The Role of a Host-Induced Arginase of *Xanthomonas oryzae* pv. *oryzae* in Promoting Virulence on Rice

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Accepted for publication 30 June 2019.

ABSTRACT

The plant bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* causes bacterial blight of rice, which is one of the most destructive rice diseases prevalent in Asia and parts of Africa. Despite many years of research, how *X. oryzae* pv. *oryzae* causes bacterial blight of rice is still not completely understood. Here, we show that the loss of the *rocF* gene caused a significant decrease in the virulence of *X. oryzae* pv. *oryzae* in the susceptible rice cultivar IR24. Bioinformatics analysis demonstrated that *rocF* encodes arginase. Quantitative real-time PCR and Western blot assays revealed that *rocF* expression was significantly induced by rice

and arginine. The *rocF* deletion mutant strain showed elevated sensitivity to hydrogen peroxide, reduced extracellular polysaccharide (EPS) production, and reduced biofilm formation, all of which are important determinants for the full virulence of *X. oryzae* pv. *oryzae*, compared with the wild-type strain. Taken together, the results of this study revealed a mechanism by which a bacterial arginase is required for the full virulence of *X. oryzae* pv. *oryzae* on rice because of its contribution to tolerance to reactive oxygen species, EPS production, and biofilm formation.

The plant bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* causes bacterial blight of rice, which was first characterized in Japan in 1884 and is one of the most destructive rice diseases prevalent in Asia and parts of Africa, Australia, and Latin America (Niño-Liu et al. 2006; Sere et al. 2005). *X. oryzae* pv. *oryzae* typically infects rice leaves through wound sites or hydathodes at the leaf tip or margin. After the pathogen propagates in the intercellular spaces of the epidermis, the bacteria colonize and spread through the plant xylem vessels where they can interact with the xylem parenchyma cells, ultimately resulting in tissue necrosis and wilting (González et al. 2012).

The *X. oryzae* pv. *oryzae*–rice interaction is an important working model to explain how pathogens inhibit host plant immune responses (Hilaire et al. 2001). Previous studies indicate that *X. oryzae* pv. *oryzae* can employ multiple virulence factors to promote its pathogenicity on rice, such as extracellular polysaccharide (EPS), biofilm, effectors secreted by the type III secretion system, cell motility, adhesion molecules, stress tolerance, and the quorum sensing (QS) system (Büttner and Bonas 2010; Yu et al. 2016). EPSs can enhance the attachment to different environmental surfaces, protect themselves from desiccation, and promote the colonization of bacterial pathogens in the host by reducing host responses (Sutherland 1988). EPS is also involved in the formation of biofilm (Vu et al. 2009). Biofilm is an important

virulence factor for bacterial pathogens to protect themselves from environment stresses such as superoxide radicals, hydrogen peroxide (H₂O₂), and high or low pH values (Marquis 1995). EPS production and biofilm formation are critical for tolerance to H₂O₂, which is an antimicrobial agent used to kill *X. oryzae* pv. *oryzae* (Bae et al. 2018). In addition, EPS production and biofilm formation in bacteria can be regulated by QS (Von Bodman et al. 1998).

Studies have shown that arginine can augment biofilm formation and polysaccharide intercellular adhesion to promote the pathogenic ability of *Staphylococcus aureus* (Zhu et al. 2007). In addition, arginine is important for the growth and biofilm development of *Streptococcus gordonii*, which is an oral bacterium (Jakubovics et al. 2015). The arginase pathway is one arm of arginine catabolism in bacterial pathogens that is encoded by the *rocABC* and *rocDEF* operons and the *rocG* gene (Calogero et al. 1994; Gardan et al. 1995). The *rocF* gene that encodes arginase is responsible for the first step of the arginase pathway, which hydrolyzes arginine to ornithine and urea (Lu 2006). Arginase was shown to be crucial for its pathogenicity of *Helicobacter pylori* in the human stomach through dysregulation of the host immune response, such as by inhibiting nitric oxide (NO) production of macrophages by directly competing with host nitric oxide synthase (NOS), protecting pathogens from reactive oxygen species (ROS) damage, inhibiting human T-cell proliferation, and reducing T-cell receptor (TCR)-zeta chain (CD3 zeta) expression (George et al. 2017; Gobert et al. 2001; Shi et al. 2014; Zabaleta et al. 2004). In addition, arginase is essential for *Leishmania donovani* survival in humans to cause disease (Boitz et al. 2017). This pathway is also employed by some pathogens to consume arginine as a carbon and nitrogen source during their infection of the host, which has been shown in *Mycobacterium tuberculosis*, *Trypanosoma cruzi*, and *Candida albicans* (Xiong et al. 2016).

Interestingly, our preliminary study of proteomics showed that RocF expression in *X. oryzae* pv. *oryzae* was induced by host rice and was identified as arginase. Yet the reason why it was induced is still unknown. Pathogenic bacteria can activate the transcription of

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Funding: This work was supported by the National Natural Science Foundation of China (grants 31571974 and 31371906).

Y. Zhang and G. Wu contributed equally to this work.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and one supplementary table are published online.

The author(s) declare no conflict of interest.

host-induced genes to disrupt host immune response and adapt to highly heterogeneous environments in host tissues (Bumann and Valdivia 2007). A study by Brown et al. (2007) indicated that the expression of genes *acrA* and *dinF* in *Ralstonia solanacearum* can be induced by toxic compounds, and these genes are required for colonization and bacterial wilt virulence in tomato. Using two-dimensional polyacrylamide gel electrophoresis, Kachroo et al. (1997) found that there is one rice-induced protein in *Magnaporthe grisea* that plays an important role in the process of infection. In addition, studies demonstrated that sRNA genes are related to the adaptation to environmental stress conditions and the pathogenicity of bacterial pathogens. For example, the expression of sRNA *IsrJ*, which controls the virulence of *Salmonella typhimurium* by regulating the translocation efficiency of virulence-associated effectors into host cells, was induced by low oxygen or magnesium within macrophages (Padalon-Brauch et al. 2008). Therefore, the host-induced genes, protein, and sRNA are required for pathogen virulence. Whether the rice-induced arginase in *X. oryzae* pv. *oryzae* is also important for promoting virulence on rice is still undetermined.

