesis without interfering with the expression level of *xanB2*. Moreover, mutations in xanthomonadin biosynthetic genes working downstream of *xanB2* often result in defective xanthomonadin production coupled with accumulation of 3-HBA and 4-HBA in the culture supernatant (data not shown). Collective findings suggest that the levels of 3-HBA and 4-HBA are dynamically modulated in the xanthomonadin differentially produced mutants Δ*rpfC* and Δ*rpfB*Δ*rpfC* by a certain unknown mechanism that remains to be elucidated in the future.

Moreover, discrepancies in bacterial pathogenesis between the *rpfB* mutants of *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* have been identified in this study. Our previous work showed that Δ*rpfB* of *X. campestris* pv. *campestris* had increased activity of extracellular enzymes and EPS production as well as enhanced virulence on Chinese radish at a moderate level (Zhou et al. 2015a). However, the present study revealed that deletion of *rpfB* in PXO99A resulted in reduced activity of extracellular amylase (Fig. 2D) and EPS production (Fig. 2C) by approximately 33 and 69%, respectively, and significantly attenuated virulence on rice cultivars IRBB3 and IR24 in a leaf-clipping assay (Fig. 3). These results are generally in line with the findings by earlier works on a marker-exchange *rpfB* mutant (*rpfB*::Tn*5*) of *X. oryzae* pv. *oryzae* KACC10859 and the *rpfB* mutant of *Xylella fastidiosa*, which showed attenuated

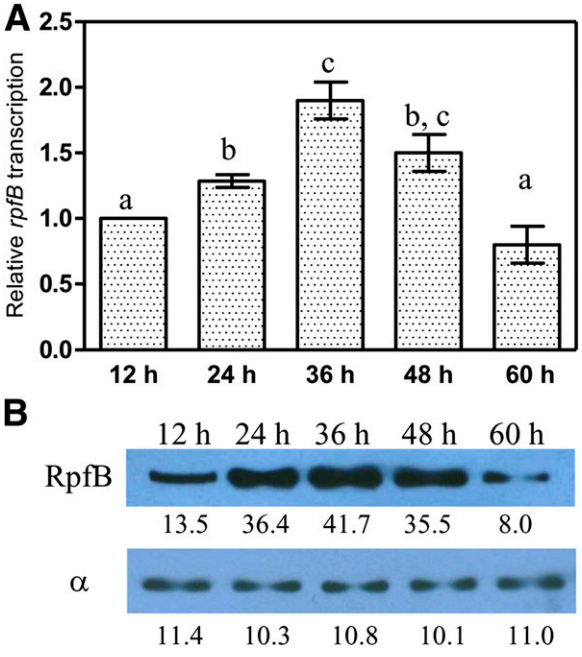

Fig. 4. Time course analysis on *rpfB* expression in *Xanthomonas oryzae* pv. *oryzae* PXO99A during growth in nutrient agar broth. **A,** Relative *rpfB* level as determined by quantitative reverse-transcription polymerase chain reaction. Amount of RNA relative to that in PXO99A harvested at 12 h postinoculation is equal to 1.0 and was normalized for population size, using 16S rRNA as endogenous control. Values are expressed as the mean and standard deviation of triplicate measurements. Different letters indicate significant differences between treatments according to least significant difference at $P = 0.05$. **B,** Western blot analysis of proteins isolated from the bacterial pellets of PXO99A harvested at the indicated time points probed with an antibody against RpfB (upper panel) and with antibody against the α subunit of RNA polymerase (lower panel). The numbers indicate signal intensity as measured by ImageJ software.

