Identification and Characterization of a Novel Gene, *hshB*, in *Xanthomonas oryzae* pv. *oryzicola* Co-Regulated by Quorum Sensing and *clp*

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ABSTRACT

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Virulence factors of *Xanthomonas oryzae* pv. *oryzicola*, the causal agent of bacterial leaf streak in rice, are regulated by a diffusible signal factor (DSF)-dependent quorum-sensing (QS) system. In this study, a novel pathogenicity-related gene, *Xoryp_010100018570* (named *hshB*), of *X. oryzae* pv. *oryzicola* was characterized. *hshB* encodes a hydrolase with a putative signal peptide, which is a homolog of imidazolone-propionase. Bioinformatic analysis showed that *hshB* is relatively conserved in the genus *Xanthomonas* but the homologous gene of *hshB* was

not found in *X. oryzae* pv. *oryzae*. Reverse-transcription polymerase chain reaction (PCR) analysis showed that *hshB* and its upstream gene, *Xoryp_010100018565* (named *hshA*), are co-transcribed in *X. oryzae* pv. *oryzicola*. Subsequent experimental results indicated that mutation of *hshB* remarkably impaired the virulence, extracellular protease activity, extracellular polysaccharide production, growth in minimal medium, and resistance to oxidative stress and bismethiazol of *X. oryzae* pv. *oryzicola*. Mutation of *clp*, encoding a global regulator, resulted in similar phenotypes. Real-time PCR assays showed that *hshB* transcription is positively regulated by *clp* and DSF, and induced by poor nutrition. Our study not only found a novel gene *hshB* regulated by DSF-dependent QS system and *clp* but also showed that *hshB* was required for virulence of *X. oryzae* pv. *oryzicola*.

The genus *Xanthomonas* comprises an important group of gram-negative phytopathogenic bacteria that infect many monocotyledons and dicotyledons worldwide (25). The bacteria penetrate the host through hydathodes, stomata, or wounds, multiplying and spreading in the intercellular space or xylem vessels. Among members of this genus, *X. oryzae* pv. *oryzicola* is the causal agent of bacterial leaf streak (BLS), one of the most serious bacterial diseases of rice (*Oryza sativa* L.) in Asia and West Africa (28).

Most phytopathogens can survive in diverse environments (26). However, virulence factors are largely expressed during plant-pathogen interactions for energy conservation through a controlled global virulence regulation network (26). Quorum sensing (QS) is a cell-to-cell communication mechanism with small signal molecules, and is an important part of the global regulatory network. Bacterial cells are able to sense and respond to their own density through a QS system, and coordinate gene expression within bacterial populations (5,26). Currently, several types of bacterial QS signals have been identified (20,26). Among them, many gram-negative bacteria regulate diverse biological functions through an N-acyl homoserine lactone (AHL)-dependent QS system; for example, *Ralstonia solanacearum* (13), *Pectobacterium carotovorum* subsp. *carotovorum* (previously *Erwinia carotovora* subsp. *carotovora*) (31), and *Pseudomonas syringae* pv. *tomato* (33). Research in the last few years has shown that the

AHL-dependent QS system plays a key role in coordinating various aspects of bacterial behavior, including biofilm dispersal, production of extracellular enzymes and polysaccharides (EPS), type 3 secretion system (T3SS), oxidative stress resistance, and antibiotic production (9,32,38,43).

Similar to the AHL-dependent QS, many species of *Xanthomonas*, such as *X. campestris* pv. *campestris*, *X. oryzae* pv. *oryzae*, and *X. oryzae* pv. *oryzicola*, have evolved a novel QS system for genetic regulation at the population level using diffusible signal factors (DSFs) (4,7,40,44). Accumulating evidence indicates that the *rpf* cluster, responsible for the DSF-dependent QS system, is conserved in *Xanthomonas* spp., the core genes of which include *rpfF*, *rpfC*, and *rpfG* (7,40,44). The *rpfF* gene encodes a putative enoyl-CoA hydratase that catalyzes biosynthesis of DSFs (40). In the model pathogen *X. campestris* pv. *campestris*, a DSF was identified as a *cis*-11-methyl-2-dodecanoic acid (42). DSFs are sensed by a unique two-component signal transduction system consisting of the sensor kinase RpfC and the response regulator RpfG (35,39). The RpfC/RpfG system couples the bacterial cell-cell communication to the intracellular regulatory networks through the second messenger cyclic di-GMP and the global regulator Clp (17,26). By the RpfC/RpfG system, DSFs regulate the expression of downstream genes involved in virulence, motility, biofilm dispersal, and extracellular enzyme and EPS production (8,18,39,44).