In this study, we found that arginase is encoded by *rocF* (accession WP_011407409) and is required for the full virulence of *X. oryzae* pv. *oryzae* on rice. Bioinformatic analysis showed that *rocF* is highly conserved among important plant-pathogenic *Xanthomonas* species. Using quantitative real-time (qRT)-PCR and Western blot analysis, we also obtained evidence showing that *rocF* expression is significantly induced by rice leaf extracts and arginine. Subsequent genetic and phenotypic studies showed that *rocF* plays an important role in the multiple virulence-related functions of *X. oryzae* pv. *oryzae*, including EPS production, biofilm formation, and H₂O₂ tolerance. To our knowledge, this is the first report of a bacterial arginase that is a functional virulence factor in a plant pathogen, and these findings will be useful in elucidating the course of events in plant-pathogen interactions.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani medium at 37°C and the *X. oryzae* pv. *oryzae* wild-type strain PXO99^A and derived mutant strains were cultured in nutrient broth (NB) medium at 28°C (Qian et al. 2013). Minimal medium XVM1 (20 mM of NaCl, 0.5 mM of phosphate, 10 mM of sucrose, and 2 mg of methionine/liter) contained macerated rice, wheat, or tobacco leaves: 20 g of

4- to 5-week-old leaves of rice, wheat, or tobacco was macerated in liquid nitrogen and used as leaf extract, which was then added to 400 ml of XVM1 medium and autoclaved to analyze *rocF* expression (González et al. 2013; Tsuge et al. 2002). Antibiotics kanamycin (50 µg/ml), spectinomycin (25 µg/ml), and gentamicin (25 µg/ml) were added to the growth medium as appropriate for selection. Plasmids were transformed into *E. coli* by heat shock and into the *X. oryzae* pv. *oryzae* wild-type strain PXO99^A by electroporation.

Bioinformatic analysis of the RocF protein. The domain organization of RocF was analyzed using SMART online software (<http://smart.embl-heidelberg.de/>). BLASTP was used to search for homologs of RocF in *Xanthomonas* species that were obtained from NCBI. DNAMAN software was used to analyze the relevant DNA sequence alignments.

Construction of the *rocF* gene deletion mutant and complementary strains. *X. oryzae* pv. *oryzae* wild-type strain PXO99^A was used as the parental strain to obtain the in-frame deletion mutant via a previously described allelic homologous recombination method (Qian et al. 2013). For complementation of *rocF*, an 1,890-bp DNA fragment containing *rocF* and its predicted promoter region was cloned into pUFR047 plasmid. The resulting constructs were transformed into the $\Delta rocF$ strain by electroporation to obtain the complementary strain. The PCR primers used in this study are listed in Supplementary Table S1.

Construction of pSS122 promoter-probe plasmid and β -glucuronidase reporter assay. To determine *rocF* promoter activity, the *rocF* promoter was predicted using Promoter 2.0 Prediction Server online software (http://www.fruitfly.org/seq_tools/promoter.html) and one 376-bp promoter was amplified by PCR using the forward and reverse primers P-*rocF*-F and P-*rocF*-R, respectively. The amplified promoter was digested with restriction endonucleases and was then integrated into the pSS122 promoter-probe vector plasmid (which was constructed from pUFR047) by T₄ DNA ligase. The recombined plasmid was then transformed into the wild-type strain by electroporation and grown on NB agar plates with gentamicin and ampicillin. In addition, the β -glucuronidase activity assay was performed as described (Pandey and Sonti 2010). Briefly, a single colony was cultured in NB liquid medium with gentamicin and ampicillin to an optical density of 1.0 at 600 nm, and 3-µl bacterial suspensions were dropped onto XVM1 medium plates containing 38 µM of 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-Gluc)/ml and different concentrations of arginine at 28°C for 5 to 7 days. At the same time, 4- to 5-week-old leaves from the susceptible rice cultivar IR24 were inoculated

TABLE 1. Bacterial strains and plasmids used in this study

Strain	Characteristics ^z	Reference
<i>Escherichia coli</i>		
DH5a	F ⁻ , ϕ 80dlacZ Δ M15, Δ (lacZYA-argF)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk-,mk+), <i>phoA</i> , <i>supE44</i> , λ^- , <i>thi-1</i> , <i>gyrA96</i>	This study
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
PXO99 ^A	Philippine race 6; wild-type strain	Qian et al. (2013)
$\Delta rocF$	<i>rocF</i> in-frame deletion mutant of strain PXO99 ^A	This study
$\Delta rocF$ (<i>rocF</i>)	$\Delta rocF$ harboring plasmid pUFR047- <i>rocF</i> (complementary strain); Gm ^R , Amp ^R	This study
PXO99 ^A (pUFZ75)	PXO99 ^A harboring plasmid pUFZ75 (GFP-labeled strain); Km ^R	This study
$\Delta rocF$ (pUFZ75)	$\Delta rocF$ harboring plasmid pUFZ75 (GFP-labeled strain); Km ^R	This study
PXO99 ^A (pSS122)	PXO99 ^A harboring plasmid pSS122; Ap ^R , Gm ^R	This study
PXO99 ^A (pSS122:: <i>rocF</i>)	PXO99 ^A harboring plasmid pSS122 with <i>rocF</i> promoter; Ap ^R , Gm ^R	This study
Plasmids		
pK18 <i>mobsacB</i>	Allelic exchange suicide vector, <i>sacB</i> oriT(RP4); Km ^R	Qian et al. (2013)
pK18- <i>rocF</i>	pK18 <i>mobsacB</i> with two <i>rocF</i> flanking fragments; Km ^R	This study
pUFR047	Broad-host-range expression vector; IncW, Gm ^R , Amp ^R , Mob ⁺ , Mob(p), lacZ α^+ , Par ⁺	Andrade et al. (2014)
pUFR047- <i>rocF</i>	pUFR047 carrying gene <i>rocF</i> with its native promoter region; Gm ^R , Amp ^R	This study
pUFR047- <i>rocF</i> -Flag	pUFR047 carrying gene <i>rocF</i> with its native promoter region and Flag label; Gm ^R , Amp ^R	This study
pUFZ75	P _{trp} -TIR-gfp cassette in pUFR034, Km ^R	Guo et al. (2015)
pSS122	Promoter-probe vector, IncW, Gm ^R , Ap ^R	González et al. (2012)
pSS122:: <i>rocF</i>	pSS122 carrying gene <i>rocF</i> with its native promoter region, Gm ^R , Ap ^R	This study