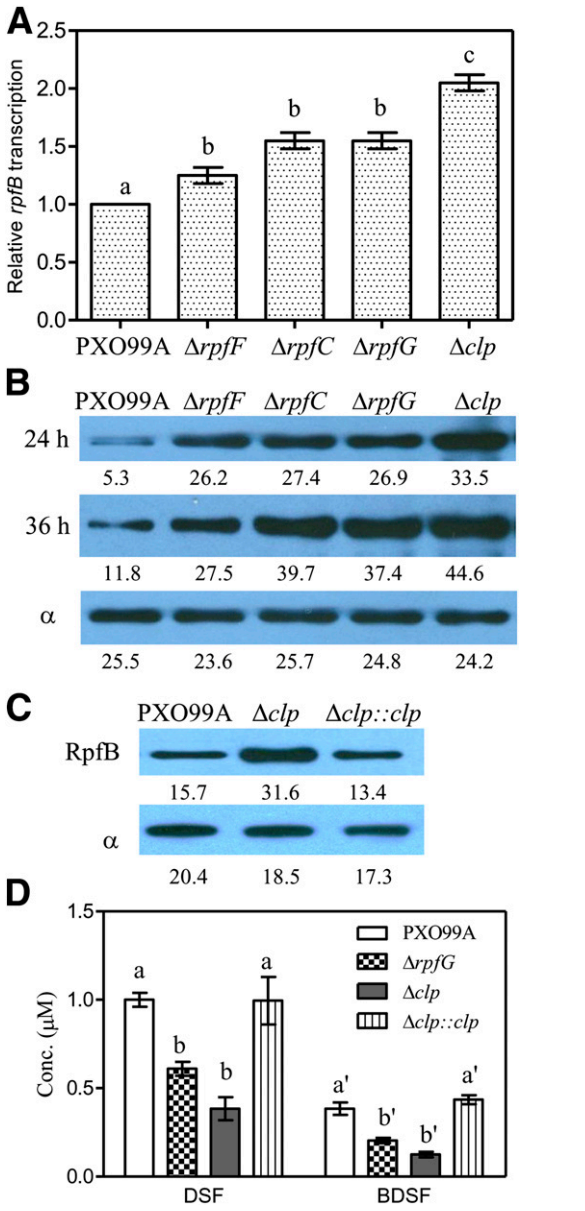

Fig. 5. The RpfC/RpfG-dependent diffusible signal factor (DSF) family signal cascade suppresses *rpfB* expression in *Xanthomonas oryzae* pv. *oryzae*. **A,** Relative *rpfB* level in the *X. oryzae* pv. *oryzae* strains PXO99A, Δ*rpfF*, Δ*rpfC*, Δ*rpfG*, and Δ*clp*, as determined by quantitative reverse-transcription polymerase chain reaction. Amount of RNA relative to that in PXO99A is equal to 1.0 and was normalized for population size, using 16S rRNA as endogenous control. **B** and **C,** Western blot analysis of proteins isolated from the bacterial pellets of test *X. oryzae* pv. *oryzae* strains grown in nutrient agar broth probed with an antibody against RpfB (upper panel) and with antibody against the α subunit of RNA polymerase (lower panel). The numbers indicate signal intensity as measured using ImageJ software. **D,** DSF and BDSF production in the *X. oryzae* pv. *oryzae* strains PXO99A, Δ*rpfG*, Δ*clp*, and Δ*clp*:*clp*. PXO99A indicates *X. oryzae* pv. *oryzae* wild-type strain PXO99A; Δ*rpfF* indicates the *rpfF* in-frame deletion mutant; Δ*rpfC* indicates the *rpfC* in-frame deletion mutant; Δ*rpfG* indicates the *rpfG* in-frame deletion mutant; Δ*clp* indicates the *clp* in-frame deletion mutant; Δ*clp*:*clp* indicates the *clp* deletion strain complemented by a single copy of *clp* in the *att*Tn7 site. Data are expressed as the means ± standard deviation of three independent assays. Different letters indicate significant differences between treatments according to least significant difference at $P = 0.05$.