In recent years, a focus of research on phytopathogenic bacteria has been the identification and functional analysis of pathogenicity-related genes that are regulated by the QS system. In *X. campestris* pv. *campestris*, 165 DSF-mediated genes were identified by microarray analysis, 133 of which were DSF-activated, and 32 were repressed (19). In addition, two-dimensional gel electrophoresis (2-DE) has also been widely used to rapidly

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identify the specific protein at the whole-proteome level. Using this method, 47 AHL-modulated proteins were identified in *P. aeruginosa*, with 20 being novel proteins (2).

In our previous research, 48 DSF-mediated proteins were identified by comparative analysis of 2-DE maps between *X. oryzae* pv. *oryzicola* wild-type strain Rs105 and *rpjF* mutant strain $\Delta rpjF$, 18 proteins of which were identified by tandem mass spectrometry (MS/MS) (44). Among these, our present study identified a novel pathogenicity-related gene, *Xoryp_010100018570* (named *hshB*), which encodes a hydrolase with putative signal peptide and is a homolog of imidazolonepropionase (HutI), and revealed its genetics and biological functions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All of the *X. oryzae* pv. *oryzicola* strains used are derivatives of the parent strain Rs105. *Escherichia coli* strains were cultivated at 37°C in Luria broth (LB) or on LB agar plates. Unless otherwise stated, the *Xanthomonas* strains were grown at 28°C in nutrient broth (NB) (beef extract at 3 g/liter, yeast extract at 1 g/liter, polypeptone at 5 g/liter, and sucrose at 10 g/liter) or on nutrient agar (NA). When required, antibiotics were added to the medium at the following final concentrations: ampicillin (Ap) at 100 µg/ml and kanamycin (Kan) at 50 µg/ml for *E. coli* and rifampicin (Rif) at 100 µg/ml and Kan at 50 µg/ml for *Xanthomonas* strains.

Generation of in-frame deletion mutants and complementation. The *X. oryzae* pv. *oryzicola* wild-type strain Rs105 was used as the parental strain for generating deletion mutants via allelic homologous recombination using the suicide plasmid pK18mobsacB (37). In-frame deletion mutants of *X. oryzae* pv. *oryzicola* were generated using the primers listed in Supplemental Table 1, as previously described (17,44). For complementation, the coding regions of these genes were amplified by polymerase chain reaction (PCR) using the primers listed in Supplemental Table 1 and cloned into the expression vector pUFR034. The resultant constructs were transferred into the corresponding mutants by electroporation.

Pathogenicity testing and determination of bacterial population in planta and minimal medium. Pathogenicity assays were performed in a glasshouse at 22 to 30°C as previously described (44,45). In brief, *X. oryzae* pv. *oryzicola* strains were cultivated in NB medium at 28°C with appropriate antibiotics.

The bacterial concentrations were adjusted to an optical density at 600 nm (OD_{600}) $\approx$ 0.1 and inoculated into leaves of 6-week-old rice plants ('Shanyou-63', susceptible to the pathogen used in this work) using an acupuncture inoculation method. Lesion lengths were measured 14 days after inoculation. Twenty-five leaves were inoculated for each *X. oryzae* pv. *oryzicola* strain in each treatment. The same experiment was repeated three times.

Growth of each *X. oryzae* pv. *oryzicola* strain in rice leaf tissue was detected by homogenizing five inoculated leaves in 9 ml of sterile water. The 6-cm-long section was cut from each inoculated leaf around the inoculated spot on days 0, 4, 8, 12, and 16 after inoculation (24). Diluted homogenates were plated on NA plates supplemented with Rif (for the wild type and mutants) or Rif plus Kan (for the complemented strains). The number of bacterial colonies on these plates was counted after incubation at 28°C for 2 days (12). Each diluted homogenate was plated on three plates, respectively. The same experiment was repeated three times.

The growth curves of *X. oryzae* pv. *oryzicola* strains in minimal medium (glucose at 5 g/liter, $[NH_4]_2SO_4$ at 2 g/liter, $MgSO_4 \cdot 7H_2O$ at 0.2 g/liter, K_2HPO_4 at 4 g/liter, KH_2PO_4 at 6 g/liter, and trisodium citrate at 1 g/liter, pH 7.0) were measured at OD_{600} . The bacterial concentrations were adjusted to $OD_{600} \approx$ 1.0; then, a 300-µl suspension was added into 30 ml of minimal medium at 28°C with shaking at 200 rpm. Each strain was inoculated into four flasks. The same experiment was repeated three times.