^z Gm^R = gentamicin, Amp^R = ampicillin, GFP = green fluorescent protein, and Km^R = kanamycin.

with the wild-type strain, which contained the pSS122 plasmid with or without the *rocF* promoter produced using the leaf-clipping method. Then the leaves from days 7 and 14 were dipped into β -glucuronidase (GUS) assay buffer containing 38 μ M of X-Gluc/ml at 37°C for 3 days. All of the leaves were subsequently dipped into ethanol for approximately 5 min, which was repeated three times until the chlorophyll in the rice leaves disappeared. Leaves that were inoculated with ddH₂O and the wild-type strain served as a negative control. At least 20 leaves were inoculated for each treatment and every treatment was repeated three times.

RNA extraction and qRT-PCR analysis. Transcription levels of genes in the PXO99^A strains that were cultured in NB liquid medium with or without H₂O₂ and XVM1 liquid medium with or without rice, wheat, or tobacco leaf extracts or 0.1 mM of amino acids were analyzed as described with some modifications (Song et al. 2017). Total RNA was extracted with TRIzol reagent (Takara, Dalian, China) according to the manufacturer's protocol. cDNA was synthesized from total RNA using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix kit (with One-Step gDNA Removal) (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. qRT-PCR was performed in the QuantStudio 6 and 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). One-hundred nanograms of cDNA of each sample was used in qRT-PCR. The relative expression ratio was calculated using the $\Delta\Delta$ threshold cycle method. 16S rRNA was used as an endogenous control and qRT-PCR was repeated three times with four independent biological replicates each time.

Western blot analysis of RocF protein expression. To test the RocF protein expression level under different conditions, one integrated PXO99^A strain was cultured as previously described (Wang et al. 2018). Briefly, we fused the Flag tag (GAT TAC AAG GAT GAC GAC GAT AAG) to the 5' terminus of *rocF* and this fragment was amplified by PCR. Then the fragment was cloned into the pUFR047 plasmid and the integrated plasmid was transformed into *X. oryzae* pv. *oryzae* wild-type strain PXO99^A to obtain a strain in which the region containing *rocF* was replaced by the FLAG-tagged fused fragment. The integrated PXO99^A *X. oryzae* pv. *oryzae* wild-type strains were cultured in 50 ml of NB liquid medium at 28°C while shaking at 220 rpm to an optical density of 1.0 at 600 nm and were centrifuged at 6,000 rpm for 5 min. Then the supernatants were discarded and the cell pellets were suspended in 50 ml of XVM1 liquid medium with or without rice, wheat, or tobacco leaf extracts or 0.1 mM of arginine at 28°C while shaking at 220 rpm for another 16-h incubation. Bacterial cells were harvested at 10,000 rpm at 4°C for 10 min, and 1 ml of radioimmunoprecipitation assay lysis buffer with 3 μ l of phenylmethanesulfonyl fluoride and 3 μ l of protease inhibitor cocktail was added to extract the total proteins. The samples were subsequently centrifuged at 10,000 rpm at 4°C for 5 min and the supernatants were transferred to new 2-ml tubes containing protein loading buffer before they were frozen at -80°C. Six micrograms of total proteins of each sample was used in Western blot analysis. Soluble proteins were separated on SDS-PAGE and transferred onto polyvinylidene difluoride membranes using a semidry blot machine (Bio-Rad). Membranes were blocked with 1× phosphate-buffered saline with Tween with 5% nonfat milk and were probed with anti-Flag mouse antibody (1:5,000) before they were incubated with horseradish peroxidase-conjugated goat antimouse secondary antibody (1:10,000). The α subunit of RNA polymerase was used as a control for sample loading. The experiment was independently repeated three times.

Virulence assays on rice. The susceptible rice cultivar IR24 was planted in a growth chamber under a cycle of 16 h of light at 28°C and 8 h of darkness at 25°C, and then the 4- to 5-week-old leaves were inoculated with PXO99^A strains in sterile distilled water at an optical density of 0.5 at 600 nm by the leaf-clipping method described previously (Wang et al. 2018). Lesion lengths were measured 15 days postinoculation. For the bacterial

population assay, 3-cm clipped leaf sections were centered on the sites that were inoculated by PXO99^A strains and were ground in a 2-ml tube with 1 ml of sterile distilled water; at least 10 leaves were collected for each sample. Bacterial populations were measured by dilution plating of 100 μ l of bacterial suspension on NB solid medium and the number of bacterial colonies was counted. At least 50 leaves were inoculated by each treatment and every treatment was repeated three times.