virulence on the susceptible rice cultivar Miyang 23ho and reduced insect colonization and transmission, respectively (Almeida et al. 2012; Jeong et al. 2008). In order to understand the underlying mechanism by which *rpfB* deletion influenced *X. oryzae* pv. *oryzae* virulence, we selected ten DSF-regulated genes as virulence-associated marker genes for expression analysis based on the results obtained in a genome-wide transcriptional analysis on the DSF-deficient mutant of Indian *X. oryzae* pv. *oryzae* BXO43 (Rai et al. 2012). qRT-PCR results revealed that expression of *PXO_04839* (encoding RND efflux membrane fusion protein MexC) was moderately downregulated by 2.5-fold ± 0.7-fold in Δ*rpfB*, compared with that in PXO99A. Expression of the other nine marker genes, including *PXO_02600* (encoding TonB-dependent receptor Bfu), *PXO_04752* (encoding chemotaxis protein CheW), *PXO_01001* (encoding structural flagella protein FliC), *PXO_04868* (encoding a putative glucoamylase), *PXO_02004* (encoding peroxiredoxin OsmC), *PXO_00466* (encoding putative two-component system regulatory protein with CheY GGDEF domain RrpX), *PXO_02671* (encoding *Xanthomonas* adhesin-like protein XadA), *PXO_01401* (encoding exopolysaccharide xanthan biosynthesis glucuronosyltransferase GumK), and *PXO_04006* (encoding Clp), was basically not affected in Δ*rpfB* (data not shown). The RND (resistance nodulation division) family efflux pumps in gram-negative bacteria, especially in those clinically important pathogens such as *Pseudomonas aeruginosa* and *Salmonella typhimurium*, extrude a broad spectrum of antibiotics and biocides from the periplasm to the outside of the cell and become one of the major contributors to intrinsic resistance (Opperman and Nguyen 2015). Therefore, reduced expression of the RND efflux membrane fusion protein encoding gene *mexC* in Δ*rpfB* might lead to detoxification defects and weaken bacterial survival under oxidative stress, especially during colonization in rice plants, which helps to get new insights in why Δ*rpfB* was less virulent than the wild-type strain PXO99A (Fig. 3). However, this is unlikely to be the sole reason. Other DSF-regulated genes responsive to *rpfB* deletion in *X. oryzae* pv. *oryzae*, albeit yet-unidentified, may contribute to *Xanthomonas* virulence in different ways, which deserves further investigation.

Glucose is the precursor of glucuronic acid, one component of EPS in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (Coplin and Cook 1990; Kumar et al. 2003) and the precursor of DSF family signals as well (Zhou et al. 2015b). Excessive production of BDSF and DSF by PXO99A Δ*rpfB* likely drained the pool of glucose that was also required for EPS biosynthesis, thus leading to reduced EPS production. As an important virulence determinant, EPS promotes *Xathomonas* pathogenesis by aiding bacterial colonization and survival, increasing the thermal death point and enhancing UV protection (Dharmapuri and Sonti 1999; El-Banoby and Rudolph 1989; Leach et al. 1957). Recently, DSF family QS signals have been shown to play crucial roles in *Xanthomonas*-plant communication by eliciting host innate immunity in several model plants, including *Arabidopsis*, *Nicotiana benthamiana*, and rice (Kakkar et al. 2015). However, the DSF-induced plant defense response could be suppressed by EPS produced by *X. campestris* pv. *campestris* (Kakkar et al. 2015). Based on these findings, we posited that reduced production of EPS and increased production of DSF family signals by PXO99A Δ*rpfB* enhanced innate immune responses in rice and resulted in attenuated virulence. However, we cannot rule out the possibility that deletion *rpfB* in *X. oryzae* pv. *oryzae* might also lead to other physiological inadequacy caused by excessive use of carbon sources and amino acids to synthesize large amount of DSF and BDSF and, thus, results in reduced production of virulence factors and, ultimately, attenuated virulence. Therefore, the detailed mechanisms remain to be experimentally verified.