DSF extraction. DSFs of *X. oryzae* pv. *oryzicola* were extracted according to a previous report (27). *X. oryzae* pv. *oryzicola* wild-type strain Rs105 was cultured to $OD_{600} = 3.0$ (stationary phase) in 200 ml of NB medium at 28°C with shaking at 200 rpm. The culture was centrifuged at 8,000 rpm for 20 min at 4°C. A total of 200 ml of ethyl acetate was added to the supernatant and fully mixed in a separating funnel. The ethyl acetate phase was concentrated by evaporation, then dissolved in 1 ml of dimethyl sulfoxide.

Quantification for EPS production. EPS production of the *X. oryzae* pv. *oryzicola* strains was measured as described previously (40). Briefly, *X. oryzae* pv. *oryzicola* strains were cultivated in NB medium at 28°C with shaking at 200 rpm for 5 days. EPS was precipitated from the culture supernatant with a twofold volume of ethanol and dried to constant weight at 55°C before measurement. Each strain was inoculated into three flasks, respectively. The same experiment was repeated at least three times.

Determination of extracellular protease activity of *X. oryzae* pv. *oryzicola* strains. To measure the extracellular activity of pro-

TABLE 1. Bacterial strains and plasmids used in this study^a

Strains and plasmids	Properties or characteristics	Source or reference
Strains		
<i>Xanthomonas oryzae</i> pv. <i>oryzicola</i>		
Rs105	Wild-type strain Rs105, Rif ^r	This laboratory
$\Delta rpjF$	<i>rpjF</i> in-frame deletion mutant of strain Rs105, Rif ^r	This laboratory
$\Delta hshA$	<i>hshA</i> in-frame deletion mutant of strain Rs105, Rif ^r	This study
$\Delta hshB$	<i>hshB</i> in-frame deletion mutant of strain Rs105, Rif ^r	This study
Δclp	<i>clp</i> in-frame deletion mutant of strain Rs105, Rif ^r	This study
$\Delta hshB(hshB)$	Mutant $\Delta hshB$ harboring plasmid pUFR- <i>hshB</i> , Rif ^r , Kan ^r	This study
$\Delta clp(clp)$	Mutant Δclp harboring plasmid pUFR- <i>clp</i> , Rif ^r , Kan ^r	This study
<i>X. oryzae</i> pv. <i>oryzae</i> PXO99A	Wild-type strain	36
<i>Escherichia coli</i> DH5 α	F ⁻ , ϕ 80d <i>lacZ</i> AM15, $\Delta(lacZYA-argF)$ U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (r _k ⁻ , m _k ⁺), <i>phoA</i> , <i>supE44</i> , λ^- , <i>thi-1</i> , <i>gyrA96</i>	This laboratory
Plasmids		
pMD19-T	Amp ^R , ColE1 origin, T Simple vector	TaKaRa
pK18mobsacB	Km ^R , oriT(RP4), <i>sacB</i> , <i>lacZα</i> , Plac, Pmbi, mobilization and counter selection	37
pK18- <i>hshA</i>	Km ^R , pK18mobsacB with two <i>hshA</i> flanking fragments	This study
pK18- <i>hshB</i>	Km ^R , pK18mobsacB with two <i>hshB</i> flanking fragments	This study
pK18- <i>clp</i>	Km ^R , pK18mobsacB with two <i>clp</i> flanking fragments	This study
pUFR034	<i>IncW</i> , Nm ^R , Km ^R , <i>Mob</i> ⁺ , <i>Mob(p)</i> , <i>lacZα</i> , PK2 replicon, cosmid	11
pUFR- <i>hshB</i>	Km ^R , pUFR034 with 1.87-kb fragment including <i>hshB</i>	This study
pUFR- <i>clp</i>	Km ^R , pUFR034 with 1.29-kb fragment including <i>clp</i>	This study

^a Rif^r, Km^R, and Amp^R indicate resistance to rifampicin, kanamycin, and ampicillin, respectively.

tease, 2 µl of bacterial culture adjusted to OD₆₀₀ ≈ 1.0 was spotted on NA plates containing 1% (wt/vol) skim milk powder. After incubation at 28°C for 36 h, protease activity was assessed according to the zones of clearing produced (35). Each strain was repeated three times. The same experiment was repeated at least three times.

Resistance to hydrogen peroxide. Experiments testing resistance to hydrogen peroxide (H₂O₂) were carried out as described below. NA plates were made containing H₂O₂ concentrations of 0, 0.1, and 0.25 mM. *X. oryzae* pv. *oryzicola* strains were cultured to mid-logarithmic phase (OD₆₀₀ = 1.0) in NB medium; then, threefold and ninefold dilutions were made. An aliquot of 5 µl of initial culture and diluted cultures for each strain were spotted on NA plates and cultured for 36 h at 28°C (23). Each strain was repeated three times. The same experiment was repeated at least three times.