Biofilm formation assays. The method used to analyze biofilm formation was described previously with some modifications (Li and Wang 2012). Briefly, the PXO99^A strains were cultured in NB liquid medium to an optical density of 1.0 at 600 nm. Forty-microliter bacterial suspensions were transferred to sterilized polystyrene tubes with 4 ml of NB liquid medium and incubated at 28°C while shaking at 220 rpm overnight. All of the tubes were subsequently kept in the chamber at 28°C for 7 days without shaking. The bacterial suspensions were moved from the tubes and washed three times with sterilized water. Biofilm formed on the tubes was visualized by staining with 0.1% crystal violet; after 20 min, excess stain was removed and the tubes were washed three times with sterilized water. The stained biofilm on the tubes was dissolved in acetic acid/ethanol (1:4, vol/vol) and the absorbance value was measured at an optical density of 590 nm through the use of an Agilent 8453 ultraviolet-visible spectrophotometer (Agilent Technologies Inc., Santa Clara, CA). The experiment was repeated three times.

The confocal laser scanning microscopy (CLSM) technology used to assay biofilm formation was described previously (Tondo et al. 2016). In brief, we transformed the pUFZ75 plasmid, which can express green fluorescent protein (GFP), into the PXO99^A strains. The GFP-labeled PXO99^A strains were cultured in NB liquid medium with 10 mM of kanamycin to an optical density of 1.0 at 600 nm; 40- μ l suspensions were transferred into 4 ml of NB liquid medium, and then 200- μ l mixed suspensions were transferred into sterilized flow chambers covered with glass slides (catalog number 155411; Lab-Tek/NUNC, Naperville, IL). All of the flow chambers were kept in a humidified polyvinylchloride box at 28°C for 2 to 3 days without shaking. Biofilm construction was visualized by CLSM (Leica Microsystems Inc., Buffalo Grove, IL) with an excitation wavelength of 488 nm and an emission wavelength of 500 to 545 nm with a 20× objective. The images were analyzed with LAS_X_Small_2.0.0_14332 software, which was provided with the confocal microscope described above. At least three biological replicates were carried out for each treatment.

Measurement of tolerance to oxidative stresses. The PXO99^A strains were cultured in NB liquid medium and grown to an optical density of 1.0 at 600 nm. Then 80- μ l bacterial suspensions of each strain were transferred into new sterilized glass bottles with 8 ml of NB liquid medium containing 0, 0.1, and 0.2 mM of H₂O₂. At the same time, bacterial suspensions of the PXO99^A strains at an optical density of 1.0 at 600 nm were diluted 5-fold (5×) and 25-fold (25×) and 3- μ l diluted bacterial suspensions were dropped onto the surface of NB solid plates with 0, 0.1, and 0.2 mM of H₂O₂. All glass bottles were inoculated at 28°C while rotating at a speed of 220 rpm and the plates were inoculated in a 28°C incubator for 2 to 3 days. Bacterial viability was analyzed by observing their growth on these plates and in these bottles. At least three biological replicates were carried out for each treatment.

Quantitative determination of EPS production. The PXO99^A strains were cultured on NB agar plates at 28°C for 2 to 3 days. Cells were then incubated in NB liquid medium at 220 rpm at 28°C to an optical density of 1.0 at 600 nm. Then 500- μ l bacterial suspensions of the relative strains were transferred to 50 ml of sterilized NB liquid medium and incubated at 28°C with constant shaking at 220 rpm for 5 days. A 50-ml bacterial suspension was centrifuged at 10,000 rpm for 10 min, the supernatant was transformed into new containers, and 100 ml of ethanol was added to precipitate the EPS for 24 h. The precipitated EPS was then dried

at 70°C and the dried EPS was weighed using a digital analytical balance. Every experiment was repeated at least three times.

Data analysis. All analysis was conducted using SPSS 14.0 software (SPSS Inc., Chicago, IL). Significant differences in virulence, bacterial population, EPS production, biofilm formation, and gene expression among different strains were determined via the hypothesis test of percentages (*t* test).

RESULTS

RocF protein is highly conserved among many important plant-pathogenic *Xanthomonas* species. To better understand *rocF*, bioinformatics analysis was performed. The *rocF* open reading frame (locus tag: *PXO_RS22625*) was 924 bp and the nucleotide position in the genome was from 4,951,804 to 4,952,727 (Fig. 1A). The RocF protein contained arginase domain (from residues 6 to 300) that was predicted by using SMART (<http://smart.embl-heidelberg.de/>); the molecular weight was 3.316 kDa, as predicted by using the ExPASy ProtParam tool (<https://web.expasy.org/protparam/>). Amino acid sequence alignment analysis demonstrated that RocF was highly conserved among the important plant-pathogenic *Xanthomonas* species, such as *X. oryzae* pv. *oryzae* KACC 10331 (accession AAW73689), *X. campestris* pv. *campestris* strain B100 (accession WP_012439504), *X. campestris* pv. *campestris* strain ATCC 33913

Fig. 1. Bioinformatics analysis of the *rocF* gene. **A**, Schematic diagram of *rocF* in the genome of *Xanthomonas oryzae* pv. *oryzae* PXO99^A. Open arrows indicate the location, length, and orientation of the open reading frames. The middle element shows the domain structure analysis of the RocF protein, which was performed using SMART online software (<http://smart.embl-heidelberg.de/>). The lower element shows that *rocF* was amplified by PCR primers P1 and P2 and was cloned into the pUFR047 plasmid for complementation of the *rocF* deletion mutant. **B**, The identity of arginase homologs in important plant-pathogenic *Xanthomonas* species. ^a*Xoo* = *X. oryzae* pv. *oryzae*, *Xoc* = *X. oryzae* pv. *oryzicola*, and *Xcc* = *X. campestris* pv. *campestris*. ^bDomains were predicted by SMART. ^cAccording to a BLASTP search.