In summary, our findings have demonstrated that the RpfB-dependent DSF family QS signal turnover system is conserved in *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* (Zhou et al. 2015a), which are genetically closely related *Xanthomonas* pathogens. However, their *rpfB* mutants have shown strikingly different virulence-associated traits, including production of EPS, xanthomonadin, and extracellular enzymes as well as virulence in host plants (Zhou et al. 2015a). These findings strongly indicate the complexity of the RpfB-dependent DSF family signal turnover system, which is likely fine-tuned to facilitate the infection process in different hosts (rice versus crucifers), even though the signal turnover activity is conservatively regulated by the RpfC/RpfG-mediated DSF signal transduction cascade via the global transcription regulator Clp and the second messenger c-di-GMP in *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* (Zhou et al. 2015a). In addition, previous research revealed considerable differences in the pattern or repertoire of DSF-regulated phenotypes among several members of genus *Xanthomonas*, including *X. campestris* pv. *campestris*, *X. oryzae* pv. *oryzae*, and *Xanthomonas oryzae* pv. *oryzicola*, although the DSF family signal molecules and DSF signaling cascade are commonly shared (He et al. 2006b; Rai et al. 2012, 2015). Therefore, the dynamic and precise control of DSF family signal level by coordinately regulating the activity of DSF biosynthetic and degradative pathways tends to be a common adaptive strategy in a strain-specific manner among different *Xanthomonas* pathogens.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in the present study are listed in Supplementary Table S1. *X. oryzae* pv. *oryzae* strains were grown at 30°C in NA (per liter: 5 g of peptone, 3 g of beef extract, 10 g of sucrose, and 1 g of yeast extract, pH 7.0). *Escherichia coli* strains were grown at 37°C in Luria Bertani medium (per liter: 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl, pH 7.0). Where required, antibiotics were used at the following concentrations (micrograms per milliliter): 20 of cefalexin, 50 of kanamycin, 20 of gentamycin, and 100 of ampicillin. Bacterial growth was determined by measuring OD_{600}.

Construction of in-frame deletion mutants and complementation.

X. oryzae pv. *oryzae* PXO99A was used as the parental strain for the generation of deletion mutants, as previously described by He et al. (2010). The primers used are listed in Supplementary Table S2. For single-copy complementation of *rpfB*, the coding region of *rpfB* plus its promoter region was amplified by PCR and was cloned in a versatile mini-Tn*7* delivery vector mini-Tn*7*T-Gm. The resulting constructs were transferred into *X. oryzae* pv. *oryzae* by electroporation and the complementation strain was selected as described by Jittawuttipoka et al. (2009).

Extraction, purification, and quantitative analysis of BDSF and DSF.

The extraction and purification of DSF and BDSF were conducted following the methods previously described by He et al. (2010). DSF and BDSF production levels were then quantified by liquid chromatography-mass spectrometry (LC-MS), following the method described by Zhou et al. (2015a). Briefly, 0.2 ml of the culture supernatant of the Δ*rpfC* strain and the Δ*rpfC* derived strains or 20.0 ml of the culture supernatant of the wild-type strain PXO99A and its derived strains was collected and extracted with equal volumes of ethyl acetate. The crude ethyl acetate extract was evaporated and dissolved in 0.1 ml of methanol for LC-MS analysis. For cellular DSF and

BDSF extraction, cell pellets were harvested from 50 ml of *X. oryzae* pv. *oryzae* cultures at 48 h after inoculation and were washed with equal volumes of 1× PBS (phosphate buffered saline) buffer. Then, the cell pellets were resuspended in 5 ml of 1× PBS buffer followed by sonication. The lysate was heated at 95°C for 5 min and the supernatant of lysate was collected by centrifugation and, then, subjected to DSF and BDSF extraction.