Tolerance to bismethiazol. Tolerance experiments were performed by subculturing *X. oryzae* pv. *oryzicola* strains (OD₆₀₀ ≈ 1.0) into NB medium at 1% inoculum, and bismethiazol was supplemented to final concentrations of 30, 60, and 100 ppm. The tolerance of bacterial cultures to bismethiazol was then measured at OD₆₀₀ 8, 16, 24, 32, 44, 56, and 68 h after inoculation. Each strain was repeated three times. The same experiment was repeated three times.

Real-time PCR and reverse-transcription PCR. To determine that the transcription of *hshB* in *X. oryzae* pv. *oryzicola* was influenced by DSF, *clp*, and nutritional conditions, relative quantitative real-time PCR was carried out using SYBR Premix EX Tag II kit (TaKaRa) in an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). 16S rRNA was used as an endogenous control. Primer sequences are listed in Table 2. Total RNA was extracted from *X. oryzae* pv. *oryzicola* wild-type strain Rs105, *ΔrpfF*, and *Δclp* at different growth stages and different nutritional conditions using the RNAiso Plus reagent (TaKaRa) following the manufacturer's instructions. To remove genomic DNA, the eluted RNA samples were treated with RNase inhibitors and DNaseI (TaKaRa). RNA integrity was confirmed by electrophoresis using 1.2% agarose gels. Then, 2 µg of each RNA sample was used to synthesize cDNA with a cDNA synthesis kit (TaKaRa). The same experiment was repeated three times.

To identify the co-transcription of *hshA* and *hshB*, three pairs of primers were designed according to the genetic organization of *hshA* and *hshB* in the genome of *X. oryzae* pv. *oryzicola* strain BLS256. Primer sequences are listed in Table 2. Reverse-transcription (RT)-PCR was performed with the following conditions: denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 50 s. Reactions were run for 38 cycles. cDNA of *X. oryzae* pv. *oryzicola* wild-type strain Rs105 was used as template for RT-PCR. The same experiment was repeated at least three times.

RESULTS

DSF regulates the transcription of *hshB* in *X. oryzae* pv. *oryzicola*. In a previous study, 18 proteins whose expression was altered by mutation of *rpjF* were identified by 2-DE and MS/MS in *X. oryzae* pv. *oryzicola* (44). Among them, we found that expression of HshB decreased fivefold in *ΔrpjF* compared with

X. oryzae pv. *oryzicola* wild-type strain Rs105. To investigate whether DSF also regulates the transcription of *hshB* in different growth stages of *X. oryzae* pv. *oryzicola*, we analyzed the expression of *hshB* by real-time PCR (Fig. 1). In this experiment, Rs105 and *ΔrpjF* were cultured in NB medium for RNA extraction at the early (OD₆₀₀ = 0.1), middle (OD₆₀₀ = 1.0), and late (OD₆₀₀ = 2.8) stages of logarithmic growth. The *ΔrpjF* culture had been added to DSF at a ratio of 1:200 3 h before RNA extraction of *ΔrpjF*+DSF. The results of real-time PCR analysis showed that the *rpjF* mutation decreased transcription of *hshB* at the early (30%), middle (49%), and late (51%) stages of logarithmic growth (Fig. 1). The transcriptional decrease of *hshB* could be completely restored by adding exogenous DSF (Fig. 1). These results demonstrate that *hshB* transcription is positively regulated by DSF in *X. oryzae* pv. *oryzicola*.

Bioinformatics analysis of *hshB* and experimental verification in *Xanthomonas* spp. The homologous sequence of *hshB* and its upstream and downstream regions were searched using BLASTN. A relatively conserved region from *nodB* to *rhgB* was present in five *Xanthomonas* spp. or pathovars investigated (Fig. 2). In this region of the *X. oryzae* pv. *oryzicola* strain BLS256 genome, *hshB* and its upstream gene *hshA* were found. Cloning and sequencing analysis showed that *X. oryzae* pv. *oryzicola* strain Rs105 possessed the homologues of *hshA* and *hshB* (100% identity to BLS256) (data not shown). The homologous genes of *hshA* and *hshB* were also found in *X. campestris* pv. *vesicatoria* (91% identity) and *X. campestris* pv. *campestris* (87% identity), respectively. However, these two homologous genes could not be found in the complete genomes of *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *citri* (Fig. 2). The difference between *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* was confirmed by Southern blot analysis (Supplemental Figure 1).