(accession NP_639266), *X. oryzae* pv. *oryzicola* BLS256 (accession AEQ98376), *X. campestris* pv. *campestris* strain 8004 (accession AAY51054), *X. gardneri* (accession WP_074058724), and *X. oryzae* pv. *oryzae* PXO86 (accession AJQ85091) (Fig. 1B).

***rocF* expression is induced by rice leaf extracts and arginine in vitro.** To determine whether *rocF* expression can be induced by rice leaf extracts, we performed qRT-PCR experiments to determine the *rocF* transcription level in XVM1 liquid medium with or without rice leaf extracts as described in the Materials and Methods. The qRT-PCR results showed that the *rocF* transcription level was approximately 4-fold higher in XVM1 medium in the presence of rice leaf extracts than in XVM1 medium; the *rocF* transcription level could also be significantly induced by nonhost plant leaf extracts, including wheat and tobacco. The same method was used to detect the *rocF* transcription level in XVM1 liquid medium with 0.1 mM of arginine. The qRT-PCR results showed that the *rocF* transcription level was approximately 5-fold higher in XVM1 medium in the presence of 0.1 mM of arginine than in XVM1 medium in the absence of arginine (Fig. 2A). We also

Fig. 2. *rocF* expression in different media. **A**, mRNA expression levels of *rocF* in different XVM1 media were detected using quantitative real-time (qRT)-PCR. Relative transcription levels of these genes were calculated by qRT-PCR with respect to the corresponding transcription levels in the wild type and the 16S rRNA as the internal standard. **B**, Analysis of RocF protein expression levels in different XVM1 media using Western blot analysis. The upper bands corresponding to the predicted size of RocF-FLAG fusion were detected by the monoclonal antibody. Anti-FLAG and the lower bands corresponding to the RNA polymerase α -subunit were detected by the specific antibody anti-RNAP. Values are the means $\pm$ SD from three independent experiments. Asterisks above the error bars indicate significant differences compared with XVM1 medium (*t* test, $**P < 0.01$). XVM1 = negative control, XVM1# = XVM1 with 0.1 mM of arginine/liter, XVM1## = XVM1 with 5 g of rice leaf extract/liter, XVM1### = XVM1 with 5 g of wheat leaf extract/liter, and XVM1#### = XVM1 with 5 g of tobacco leaf extract/liter.

measured the *rocF* transcription level in XVM1 medium containing seven different amino acids at a concentration of 0.1 mM. The results showed that *rocF* expression was significantly induced by glutamate and ornithine, which were metabolites from arginine, but not by histidine, methionine, proline, phenylalanine, and leucine (Supplementary Fig. S1). These results indicate that *rocF* expression can be significantly induced by rice leaf extracts and arginine.

Because *rocF* expression can be significantly induced by rice leaf extracts and arginine at the transcription level, we then investigated whether RocF protein expression can be induced at the same conditions by using Western blot. The integrated PXO99^A strain was constructed in which the 5'-terminus of *rocF* was labeled with a FLAG tag as described in the Materials and Methods. The PXO99^A strain with FLAG-tagged *rocF* was cultured in 50 ml of XVM1 liquid medium with or without rice leaf extracts or 0.1 mM of arginine, and the total proteins were extracted and analyzed as described in the Materials and Methods. Western blot analysis showed that the presence of rice leaf extracts or arginine resulted in a significant increase in RocF expression compared with that observed in cells grown in XVM1 medium, and detectable bands corresponding to the predicted size of the RocF-FLAG fusion protein were observed. At the same time, RocF protein expression was also significantly induced by wheat and tobacco leaf extracts. Under the same test conditions, expression of the α subunit of RNA polymerase was analyzed as an internal control (Fig. 2B). These data indicate that the expression of *rocF* and RocF can be induced by host and nonhost leaf extracts and arginine maybe the potential inducer.

Promoter activity of *rocF* is induced by arginine during pathogen infection. To further confirm that the *rocF* promoter was also functional in rice leaves, β -glucuronidase activity of the PXO99^A strains with or without the pSS122 plasmid containing the *rocF* promoter in rice leaves was determined as described in the Materials and Methods. Very light indigo-blue staining was observed in major longitudinal veins of rice leaves 7 days post-inoculation of the PXO99^A strain with the pSS122 plasmid containing the *rocF* promoter, but no staining was observed in major longitudinal veins of rice leaves that were infected by the PXO99^A strains with or without the pSS122 reporter plasmid. Staining intensity increased during the development of rice. Intense indigo-blue staining was observed in major longitudinal veins of rice leaves 14 days postinoculation of the PXO99^A strain with the pSS122 plasmid containing the *rocF* promoter, but indigo-blue staining still could not be observed in major longitudinal veins of rice leaves (Fig. 3A). These results demonstrate that *rocF* promoter activity may be increased by components of the rice leaves during the infection of the PXO99^A strain.

qRT-PCR and Western blot results showed that *rocF* expression was significantly induced by arginine; therefore, we speculated that the arginine in rice leaves is the component to increase *rocF* activity. Therefore, we determined *rocF* activity on XVM1 medium plates with or without 0.1 mM of arginine. Indigo staining was observed for the PXO99^A strain with the pSS122 plasmid containing the *rocF* promoter, whereas no staining was observed for the PXO99^A strain with or without the pSS122 plasmid (Fig. 3B). These results suggest that activity of the *rocF* promoter is significantly induced by arginine during pathogen infection.