Five microliters of the condensed samples were applied to an ultra performance liquid chromatographic system (Agilent 1290 Infinity) on a Zorbax XDB C18 reverse phase column (4.6 × 150 mm, temperature-controlled at 30°C) and were eluted with methanol and water (80:20, vol/vol) at a flow rate of 0.4 ml·min^{-1} in a diode array detector (Agilent G4212A). Data were acquired in the centroid mode using Agilent MassHunter Workstation data acquisition software (revision B.04). BDSF and DSF levels in the culture supernatant were quantified using the extracted ion intensity (EI) from the LC-MS chromatogram according to the following formula: BDSF (μM) = $8.61 \times 10^{-6} \times$ EI – 0.20, DSF (μM) = $9.09 \times 10^{-6} \times$ EI – 0.04. The formula was derived from a dose–EI plot in the LC-MS chromatogram using various dilutions of synthetic BDSF and DSF signal molecules with correlation coefficients (R^2) of 0.998 and 0.996, respectively.

Quantitative determination of extracellular enzyme activity and EPS production.

The method for quantitative determination of EPS production was previously described by Zhou et al. (2013b). The extracellular enzyme activity of protease in *X. oryzae* pv. *oryzae* strains was measured essentially as described by He et al. (2006b). The activity of extracellular amylase and cellulase was determined following the methods described by Zhu et al. (2011).

Virulence assay on rice.

The virulence of *X. oryzae* pv. *oryzae* strains on two rice cultivars, IRBB3 and IR24, was estimated by leaf-clipping, as previously described (Zhou et al. 2013b). Briefly, the bacterial pellets from the culture at an OD_{600} of 1.6 were resuspended in sterile PBS buffer to an OD of 0.1 and were inoculated into adult rice plants by leaf-clipping, for lesion length measurement. Twenty leaves for each test strain were inoculated. Lesion length was measured 2 weeks after inoculation. Each strain was tested in at least three separate experiments.

Total RNA extraction and quantitative RT-PCR analysis.

Total RNAs were isolated using the RNeasy mini-prep kit (Qiagen). The PrimeScript RT reagent kit was used for qRT-PCR analysis. Quantification of gene expression and melting curve analysis were performed using Mastercycler ep Realplex 4S (Eppendorf) and SYBR Premix Ex Taq (Takara). The constitutively expressed 16S rRNA gene was used as a reference to standardize all samples and replicates.

Protein purification and Western blot analysis.

Clp protein was expressed and purified following the method described elsewhere (Tao et al. 2010). The protein samples were stored as aliquots in 20% (vol/vol) glycerol at –20°C until use. Western blot analysis was performed as described by Green and Sambrook (2012). The polyclonal antisera against the RpfB protein derived from *X. campestris* pv. *campestris* XC1 was used in the Western blot analysis as described by Zhou et al. (2015a). The hybridization signal was detected using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific Pierce USA). The monoclonal antibody against α subunit of RNA polymerase (NeoClone) was used as a control for sample loading.

EMSA and DN*ase* I footprinting assay.

The *rpfB* promoter region covering 300 bp upstream of the translational start codon P_{rpfB} was cloned into the T-vector of pTA2 (Toyobo) as template. Fluorescent FAM-labeled P_{rpfB} probe was amplified by PCR. EMSA was performed using LightShift chemiluminescent EMSA kit (Thermo Scientific Pierce USA). DN*ase* I footprinting assays were performed as described by Wang et al. (2012).

Site-directed mutagenesis on Clp binding motif.

The *rpfB* promoter region P_{rpfB} was cloned into pTA2 for further point mutation using the QuickChange site-directed mutagenesis kit (Agilent Technologies). The mutated P_{rpfB} was further cloned into the suicide vector pK18mobsacB and the resulting recombinant construct was mobilized into *X. oryzae* pv. *oryzae* by electroporation. The strains with point mutations in P_{rpfB} in the chromosome were verified by PCR amplification and DNA sequencing.

Statistical analysis.

P values were determined by an unpaired Student's *t* test with two groups, assuming unequal variances by using the Microsoft Office Excel data analysis tool. $P < 0.05$ was considered statistically significant.

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