hshA and *hshB* share the same transcriptional orientation (Fig. 2), with overlap of 4 bp in *X. oryzae* pv. *oryzicola*. RT-PCR analysis confirmed that these two genes belonged to an operon in *X. oryzae* pv. *oryzicola* strain Rs105 (Fig. 3). Bioinformatics analysis showed that both HshA (ZP_02244585.1) and HshB

Fig. 1. *hshB* transcription is positively regulated by a diffusible signal factor (DSF). Differential expression of *hshB* was confirmed using relative quantitative real-time polymerase chain reaction. Total RNA was extracted from *Xanthomonas oryzae* pv. *oryzicola* strains Rs105, *ΔrpjF* (lost the ability to synthesize DSF), and *ΔrpjF*+DSF (*ΔrpjF* cultures with added exogenous DSF) at the early (optical density at 600 nm [OD₆₀₀] = 0.1), middle (OD₆₀₀ = 1.0), and late (OD₆₀₀ = 2.8) stages of logarithmic growth, respectively. Vertical bars represent standard errors of the means.

TABLE 2. Primers for quantitative real-time polymerase chain reaction (qPCR) and reverse-transcription (RT)-PCR

Gene fragment (method or purpose)	Primer sequence for 5' sequence	Primer sequence for 3' sequence
16S rRNA	5'-AATGGGCGCAAGCCTGATC-3'	5'-AACCACCACCTACGCACGC-3'
<i>hshB</i> (qPCR)	5'-CTCGATCAACTGGTTGCCGAAG-3'	5'-CCAGATATTTCGCAAGGACGC-3'
<i>clp</i> (qPCR)	5'-TTCCAGACCAGCCTGTGCGC-3'	5'-CTTTCCGTGTAGTGTGCGAGCAG-3'
<i>hshA</i> (RT-PCR)	5'-AGAACGCACCGCAGAAGAAG-3'	5'-CAAGCATGAAGTCCGATACC-3'
<i>hshB</i> (RT-PCR)	5'-CGTGATCGCGACATTGGTC-3'	5'-GTTCCACCTGCACCACGATG-3'
<i>hshAB</i> (RT-PCR)	5'-GATGGGCGATGTGGTGGTG-3'	5'-ATGCCGATCTCGGTGATGC-3'

(ZP_02244586.1) were the homolog of HutI and were involved in secondary metabolites biosynthesis, transport, and catabolism. Furthermore, bioinformatics results also indicated that both HshA and HshB might be secreted hydrolases with a putative signal peptide at the N terminal. However, mutation of *hshA* did not influence virulence and virulence-associated phenotypes (e.g., EPS production and extracellular protease activity) in *X. oryzae* pv. *oryzicola* (data not shown).

Involvement of both *hshB* and *clp* in the pathogenicity of *X. oryzae* pv. *oryzicola* in the host of rice. In the present study, six of these genes were successfully deleted using homologous double-crossover protocol. Among six genes, mutation of *hshB* (named $\Delta hshB$) led to complete loss of virulence of *X. oryzae* pv. *oryzicola* compared with the wild-type strain Rs105 (average lesion length, 3.05 ± 0.40 cm) (Fig. 4A and B). The virulence of $\Delta hshB$ was rescued by the complementary vector pUFR-*hshB* containing the *hshB* gene of *X. oryzae* pv. *oryzicola* (average lesion length, 2.09 ± 0.27 cm) (Fig. 4A and B). The bacterial growth curves in inoculated rice leaves were consistent with the results from the lesion measurement experiments (Fig. 4C). These results demonstrate that *hshB* is required for virulence of *X. oryzae* pv. *oryzicola*.

Gene *clp* encodes a global transcriptional regulator, Clp, which is a key node in the intricate DSF signaling network (17,26). To explore whether *hshB* is also regulated by *clp* in *X. oryzae* pv. *oryzicola*, the *clp* deletion mutant (named Δclp) was constructed. Mutant Δclp caused very short lesions (average, 0.61 ± 0.19 cm) compared with the wild-type strain Rs105 (average, 3.32 ± 0.56 cm) in the rice leaves (Fig. 4D and E). Compared with the wild-type strain Rs105, the bacterial population of Δclp in the rice leaves also decreased (Fig. 4F). The virulence of the mutant Δclp was restored by introducing the complementary vector pUFR-*clp* into the Δclp (Fig. 4D to F). These results demonstrate that *clp* is also required for the full virulence of *X. oryzae* pv. *oryzicola*.