***rocF* is required for the full virulence of *X. oryzae* pv. *oryzae*.** A *rocF* deletion mutant strain and a complementary strain, $\Delta rocF$ and $\Delta rocF(rocF)$, respectively, were constructed to investigate the potential biological functions of *rocF* in PXO99^A. The qRT-PCR results showed that the corresponding region of *rocF* was deleted successfully (Supplementary Fig. S2). The virulence assay for PXO99^A strains in the susceptible rice cultivar IR24 was performed by the leaf-clipping method (Guo et al. 2015). Lesion lengths were measured 15 days postinoculation (Fig. 4A) and the data are presented in Figure 4B. The wild-type strain caused a lesion

length of 26 ± 0.8 cm, whereas the $\Delta rocF$ strain caused a lesion length of just 12.5 ± 1.0 cm in all infected leaves. These data indicate that the virulence of PXO99^A is significantly decreased after deletion of *rocF*. The virulence of the $\Delta rocF$ strain in the leaves of susceptible rice cultivar IR24 was restored in the complementary strain $\Delta rocF(rocF)$, which caused a lesion length of 23 ± 1.2 cm. At the same time, we assayed the colonization of PXO99^A on rice leaves at 0, 8, and 15 days postinoculation. Growth of the $\Delta rocF$ strain was significantly reduced on rice leaves compared with the growth of the wild-type strain and the reduced bacterial population was restored to wild-type levels by complementation (Fig. 4C). To determine whether deletion of *rocF* resulted in a decrease in the proliferation of PXO99^A, we tested their growth rates in NB liquid medium. There was no difference among the wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains in their ability to grow in NB liquid medium (Supplementary Fig. S3). These results indicate that deletion of *rocF* does not influence the viability of PXO99^A. Three replicates were used for each treatment and the experiment was repeated three times. Overall, these results demonstrate that a functional *rocF* is required for PXO99^A to achieve full virulence on rice.

Fig. 3. GUS activity determination of the *rocF* promoter in rice leaves and under arginine conditions. **A**, GUS activity determination of the PXO99^A strains with or without the pSS122 plasmid containing the *rocF* promoter-infected rice leaves stained by GUS assay buffer containing 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-Gluc). The leaves were acquired 7 and 14 days postinoculation (DPI). The wild-type strain without the pSS122 vector and ddH₂O-infected leaves were used as the negative control. Indigo-blue staining indicates increased GUS activity of the *rocF* promoter. **B**, GUS activity determination of the PXO99^A strains with or without the pSS122 plasmid containing the *rocF* promoter under the XVM1 medium with or without 0.1 mM of arginine and X-Gluc. Indigo staining indicates increased activity of the *rocF* promoter. The experiment was repeated three times. XVM1 = XVM1 without 0.1 mM of arginine and XVM1⁺ = XVM1 with 0.1 mM of arginine.

Deletion of *rocF* reduces EPS production by *X. oryzae* pv. *oryzae*. EPS formation is associated with attachment to different environmental surfaces and EPS represents an essential factor in the virulence of pathogenic bacteria (Neo et al. 2010). This finding promoted us to determine whether the deletion of *rocF* affects EPS production in PXO99^A. The wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains were grown in NB liquid medium for 5 days, and EPS was extracted from the cultures and quantified as described in the Materials and Methods. The results showed that EPS production was reduced by approximately 50% in the $\Delta rocF$ strain compared with the wild-type strain and there was no significant difference in EPS production between the $\Delta rocF(rocF)$ strain and wild-type strain (Fig. 5A). In addition, our qRT-PCR data showed that deletion of *rocF* caused significantly decreased expression of genes related to the polysaccharide biosynthesis process, including *PXO_RS15910* (*GumC*), *PXO_RS14800* (α -L-fucosidase), *PXO_RS11405* (sugar ABC transporter permease), *PXO_RS11410* (multiple sugar transport system substrate-binding protein), *PXO_RS08055* (L-arabinonolactonase), *PXO_RS10635* (multiple sugar transport system ATP-binding protein), and *PXO_RS14820* (glycoside hydrolase family 3) (Fig. 5B). These data demonstrate that *rocF* plays an important role in the EPS production of PXO99^A.

Loss of *rocF* affects the biofilm formation of *X. oryzae* pv. *oryzae* on abiotic surfaces. EPS is involved in the formation of biofilm, which is a virulence factor in many plant-pathogenic

bacteria (Danhorn and Fuqua 2007; Vu et al. 2009). Because the deletion of *rocF* reduced EPS production, it is likely that *rocF* is related to the biofilm formation in PXO99^A. To test this hypothesis, we determined biofilm formation in the wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains of PXO99^A on the surface of polystyrene tubes. The results showed that biofilm formation on the polystyrene tube surface by the $\Delta rocF$ strain was significantly reduced compared with that formed by the wild-type strain (Fig. 6A). The level of crystal violet staining, which indicates biofilm formation, in the $\Delta rocF$ strain was reduced by approximately 40% compared with that produced by the wild-type strain (Fig. 6B); crystal violet staining of biofilm produced by the complemented strain was restored to the level observed for the wild-type strain. These results indicate that *rocF* contributes to the biofilm formation of PXO99^A on the surface of polystyrene tubes. To further validate these results, we used CLSM technology to observe the biofilm formation of PXO99^A strains as described in the Materials and Methods. The results showed that the $\Delta rocF$ strain produced a less organized biofilm than the wild-type strain. At the same time, the biofilm thickness produced by the $\Delta rocF$ strain was approximately 20 to 30 μ m, which was much thinner than the approximately 50- to 60- μ m-thick biofilm produced by the wild-type strain (Fig. 6C). These data indicate that the deletion of *rocF* negatively influenced biofilm formation. Therefore, the data from these two assays demonstrate that *rocF* is required for biofilm formation of PXO99^A.

***rocF* contributes to the H₂O₂ stress tolerance of *X. oryzae* pv. *oryzae*.** During infection, plant bacterial pathogens produce EPS and biofilm to protect them against the damage caused by environmental stresses such as H₂O₂ (Vu et al. 2009). Therefore, we carried out the experiments as described in the Materials and Methods to investigate the role of *rocF* in resistance to H₂O₂. The results showed that the growth of the $\Delta rocF$ strain was weaker on 0.1- and 0.2-mM H₂O₂ plates than the growth of the wild-type strain (Fig. 7A). A similar result was found in 8 ml of NB liquid medium with 0, 0.1, and 0.2 mM of H₂O₂ (Fig. 7B). In addition, the growth rate of the $\Delta rocF$ strain was significantly inhibited in the presence of 0.2 mM of H₂O₂ in NB liquid medium compared with the wild-type and $\Delta rocF(rocF)$ strains (Fig. 7C). All of the results indicated that the $\Delta rocF$ strain was significantly more sensitive to H₂O₂ than the wild-type strain. In addition, the complementary strain could fully rescue their tolerance to H₂O₂. At the same time, genes involved in H₂O₂ detoxification and adaptation, including *PXO_RS17325* (catalase HPII), *PXO_RS22555* (catalase), *PXO_RS05475* (*ahpC*), *PXO_RS05465* (*LysR* family transcription regulator), and *PXO_RS06520* (*Fur* family transcription regulator), were measured. Their transcription levels were determined by qRT-PCR in the wild-type strain and the $\Delta rocF$ strain in the presence of 0.1 mM of H₂O₂. The results showed that the expression of these genes was significantly reduced in the $\Delta rocF$ strain compared with that in the wild-type strain (Fig. 7D). Taken together, these data demonstrate that *rocF* is indeed involved in the H₂O₂ stress tolerance of PXO99^A.

DISCUSSION

In this study, we identified a novel *X. oryzae* pv. *oryzae* virulence gene, *rocF*, which encodes arginase. A *rocF* mutant showed dramatically reduced virulence on rice. The results of this study indicate that decreased EPS production, reduced biofilm formation, and enhanced H₂O₂ sensitivity are major reasons for the deficiency in virulence of the *rocF* mutant.

EPS is an important virulence determinant in *Xanthomonas* spp. during the infection of pathogens. EPS can create an environment for the growth and spread of pathogens in planta and can protect pathogens against toxic compounds from hosts to enhance pathogenicity (e.g., H₂O₂) (Guo et al. 2015). The *detR* mutant showed reduced virulence of *X. oryzae* pv. *oryzae*, which resulted from a reduction of EPS and intolerance to ROS (Nguyen et al.

Fig. 4. Results of the pathogenicity test of *Xanthomonas oryzae* pv. *oryzae* on host rice. **A**, Disease symptoms caused by the wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains on 4- to 5-week-old susceptible rice cultivar IR24 leaves. Representative pictures were taken 15 days postinoculation. Sterilized water-inoculated rice leaves served as a negative control. **B**, Lesion length caused by the wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains ($OD_{600} = 0.5$) on 4- to 5-week-old susceptible rice cultivar IR24 leaves by using a leaf-clipping method. The lesion length was calculated 15 days postinoculation. **C**, Bacterial populations of the wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains ($OD_{600} = 0.5$) on 4- to 5-week-old susceptible rice cultivar IR24 leaves by using a leaf-clipping method. The inoculated rice leaves for extracting bacteria were harvested after 0, 8, and 15 days, homogenized in sterilized water, and serially diluted on nutrient broth solid plates to count the number of bacterial populations (in CFU). The bacterial populations were presented as the log CFU per leaf. Values are the means $\pm$ SD from three independent experiments ($n = 50$). Asterisks above the error bars indicate significant differences compared with the wild-type strain (t test, ** $P < 0.01$). $\Delta rocF$ = *rocF* deletion mutant strain, $\Delta rocF(rocF)$ = complementary strain, and OD_{600} = optical density at 600 nm.

2016). Deletion of *rocF* in *X. oryzae* pv. *oryzae* also resulted in the loss of EPS production; at the same time, the transcription level of *PXO_RS15910* (*gumC*) was decreased in $\Delta rocF$ compared with the wild type. *GumC* is one of the important *gum* genes that is responsible for transport and polymerization and is required for EPS synthesis; mutation of this gene showed reduced virulence (Kim et al. 2009). Therefore, we conclude that *rocF* plays an important role in EPS biosynthesis and virulence by regulating *gumC* transcription. Another key factor contributing to the loss of virulence of the *rocF* mutant is reduced biofilm formation. *X. oryzae* pv. *oryzae* can adhere to the plant surface and then invade the intercellular space of the host plant cells to acquire nutrition and impair plant defense responses. Biofilm is potentially associated with adhesion to surfaces during infection and colonization and is important for tolerance to environmental stresses of *X. oryzae* pv. *oryzae* (Boher et al. 1997; Crossman and Dow 2004; Vu et al. 2009). Our results showed that the deletion of *rocF* caused less formation of biofilm on the surface of polystyrene tubes. Therefore, we conclude that *rocF* is crucial for the formation of biofilm to regulate the virulence of *X. oryzae* pv. *oryzae*. *X. oryzae* pv. *oryzae* successfully infects rice depending on the ability to counteract H_2O_2 , and its catalases are essential for the detoxification process of H_2O_2 . Yu et al. (2016) reported that the OxyR regulated catalase CatB functions as an important factor to detoxify H_2O_2 in order to promote the bacterial pathogenesis of *X. oryzae* pv. *oryzae* on rice. In our study, $\Delta rocF$ was hypersensitive to H_2O_2 and a very small bacterial population survived under the condition of 0.2 mM of H_2O_2 . In addition, we measured the transcription levels of catalases in $\Delta rocF$ because catalase is required for the detoxification of H_2O_2 in *Xanthomonas* spp. during their early stages of infection. qRT-PCR results indicated that the transcription levels of *PXO_RS17325* (catalase HP11), *PXO_RS22555* (catalase), and *PXO_RS05475* (*ahpC*) were deregulated. These findings led us to conclude that the *rocF* mutant caused dramatically reduced virulence of *X. oryzae* pv. *oryzae* on rice, maybe owing to the deregulation of detoxifying enzymes such as catalases.