***hshB* is required for normal growth of *X. oryzae* pv. *oryzicola* in minimal medium.** $\Delta hshB$ exhibited a severe growth deficiency compared with the wild-type strain Rs105 (Fig. 5A) but the growth of Δclp was little different from that of Rs105 (Fig. 5A). The growth deficiency of $\Delta hshB$ was restored in the complemented strain $\Delta hshB(hshB)$ (Fig. 5A). However, in the rich medium NB, no significant differences were observed on the

growth of $\Delta hshB$, Δclp , and Rs105 (data not shown). To further analyze why the growth of $\Delta hshB$ was severely inhibited in minimal medium, we investigated transcription of *hshB* using real-time PCR for Rs105 cultured in the rich medium NB and in minimal medium. The results showed that the transcription of *hshB* was 43.3-fold upregulated in minimal medium compared with that in NB medium (Fig. 5B), further demonstrating that *hshB* is required for normal growth of *X. oryzae* pv. *oryzicola* in minimal medium.

Both *hshB* and *clp* regulate EPS production and extracellular protease activity of *X. oryzae* pv. *oryzicola*. EPS and extracellular protease, which are secondary metabolites of plant pathogens, are important virulence factors in *Xanthomonas* spp. (16,35). The mutations of *hshB* and *clp* dramatically impaired EPS production and extracellular protease activity (Fig. 6A and B). The EPS production of the *hshB* mutant strain $\Delta hshB$ decreased 33.7% compared with that of the *X. oryzae* pv. *oryzicola* wild-type strain Rs105, and that of the *clp* mutant strain Δclp decreased by 57.7% (Fig. 6A). Both $\Delta hshB$ and Δclp only produced smaller zones of clearing on NA plates containing 1% (wt/vol) skim milk powder compared with Rs105 (Fig. 6B), which showed that both *hshB* and *clp* positively regulated the extracellular protease activity of *X. oryzae* pv. *oryzicola*. The phenotypes of $\Delta hshB$ and Δclp were restored in the complemented strains $\Delta hshB(hshB)$ and $\Delta clp(clp)$, respectively (Fig. 6A and B).

Both *hshB* and *clp* contribute to resistance of *X. oryzae* pv. *oryzicola* to oxidative stress and bismerthiazol. To assess the contributions of *hshB* and *clp* to the *X. oryzae* pv. *oryzicola* response to oxidative stress, we tested the sensitivity of $\Delta hshB$ and Δclp to H_2O_2 using a stress plate assay. The results indicated that both $\Delta hshB$ and Δclp were more sensitive to H_2O_2 compared with the wild-type strain Rs105 (Fig. 7A). Even 0.1 mM H_2O_2 severely repressed growth of $\Delta hshB$ and Δclp ; however, Rs105 was able to grow normally at several dilutions of H_2O_2 (Fig. 7A). These phenotypes were restored in the complemented strains (Fig. 7A).

Bismerthiazol has been widely used to control rice bacteria diseases caused by *X. oryzae* pv. *oryzicola* and *X. oryzae* pv.

Fig. 2. Comparisons of the *nodB*-*rhgB* interspace region containing *hshA* and *hshB* in five *Xanthomonas* pathovars. Colored arrows display the orientations and genetic organization of genes in the *nodB*-*rhgB* region. Gene size and organization is based on the complete genome sequence from the GenBank database, with accession numbers AAQN000000000 for *Xanthomonas oryzae* pv. *oryzicola* strain BLS256, AM039951 for *X. campestris* pv. *vesicatoria* strain 85-10, AE008922 for *X. campestris* pv. *campestris* strain ATCC 33913, CP000967 for *X. oryzae* pv. *oryzae* strain PXO99A, and AE008923 for *X. axonopodis* pv. *citri* strain 306.

Fig. 3. Reverse-transcription polymerase chain reaction (RT-PCR) analysis showed that *hshA* and *hshB* are co-transcribed. **A**, Genetic organization of *hshA* and *hshB*. A, B, and AB fragments were amplified by primers listed in Table 2. **B**, RT-PCR and PCR were performed by same primer pairs, and the same RNA samples were used for PCR analysis to preclude the possibility of contaminated genomic DNA in the purified total RNA samples. A: *hshA*; B: *hshB*; AB: *hshAB*.

Fig. 4. Virulence assays and growth of *Xanthomonas oryzae* pv. *oryzicola* wild-type strain Rs105 and its derivative strains in rice. **A and D**, Lesion lengths on rice leaves inoculated with *X. oryzae* pv. *oryzicola* strains 14 days after inoculation. **B and E**, Disease symptoms on rice leaves inoculated with *X. oryzae* pv. *oryzicola* strains 14 days after inoculation. **C and F**, Growth of *X. oryzae* pv. *oryzicola* strains on rice leaves. Rs105: the wild type; $\Delta hshB$: *hshB* mutant; $\Delta hshB(hshB)$: $\Delta hshB$ complemented with pUFR-*hshB*; Δclp : *clp* mutant; $\Delta clp(clp)$: Δclp complemented with pUFR-*clp*. Vertical bars represent standard errors of the means.