Although EPS production, biofilm formation, and H_2O_2 tolerance contribute to the virulence of *X. oryzae* pv. *oryzae* on rice, more work will be needed to increase our understanding of the underlying molecular mechanisms of their regulation in *X. oryzae* pv. *oryzae*. A previous study showed that the metabolic enzyme fructose-1,6-

bisphosphate aldolase can combine the promoter region of *katG* and *rpoA* to regulate their transcription in pathogenic *Francisella* bacteria (Ziveri et al. 2017). In our study, *rocF* encoded arginase, which is an arginine metabolic enzyme, and the transcription levels of genes related to EPS biosynthesis or H_2O_2 detoxification were reduced. This led us to conclude that the metabolic enzyme arginase

Fig. 5. Effects of *rocF* deletion on extracellular polysaccharide (EPS) production by *Xanthomonas oryzae* pv. *oryzae*. **A**, Determination of EPS production by the wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains grown in nutrient broth (NB) liquid medium after 5 days with shaking at 220 rpm at 28°C. The bacterial cultures were collected for the determination of EPS production. **B**, Quantitative real-time (qRT)-PCR determination of the transcription levels of seven sugar transport and biosynthesis genes in the wild-type and $\Delta rocF$ strains grown in NB liquid medium. The relative transcription levels of these genes were calculated by qRT-PCR with respect to the corresponding transcription levels in the wild type and 16S rRNA as the internal standard. Values are the means $\pm$ SD from three independent experiments. Asterisks above the error bars indicate significant differences compared with the wild-type strain (*t* test, ***P* < 0.01). $\Delta rocF$ = *rocF* deletion mutant strain and $\Delta rocF(rocF)$ = complementary strain.

Fig. 6. Effects of *rocF* deletion on biofilm formation by *Xanthomonas oryzae* pv. *oryzae*. **A**, Biofilm formation by the wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains on polystyrene tubes stained with crystal violet. **B**, Biofilm production quantification of the wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains on polystyrene tubes corresponding to **A**. The OD₅₉₀ value was measured after the stained tubes were incubated with ethanol-acetone. **C**, Confocal laser scanning microscopy was performed to observe the three-dimensional structure of biofilm formation by the wild-type and $\Delta rocF$ strains. Images were obtained using a 20 $\times$ objective. Values are the means $\pm$ SD from three independent experiments. Asterisks above the error bars indicate significant differences compared with the wild-type (*t* test, ***P* < 0.01). $\Delta rocF$ = *rocF* deletion mutant strain, $\Delta rocF(rocF)$ = complementary strain, and OD₅₉₀ = optical density at 590 nm.

Fig. 7. Observation of *Xanthomonas oryzae* pv. *oryzae* resistance to hydrogen peroxide (H_2O_2). **A**, The wild-type, $\Delta rocF$, and $\Delta rocF(rocF)$ strains were precultured to the exponential phase of the indicated value ($OD_{600} = 1.0$) in nutrient broth (NB) liquid medium as the initial cultures (1X), which were then diluted 5-fold (5X) and 25-fold (25X). Three-microliter suspensions of the initial cultures and diluted cultures for each strain were dropped onto NB solid medium plates with 0, 0.1, and 0.2 mM of H_2O_2 to observe their growth defects. **B**, Eight-microliter suspensions of the initial cultures in **A** were transformed into 8 ml of NB liquid medium with 0, 0.1, and 0.2 mM of H_2O_2 to observe their growth defects. Pictures were taken of each sample at 0, 24, and 48 h. **C**, Twenty-microliter suspensions of the initial cultures in **A** were transformed into 20 ml of NB liquid medium with 0.2 mM of H_2O_2 to observe their growth rates. The value of each sample at OD_{600} was measured every 2 h. **D**, mRNA expression levels of genes associated with detoxification and adaptation to H_2O_2 in the wild-type and $\Delta rocF$ strains grown in NB liquid medium with 0.1 mM of H_2O_2 were determined using quantitative real-time (qRT)-PCR. Relative transcription levels of these genes were calculated by qRT-PCR with respect to the corresponding transcription levels in the wild type and 16S rRNA as the internal standard. Values are the means $\pm$ SD from three independent experiments. Asterisks above the error bars indicate significant differences compared with the wild-type strain (t test, $**P < 0.01$). $\Delta rocF$ = *rocF* deletion mutant strain, $\Delta rocF(rocF)$ = complementary strain, and OD_{600} = optical density at 600 nm.

can also possibly combine the promoter region of these genes to regulate their transcription.

Arginine has also been characterized as an important nitrogen source for plants (Slocum 2005). More importantly, arginine is a common substrate used by NOS to produce NO, and NO is a diffusible molecular messenger that plays a key role in the rapid induction of the plant immune response (Vidhyasekaran 2014). Whether the *rocF* mutant caused dramatically reduced virulence of *X. oryzae* pv. *oryzae* on rice is associated with the metabolic of arginine then inhibit the NO production of rice requires further study.

In summary, this study demonstrated that after *X. oryzae* pv. *oryzae* infects rice, the arginine in rice is used by *X. oryzae* pv. *oryzae* to induce *rocF* expression, which contributes to EPS production, biofilm formation, and H_2O_2 detoxification. Notably, all of these functions are major factors that contribute to the reduced virulence of the *rocF* deletion mutant strain. To our knowledge, this is the first report of a bacterial arginase that is a functional virulence factor in a plant pathogen. This study not only identified a conserved protein, RocF, as a virulence determinant in *X. oryzae* pv. *oryzae*, but it is also helpful to our understanding of the pathogenic

mechanisms of *X. oryzae* pv. *oryzae*. In future studies, it will be interesting to determine how *rocF* contributes to the expression of important genes in EPS production and H_2O_2 detoxification and adaptation.

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