Fig. 5. *hshB* is required for normal growth of *Xanthomonas oryzae* pv. *oryzicola* in minimal medium. **A**, Growth curves of *X. oryzae* pv. *oryzicola* strains were determined in minimal medium. **B**, *hshB* expression analysis using relative quantitative real-time polymerase chain reaction. Total RNA was extracted from strain Rs105 cultured in minimal medium (MMX) and rich medium (NB). Vertical bars represent standard errors of the means.

Fig. 6. Both *hshB* and *clp* play important roles in the regulation of virulence factor production. **A**, Measurement of polysaccharide production by *Xanthomonas oryzae* pv. *oryzicola* wild-type strain Rs105 and its derivatives. Vertical bars represent standard errors of the means. **B**, Determination of extracellular protease activity of *X. oryzae* pv. *oryzicola* strains on nutrient agar plates containing 1% (wt/vol) skim milk powder.

oryzae. To examine whether mutations of *hshB* and *clp* influence bismethiazol resistance of *X. oryzae* pv. *oryzicola*, we investigated growth curves of *X. oryzae* pv. *oryzicola* strains in NB medium supplemented with several concentrations of bismethiazol (30, 60, and 100 ppm) at 28°C with shaking at 200 rpm. The results showed that growth of $\Delta hshB$ and Δclp was severely inhibited by 60 ppm bismethiazol compared with the wild-type strain Rs105 (Fig. 7B and C). In all *X. oryzae* pv. *oryzicola* strains, growth was little affected at 30 ppm bismethiazol but completely inhibited at 100 ppm (data not shown).

The global regulator Clp modulates expression of *hshB* in *X. oryzae* pv. *oryzicola*. Previous studies suggested that DSF activated the transcriptional regulator Clp and then modulated the expression of many downstream genes (5,26). To investigate whether *clp* plays a role in regulating the expression of *hshB*, we assessed the transcript level of *hshB* in Rs105 and Δclp using real-time PCR. The results showed that the transcript level of *hshB* was 40-fold downregulated in Δclp compared with that in Rs105 (Fig. 8A). In contrast, the transcript level of *clp* was only 3.1-fold downregulated in $\Delta hshB$ compared with that in Rs105 (Fig. 8B). These results indicated that the *clp* positively regulated the expression of *hshB* in *X. oryzae* pv. *oryzicola*.

DISCUSSION

Many biological processes of phytopathogens are controlled through intricate regulatory networks of gene expression (26,42). Identifying the key genes in the global regulatory network and positioning them in this network are vital to understand host-pathogen interactions (16,22,26,34). In a previous study, we found that expression of HshB was positively regulated by DSF (44). The results of this work further demonstrate that transcrip-

tion of *hshB* is also positively regulated by DSF in *X. oryzae* pv. *oryzicola* (Fig. 1). The accumulated evidence indicates that the global transcriptional regulator Clp is located downstream of the two-component system RpfC/RpfG in the DSF signaling pathway (17,26), which modulates the transcriptional expression of numerous genes involved in, for example, virulence and extracellular enzyme and EPS biosynthesis, either directly or indirectly (17). The present study indicates that the *clp* mutant possesses phenotypes similar to those of the *hshB* mutant (Figs. 4 and 7). The overlapping functions of *clp* and *hshB* suggest that a hierarchical relationship might exist between *clp* and *hshB* in the DSF signaling network of *X. oryzae* pv. *oryzicola*. Transcription analysis of *clp* and *hshB* indicates that the influence of *clp* on the transcript level of *hshB* is 12.7-fold compared with that of *hshB* on the transcript level of *clp* in *X. oryzae* pv. *oryzicola* (Fig. 8). This indicates that *clp* might locate upstream of *hshB*, and directly or indirectly regulates the expression of *hshB*.

BLASTN analysis of *hshB* showed homologues in *X. campestris* pv. *vesicatoria* strain 85-10 (90% identity) and *X. campestris* pv. *campestris* strain ATCC 33913 (85% identity). However, the homologue of *hshB* was not found in three published genome sequences of *X. oryzae* strains PXO99A, KACC10331, and MAFF 311018. *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* are two pathovars of *X. oryzae* that cause bacterial leaf streak and bacterial blight of rice, respectively (28). *X. oryzae* pv. *oryzicola* typically enters the rice leaf through stomata and colonizes intercellular spaces of the parenchyma (30). In contrast, *X. oryzae* pv. *oryzae* penetrates the leaf mainly through hydathodes at the leaf tip and leaf margin, and spreads in the xylem (29,30). The genetic difference of *hshB* between *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* might be the result of adaptive evolution for different microenvironments of colonizing in rice leaves.

Fig. 7. Mutations of *hshB* and *clp* impair resistance to H_2O_2 and bismethiazol in *Xanthomonas oryzae* pv. *oryzicola*. **A**, *X. oryzae* pv. *oryzicola* strains were grown on nutrient agar plates with H_2O_2 (0, 0.1, and 0.25 mM). Each *X. oryzae* pv. *oryzicola* strain was diluted threefold and ninefold. **B**, *X. oryzae* pv. *oryzicola* strains were grown in nutrient broth (NB) medium. **C**, *X. oryzae* pv. *oryzicola* strains were grown in NB medium with bismethiazol at 60 ppm. Vertical bars represent standard errors of the means.

Analysis of genome sequences indicated that *hshB* is likely part of a two-gene operon, together with its upstream gene *hshA*. This was demonstrated by RT-PCR in the current study (Fig. 3). In *X. oryzae* pv. *oryzicola*, the stop codon of the *hshA* open reading frame (ORF) overlaps with the start codon of the *hshB* ORF. This kind of arrangement had been observed in some bacteria for genes having closely related functions (6,10). Both HshA and HshB are homologues of HutI but the comparative analysis showed that the identity was only ≈ 16.3 to 17.2% among HshA, HshB, and HutI at the amino acid level. HutI is a member of the histidine utilization operon (*hut*). The histidine operon is highly conserved in bacteria, including genus *Xanthomonas*, which is the histidine degradation pathway (21). This study showed that both HshA and HshB possessed a putative signal peptide, which might be secreted proteins by the type II secreted system. But HutI did not have signal peptide. Compared with the wild strain Rs105, the mutation of *hshA* could not influence the growth of *X. oryzae* pv. *oryzicola* in minimal medium (data not shown) but the *hshB* mutant could not grow in minimal medium (Fig. 5A). Histidine was ultimately catabolized to L-glutamic acid and formamide by the histidine operon. The growth of the *hshB* mutant in minimal medium could not be restored by adding L-histidine (Supplemental Figure 2) but could be restored by adding L-glutamic acid and formamide. These results showed that HshB might take part in the catabolism of histidine.

Fig. 8. Transcription of *hshB* is positively regulated by *clp* in *Xanthomonas oryzae* pv. *oryzicola*. **A**, *hshB* expression analysis using relative quantitative real-time polymerase chain reaction (PCR). **B**, Gene *clp* expression analysis using relative quantitative real-time PCR. Total RNA was extracted from strains Rs105, Δclp , and $\Delta hshB$ cultured in minimal medium, respectively. Vertical bars represent standard errors of the means.

The oxidative burst, which primarily produce H_2O_2 and superoxide (O_2^-) at the site of attempted invasion (1,3), is one of the most rapid plant defense responses at early stages of plant-pathogen interactions (14,41). Similar to many other pathogens (23,41), *X. oryzae* pv. *oryzicola* must overcome the oxidative stress response generated by the rice cells for successful invasion. The results of this study demonstrate that both *hshB* and *clp*, especially *hshB*, make a significant contribution to resistance to oxidative stress (Fig. 7A). In this present study, we found that the resistance to oxidative stress sharply decreased at reduced population densities of *X. oryzae* pv. *oryzicola* strains (Fig. 7A), suggesting that resistance to oxidative stress might be involved in the DSF-dependent QS of *X. oryzae* pv. *oryzicola*. Previous investigations also revealed the relationship between resistance to oxidative stress and QS in *X. campestris* pv. *campestris* and *P. aeruginosa* (15,17). In addition, the bismerthiazol resistance further demonstrates that both *hshB* and *clp* help *X. oryzae* pv. *oryzicola* to resist diverse environmental pressures, and provides a new clue for understanding the mechanism of bismerthiazol.

In conclusion, we identified a novel pathogenicity-related gene in *X. oryzae* pv. *oryzicola*, *hshB*, which encodes a putative secreted protein. Our data show that *hshB* is involved in virulence, EPS production, extracellular protease activity, growth in minimal medium, and resistance to oxidative stress and bismerthiazol of *X. oryzae* pv. *oryzicola*, and that *clp* also possesses similar functions. We present evidence that *clp* positively regulates the expression of *hshB* and that it might locate upstream of *hshB* in the global regulatory network of *X. oryzae* pv. *oryzicola* gene expression.

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